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Systems Biology Approaches to the Computational Modelling of Trypanothione Metabolism in *Trypanosoma brucei*

by

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Abstract

This work presents an advanced modelling procedure, which applies both structural modelling and kinetic modelling approaches to the trypanothione metabolic network in the bloodstream form of *Trypanosoma brucei*, the parasite responsible for African Sleeping sickness. Trypanothione has previously been identified as an essential compound for parasitic protozoa, however the underlying metabolic processes are poorly understood. Structural modelling allows the study of the network metabolism in the absence of sufficient quantitative information of target enzymes. Using this approach we examine the essential features associated with the control and regulation of intracellular trypanothione level. The first detailed kinetic model of the trypanothione metabolic network is developed, based on a critical review of the relevant scientific papers. Kinetic modelling of the network focuses on understanding the effect of anti-trypanosomal drug DFMO and examining other enzymes as potential targets for anti-trypanosomal chemotherapy.

We also consider the inverse problem of parameter estimation when the system is defined with non-linear differential equations. The performance of a recently developed population-based PSwarm algorithm that has not yet been widely applied to biological problems is investigated and the problem of parameter estimation under conditions such as experimental noise and lack of information content is illustrated using the ERK signalling pathway. We propose a novel multi-objective optimization algorithm (MoPSwarm) for the validation of perturbation-based models of biological systems, and perform a comparative study to determine the factors crucial to the performance of the algorithm. By simultaneously taking several, possibly conflicting aspects into account, the problem of parameter estimation arising from non-informative experimental measurements can be successfully overcome. The reliability and efficiency of MoPSwarm is also tested using the ERK signalling pathway and demonstrated in model validation of the polyamine biosynthetic pathway of the trypanothione network.

It is frequently a problem that models of biological systems are based on a relatively small amount of experimental information and that extensive *in vivo* observations are rarely available. To address this problem, we propose a new and generic methodological framework guided by the principles of Systems Biology. The proposed methodology integrates concepts from mathematical modelling and system identification to enable physical insights about the system to be accounted for in the modelling procedure. The framework takes advantage of module-based representation and employs PSwarm and our proposed multi-objective optimization algorithm as the core of this framework. The methodological framework is employed in the study of the trypanothione metabolic network, specifically, the validation of the model of the polyamine biosynthetic pathway. Good agreements with several existing data sets are obtained and new predictions about enzyme kinetics and regulatory mechanisms are generated, which could be tested by *in vivo* approaches.

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Declaration

This thesis has not been previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree

All the work reported in this thesis has been performed by myself, except where otherwise stated. Other sources are acknowledged explicitly in the text. A bibliography is appended.

Xu Gu

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Chapter 1

Introduction

1.1 Scope of the Thesis

To understand complex biological systems, an integration of experimental and computational research is required. The emerging field of Systems Biology provides a powerful foundation and established scientific methods to enable the understanding of biological pathways at the system level. The best way to achieve a system-level understanding is via the use of computational modelling. However, constructing mathematical models for poorly understood biological systems with a large number of components is not a straightforward process. Standard engineering methodologies for computational modelling are challenged and an integrated and iterative approach is necessary for studying biological systems.

In this thesis, the challenge of computational modelling of complex biological systems is investigated, when the prior knowledge about the system is incomplete and the available experimental data is sparse. We propose a new methodological framework to address this challenge. This framework takes advantage of a decompositional approach, which integrates metabolic modelling with global optimization to simultaneously explore the model structure and kinetic parameters. We illustrate the feasibility of the proposed methodological framework on solving an important biological system that causes a serious illness – the trypanothione metabolic pathway in *Trypanosoma brucei*, a parasite that causes *African Sleeping sickness*. To pursue this goal, some existing scientific methods are reviewed and then adapted and extended in an appropriate way for use in solving the problem of interest.

The following sections give an overall introduction to the relevant background

of the research subject.

1.2   Systems Biology

> *A big challenge for computer scientists who are considering getting involved in Systems Biology, in addition to the requirement of good level of biological foundation, is to keep open-minded and be creative in the design of modern methodologies to make a contribution to biological and computer science domain. — Eberhard O. Voit*

Systems Biology (Kitano 2002b) is an interdisciplinary field that applies established scientific methods to build mathematical models of biological systems and to address associated biological problems. The subject provides a vital interface between biologists and computer scientists, applied mathematicians and statisticians to support the development of a unified understanding of the biological processes involved.

Systems Biology is becoming very popular as it is widely recognized that, in biology, dynamic behavior of the whole system may not be easily deduced from collective descriptions of individual parts, and can only be achieved by a systematic approach with assistance of advanced computing technology. The exponential growth of biological knowledge offers the possibility to perform various computational analyses on one organism or across different organisms.

Computational Modelling is an integral component of Systems Biology (Kitano 2002a). In many publications, the terminologies of mathematical modelling and computational modelling have been used interchangeably, causing confusion to prospective practitioners of this field. Recently, a fierce discussion on 'dichotomies between computational and mathematical models' between Fisher and Henzinger (Fisher and Henzinger 2008) and Hunt et al. (Hunt et al. 2008) points out that the concepts can only be properly interpreted in the context in which the model is used.

We hold the view that mathematical modelling and computational modelling represent two cornerstones of scientific research that are complementary to each other. Both terminologies intend to answer critical questions concerning system behavior when the systems are represented in mathematical formats. Additionally, computational modelling reflects a rigorous procedure for investigating the structure and dynamic regulation of biological systems and developing design principles of the systems by computationally executing mathematical models.

This thesis presents a computational modelling procedure to study the biological pathway of interest, which involves constructing mathematical models and devising powerful techniques for the purpose of the study.

1.3 Computational Modeling

Computational Modelling (Bower and Bolouri 2001) is a commonly used approach to explain and predict system behavior via a variety of mathematical calculations and established scientific methods. Mathematical models are approximate and standardized representations of the knowledge of the underlying processes (de Jong 2002). In the context of biological systems, mathematical models illustrate a number of different components, rates at which these components interact and physical laws that govern the reactions. Good models can be adopted to supplement or even to replace *in vivo* or *in vitro* experiments for the interpretation and hypothesis of biological phenomenon (Voit 2000).

Computational Systems Biology (Kitano 2002a) suggests a methodological framework for constructing mathematical models, as illustrated in Figure 1.1. This modelling methodology is also described as an *analytical* approach. As defined by Söderström and Stoica (Söderström and Stoica 1989), the analytical approach relies on physical insights to elucidate the dynamic behavior of a phenomenon. This is in contrast to the other approach, the *experimental* approach, where mathematical models are a parameterized function and model parameters are assigned with suitable numerical values by fitting the model to experimental data. Application of the experimental approach to the construction of mathematical models is defined as *System Identification*; the subject is discussed in detail in Section 1.5.

Following the analytical approach for mathematical model construction, one starts from *Requirements Capture* for a global identification of the system through the collection of knowledge regarding the structure and regulation of the system, based on which an initial model topology is proposed and the inputs of the system (initial values of system components and kinetic parameters of chemical reactions) are defined. The second step of *Model Construction* determines the modelling formalism to be applied. In this step, correctness of solution methodologies has to be ensured, for example, using continuous modelling methods to model discrete systems is obviously wrong. Assumptions must be made in this step in order to constrain a system within a feasible boundary.

After a mathemtical model is formulated, *Simulation* can be carried out. As defined by (de Jong 2002), simulation is the process of exhibiting information contained in the model, which often refers to the description of dynamic behaviour of the system. Simulation provides us the abstraction of biological systems, with which our knowledge about the systems can be consolidated. Data produced from *in silico* simulation will have to be carefully compared with experimental observations for *Model Validation*. A critical question to be answered in the step of model validation is 'does the model adequately describe the system of interest?' Through model validation we gain confidence in the model that it is useful not only for reproducing measured dynamics but also for predicting system behavior.

An inconsistency between model results and observations indicates deficiencies in the model, which prompts the process of model refinement, where a new model structure may be designed and the relevant *in vivo* experiments may have to be planned. A cyclic workflow from requirement collection to model validation is often required in order for a final model to be satisfactory. By the end of the building cycle, a model that is an adequate representation of reality can be developed. This is followed by *Model Analysis* in order to study the systemic properties, for instance, parameter sensitivity analysis. Little can be gained by using an inadequate model for analysis.

However, one critical limitation of the analytical approach is the dependence of model construction on a substantial amount of information being available. An attempt to construct a mathematical model with a complete mechanistic description of the system is impractical and in some cases, mathematical modelling can only be enabled on a portion of the system. This is particularly the case when studying new biological processes.

Biological data that can be detected in experiments is usually limited. Computational simulation without a complete initial status is not allowed. Promisingly, given the system outputs, the inputs can be predicted via *backward* simulation, assuming the model structure is known. The process of approximating parameters that are not available from biological experiments is defined as *Parameter Estimation*, which is sometimes known as the *Inverse Problem*. Parameter estimation is one building block of the model validation procedure, and is therefore an important research problem in Systems Biology.

In forward simulations, the well-posedness of the problem, i.e. the existence, uniqueness and stability of the solution, is often assumed. However, for backward

simulations, it is well-known that ill-posedness is generic (Moles et al. 2003). A theoretical verification by Sontag (Sontag 2006) shows that, in order to sufficiently identify r parameters, as many as $2r+1$ experimental measurements must be available. It is therefore not surprising that for some systems parameter estimation is the modelling step that requires the most effort. An experimental study of the global optimization method in solving the parameter estimation problem is presented in Chapter 5.
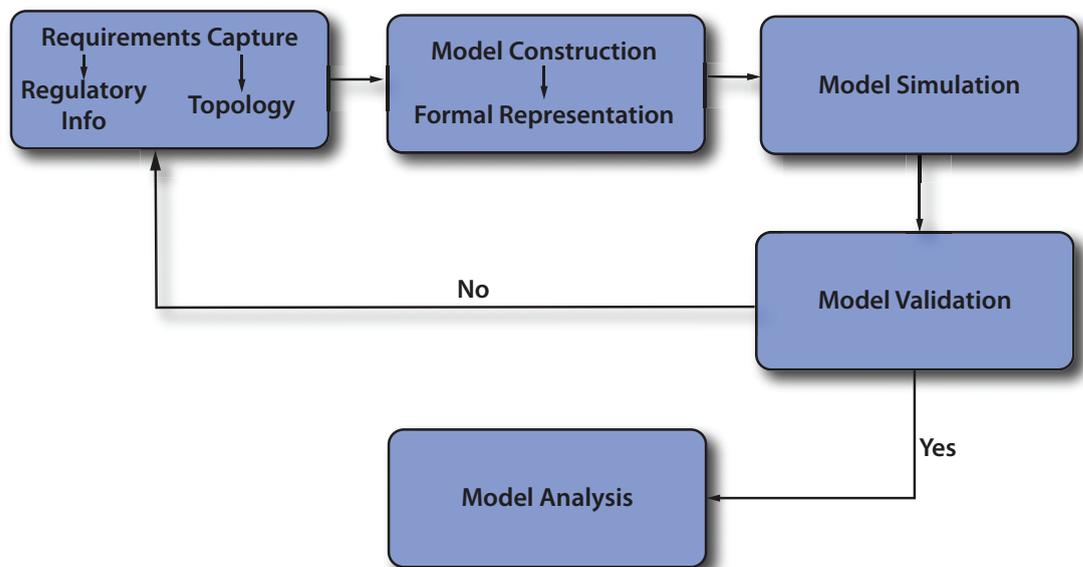
Figure 1.1: Five basic steps of standard computational experimentation.

Importantly, two aspects should be addressed prior to a modelling procedure, which are concerned with identifying the problem and stating the purpose or the intended use of the model. An explicit definition of these two aspects will help clarify the applicability of the model.

The problem definition is concerned with the identification of biological questions to answer and the measurements available and suitable for the intended use of the model. *The purpose of modelling* differs in the aspect of whether the modelling aims at seeking a model to reproduce what has already been observed or to make predictions about the system before *in vivo* experiments are carried out. For example, both steady-state metabolic fluxes and time-dependent concentrations are suitable for investigating the metabolism, giving rise to two major perspectives underpinning the modelling of metabolic systems, namely *Structural Modelling* and *Kinetic Modelling*. However, the drawback of the

former is the limited predictive power in studying the system behaviour under different conditions, which is not compatible with the modelling effort that involves the formulation of mechanistic hypotheses about the system dynamics. The subject of interest is discussed in detail in Section 1.4.

The availability of the prior knowledge determines the level of detail or abstraction of the kinetic model of the system. Two types of rate equations, as defined by Westerhoff et al. (Westerhoff et al. 2009), are phenomenological equations and mechanically-precise equations. When we attempt to seek the mechanism responsible for a particular biological phenomena, phenomenological equations may be sufficient and they could enable simpler or even analytical solutions of the system. However, when attempting to seek for a mechanism that is actually responsible, precise equations for the system components are required. Application of phenomenological equations to approximate the dynamic behavior of all the system components belongs to the field of *System Identification*. Discussion on the subject of interest is continued in Section 1.5.

Computational modelling of complex biological systems is an interesting challenge. Biological complexity is embodied in the non-linearity of enzyme kinetics and mutual interactions with the environment (Westerhoff et al. 2009). In the context of computational modelling, the problem of complexity appears in two facets with regard to *Dimensionality* and *Uncertainty*.

Dimensionality, as the term indicates, refers to the fact that a large number of system components are usually interconnected within a tangled complex web. Uncertainty arises from two main sources – model structure and parameter values. Parameters (including initial condition and kinetic parameters) are often unknown and imprecise. Parameter estimation through a small sample or non-representative samples causes large variations in the estimated solutions. Uncertainty in model structure is concerned with whether the model captures the right mechanism. An incorrect model structure can impact the accuracy of parameter estimates, and as a result, the reliability of model predictions.

It is noteworthy that mathematical models evolve as the knowledge about the systems increases. It is worthwhile to retain multiple models of the same system with different levels of detail and abstraction, thus the most appropriate model can be selected for the tasks at hand.

1.4 Metabolic Modelling

The approaches applied at various stages of computational modelling are varied in the amount and type of experimental data available and the intended use of the model, as elucidated in Section 1.3. The general aim of computational modelling of metabolic systems falls into two primary categories of studying time-invariant (i.e. metabolic flux distribution) and time-dependent behavior (i.e transient metabolite concentrations). The so-called *Structural Modelling* and *Kinetic Modelling* approaches constitute two main approaches of metabolic engineering (Schuster et al. 1999).

The two main types of modelling are not contradictory but rather complementary to each other. Structural modelling is a relatively straightforward process that takes the stoichiometry and reversibility of the involved reactions as the only inputs. Heinrich et al. (Heinrich et al. 1977) stated that a clear description of the metabolic flux distribution in the system is vital for the understanding of metabolic regulation. As the knowledge required for structural modelling is primarily the stoichiometry of the system, this modelling approach can be regarded as a precondition for kinetic modelling, with which the non-stiochiometric information, – i.e. enzymatic kinetics, is incorporated.

Both approaches have their own merits. Breitling et al. (Breitling et al. 2008) argued that structural models can be used to predict mutant growth phenotypes and wrong predictions can guide iterative model improvement. On the contrary, kinetic modelling requires the enzyme kinetics and regulatory information; however, such detailed information has proved to be difficult to obtain. Parameter estimation is also a complex problem for kinetic modelling when data is missing. This is however not necessary for structural modelling, resulting in a more soluble problem with this approach.

The thesis presents an advanced modelling procedure using both modelling approaches for the metabolic pathway under study. The constructive evaluation of both metabolic models can serve the purpose of chemotherapeutic target validation and anti-parasitic drug discovery. The computational investigation aims to gain valuable insights and generate good predictions about the biological system under consideration through collectively exploring the steady-state properties and individual dynamic events of the system.

1.5   System Identification

In many cases mathematical model construction starts with the application of basic physical laws (i.e. mass action) to the process being studied, followed by using a modelling formalism (i.e. ordinary differential equations) to elucidate the relations between variables. Given complete physical knowledge and detailed quantitative information about the system, a correct model can, in theory, be constructed and all the model parameters can be determined numerically. However, this situation is very rare in the context of biological applications, where our prior knowledge is restricted by sparse quantitative information and incomplete physical aspects of the underlying processes. In such cases, it is necessary to use identification techniques.

System identification is defined by Ljung (Ljung 2008) as "the art and science of building mathematical models of systems from observed input-output data". Two broad branches of system identification are *structure identification* and *parameter estimation* (Söderström and Stoica 1989). Structure identification is concerned with finding a suitable model structure, within which a good model can be determined, and parameter estimation is defined as, given a structure and a set of experimental data, the determination of model parameters that govern the dynamic behaviour of the system. In practice, the exploration of structure and parameters are often carried out iteratively, where a model structure is chosen and the corresponding parameters are estimated.

The need for system identification has become increasingly common in the fields of science and technology. The procedure of system identification is characterized by four basic ingredients in sequence according to Söderström and Stoica (Söderström and Stoica 1989) and Ljung (Ljung 2008):

1. *Requirement of experimental observations*; this refers to performing cell culture experiments to produce experimental data.

2. *Determination of an appropriate model*; this is the single most important step in the identification process. It concerns looking for a model structure to approximate the observed input-output relationship of the system. Models with different mathematical representations, which differ in the level of prior information contained, can be formed.

3. *Decision of a criterion of fit*; this refers to defining a fitting criterion, for example a least squares criterion (the residual between the model predic-

tions and experimental observations) and applying a parameter estimation process that attempts to match a particular data set against each candidate model. A particular model that can best describe the data set is selected. An appropriate definition of the criterion is critical for the estimation process.

4. *Evaluation and Validation*; this step is concerned with testing whether the model is an appropriate representation of the system when it is used with other data sets. An iterative refinement procedure is required depending on the acceptable accuracy of the model in approximating the true description.

The step of *Evaluation and Validation* in the system identification procedure should proceed according to the purpose of the modelling. Predictive models should be evaluated in terms of the goodness of fit in reproducing measured data, which provides evidence of the credibility of the model. When the purpose of the modelling is to develop predictive models, the model performance has to be validated on interpreting *fresh data*, which is a data set not used for the training process. Once the model is evaluated with estimates that are valid and the predictability is assessed as reliable, the model is considered to be sufficiently relevant in describing the underlying processes and is ready to be applied to its intended use. If this is not the case, alternative model structures must be considered, unknown parameters of the model have to be estimated and new model has to be validated.

System identification depends on the availability of sufficient experimental observations. One disadvantage of the model obtained by system identification is the limited physical insight provided, since in most cases the parameters of the model have no direct physical meaning. Dynamic models pose the most challenging identification problem due to the non-linear nature and extensive computational resources required. Concepts related to system identification will be introduced in detail in Chapter 2.

1.6 Trypanothione Metabolic Pathway

Trypanosoma brucei, a protozoan parasite, is the causative agent of the fatal disease *African Sleeping sickness*. *Trypanosoma brucei* is transferred between its mammalian hosts by bites of the tsetse fly, which lives within the bloodstream

of the mammalian host (bloodstream form) and the midgut of the fly (procyclic form). The disease is endemic in certain regions of Africa and infects millions of people annually (Fairlamb 2003). Background information on human African trypanosomiasis has been reported (Table 1.1). However, compared with these numbers, research towards understanding the parasite is still insufficient and more work remains to be done.

Human African Trypanosomiasis	
Number infected	0.5 million
Deaths per year	50,000
Disability adjusted life years	1,598,000
Distribution	Sub-Saharan Africa
Causative organisms	*T.brucei rhodesiense* & *T.brucei gambiense*
Vector	Tsetse fly (*Glossina*)
Natural habitat	Forested rivers & shores (*gambiense*)
	Savannah (*rhodesiense*)
Natural host	Ungulates & other mammals (*rhodesiense*)
	Mainly man only (*gambiense*)

Table 1.1: Data on human African trypanosomiasis (Barrett et al. 2003).

Drug development against human African trypanosomiasis has become a major public concern due to toxicity, efficacy and availability problems with current drug treatments (Muller et al. 2003, Turrens 2004). Identification of potential drug targets within the parasites is an invaluable tool for designing chemotherapeutic agents against the parasitic diseases. The challenge in drug design arises from the similarity of metabolic pathways in parasitic protozoans and the mammalian host. Anti-parasitic drugs that are efficient, non-toxic and affordable are urgently required.

Trypanothione was discovered to be unique to trypanosomatids (Fairlamb et al. 1985) and has been a major focus of trypanosome research. Several potential drug targets that result in the depletion of trypanothione, and consequently, inhibition of the cell growth, have been investigated. The polyamine biosynthetic sub-pathway is of vital importance to the survival of the trypanosomatid parasite and is a validated drug target for treatment of the disease. α-DL-Difluoromethylornithine (DFMO), the only new drug licensed for treatment of African Sleeping sickness in 50 years, inhibits ornithine decarboxylase which catalyzes the initial step in polyamine biosynthesis (Fairlamb 2003). Glycolysis in bloodstream-form of *Trypanosoma brucei* has also been verified as a conve-

nient context for studying the prospects for using enzyme inhibitors as antiparasitic drugs (Eisenthal and Cornish-Bowden 1998, Albert et al. 2005, Bakker et al. 1997, Bakker 1998).

This work presents the first attempt at kinetic modelling of the trypanothione metabolism in the parasitic organism. While some of these metabolic components have been studied in other cell types (Rodriguez-Caso et al. 2006) and are relatively well understood, comparatively little work has been done on these components in trypanosomal cells. A systematic investigation of various aspects of the trypanothione metabolism will benefit the development of efficient chemotherapeutic drugs that can exert clinical functions in a consistent and robust manner.

A mechanistic modelling approach is designed to construct the kinetic model of the trypanothione metabolic pathway, which supports a simultaneous investigation of the suitability of model structure and the exploration of missing parameters. Our *in silico* investigation focuses on understanding the effect of anti-trypanosomal drug DFMO and examining other enzymes as potential targets for anti-trypanosomal chemotherapy. The kinetic modelling starts with an extensive literature research on the physical basis of the cell functions and available quantitative information on the system components and their interactions. Substantial knowledge of the network topology and enzymatic reactions makes the kinetic modelling possible, yet significant numbers of parameters are unknown. This poses severe difficulties to standard engineering methodologies for the study of system behaviour and model-based interpretation of the experimental data.

1.7 Thesis Statement

The best way to understand complex networks of biological pathways is through the use of computational modelling. Coupled with experimental data, computational modelling aims to enable the construction and interpretation of complex systems in a sound and integrated environment. A major problem for such modelling is the uncertainty and incompleteness of prior knowledge and experimental observations of the system of interest.

We present a methodological framework based on the principles of Computational Systems Biology and System Identification to guide the establishment of mechanistic mathematical models. We demonstrate that the methodologi-

cal framework, which integrates model decomposition with metabolic modelling and global optimization is advantageous in tackling the problem of uncertain and partial representation of biological systems. We propose a novel approach for applying a multi-objective optimization scheme to the validation of perturbation-based models of biological systems and demonstrate that it is a promising strategy for model validation with an integrated study of different system states. The novel validation approach can be generalized for application to various real-world multi-objective optimization problems.

We demonstrate the methodological framework using the computational modelling of the trypanothione metabolic pathway in the bloodstream form of *Trypanosoma brucei*. The novel model validation approach is tested on a signal-transduction pathway and applied to the validation of the trypanosome polyamine biosynthetic sub-pathway. The proposed approaches enable a systematic evaluation of the kinetic model of the trypanothione metabolic pathway, a consistent interpretation of the underlying biological processes and *in silico* hypotheses of uncovered kinetic mechanisms.

1.8 Thesis Contributions

A list of contributions of the thesis is as follows:

- The first structural model of the trypanothione metabolic pathway in blood-stream-form *Trypanosoma brucei*, presented in Chapter 3. This model supports the study of metabolic capabilities of trypanosomes to support cell growth and the rational identification of potential drug targets. By means of structural modelling, the correlation between structural and functional characteristics of the pathway is unravelled, which assists in the initialization of the proposed decompositional approach in Chapter 7.

- The first kinetic model of the trypanothione metabolic pathway in blood-stream-form *Trypanosoma brucei*, proposed in Chapter 4. The kinetic model is constructed based on information gleaned from the experimental biology literature, and represented by two functionally independent sub-networks derived in Chapter 3. This kinetic model is comprehensively studied and strategically refined in Chapter 7.

- One of the first experimental studies to investigate the performance of the single objective optimization algorithm PSwarm on a complex real world

problem, detailed in Chapter 5. PSwarm is a newly developed population-based evolutionary algorithm, which has not yet been widely applied to solve biological systems; in this case the complex model of a signal transduction pathway is examined.

- A novel and generic approach, MoPSwarm, for the validation of perturbation-based models of biological systems, proposed in Chapter 6. Our proposal takes advantage of the multi-objective optimization scheme and has the potential to solve non-linear and dynamic real-world applications. The usefulness of MoPSwarm is illustrated on the complex model of a signal-transduction pathway, which enables an effective computation on the parameter space for system dynamics constrained by multiple conditions. Reliability of the proposed approach is demonstrated on the validation of the model of polyamine biosynthetic sub-pathway of the trypanothione metabolic pathway.

- A methodological framework, proposed in Chapter 7, for addressing the challenges of computational modelling when the prior knowledge of the system is incomplete and the experimental data is sparse. The framework comprises a decompositional approach via an optimization-based study to the examination of structure correctness of kinetic models. This methodological framework is generic to any modelling formalism and independent of the optimization algorithms used.

- Biologically, a regulatory link between the transporter enzyme of exogenous arginine, intracellular arginase and intracellular ODC of the trypanothione metabolic pathway is hypothesized and validated *in silico*. Unknown kinetic parameters of the polyamine biosynthetic sub-pathway are estimated to be tested by *in vivo* approaches.

1.9   Outline of the Dissertation

This thesis is structured as follows. The connections between these chapters reflect the systematic development of the thesis from the initial motivation.

In Chapter 2 we give an overview of the background and details about the theory of computational modelling, with special attention given to metabolic pathways.

In Chapter 3 we provide a comprehensive compilation and description of reactions pertinent to the modelling of trypanothione metabolism. We describe the construction of a structural model of the trypanothione pathway, using the information obtained from a thorough review of the relevant literature. The model is designed based on topological information and analyzed with theoretical tools and concepts. We employ established methods of structural analysis to study topological properties and the growth capabilities of the pathway. We derive functional modules of the system that can operate at steady-state for the kinetic study in Chapter 7.

In Chapter 4 we describe the construction of the first kinetic model of the trypanothione metabolic pathway based on the kinetics of enzymes and metabolites obtained from the literature. The model is built based on the standard Michaelis-Menten law with non-linear regulation of enzyme kinetics explicitly formulated.

In Chapter 5 we summarize the state-of-the-art of Particle Swarm optimization and investigate the performance of the PSwarm implementation of the algorithm on solving the non-linear optimization problem of the complex model of a signal-transduction pathway. We conduct a scientific investigation of parameter estimation problems in various scenarios when the observation data is characterized with different levels of information content and noise. The experimental results motivate us to seek a better solution for optimization problems when system parameters are constrained by more than one state of the system.

In Chapter 6 we propose a novel approach for applying a multi-objective optimization scheme that accounts for more than one state of the system. A number of strategies critical to the multi-objective optimization are discussed via a comparative study. Satisfactory simulation results for the signal-transduction pathway were obtained using the proposed approach, demonstrating the reliability and utility of the algorithm for model validation.

In Chapter 7 we propose a methodological framework for the system identification of a poorly understood system – the trypanothione metabolic pathway, whereby the problems of structure identification and parameter estimation are simultaneously explored. The relationship between topological and functional modules observed in Chapter 3 guides the decompositional procedure and directs the search for incorrect mathematical representation in an efficient manner. The multi-objective optimization approach developed in Chapter 6 successfully solves a structurally-correct sub-system, namely the polyamine biosynthetic

sub-pathway. The methodological framework is demonstrated to be useful for tackling the challenge of system identification of poorly understood systems.

We review our results and achievements and discuss ideas for future work in Chapter 8.

1.10 Publications

We intend to submit the following aspects of this work:

1. Mathematical modelling of polyamine metabolism in *Trypanosoma brucei*. *Journal of Biological Chemistry*.

2. Multi-objective optimization algorithm MoPSwarm – a reliable approach for model validation of biochemical systems. *PLoS Computational Biology*.

Other publications during the period of this research include

1. D. Gilbert, Hendrik Fuβ, X. Gu, R. Orton, S. Robinson, V. Vyshemirsky, M. Kurth, C. S. Downes and W. Dubitzky (2006). Computational methodologies for modelling, analysis and simulation of signalling networks. *Briefings in Bioinformations* **7**(4), 339–353.

2. D. Gilbert, M. Heiner, S. Rosser, R. Fulton, X. Gu and M. Trybilo (2008). A Case Study in Model-driven Synthetic Biology. In *Biologically Inspired Cooperative Computing: BICC 2008*. IFIP Springer **268**, 163–175.

3. X. Liu, J. Jiang, O. Ajayi, X. Gu, D. Gilbert and R. Sinnott (2008). BioNessie – A Grid Enabled Biochemical Networks Simulation Environment. Studies in Health Technology and Informatics IOS Press **138**, 147–157. *Global Healthgrid: e-Science Meets Biomedical Informatics – Proceedings of HealthGrid 2008*, edited by Solomonides et al..

4. X. Gu, M. Trybilo, S. Ramasy, R. Fulton, S. Rosser and D. Gilbert (2008). Engineering a novel self-powering electrochemical biosensor. *IET Synthetic Biology* (*submitted*).

Chapter 2

Background and Related Work

In this chapter we review the theory and related research in the domain of computational modelling of biological systems. Current challenges related to the research studies are discussed.

2.1 Biological Systems

Biological systems are non-linear and complex networks, where the interaction of different pathways and dynamics of information processing within the pathway produces a multitude of biological outputs. A living organism relies on these pathways to accommodate internal or environmental changes via a variety of cellular behaviour, for instance, signal transduction, feedback regulation and communication among cells.

Biological pathways are classified into three categories. *Metabolic pathways* exist within cells, which emerge from interactions between locally-transcrib-ed proteins to perform two essential activities including the generation of energy (i.e. ATP) and relying on the energy to construct larger organic molecules (i.e. proteins and nucleic acids). *Signalling pathways* refer to the movement of signals from outside the cell to its intracellular response mechanisms through a series of phosphorylation events, which triggers specific patterns of gene expression. *Gene regulatory pathways* control a host of processes of gene expression in response to intracellular signals. Interactions between the three categories of pathways unify these processes and bring out the emergent behaviour of the whole organism.

In this section, we focus on metabolic and signal-transduction pathways in accordance with the biological application of interest. A comprehensive overview of the signalling pathways can be found in (Cho and Wolkenhauer 2003, Neves

and Iyengar 2002, Berg et al. 2002, Lauffenburger 2000, Heinrich et al. 2002). Signal-transduction pathways mediate the sensing and processing of stimuli. It follows a broadly similar course that can be viewed as a molecular circuit. These molecular circuits detect, amplify, and integrate diverse external signals to generate responses such as changes in enzyme activity, gene expression, or ion-channel activity (Berg et al. 2002).

2.1.1 Metabolic Pathway

A metabolic pathway is the collection of enzymatic processes that produce energy used by the cell and a number of other molecules (Fell 1997).

An individual metabolic pathway involves biosynthesis and biodegradation catalysed by enzymes. These two types of reactions occur in a completely opposite way: most synthetic reactions require energy and often involve the breakdown of adenosine triphosphate (ATP), whereas degradative reactions eventually generate ATP. An enzyme-substrate interaction is the elementary unit of a metabolic pathway. It is common that an enzyme can react with multiple reactants and a single reaction can be catalyzed by more than one enzyme. Catalytic activities of enzymes are primarily regulated by two processes, including conformational modification and peptide-bond cleavage.

A better understanding of the metabolism has many applications (Karp and Mavrovouniotis 1994), which range from bioprocess engineering, aiming at creation of novel metabolic processes for optimal cellular productions to health-related areas, concerned with designing potent drugs than can effectively intervene with the metabolism. Towards this end, a number of techniques have been developed and successfully applied to studying several metabolic pathways. Metabolic flux analysis is a frequently used methodology for an accurate quantification of the magnitude of pathway fluxes at a steady state participating in overall cellular functions (see Stephanopoulos 1999). Introductions to the general concepts relevant to metabolic systems are continued later in this chapter.

2.1.2 Gene Regulatory Pathway

Gene regulatory pathways, as defined by de Jong (de Jong 2002), concerns regulatory interactions between genes and gene-products. The pathway controls the process of gene expression, which occurs in two steps, namely *transcription* (from DNA to RNA) and *translation* (from RNA to protein). An operon is an impor-

tant functional unit of transcription and genetic regulation. An operon comprises a single promoter, the transcription factor binding sites that modulate the rate of transcription initiation at that promoter, the genes that are transcribed from the promoter and the transcription terminator (Karp et al. 2002).

A typical process of gene expression is as follows: an initiating signal gives rise to the activation of a protein called a transcription factor, then the factor simultaneously binds DNA and an RNA polymerase, which triggers the transcription of DNA to mRNA and translation of mRNA to proteins. Transcription rate can be modulated by two types of proteins, namely *depressors* and *activators*, which exhibit opposite functions in controlling the activity of RNA polymerase, and in consequence, the gene expression level. Regulation of gene expression is not only determined by genes *per se* but also dependent on their relative spatial location along the operon. For instance, genes that show regional similarities are likely to be expressed at the same time. The spatial arrangement of genes is therefore an useful message that can be used to elucidate temporal gene expression patterns.

Multi-level expression of genes renders gene regulatory pathways extremely complicated, which makes computational modelling of the pathways necessary. Intensive scientific research has been carried out in this area. De jong (de Jong 2002) conducted a literature survey about work done in analysis and simulation of gene regulatory pathways, including methods such as Bayesian networks, partial differential equations and rule-based models. Thieffry and Thomas (Thieffry and Thomas 1998) discussed some qualitative tools for the dynamic analysis of gene regulatory pathways, where the authors argued that logical formalisms can be an interesting alternative to differential approaches because in most cases qualitative descriptions of biological systems are often available.

Wessels et al. (Wessels et al. 2001) compared different genetic approaches (e.g. pair-wise methods) that rely on high-throughout gene expression data for the modelling of gene regulatory pathways. An up-to-date review by Crampin (Crampin 2006) focused on the issues arising in the attempt to identify regulatory pathways directly from high-throughout gene expression data measured using DNA microarray technology and quantitative PCR. Quantitative high-throughout measurement of gene expression makes large-scale gene expression analysis possible.

Overall, the complexity of biological systems lies in the multitude of sometimes subtle ways that different types of biological pathways interconnect. Therefore, in almost all cases, it is hard or impossible to construct complete mathe-

matical models – mathematical models are only approximate representations of biological systems with various degrees of accuracy.

2.2 Metabolic Network and Regulation

Metabolic pathways are an essential key to the systemic behaviour of a biological cell, as they describe a multitude of enzymatic reactions carrying out various cellular functions. An understanding of metabolic network and regulation is of practical importance to the development of new pharmaceutical approaches. *In vitro* investigation of the inhibitory potential of chemical compounds on network enzymes is the primary approach for drug target identification. This approach can be facilitated by *in silico* simulation of enzyme activities in response to external stimuli.

A reaction network is characterized by three aspects (Sauro et al. 2006), including the number of chemical species and processes, the sequence of their interactions and the rate laws governing the elementary reaction velocities. There are two families of kinetic rate laws that can be used to model the behaviour of enzyme reactions, namely *Mass Action* and *Michaelis Menten*. The following section introduces both rate laws according to the description by Cook and Cleland (Cook and Cleland 2007).

The Rate Laws of Mass Action and Michaelis Menten

The law of mass action states that the rate of a chemical reaction is proportional to the product of the substrate concentrations raised to a given power. Given a simple reversible enzymatic pathway, where substrate S reacts to yield product P by the catalysis of enzyme E, mass action kinetics model the pathway in a two-step reaction as shown below:

$$S + E \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} P + E \tag{2.1}$$
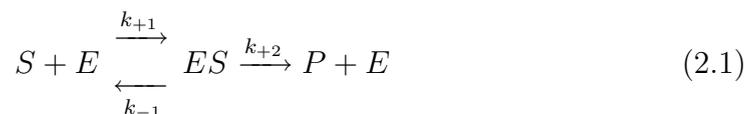
The first reaction depicted with double arrow is a reversible reaction reflecting the reversible binding and unbinding of the enzyme E and the substrate S, where k_{+1} and k_{-1} are rate constants for forward and backward processes respectively. The second reaction is an irreversible reaction in which the enzyme-substrate complex ES is irreversibly converted into product P and enzyme E with rate

constant k_{+2}. The set of differential equations for state variables E, S, ES and P is constructed as follows. During the whole process, the total enzyme concentration $[E_0]$ remains constant satisfying the formula $[E_0] = [E] + [ES]$.

$$\frac{d[E]}{dt} = -k_{+1}[S][E] + k_{-1}[SE] + k_{+2}[SE]$$

$$\frac{d[S]}{dt} = -k_{-1}[S][E] + k_{-1}[SE]$$

$$\frac{d[ES]}{dt} = k_{+1}[S][E] - k_{-1}[SE] - k_{+2}[SE]$$

$$\frac{d[P]}{dt} = k_{+2}[SE]$$

Michaelis-Menten kinetics, defined by Henri Michaelis and Maude Menten, are a commonly used and powerful rate law for modelling the enzymatic mechanisms of metabolic pathways, which quantitatively describe the effect of substrate concentration on enzyme reaction rate.

Michaelis-Menten kinetics is based on quasi-steady-state approximations. It states that after an initial fast transient, the enzymatic reaction enters a slowly changing regime where the dependent variables are assumed to be in instantaneous equilibrium (Schness and Mendoze 1997). This implies that the concentration of the intermediate complex $[ES]$ is in a quasi-steady state with regard to substrate $[S]$ and product $[P]$. The substrate concentration is assumed to be much larger than the enzyme concentration ($[E_0]/[S] << 1$), which ensures that the enzyme can be saturated with substrates. Under this assumption, the concentration of the intermediate complex remains approximately constant, i.e. $d[ES]/dt \approx 0$. With this knowledge, we can solve for $[ES]$ in terms of $[S]$ yielding

$$[ES] = \frac{[E_0][S]}{K_m + [S]} \tag{2.2}$$

where K_m is the half-saturation constant defined as the substrate concentration at which the reaction rate reaches half of its maximum value:

$$K_m = \frac{k_{-1} + k_2}{k_1} \tag{2.3}$$

Thus, we derive the differential equation of $[S]$:

$$\frac{d[S]}{dt} = -\frac{V_{max}[S]}{K_m + [S]} \tag{2.4}$$

Therefore, modelling the sample pathway with Michaelis-Menten kinetics results in a one-step reaction, as shown below:

$$S \xrightarrow{V_f[S],K_{m1}} \underset{V_b[P],K_{m2}}{\longleftarrow} P \tag{2.5}$$

This scheme results in the model given below, which contains two variables S and P and four parameters including V_f and V_b representing the maximum rate of the forward and backward reactions, and K_{m1} and K_{m2} representing the half-saturation constants:

$$\frac{d[P]}{dt} = \frac{V_f[S]}{K_{m1}+[S]} - \frac{V_b[P]}{K_{m2}+[P]}$$

$$\frac{d[S]}{dt} = \frac{V_b[P]}{K_{m2}+[P]} - \frac{V_f[S]}{K_{m1}+[S]} \tag{2.6}$$

Non-linearity of enzymatic reactions is often characterized with multi-activation mechanisms where multiple substrates may bind to an enzyme to moderate its activity. A generic rate equation of the enzyme catalyzing a two-substrate reaction (S_1 and S_2) is expressed as follow

$$V_{enzyme} = V_{max} \cdot \frac{[S_1]}{K_{m1}+[S_1]} \cdot \frac{[S_2]}{K_{m2}+[S_2]} \tag{2.7}$$

Overall, Michaelis-Menten kinetics and mass action kinetics are fundamentally equivalent as the former can be derived from the latter. Mass action kinetics allow the description of pathway dynamics to the level of detail of concentration of enzyme-substrate complex ($[ES]$). However, as we are only interested in the dynamics of substrates ($[S]$) and products ($[P]$) of the reactions, Michaelis-Menten kinetics are a preferable scheme since fewer differential equations need to be formulated and are therefore applied in this thesis.

Metabolic Regulation

Tyson et al. (Tyson et al. 2003) explored the physiological responses of cells to external and internal stimuli, which are governed by genes and proteins interacting in complex signalling pathways. Diverse types of responses can be created by embedding these signal-response elements in pathways.

Cell dynamics and behaviour are characterised by *hyperbolic responses, sig-*

moidal responses (ultrasensitive response) and hysteresis (Sontag 2005). Sigmoidal responses are characteristics of many signalling cascades, which display the so-called ultrasensitive response to inputs. Sigmoidal responses can be modelled using the Hill function, where the Hill coefficient measures the degree of cooperativity between subunits that bind the ligand in multi-subunit proteins. Hysteresis describes the phenomenon in which the actual steady state depends on the history of the system. One of the main roles of such hysteric behaviour is in producing oscillations (Blasius et al. 1998). Feedback loops are recognized as an important mechanism in the regulation and control of biological functions, which can be treated as modifiers to stabilize, destabilize, sensitize or de-sensitize the dynamic behaviour of a process (Wolkenhauer et al. 2004).

The stimulus-response representation of a molecular system is necessary to understand the dynamic interactions among the components that constitute a pathway (Wolkenhauer et al. 2004). The fundamental building blocks of metabolic pathways are enzymatic reactions, which characterize a diverse range of enzyme-substrate interactions that fulfill identifiable metabolic functions. Based on years of research, mathematical formulations describing these non-linearities have been developed.

Figure 2.1 presents three typical shapes of the dependence of enzymatic velocity on substrate concentration, including the standard Michaelis-Menten mechanism, substrate inhibition and the Hill function.

Conceptually, any substrate that causes a decrease in the production state of product as its concentration increases will lead to a reaction that displays substrate inhibition kinetics (shown in Figure 2.1(b)). The Hill function corresponds to a particular biological phenomena – cooperative binding. In cooperative binding, the binding of the substrate at one site (of an enzyme with multiple active sites) increases or decreases the affinity for the substrate at other sites. This is defined as *positive cooperativity* and *negative cooperatively*, respectively. As shown in Figure 2.1(c), when the Hill coefficient n gets higher, a steeper non-linearity is produced. In the cases of substrate inhibition and Hill equation, values assigned for n are for illustrative purposes only.

Rate expressions corresponding to the curves are given in Equations (2.8) to (2.10). From these components, metabolic pathways can produce regulatory dynamics of great complexity. Many enzymes in metabolic pathways are subject to feedback regulations, where an end-product activates or inhibits the enzyme activity by binding to a separate site. A variety of feedback loops can further
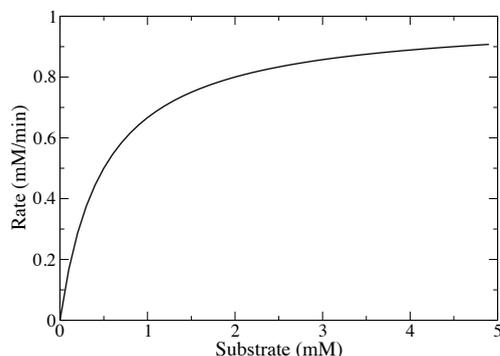
(a) Michaelis-Menten mechanism
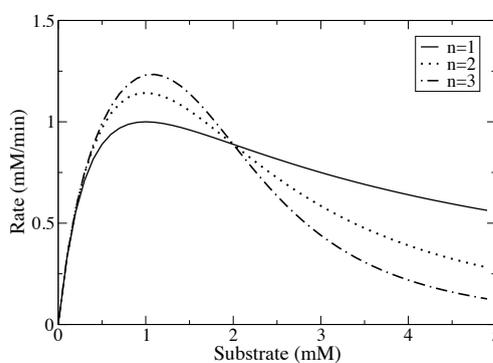
(b) Substrate Inhibition
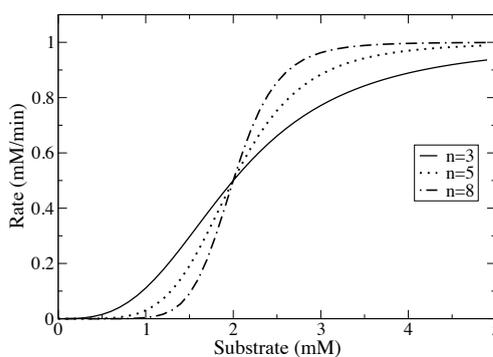
(c) Hill Equation

Figure 2.1: Three typical shapes of the dependence of the enzyme velocity on the substrate concentration (in the range 0 to 5 mM) are shown schematically. (a): Michaelis-Menten mechanism. (b): Michaelis-Menten mechanism plus substrate inhibition with $n = 1$ (*continuous line*), $n = 2$ (*dotted line*), and $n = 3$ (*segment and dotted line*). (c): Hill equation with $n = 3$ (*continuous line*), $n = 5$ (*dotted line*), and $n = 8$ (*segment and dotted line*). The three rate expressions are given in Equations (2.8) to (2.10).

complicate the regulation and control of metabolic pathways.

(a) Michaelis-Menten kinetics (2.8)

$$\text{Rate} = \frac{V_{max} \cdot \dfrac{[S]}{K_m}}{1 + \dfrac{[S]}{K_m}}, \quad V_{max} = 1, \ K_m = 0.5$$

(b) Substrate Inhibition (2.9)

$$\text{Rate} = \frac{V_{max} \cdot \dfrac{[S]}{K_m}}{1 + \dfrac{K_m}{[S]} + (\dfrac{[S]}{K_{si}})^n}, \quad V_{max} = 2, \ K_m = 0.5, \ K_{si} = 2, \ n = 1, \ 2, \ 3$$

(c) Hill Equation (2.10)

$$\text{Rate} = \frac{V_{max} \cdot (\dfrac{[S]}{K_m})^n}{1 + (\dfrac{[S]}{K_m})^n}, \quad V_{max} = 1, \ K_m = 2, \ n = 3, \ 5, \ 8$$

2.3 Mathematical Modelling Formalism

Modelling is an advanced technique for the study of dynamic interactions between cellular components. Complexity of biological systems makes it necessary to take advantage of formalisms to model and study the systems, for gaining both better comprehension and experimentally testable predictions. A rapid accumulation of data on biological molecules enables the development of mathematical models to represent and analyze biological systems.

Construction of mathematical models involves quantitative considerations of interaction characteristics of biological systems as well as a multitude of information regarding the concentrations and rates of the system. Mathematical models can be classified into a variety of types according to different criteria, some of which are described below:

- *Continuous versus discrete*: Continuous models are used to model continuous systems, where variables (i.e. concentration of chemical species) undergo smooth changes, whilst discrete models are used to model discrete

systems where changes in variables occur in discrete steps (i.e. number of molecules or a countable number of points in time).

- *Deterministic versus stochastic*: Given a fixed set of initial conditions, a deterministic model always produces the same output, whilst a stochastic model takes randomness or probability distributions into account such that for a given input, the outcome of the model is not uniquely determined but takes a range of possible values.

- *Static versus dynamic*: Models are said to be dynamic if their behaviour varies with time, and time therefore enters as an independent variable (i.e. differential equation). Models are static if their behaviour is constant and does not vary with time (i.e. mass-balance equations).

- *Quantitative versus qualitative*: Quantitative models are designed to study time dependent behaviour, whereas qualitative models are mainly used to identify high-level properties, such as structure and global functions of biological systems. Qualitative models may contain quantitative variables, which are however used for qualitative rather than numerical reasoning about relationships between system components.

In this thesis, we focus on continuous deterministic mathematical models by means of ordinary differential equations (ODE), which are a widespread formalism to model dynamic systems in science and technology. The ODE formalism models time-dependent behaviour of system variables with respect to the independent variable. In the context of biology, the independent variable is usually time and dependent variables (so-called state variables) are measurable quantities (i.e. concentrations of biological entities), which have non-negative values. Models defined with ODEs have been employed to quantitatively analyze metabolic pathways, for example, the glycolysis pathway in bloodstream-form *Trypanosoma brucei* (Bakker 1998), the polyamine metabolic pathway in mammalian cells (Rodriguez-Caso et al. 2006) and the glutathione metabolic pathway in liver cells (Reed et al. 2008). ODE-based modelling and simulation has also been performed on the ERK (Extracellular Signal Regulated Kinase) cascade of the MAPK (Mitogen-Activated Protein Kinase) pathway, which transduce a variety of external signals to generate a wide range of cellular responses (Orton et al. 2005).

In this section, we give a detailed explanation of ODE-based modelling techniques. However, an alternative technique of stochastic modelling is also outlined for the sake of comparison. Generally speaking, stochastic approaches are used to model components that are present in small molecule numbers (i.e. promoters and transcription factors), whilst deterministic approaches are suitable when components are present in high concentrations (i.e. proteins).

2.3.1 Ordinary Differential Equations

Dynamic cellular processes are frequently described using sets of differential equations. Of all types of differential equations, ODEs are the most commonly used technique to describe and explore dynamics of a specified natural system, in particular, in the modelling and analysis of biological systems.

Equations relating an unknown function and one or more of its derivatives is called a differential equation (Ascher et al. 1995). Studying system behaviour by means of differential equations comprises the following steps:

- to formulate the differential equation that can describe a specific system

- to find an appropriate solution of that equation

- to understand the system by interpreting the solution

Construction of an ODE model of metabolic pathways is illustrated by an example. Consider the simple metabolic pathway:

$$S_s \xrightarrow{v_1} S_i \xrightarrow{v_2} S_p \tag{2.11}$$

where S_i is the intermediate component of the pathway, concentrations of which can vary depending on enzyme activities and concentration of substrate (S_s) and product (S_p). Intracellular chemical reactions are catalyzed by enzymes E_{s_1} and E_{s_2} at rates v_1 and v_2 respectively. The differential equation of the state variable S_i is expressed as the difference of incoming and outgoing rate velocities governed by a specific kinetic law (i.e. Michaelis-Menten kinetics)

$$\frac{d[S_i]}{dt} = v_1 - v_2 \tag{2.12}$$

Due to the non-linear nature, computational calculation is required to integrate differential equations in order for essential information underlying the

model structure to be properly interpreted. General methods of numerical integration include the Euler method and Runge-kutta Method; relevant materials are available elsewhere (see Press et al. 2002).

A large number of software tools have been developed for the simulation of dynamic models specified in terms of ODEs. The software tools differ in two aspects, including the underlying techniques applied and capabilities supported. Matlab is a general-purpose mathematical environment that is widely used in the physical and engineering sciences. A major benefit of the Matlab environment is the comprehensive library of mathematical and graphical functions, enabling convenient visualization, analysis and optimization of biological models. A comprehensive review on modelling tools and resources is given by Gilbert et al. (Gilbert et al. 2006).

2.3.2 Stochastic Master Equations

The number of molecules participating in elementary reactions can vary by orders of magnitude (Shampine et al. 2000). ODEs are appropriate to model systems with a large number of molecules involved, in which case the reaction probability can be assumed to be independent of the details of collisions between molecules. When modelling biological systems that contain only a few molecules, the discrete nature of the number of molecules cannot be ignored. It thus may be useful to develop models that are both discrete and stochastic in order to accurately describe the random occurrence of molecule collisions. One of the frequently used models is stochastic master equations, which has the potential to describe a wide range of phenomena.

The stochastic formalism decompose biological pathways into elementary reactions with probabilities are variables to describe the state of the system. A joint probability distribution, $P(X_1, \ldots, X_N, t)$, is determined by the probability of individual molecular species i having X_i number of molecules. Suppose that there are M different reactions in the system, the change over time of the probability distribution is expressed in the following equation, based on the descriptions given by Baldi and Hatfield (Baldi and Hatfield 2002):

$$P(X_i, \ldots, X_N, t + \Delta t) = P(X_i, \ldots, X_N, t) \left(1 - \sum_{j=1}^{M} \alpha_j \Delta t \right) + \sum_{j=1}^{M} \beta_j \Delta t \quad (2.13)$$

where $\alpha_j \Delta t$ is the probability that reaction j will occur in the interval Δt given

the system in the state $X_1, \ldots, X_N$ at time t and $\beta_j \Delta t$ is the probability that reaction j will bring the system to the state $X_1, \ldots, X_N$ in the interval Δt. Taking the limit as $\Delta t \to 0$ gives the *master equation*, which describes the probability distribution of all possible states at all times, defined as follows:

$$\frac{\partial P}{\partial t} = \sum_{j=1}^{M} (\beta_j - \alpha_j P) \tag{2.14}$$

Using analytical methods to analyze the master equations would be extremely difficult, given the amount of information required for the stochastic formalism (i.e. the number of chemical species involved and the number of molecules each species contains). Stochastic simulation techniques, for instance, Gillespie's method (Gillespie 1977), are required to solve the equations.

Overall, ODE models and stochastic models are concentrated on different objectives when studying dynamic systems. As stated by de jong (de Jong 2002), ODEs determine the transient changes of system variables with respect to time, whilst master equations examine the probability that a shift in the state of the system occurs.

2.3.3   Computational Simulation

Computational simulation is an essential tool for the study of complex systems, where a purely empirical approach is infeasible. The type of simulation applied should correspond to the type of mathematical model to be solved. In stochastic simulations, concentrations (in terms of numbers of molecules) are treated as random variables. In contrast, ODE-based approaches are deterministic and concentrations are obtained by solving the differential equations (Ullah et al. 2006). By means of computational simulation, an intuitive impression of how these equations relate to dynamic responses can be gained. With valid models, simulation results are able to make predictions about possible behaviour of biological systems (Endy and Brent 2001).

There can be many circumstances where computational simulation is necessary. Neelamkavil (Neelamkavil 1987) stated that simulation is necessary when there is a need to study the past, present, or future behaviour of the system in real time, such as side-effects of new drugs. Computational simulation enables us to investigate the sensitivity of biological systems to parameter changes such as environmental noise and minor mutations (Liu et al. 2008). Kitano

(Kitano 2002a) highlighted that noise tolerance is exhibited by several biological systems that feature oscillatory behaviour and transcriptional regulations, however the degree of tolerance can only be quantitatively evaluated using computational simulations. For example, the robustness of segmentation during *Drosophila* embryogenesis in response to various kinetic parameters has been investigated via computational simulation (von Dassow et al. 2000).

Exploration of the time-dependent behaviour of biological processes is one of the most frequent applications of dynamic models (Wiechert 2002). Huang and Ferrell (Huang and Jr. 1996) developed differential equation models of MAPK signalling pathway to study the role of negative feedback mechanisms in governing ultrasensitivity in the pathway. Mechanisms regulating MAPK pathway dynamics were further examined by Asthagiri and Lauffenburger (Asthagiri and Lauffenburger 2001), where the authors implemented feedback mechanisms in a mathematical model of the pathway and demonstrated that with negative feedback regulations, the model yielded a complete signal adaptation to its direct target. Cho et al. (Cho et al. 2003) developed an ODE model to investigate the influence of RKIP (the Raf Kinase Inhibitor Protein) on ERK signalling pathway. Refer to (Hoppensteadt and Peskin 2002) for more interesting applications.

Mathematical models are a valuable tool for organising data and the examination of complex biological interactions (Bailey 1998). In the context of biology, mathematical models integrate biological data at various levels (including transcriptome, proteome and metabolome) and provide an unambiguous description of biological systems. A mismatch between simulation results and experimental observations can indicate deficiencies in the model, suggest modifications to the model and guide new biological experiments. For example, as found by Orton et al. (Orton et al. 2005), computational simulation of the Schoeberl MAPK model (published by Schoeberl et al. (Schoeberl et al. 2002)) revealed that the negative feedback loop from ERK-PP to SOS is necessary. Developing sound models with predictive power is central to the domain of computational modelling.

After a valid model is constructed, formal analysis can be carried out to study interesting properties of the system. Model analysis comes into various forms with different objectives. It can be used to understand system structure or to investigate system dynamics. In reality, analysis of dynamics and structure are overlapping processes and are beneficial to each other (Kitano 2002b).

Application of analytical methods depends on the amount and type of biological knowledge incorporated in the model (Kitano 2002b). For example, static

models that are based on graph theory are completely adequate for steady-state analysis, since only the network topology is required. However, static models are incompatible with simulation-enabled analyses (e.g. parameter sensitivity analysis), where a dynamic descriptions of the system is necessary.

2.4 System Identification

System identification is defined by Ljung (Ljung 2008) as "the art and science of building mathematical models of systems from observed input-output data". Two broad branches of system identification are *Structure Identification* and *Parameter Estimation* (Söderström and Stoica 1989). Structure identification is concerned with finding a suitable model structure, within which a good model is to be decided upon, and parameter estimation deals with, given a structure and a set of experimental data, how the model can be fitted to observation data in the best possible way. In this section, we focus on the subject of structure identification. Computational issues associated with global optimization in the context of single-objective optimization are studied in detail in Chapter 5. A multi-objective optimization approach for parameter estimation is investigated in Chapter 6.

According to different levels of initial knowledge about the system and the purpose of modelling, mathematical model representations can be classified into three general categories, which are described in generic terms below and as summarized in the reviews (Ljung 2007, Sontag 2006, Sjöberg et al. 1995).

1. *White Box model*: This is the case when physical insight about the system is complete and all the conditions are known quantitatively, indicating that it is possible to construct the model entirely from prior knowledge.

2. *Black Box model*: This is the case when no physical insight is available or used. A black-box model is a parametrized description of the process, and all parameters are estimated from measurements performed on the process without taking into account any prior knowledge about the process.

3. *Grey Box model*: This is the case when physical insight about the system is incomplete and unknown quantitative conditions have to be determined from observed data. According to the type and level of information available for the task, grey-box models can be further divided into the following sub-classes:

- A mechanistic description of the system can be built on existing physical knowledge about the system (i.e. rate laws and interconnection relationships). When the model structure is defined, the identification process is then to estimate the parameters from observed data, which are unknown physical constants in the description.

- Mechanistic descriptions can be only obtained for some elementary parts of the system. To construct an entire model, black-box structures representing non-linear transformations between system components are necessary to approximate the ambiguously defined or unknown dynamics.

The idea of designing white-box models (so-termed mechanistic models) is to base the model construction on physical cellular functions, thus the results of model simulation and prediction can be directly applied to actual biological systems. The development of white-box models in order to understand the systems of life is the fundamental question addressed by computational systems biology (Kitano 2002a). A detailed description of biological systems allows the performance of simulated experiments to predict unobserved properties. However, a major weakness of this approach is the empirical nature and the essential relations are often difficult to extract (Heinrich et al. 1977). Constructing a white-box model for complex dynamic systems is an extremely difficult task as in most cases the knowledge about the system is at best partial.

System identification is concerned with the development and analysis of methods for performing black-box modelling (Söderström and Stoica 1989). The black-box model representations appear to be more beneficial in modelling biological applications that are poorly defined. Black-box models are relatively easy to construct and use (Söderström and Stoica 1989), and can be approximated in the form of, for example, power series polynomials, fuzzy logic or neural networks. A difficult problem in system identification is to find an appropriate mathematical form, in particular when the dynamics of the system are non-linear.

Basically, black-box modelling looks for a relationship between past observations and future outputs. The area of black-box modelling is very diverse, and covers topics from mathematical approximation theory; specifically, estimation theory, regression and classification methods (Sjöberg et al. 1995).

Sontag et al. (Sontag et al. 2004) attempted to determine reaction mecha-

nisms of biological systems from the measurements of time-course species concentrations. The authors developed quantitative techniques to identify causal relationships between species by monitoring time-dependent cellular responses to perturbations. The basic idea is to analyze the direct effect of a small change in one network node on the activity of another node, while keeping all remaining nodes fixed. The network responses to perturbations are estimated using the partial derivatives that quantify the influence of each variable upon the rate of change of other variables. The big advantage of this approach is that it is also applicable to the situations where some network nodes cannot be perturbed; this limit is tackled by applying more independent perturbations to other nodes to which perturbations are allowed. Thus, an increase in the node connections does not necessary change the number of required perturbation experiments and calculations.

Gardner et al. (Gardner et al. 2003) applied multiple linear regressions to the system identification of a nine-gene sub-network of the SOS pathway in *Escherichia coli*. Regulatory coefficients for each pairs of genes were determined, whereby the interconnection map and the key regulatory functions between gene products in the network were identified. In the work by Gardner et al., no prior knowledge was claimed to be necessary, but still 36 combinations of seven perturbations was used. Geier et al. (Geier et al. 2007) addressed the problem of model construction of gene-regulatory networks from knockout data. The authors compared the performance of different network reconstruction methods and investigated the impact of data size and observation noise on the construction of gene regulatory networks. They found that error rates during the construction process increased with an elevation in the noise level and a decrease in the data size.

Moreover, Crampin et al. (Crampin, McSharry and Schnell 2004) and Srividhya et al. (Srividhya et al. 2007) considered the problem of inferring kinetic mechanisms for chemical reactions from time series data using polynomial models of chemical reactions based on mass action kinetics. Kocijan et al. (Kocijan et al. 2003) also took advantage of the black-box identification approach formulated as a Gaussian process model to study the model-based predictive control problem. Refer to the works (Lauwers et al. 2007, Liu and Wang 2008, Kemna and Mellichamp 1995) for more examples of the system identification of biological systems.

The biggest disadvantage of model construction via black-box modelling is

the implicit relationship with physical reality (Heinrich et al. 1977). This is to say that any arbitrary black-box model can be made to fit experimental observations, however this does not necessarily mean that they represent a physically correct model of the system. Söderström and Stoica (Söderström and Stoica 1989) pointed out that models obtained by system identification in some cases have limited validity (for example, they are valid only for a certain working point) and parameters included are used only as tools to give a good description of the system's behaviour. The amount of available experimental data and the non-linearity of biological systems are the deciding factors when selecting an appropriate model approximation. An overview of system identification techniques for model construction from time series data is given by Crampin et al. (Crampin, Schnell and McSharry 2004).

In contrast, grey-box modelling can relieve some of the drawbacks arising from black-box modelling and still maintain dynamical flexibility (Tan and Li 2002, Oussar and Dreyfus 2001). We consider grey-box models as a combination of white-box and black-box models. Tulleken (Tulleken 1993) argued that grey-box modelling can benefit the advanced control design, which requires a model that can adequately describe the mechanisms of the underlying process. Tulleken proposed an approach to solve the problem of statistical estimation where a linearly parametrized dynamic regression model was used. Tan and Li (Tan and Li 2002) employed Padé approximation in the form of a regressive function as the black-box structure to solve two non-linear chemical processes. Tullenken (Tulleken 1991) applied this type of modelling to the model construction of a batch reactor system and indicated that the grey-box strategy could improve the statistical identification results considerably.

As highlighted by Heinrich et al. (Heinrich et al. 1977), there are compromises among the three modelling representations in the use of models that are as simple as possible, yet are still consistent with the real system. Simplification of a model can be achieved through the reduction of the number of variables or parameters. One useful model reduction technique is based on topological reduction of metabolic pathways, through which adjacent reactions may be lumped into a single overall reaction (Heinrich et al. 1977). This technique is applied in the structural modelling of the trypanothione metabolism in Chapter 3.

In summary, a sound metabolic model is necessary for achieving a global understanding of the capabilities of metabolic processes. However, no automatic reconstruction methods can be directly used to discover currently un-

known metabolic reactions (Breitling et al. 2008). Breitling et al. summarized a number of technological advances that could allow *de novo* reconstruction of large unexplored parts of the metabolic map directly from experimental observations. Breitling et al. (Breitling et al. 2008) highlighted a high-accuracy mass-spectrometry approach, which has been applied to the study of *T. brucei*. The authors argued that this approach can accurately predict the potential connectivity between related metabolites, thus enables a precise *de novo* reconstruction of an entire hypothetical metabolic map. Recently developed software by Jourdan et al. (Jourdan et al. 2008) enables visualization of mass-spectrometry data obtained from this method and allows basic analysis of the resulting network.

In many instances, sufficient physical knowledge and quantitative information for constructing a mechanistic mathematical model of the entire system is difficult or impossible to obtain. We take advantage of the concept of grey-box models in the kinetic modelling of a sub-pathway of the trypanothione metabolism (the polyamine biosynthetic sub-pathway), as illustrated in Chapter 7. Within the grey-box model, a white-box representation is applied to describe known physical properties of the system and a black-box representation is used to approximate the unknown dynamics.

2.5 Network Modularity

Cells can be seen as composed of a large number of self-contained reaction subsystems, so-called 'modules', which carry out specific biological processes. Cell behaviour thus can be understood in terms of 'modules' interacting via cascades and feedback regulations in a complex web, which is defined by Sontag et al. (Sontag 2006) as one of the important themes in current molecular biology. Sub-systems can be thought of as black boxes that process time-dependent input signals (i.e. forcing functions) or external signals (i.e. stimuli) into output responses (i.e. measurements provided by biological reporter devices). Model decomposition techniques facilitate the study of emergent properties of interconnections, thus benefiting the model construction of large-scale biological systems on modular bases (Kholodenko et al. 2002).

From the perspective of molecular biology, a module can be considered as a group of biological components that are spatially isolated and/or functionally independent, and classified as topological and functional modules, correspondingly. From a purely topological perspective, hierarchical modularity is evident

in metabolic networks in all organisms (Ravasz et al. 2002). Topology-based modules are comparatively straightforward to derive. Since they are based on graph theory, minimal prior knowledge about biological function or evolution is required.

Centrality is a core concept for the study of topological modules. Edge betweenness is the most prominent measure of centrality, which determines the relative importance of an edge within a graph, and therefore the density of sub-networks of a complex network system (Girvan and Newman 2001). The measure of edge betweenness favors edges lying between sub-networks and disfavors edges lying inside sub-networks. There are various implementations of this measure. One of the standard variants is *shortest-path betweenness*, which finds the shortest paths between all pairs of vertices and counts how many run along each edge (Newman 2001). Zhao et al. (Zhao et al. 2007) applied this decompositional approach to examine the co-evolution of topological modules of metabolic networks in *H. sapiens*, where they used the simulated annealing algorithm proposed in (Guimera and Amaral 2005) to develop the modules. In spite of the prevalence of topological modularity in networks, network modularization should rely on functional characterization of biological components (Sauro et al. 2006). Hartwell et al. (Hartwell et al. 1999) argued that the identification of functional modules is a crucial level of abstraction in the modelling of biological systems. However, how to functionally modularize a network is the key problem.

Ravasz et al. (Ravasz et al. 2002) proposed a decompositional approach for the classification of topological modules, where clustering coefficients among substances are calculated to determine the number and degree of clustering. The coefficients reflect the The authors attempted to establish a relation between topological modules and the known functional properties of the metabolites, and pointed out that biological verification of the relation remains to be the critical issue. Further experiments and theoretical analyses are needed in order to understand the potential relation between the identified hierarchical architecture and functionally relevant sub-networks.

The concept of modularity has been applied to solve the parameter estimation problem of biological systems, by which the complexity of the problem can be largely reduced. Van Riel and Sontag (van Riel and Sontag 2006) developed a 'dependent-input' approach for the identification of unknown system parameters. This approach allows a part of the system to be studied independent of the rest

of the cell by replacing the un-modelled dynamics (of the rest of the system) with the measured dependent inputs. A critical challenge with regard to the use of the 'dependent-input' approach lies in how to represent the modules to render the predictive power, since the ultimate goal is not only to examine the underlying assumptions but also to make predictions to be tested experimentally.

Bentele et al. (Bentele et al. 2004) took advantage of network modularity for parameter estimation in the apoptotic signalling network. Sensitivity analysis that describes changes of molecule concentrations as result of changes in the values of parameters (so-called local sensitivity analysis) is applied to identify clusters that contain a subset of molecules whose concentrations depend on a subset of parameters only. The parameter estimation problem was solved via a two-level hierarchy, where at the upper level, global parameters that belong to more than one cluster are estimated and at the lower level, remaining local parameters belonging to individual subsets are estimated separately from each cluster. At the lower level, estimation of the local parameters is dependent on the values of the global parameters solved from the upper level. However, this approach may be computationally expensive, as sensitivity analysis has to be performed and evaluated within the parameter estimation space after each iteration step.

In summary, network modularity provides a convenient way to examine the relationship between topological and functional building blocks that accomplish specific cellular functions, which assists in the analysis of system behaviour and exploration of unknown systemic properties. Within a 'modular' framework, the challenge of understanding the complex network of molecular interactions can be facilitated (Lauffenburger 2000). We take advantage of the concept of network modularity in the modelling of the trypanothione metabolic pathway. In Chapter 7, we propose an optimization-based decompositional approach that is different from the previously discussed approaches to guide the investigation of model structure correctness of the pathway.

2.6   Discussion

The study of biological pathways is becoming the reference framework for understanding the dynamics of many intercellular and intracellular processes. As most pathways of interest involve components connected through interlocking loops, an intuitive understanding of their dynamics is hard to obtain. It is therefore

necessary to apply formal methods to modelling and analysis of these pathways.

There are many different types of models that can be used to study biological systems, and hence question of which model should be selected might arise. The type of model is determined by the purpose of model building, for example, whether it is to obtain an in-depth understanding, to make predictions about novel behaviour, or to suggest design principles for biological experiments. Even though dynamic models are widely adopted to quantitatively study biological systems, the merits of (graph-based) static models should not be overlooked. Static models are suitable for studying topological properties and time-invariant behaviour of biological systems, which can help concentrate on the essential part of the system even without dynamic simulation. Conclusions drawn from static models can be both qualitative and quantitative such as the description of system outputs in response to environmental or genetic modifications.

In this thesis, we focus on deterministic continuous models of chemical reactions to study the dynamic properties of the trypanothione metabolic pathway. There are at least three possible arguments for the use of stochastic models for chemical reactions (Erdi and Toth 1988), which can take the inherently random phenomena of processes into account, however stochastic models are not appropriate for representing the biological system of interest, as the chemical reactions are assumed to not occur randomly and a set of inputs of the system will result in an unique set of outputs. Moreover, for many classes of phenomena the stochastic model is only slightly 'better' than the deterministic approach, while the mathematics of the stochastic model is considerably more complicated (Erdi and Toth 1988). There is thus little reason to complicate the problem for only a small gain in accuracy. ODEs, which are well known for their unambiguous mathematical expression and compatibility with quantitative analysis, are applied to model the metabolic processes of interest and to address the importance of deterministic kinetics.

Chapter 3

Structural Modelling of Trypanothione Metabolism in Bloodstream-form *Trypanosoma brucei*

In this chapter we investigate how the structural modelling approach can benefit the understanding of important physiological characteristics of the trypanothione metabolic pathway in the absence of detailed kinetic information and rational identification of potential drug targets. We discuss the usefulness of structural modelling and explore the steady-state properties and growth capabilities of the pathway. The system properties such as network robustness and flux control coefficients are also studied and interpreted biologically.

3.1 Overview

Metabolic models are used to capture and reproduce the essential behaviour of the functions of metabolic systems. One commonly-used approach to studying complex systems is dynamical simulation. A complete description of dynamic properties requires the knowledge of intricate regulatory features. However, the regulatory information required to establish such detailed kinetic models are rarely available (Stelling et al. 2002).

Knowledge about the trypanothione metabolic pathway is currently restricted to a qualitative view and the network topology (also termed as structural topol-

ogy and used interchangeably) is better understood than its numerical aspects. The structural modelling approach, based on the network stoichiometry and reversibility of reactions, enables us to study the metabolic pathway in the absence of kinetic information. Network stoichiometry is related to the proportions of reactants and products that take part in a reaction. This approach is conducted through analyzing the stoichiometric matrix with established techniques that compute a set of routes satisfying certain conditions.

In this chapter, we formulate a model of the trypanothione metabolic pathway represented as a stoichiometric matrix, and perform structural analysis of this model, to gain insights into the critical properties of the pathway. We first give some background on the model of *T. brucei* and anti-trypanosomal drug development therein. We then investigate the trypanothione metabolism from a pathway perspective, which illustrates how the conservation constraints and enzyme subsets can be deduced through a rigorous analysis of the stoichiometric matrix. We also present a computational investigation into the detection of network gaps and study system robustness with respect to different structural topologies to quantitatively measure the network flexibility correspondingly.

Next, we demonstrate the usefulness of *Metabolic Flux Analysis* for the estimation of steady-state flux distributions with respect to specific optimization criteria. Essentiality of gene products in the pathway for supporting given objective functions are assessed when individual reactions are knocked out or environmental perturbations are applied. Essential enzymes identified are reckoned to have considerable potential for chemotherapy. The advantages and limitations of the structural modelling in general, and the issues specific to modelling the trypanothione metabolic pathway are summarized in Section 3.6.

3.2 Biological Background

A schematic representation of the trypanothione metabolic pathway in *T. brucei* is depicted in Figure 3.1, which is formulated based on biological information available from the literature and in collaboration with biologists studying *T. brucei*. To the best of our knowledge, this schematic representation takes into account all known elementary interconnections of the system as observed in biological experiments. We also incorporated biological information derived from the counterparts in other parasitic organisms (i.e. *Leishmania* and *T. cruzi*) into the model description. The chemical reactions that have been observed in other
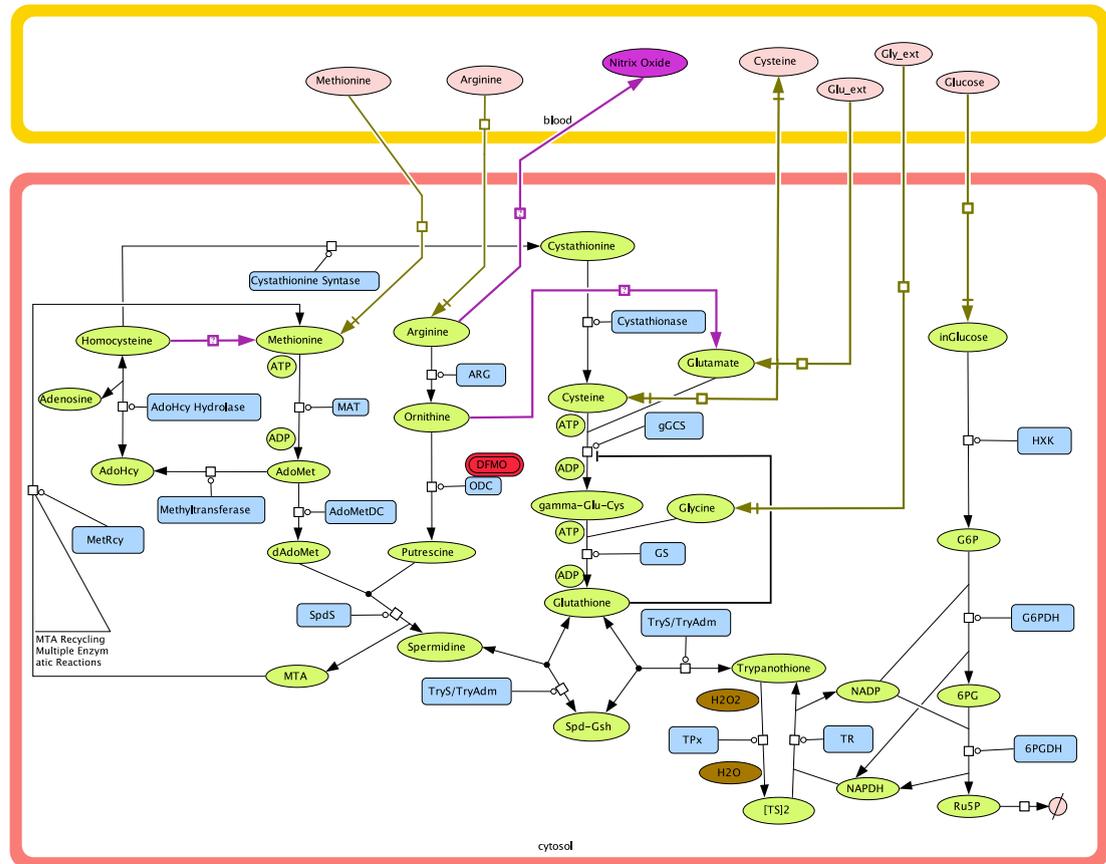
Figure 3.1: A complete schematic representation of the trypanothione metabolism in *T. brucei*. In the cytosolic compartment, variable metabolites with time-dependent concentrations are nodes in green and constant metabolites with fixed concentrations at their physiological levels are nodes in brown. Boxes in blue are the enzymes catalyzing each elementary step in the pathway, where the drug 'DFMO' is highlighted besides the enzyme it inhibits. In the blood compartment, external metabolites are nodes in pink, and the unconfirmed component is the node in purple. The concentrations of both constant and external metabolites are fixed at their physiological levels. Edges with one-way arrows denote irreversible reaction steps, indicating directions of net reaction fluxes, and those with two-way arrows denote reversible steps. Edges in purple, assigned with question marks represent the presence of reactions that have not been evidenced in *T. brucei*. A comprehensive description of the enzymes contained in this figure is presented in Chapter 4.

parasites but not in *T. brucei* are indicated with a question mark in the diagram.

Extensive research into trypanosomes over the past two decades has been devoted to target identification for chemotherapy of trypanosomal infections. Selective toxicity is the main principle of drug action when killing parasites,

that is, drugs must be toxic to the parasite than to the host (Bakker 1998). Enzymes and metabolites unique to parasites are good candidate targets for anti-parasitic drug development (Barrett et al. 2003, Krauth-Siegel et al. 2005).

In spite of knowledge about structure and biosynthetic routes for some inhibitory compounds, drugs that are suitable for clinical trials are still difficult to obtain (Muller et al. 2003). Any new drug that can be developed and taken into the phase of clinical application has to be examined via a rigorous drug validation process. This involves testing the drug efficacy with a large number of substances and long-term collaborations between research institutes and drug development companies (Keiser et al. 2001).

The lack of success in anti-parasitic drug design can be attributed to several factors, for example, resistance to drug efficacy. Drug resistance refers to the ability of organisms to withstand a drug to which they are normally vulnerable. Considerable research has been performed towards the understanding of DFMO resistance in a number of cell types and microorganisms, including Ehrlich ascites cells (Alhonen-Hongisto et al. 1980), *L. infantum* (Fouce et al. 1991, Basselin et al. 1996) and procyclic-form *T. brucei* (Phillips et al. 1987). Another obstacle in anti-parasitic drug design is that cells have developed a range of self-repairing systems that could counteract the inhibitory effect. Thus, to enhance the *in vivo* drug efficacy, anti-trypanosomal therapy should aim at more than one chemotherapeutic target. In this way, an adequate depletion of the metabolites pertinent to cell proliferation is likely to be achieved.

3.3   Metabolic Balancing

The steady-state properties of metabolic fluxes are important for understanding the regulation of metabolism. In metabolism, the basic components are metabolites and reactions. Metabolites are converted by biochemical reactions; or they are transported between compartments or exchanged with the environment.

The degree to which pathways participate in various cellular and metabolic process can be quantified using metabolic fluxes, which measure the reaction rate of the pathway. It is thus of considerable importance as a metric in metabolic engineering. Metabolic Flux Analysis is a methodology that allows flux values to be determined from intracellular reactions via a stoichiometric model and the incorporation of appropriate mass balances (Stephanopoulos et al. 1998).

In this section, we first give an introduction to the theory of mass balancing,

and then present our work on the construction of mass-balance model for the trypanothione metabolic pathway and the associated structural analysis applied.

3.3.1 Theory

The starting point for structural analysis is the reaction network stoichiometry, which indicates how substrates are converted to metabolic products and biomass constituents. To enable the development of a mass-balance model (also termed as structural model in this thesis), definitions of all the metabolites and reactions of the network are required.

Biological systems are often open systems interacting with the environment through the exchange of mass and/or energy. The external sources with which the systems exchange fluxes are normally set as constants. Internal rate constants in our analysis refer to the net rate of enzymatic reactions within the system. The term 'net rate' is mainly designated for reversible reactions, which denotes the difference between the rates of forward and backward reactions.

A metabolic model accounts for the interactions of metabolites and enzymes. It is a list of coupled biochemical reactions, representing the network that the reactions form by connecting the metabolites. The main concepts in setting up mass-balance models are now explained in detail and illustrated through an example.

Consider the simple pathway:

$$X_{in} \xrightarrow{b_1} S_s \xrightarrow{v_1} S_i \xrightarrow{v_2} S_p \xrightarrow{b_2} X_{out} \tag{3.1}$$
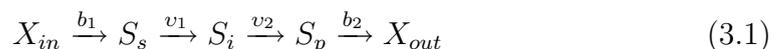
The two species, X_{in} and X_{out}, are boundary species, fixed by the experiment. Assimilation and export of the boundary species are catalyzed by enzymes E_{b_1} and E_{b_2} at rates b_1 and b_2 respectively; they are so-called external fluxes. The species S_s, S_i and S_p are state variables of the system (so-called internal metabolites), which can change depending on enzyme activities, including kinetic constants and concentrations of the boundary species. Reactions of the internal metabolites are catalyzed by enzymes E_{v_1} and E_{v_2} at rates v_1 and v_2 respectively; these are so-called internal fluxes.

To give a concrete example, let us assume the external reaction rates, b_1 and b_2, and internal reaction rates, v_1 and v_2, are given by two Michaelis-Menten rate laws and two Mass-Action rate laws; all reactions are irreversible so that the rates considered are net rates of the reactions. The rate of reactions for the

internal reactions are

$$v_1 = k_{v_1} \cdot S_s \tag{3.2}$$

$$v_2 = k_{v_2} \cdot S_i \tag{3.3}$$

where k_{v_1} and k_{v_2} stand for the rate constants of the reactions. The rate of change of S_i is given by:

$$\frac{dS_i}{dt} = v_1 - v_2 \tag{3.4}$$

and at steady sate $v_1 = v_2$. Given this condition, the steady state concentration of S_i can be easily shown to be:

$$S_i = \frac{k_{v_1} \cdot S_s}{k_{v_2}} \tag{3.5}$$

For a linear chain pathway, any rate can be used to compute the flux since all steps carry the same flux. Therefore, in our example pathway, the individual reactions can be lumped into one step describing a direct conversion of substrate S_s into product S_p with a forward reaction rate v_f. This lumping does not change the total number of degrees of freedom because the removal of one reaction rate v_2 is also accompanied by the elimination of one mass balance (that of S_i).

A significant reduction on network topology can be achieved with such a technique by lumping unknown rates of reactions into a linear sequence. This is equivalent to network simplification by considering only metabolites serving as network branch points, which refer to the points at which incoming flow is split into more than one outgoing stream.

In theory, a steady-state flux is a function of all kinetic constants in the pathway. In the example, the steady-state flux, J_2 through the reaction catalyzed by E_{v_2} can be computed from the rate $v_2 = k_{v_2} \cdot S_i$ and by substitution yields

$$J_2 = k_{v_2} \cdot \frac{k_{v_1} \cdot S_s}{k_{v_2}} = k_{v_1} \cdot S_s \tag{3.6}$$

In general, the rates can be expressed as functions of their influencing factors. For most systems, however, the algebraic analysis just described is not possible because the mathematical expressions involved become too complex. Thus, the system can only be solved with the aid of computers.

Once all the reactions and external fluxes are identified, a mass-balance model can be derived for all the metabolites in the network. At steady state, the set of

equations describing the changes of metabolite concentrations is equal to zero

$$\frac{dS_i}{dt} = \sum \text{Inflows} - \sum \text{Outflows} = 0 \tag{3.7}$$

The mass-balance model of a metabolic pathway is described using the stoichiometric matrix, $\mathbf{S}$, relating the flux rates of enzymatic reactions, v, to time derivatives of metabolite concentrations, X, as

$$\frac{dX_m}{dt} = \mathbf{S} \cdot v \tag{3.8}$$

$$v = [v_1 \; v_2 \; \cdots \; v_{n_{int}} \; e_1 \; e_2 \; \cdots e_{n_{ext}}]^T \tag{3.9}$$

where v_i and e_i represent the internal and exchange fluxes of the system.

At steady state, the change in the amount of a component over time across all reactions within the system is zero. The set of differential equations (Equation (3.8)) is then written in the form

$$\frac{dX}{dt} = \mathbf{S} \cdot v = 0 \tag{3.10}$$

$\mathbf{S}$ is a $m \times n$ matrix of all feasible fluxes. m and n represent the number of internal components and reactions, respectively. In the context of metabolism, internal metabolites are quantified with transient concentrations over time, while external metabolites have concentrations fixed at certain amount.

Steady-state simulation of a mass-balance model computes the solutions that satisfy the cellular constraints of each balance equation of the system. Since m is usually far fewer than n, Equation (3.10) is typically an under-determined system. As defined by Stephanopoulos et al. (Stephanopoulos et al. 1998), if the number of unknown variables is greater than the number of equations defining the system, then the system is under-determined. The consequence of this is that there exists an infinite number of solutions to the system. In order to determine a unique solution for the system, it is necessary to apply additional constraints.

One way to obtain additional constraints is to measure unknown fluxes for the metabolic pathway. However, when the exact flux values are not available, ranges of allowable flux values are commonly incorporated as additional constraints (Kauffman et al. 2003). When the constraints are not sufficient for direct calculation, Linear Programming (LP) can be used to estimate the in-

tracellular flux distribution. The usefulness of this approach in studying the trypanothione metabolism is illustrated in Section 3.5.1.

3.3.2 Mass-balance Model Construction

The first step towards structural model construction is to define all the metabolites and associated metabolic reactions. The trypanothione metabolic pathway (Figure 3.1) contains 26 internal metabolites and 32 reactions, mediated through 28 proteins. In Section 3.3.1, we stated that a significant reduction in network topology can be achieved by lumping (unknown) rates of reactions in linear sequence. The concept of Enzyme Subsets suggests that enzymes belonging to the same subset can be lumped without losing any topological information (see detailed explaination in Section 3.3.4).

Work by Varma and Palsson (Varma and Palsson 1993) has shown that a system can also be intuitively simplified by removing the intermediate reactions where the source reactant and product are involved in only one reaction. This technique is employed for the system simplification of the trypanothione pathway, which, in this way, reduced the original pathway into one comprising 18 internal metabolites, 6 extracellular entities and 21 reaction fluxes.

The graphical representation of the reduced pathway is presented in Figure 3.2. Edges and component(s) with no explicit experimental evidence in *T. brucei* were removed from the diagram and will not be considered. The list in Tabel 3.1 associates reactions in Figure 3.2 with the fluxes going through.

The stoichiometric matrix (3.12) was constructed solely based on the network stoichiometry and irreversibility of the reactions. Regulations of the enzyme kinetics are not considered in the mass-balance model. Flux notations v_i and b_j denote the exchange of fluxes between variable metabolites and with the environment respectively. In the case where the external fluxes are known, the rate of change of flux constraints can be identified precisely.

The entries in each column correspond to the stoichiometric coefficients of the metabolites of each reaction. By convention, negative values are given for reactants and positive values are given for products. The concentration of metabolites is denoted by brackets. The ith row of the matrix defines the participation of a particular metabolite across all metabolic reactions, and the jth column provides the stoichiometry of all metabolites in that reaction. We have
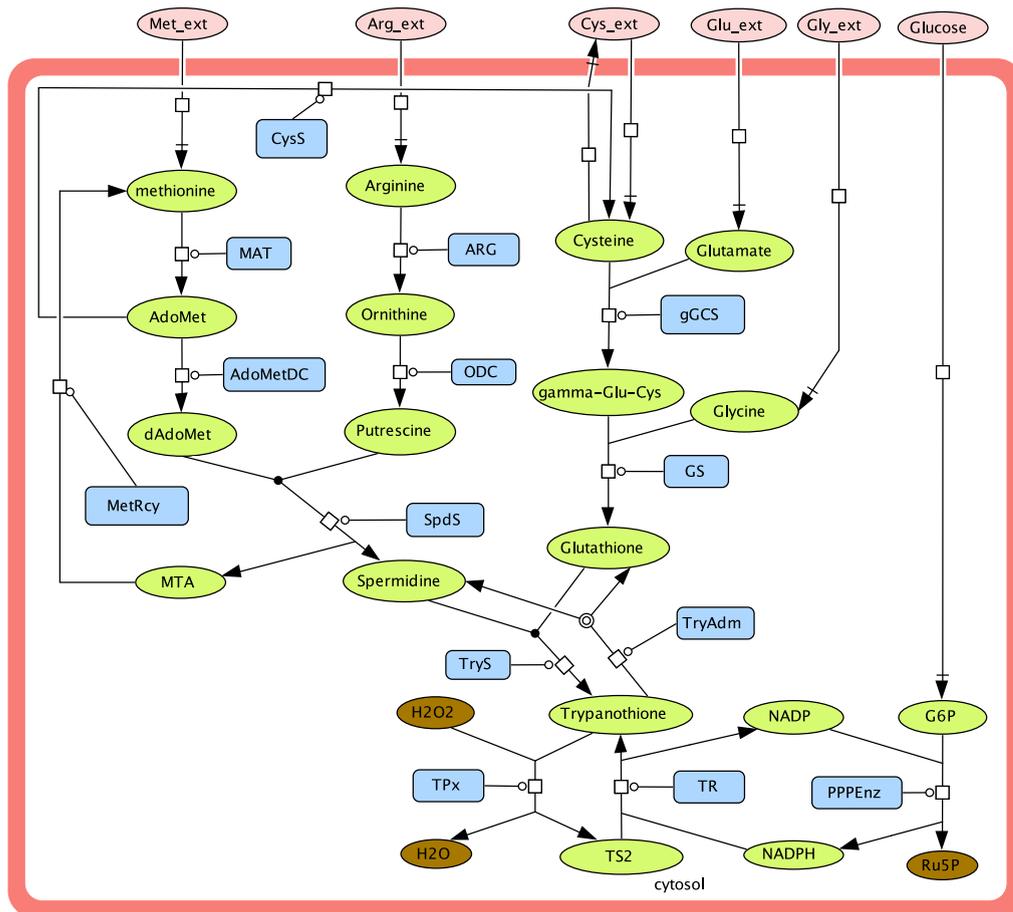
Figure 3.2: Graphical representation of the trypanothione metabolism for structural modelling. Internal metabolite pools are nodes in green and external ones are nodes in pink. Edges with one-way arrow denote irreversible reaction steps, indicating the direction of net reaction fluxes. Metabolites in brown are constant variables of the pathway, whose concentrations are fixed at their physiological levels. Abbreviations of intracellular enzymes: ARG, arginase; ODC, ornithine decarboxylase; SpdS, spermidine synthase; MAT, adomet synthase; AdoMetDC, adomet decarboxylase; CysS, cysteine synthetase; MetRcy, methionine recycling enzyme; gGCS, γ-glutamylcysteine synthetase; GS, glutathione synthetase; TryS, trypanothione synthetase; TryAdm, trypanothione amidase; TPx, trypanothione peroxidase; TR, trypanothione reductase; PPPEnz, pentose phosphate pathway enzymes.

$$\left[\frac{d[Arg]}{dt} \quad \frac{d[Orn]}{dt} \quad \frac{d[Met]}{dt} \quad \frac{d[AdoMet]}{dt} \quad \frac{d[Put]}{dt} \quad \frac{d[dAdoMet]}{dt} \quad \frac{d[Spd]}{dt} \quad \frac{d[MTA]}{dt}\right.$$

$$\frac{d[Cys]}{dt} \quad \frac{d[Glu]}{dt} \quad \frac{d[Gly]}{dt} \quad \frac{d[gGluCys]}{dt} \quad \frac{d[GSH]}{dt} \quad \frac{d[TSH]}{dt} \quad \frac{d[TS_2]}{dt}$$

$$\left.\frac{d[G6P]}{dt} \quad \frac{d[NADPH]}{dt} \quad \frac{d[NADP]}{dt}\right]^T =$$

$$\mathbf{S_{tb}} \cdot [v_1 \quad v_2 \quad v_3 \quad v_4 \quad v_5 \quad v_6 \quad v_7 \quad v_8 \quad v_9 \quad v_{10} \quad v_{11} \quad v_{12} \quad v_{13} \quad v_{14} \quad b_{arg}$$

$$b_{met} \quad b_{cys} \quad b_{eff} \quad b_{glu} \quad b_{gly} \quad b_{glc}]^T \tag{3.11}$$

where

No.	Label	Reaction Name	Stoichiometry
1	v_1	Arginase	arginine → ornithine
2	v_2	Ornithine decarboxylase	ornithine → putrescine
3	v_3	AdoMet synthase	methionine → AdoMet
4	v_4	AdoMet decarboxylase	AdoMet → dAdoMet
5	v_5	Cysteine synthase	AdoMet → cysteine
6	v_6	Spermidine synthase	putrescine+dAdoMet → spermidine+MTA
7	v_7	MTA recycling	MTA → methionine
8	v_8	γGCS	cysteine+glutamate → γGluCys
9	v_9	Glutathione synthetase	γGluCys+glycine → Glutathione
10	v_{10}	Trypanothione synthetase	spermidine+2glutathione → trypanothione
11	v_{11}	Trypanothione hydrolysis	trypanothione → spermidine+2glutathione
12	v_{12}	Trypanothione perioxidase	trypanothione → TS_2
13	v_{13}	Trypanothione reductase	TS_2+NADPH → trypanothione+NADP
14	v_{14}	PPP Enzymes	G6P+NADP → Ru5P+NADPH
15	b_{arg}	Arginine transport	Ext_{arg} → arginine
16	b_{met}	Methionine transport	Ext_{met} → methionine
17	b_{cys}	Cysteine uptake	Ext_{cys} → cysteine
18	b_{eff}	Cysteine excretion	cysteine → Ext_{cys}
19	b_{glu}	Glutamate uptake	Ext_{glu} → glutamate
20	b_{gly}	Glycine uptake	Ext_{gly} → glycine
21	b_{glc}	Glucose uptake	Ext_{glc} → glucose

Table 3.1: Reaction names and stoichiometries of the model shown in Figure 3.2. All reactions are represented as irreversible reactions with one-way arrows.

$$
\mathbf{S_{tb}} =
\begin{bmatrix}
-1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 \\
1 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & -1 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 1 & -1 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 1 & 0 & 0 & 0 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 1 & 0 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & -1 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 1 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 1 & 0 & 0 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & -1 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 0 & 0 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & -2 & 2 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & -1 & -1 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 \\
0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & -1 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
\end{bmatrix}
$$

$$(3.12)$$

With the above information, the mass-balance equations of the trypanothione system can be formulated as below:

$$
\begin{aligned}
X_1 &= \frac{d[Arg]}{dt} &= 0 = \ & b_{arg} - v_1 \\
X_2 &= \frac{d[Orn]}{dt} &= 0 = \ & v_1 - v_2 \\
X_3 &= \frac{d[Met]}{dt} &= 0 = \ & b_{met} - v_3 + v_7 \\
X_4 &= \frac{d[AdoMet]}{dt} &= 0 = \ & v_3 - v_4 - v_5 \\
X_5 &= \frac{d[Put]}{dt} &= 0 = \ & v_2 - v_6 \\
X_6 &= \frac{d[dAdoMet]}{dt} &= 0 = \ & v_4 - v_6 \\
X_7 &= \frac{d[Spd]}{dt} &= 0 = \ & v_6 - v_{10} + v_{11} \\
X_8 &= \frac{d[MTA]}{dt} &= 0 = \ & v_6 - v_7 \\
X_9 &= \frac{d[Cys]}{dt} &= 0 = \ & b_{cys} - b_{efcys} + v_5 - v_8 \\
X_{10} &= \frac{d[Glu]}{dt} &= 0 = \ & b_{glu} - v_8 \\
X_{11} &= \frac{d[Gly]}{dt} &= 0 = \ & b_{gly} - v_9 \\
X_{12} &= \frac{d[gGluCys]}{dt} &= 0 = \ & v_8 - v_9 \\
X_{13} &= \frac{d[GSH]}{dt} &= 0 = \ & v_9 - 2 \cdot v_{10} + 2 \cdot v_{11} \\
X_{14} &= \frac{d[TSH]}{dt} &= 0 = \ & v_{10} - v_{11} - v_{12} + v_{13} \\
X_{15} &= \frac{d[TS_2]}{dt} &= 0 = \ & v_{12} - v_{13} \\
X_{16} &= \frac{d[G6P]}{dt} &= 0 = \ & b_{glc} - v_{14} \\
X_{17} &= \frac{d[NADPH]}{dt} &= 0 = \ & -v_{13} + v_{14} \\
X_{18} &= \frac{d[NADP]}{dt} &= 0 = \ & v_{13} - v_{14}
\end{aligned}
$$

$$(3.13)$$

Once the mass-balance model is defined, a theoretical analysis can be performed. In the following sections, the general concepts of *Conserved Moieties*

and *Enzyme Subsets* are studied for the trypanothione metabolic pathway.

3.3.3 Conserved Moieties

It is necessary for the stoichiometric matrix to have sufficient information content in order, for a steady state, to be possible in mathematical terms. In mathematical terms, this implies that all equations are linearly independent. In other words, no rows may be a linear combination of, or can be solved from, the remaining ones. In order to supply enough information for a steady-state calculation, the first step is to ascertain whether there are groups of balance equations that can sum to zero, which refers to the study of conserved moieties of the system. According to conservation moieties, a reduced matrix can be obtained by clamping 'abundant' balance equations. Such implementation leads to a determined system of balance equations that contains enough information to compute a steady state, though in this case, a unique steady state is not guaranteed.

Conserved moieties are defined by Heinrich and Schuster (Heinrich and Schuster 1996) as components such as atoms and molecules that are preserved within the system and does not degrade over time, for example, in a cyclic reaction. This can be expressed by equations, namely the so-called conservation relationships. The metabolites contributing to a conservation relationship depend on each other, and their total concentration is fixed (Hofmeyr 1986).

Conserved moieties in metabolic systems reveal themselves as linear dependencies in the rows of stoichiometry matrix. This can be detected by inspecting the rows of $\mathbf{S_{tb}}$, examining if the rank of $\mathbf{S_{tb}}$ is less than m, which indicates the number of variable metabolites of the system. Rank of $\mathbf{S_{tb}}$ with $rank(\mathbf{S_{tb}})$ gives the number of linearly dependent metabolites. Whenever the system exhibits conserved moieties, it gives $rank(\mathbf{S_{tb}}) < m$. All the relations have been determined when the number of variable metabolites is equal to the rank. The embedded function rank from Matlab (http://www.mathworks.com/) was employed and gave a rank of 17 for the system with 18 variable metabolites, indicating one conserved relationship.

In our system, the conserved moiety is the redox couple NADP/NADPH, which is integral for trypanothione synthesis and regeneration. NADP is formed when NADPH is consumed and *vice versa*. The row for NADP in $\mathbf{S_{tb}}$ is dependent on the row for NAPDH, exhibiting the opposite signs of the coefficients.

This is true even during transients and not only in steady state;

$$X_{17} + X_{18} = 0$$

This implies moiety conservation, such that

$$[NADP] + [NAPDH] = constant \tag{3.14}$$

In this way, the moiety conservation reduces the balance equations in Equation (3.13) to a set of 17 (denoted as $\mathbf{S_{tb}^{rd}}$), which supplemented by one conservation equation. To check if no conserved equations can be further deduced from $\mathbf{S_{tb}^{rd}}$, which describes mass balance equations for each of the 17 metabolites (denoted as m^{rd}), we calculate the rank of the reduced matrix. The calculation returns an identical value to the number of variable metabolites and the equality $rank(\mathbf{S_{tb}^{rd}}) = m^{rd}$ is satisfied. The result indicates that the system of balance equations has been correctly replaced by all possible conservation equations, and the first step towards steady-state simulation has been successfully accomplished. The number of degrees of freedom of the system is calculated to be $m^{rd} - rank(\mathbf{S_{tb}^{rd}}) = 0$.

3.3.4 Enzyme Subsets

Enzyme subsets can be derived directly from mass-balance equations. Enzyme subsets (Pfeiffer et al. 1999) are groups of enzymes that have constant flux ratios whenever the system is in steady state. This concept is useful when studying gene expression or metabolic regulation, and groups of enzymes with the same evolutionary pattern are of particular interest. Pfeiffer et al. (Pfeiffer et al. 1999) indicate that enzymes in any one branch carry the same steady-state flux and are likely to be expressed simultaneously. Schuster et al. (Schuster, Klamt, Weckwerth, Moldenhauer and Pfeiffer 2002) found that there is a correlation between the relative changes of gene expression for the enzymes classified into the same subset in yeast central metabolism during the diauxic shift.

Enzyme subsets with more than one reaction are of interest. Trivial subsets with only one reaction are not considered in further analysis. Three non-trivial subsets are identified for the trypanothione system using *METATOOL* (Pfeiffer et al. 1999, von Kamp and Schuster 2006).

Metabolic reactions are often present in linear combinations, so identifying

Index	Label	Chemical Reaction
1	$v_3,\ v_5,\ b_{met}$	MAT, CysS, Methionine transport
2	$v_{10},\ v_{11}$	TryS, TryAdm
3	$v_{12},\ v_{13},\ v_{14},\ b_{glc}$	TPx, TR, PPPEnz, Glucose transport

Table 3.2: Enzyme subsets of the trypanothione pathway.

a portion of them can assist in deriving the values of the remaining ones. This information is valuable for biologists in designing experiments in an economical way. Based on the enzyme subsets in Table 3.2, for example, measurement of the steady-state flux v_{12} can aid in determining the values of other fluxes in the group $(v_{13},\ v_{14},\ b_{glc})$.

A special case of enzyme subsets is one that contains all the network gaps. *Network gaps* (Schuster and Schusters 1990) were first introduced to describe the situation where there are reactions that have a net flux of zero after the system has reached steady state. These subsets are less interesting *per se*, as the reactions contained do not show any interactions with other parts of the system.

Another concept that enzyme subsets leads to is the elementary mode, which studies the set of reactions that can sustain a steady-state flux when the remaining reactions function properly. From the perspective of elementary modes, network gaps are rather important.

3.4   Elementary Mode Analysis

Schuster et al. (Schuster et al. 2000) stated that an *elementary mode* is the "minimal set of enzymes that can operate at steady state with all irreversible reactions operating under normal conditions". Elementary modes are not decomposable, which means that no reactions can be removed from the mode without violating the steady state condition.

With the elementary mode approach, network complexity can be reduced to a minimal set of reactions. For example, it can help detect which of several pathways is preferred by the organism to consume a particular substrate. Another very similar concept called *Extreme Pathways* has been introduced recently, which is the systemically independent subset of the elementary modes. A comparison of these two principles is presented by Klamt and Stelling (Klamt and Stelling 2003).

A calculation procedure for the computation of elementary modes is given by (Schuster, Hilgetag, Woods and Fell 2002), where external metabolites representing the inputs and outputs are necessary for elementary modes to be calculated. In our analysis, we applied *METATOOL*, which has been used in many applications to calculate elementary modes.

One prominent role for elementary modes is to reveal missing links or network gaps in the metabolism of organisms. For example, by means of the elementary mode analysis, Schuster et al. (Schuster et al. 1999) found a missing link in the metabolism of *M. hominis* by detecting that some experimentally-verified enzymes were not contained in any elementary modes of the system.

Four elementary modes were detected by *METATOOL* for the metabolic model expressed by Equation (3.13). They correspond to four different metabolic functions, as shown in Table 3.3.

Mode	Name	Participating steps
EM1	trans-sulphuration sub-pathway	b_{met}, v_3, v_5, b_{eff}
EM2	trypanothione synthesis-hydrolysis	v_{10}, v_{11}
EM3	trypanothione redox cycle	v_{12}, v_{13}, v_{14}, b_{glc}
EM4	cysteine export-assimilation	b_{cys}, b_{eff}

Table 3.3: Elementary modes of the trypanothione model shown in Figure 3.2.

The above analysis reveals that there are missing links or network gaps in the pathway, as the enzymatic reactions corresponding to fluxes v_1, v_2, v_4, v_6, v_7, v_8, v_9, b_{arg}, b_{glu}, b_{gly} and b_{glc} carry zero net flux. However, experimentally, it has been verified that exogenous arginine plays a critical role in cell growth as well as the other enzymes on the spermidine biosynthesis path. These indicate that some existing intermediates of the pathway would not have been generated.

A biological pathway can be defined in terms of elementary modes, which are derived from the network topology and uniquely define it (Papin et al. 2003). By taking advantage of this unique feature, we proposed an original approach to detect network gaps and seek potentially suitable topologies. The approach was programmed in Matlab and dependent on *METATOOL* for the calculation of elementary modes for a given network topology. Structural malfunction can sometimes be examined through an intuitive analysis. The automatic investigation presented here is advantageous in exploring network topologies from the perspective that diverse results can be calculated with less computation time.

The following paragraphs briefly introduce the rationale of our approach. Supplementary information is given in Appendix B.1.

3.4.1 An Original Approach to the Identification of Network Gaps

Network gaps are caused by two factors in general. First, there are 'dead' metabolites in metabolic systems that are constantly produced but not consumed in other reactions. Schuster and Schuster (Schuster and Schusters 1990) indicated that treating the 'dead' metabolites as external components could be a solution. Second, topologically, missing reactions prevent a steady-state flux going through a certain group of metabolites, even though each metabolite in the group is characterized with both incoming and outgoing fluxes. The first factor can be scrutinized with relative ease by inspection of the network diagram. The second factor is often hidden and analytical solutions are sometime hard to obtain.

The challenge in identifying network gaps is the lack of rules to follow. To make the computational investigation feasible, we constrain the missing reactions to internal metabolites participating in other metabolic functions. Almost all the well-known biosynthesis reactions of the internal metabolites have been incorporated into the pathway; hence, no further investigation will be applied to this aspect. Given such constraints, there are still several possibilities. For example, the missing reactions outgoing from the internal metabolites and terminating on unknown sources can occur independently or in pairs, or missing reactions outgoing from internal metabolites can be recycled to regenerate other existing metabolite(s).

To narrow down the search, our approach uses the following assumption: in addition to the existing reactions, there are also reactions of internal metabolites participating into other metabolic functions and these reactions occur in isolation, one reaction at a time, which we term 'single-branch' addition. Biologically, the missing reactions may refer to enzymatic or decay reactions of the metabolites, which are assumed as irreversible reactions in our analysis. *In silico* results of the 'single-branch' addition indicate that only the addition of a branch outgoing from the metabolites trypanothione or oxidized trypanothione (TS2) can enable a full coverage of the enzymatic reactions in the elementary modes.

We also investigate 'double-branch' addition to examine the network topology

when the outgoing branches of the internal metabolites are assumed to occur in pairs (see details in Appendix B.1). *In silico* results of the 'double-branch' addition returned 30 pairs, the addition of which to the original topology results in 'gap-free' topologies. Of the 30 pairs, 28 contain either trypanothione or TS2 and one special case where the pair consists of both trypanothione and TS2. The above observations are equivalent to the findings from the 'single-branch' addition that outgoing branches from trypanothione or TS2 are essential for the system to be 'gap free'. The last pair is spermidine and glutathione. As this pair contains neither trypanothione nor TS2 we retain it for further analysis.

In summary, the computational investigation of network gaps discovered three topologies where the enzymatic reactions of biological importance are all properly used in metabolism. The three network topologies are highlighted and labelled in Figure 3.3.

Elementary modes are useful in that they allow important cellular properties to be extracted from the network topology, for example, mutant phenotypes and network robustness. The elementary modes derived from the identified network topologies **ST1** to **ST3** are shown in Table 3.4.

By definition, elementary modes indicate the number of independent steady-state fluxes that can exist in a system. If this is zero, the system cannot sustain any steady-state flux. The elementary modes in Table 3.4 indicate that all the three structural topologies can carry steady-state fluxes. As all of the modes are irreversible, which means that no reversible reactions are contained, no negative fluxes are allowed for any of them.

Elementary flux modes correspond to different basic functions that a biochemical system is able to fulfill. Four common elementary modes were identified for all structural topologies, namely trans-sulphuration sub-pathway, trypanothione redox cycle, trypanothione regeneration and cysteine transport. It should be noted that of the six elementary modes, EM5 – cysteine transport is the only elementary mode that depends entirely on the chemical exchange between exogenous cysteine and endogenous cysteine, therefore the flux carried by this elementary mode always remains constant. As a result, this mode cannot be analyzed and will not be further discussed.

Before proceeding with further analysis, the three numerical candidates selected from the computational approach must be checked for biological relevance. Detailed inspection of the elementary modes unravels structural flaws. It is well-known that the intracellular level of trypanothione is determined by

ST	Mode	Name	Elementary Steps
ST1	EM1	trypanothione biosynthetic sub-pathway	b_{arg}, b_{met}, v_1, v_2, v_3, v_4, v_5, v_6, v_7, v_8, v_9, v_{10}, b_{glu}, b_{gly}, v_{tshout}
	EM2	trans-sulphuration sub-pathway	b_{met}, v_3, v_5, b_{eff}
	EM3	trypanothione redox cycle	v_{12}, v_{13}, v_{14}, b_{glc}
	EM4	trypanothione regeneration	v_{10}, v_{11}
	EM5	cysteine transport	b_{cys}, b_{eff}
	EM6	trypanothione biosynthetic sub-pathway	b_{arg}, b_{cys}, v_1, v_2, v_3, v_4, v_6, v_7, v_8, v_9, v_{10}, b_{glu}, b_{gly}, v_{tshout}
ST2	EM1	biosynthesis of reduced trypanothione	b_{arg}, b_{met}, v_1, v_2, v_3, v_4, v_5, v_6, v_7, v_8, v_9, v_{10}, v_{12}, b_{glu}, b_{gly}, v_{ts2out}
	EM2	trans-sulphuration sub-pathway	b_{met}, v_3, v_5, b_{eff}
	EM3	trypanothione redox cycle	v_{12}, v_{13}, v_{14}, b_{glc}
	EM4	trypanothione regeneration	v_{10}, v_{11}
	EM5	cysteine transport	b_{cys}, b_{eff}
	EM6	biosynthesis of reduced trypanothione	b_{arg}, b_{cys}, v_1, v_2, v_3, v_4, v_6, v_7, v_8, v_9, v_{10}, v_{12}, b_{glu}, b_{gly}, v_{ts2out}
ST3	EM1	spermidine biosynthetic sub-pathway	b_{arg}, v_1, v_2, v_3, v_4, v_6, v_7, v_{spdout}
	EM2	trypanothione redox cycle	v_{12}, v_{13}, v_{14}, b_{glc}
	EM3	trypanothione regeneration	v_{10}, v_{11}
	EM4	glutathione biosynthetic sub-pathway	b_{cys}, b_{glu}, b_{gly}, v_8, v_9, v_{gshout}
	EM5	cysteine transport	b_{cys}, b_{eff}
	EM6	glutathione biosynthetic sub-pathway	b_{met}, v_3, v_5, v_8, v_9, b_{glu}, b_{gly}, v_{gshout}
	EM7	trans-sulphuration sub-pathway	b_{met}, v_3, v_5, b_{eff}

Table 3.4: Elementary modes of the identified structural topologies of the trypanothione pathway summarized in Figure 3.3. **ST** stands for the structural topology.
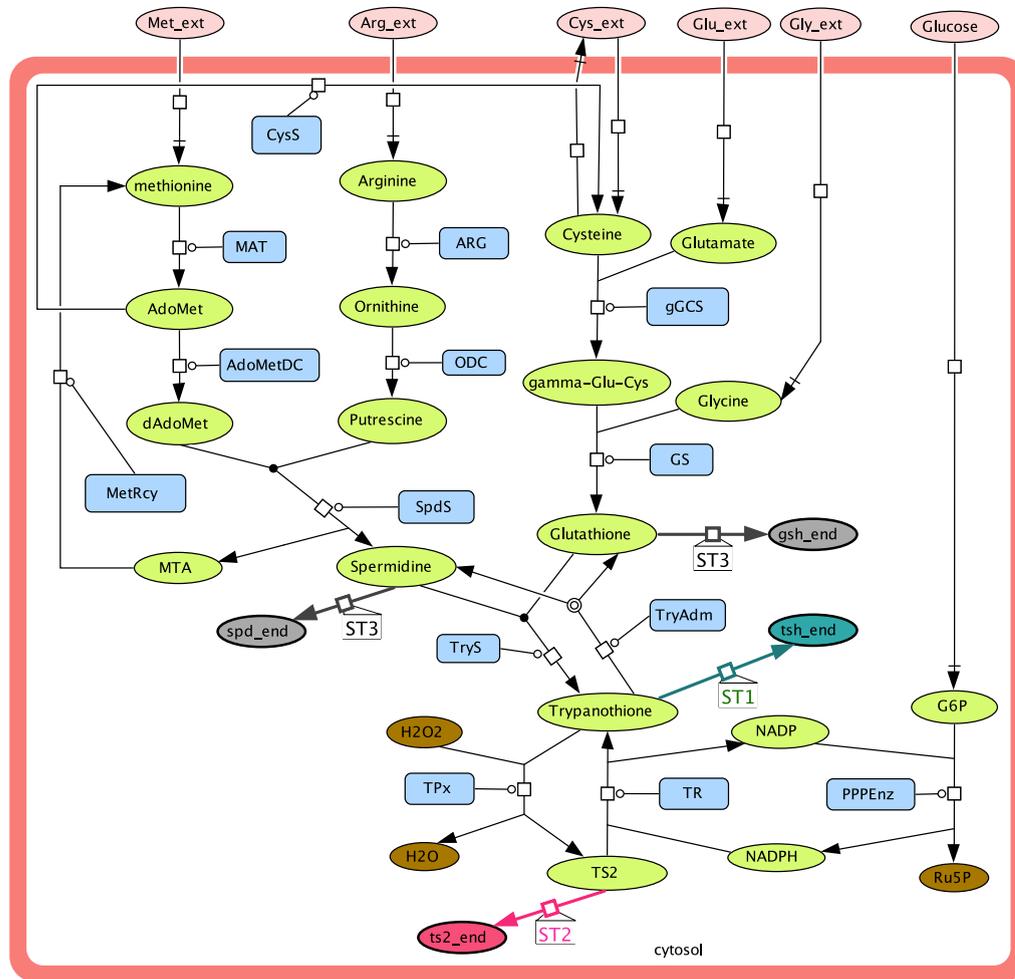
Figure 3.3: 'Gap-free' structural topologies of the trypanothione pathway. Additional branches outgoing from certain metabolites are labelled with **ST1**, **ST2** and **ST3** and highlighted in dark green, red and grey, where the associated ovals, namely tsh_end, ts2_end, spd_end and gsh_end, are treated as constant components.

assimilation of exogenous metabolites and intracellular enzymes activity. EM3 from **ST3** contradicts biological evidence that trypanothione is independent of external factors and can constantly operate on its own via the reactions v_{10} and v_{11}. As a consequence, the structural topology, **ST3**, will not be considered further. Our analysis concentrates on studying the systematic properties of the trypanothione pathway with structural topologies **ST1** and **ST2** owing to their biological relevance of the experimental observations.

3.4.2   Network Robustness

Elementary mode analysis is an established tool for studying system properties of metabolic pathways. According to Stelling et al. (Stelling et al. 2002), system properties can be summarized as network diversity, robustness and redundancy. The above concepts are similar properties in the sense that they are all determined by the quantity of elementary modes when individual reactions are knocked out.

In the work by Stelling et al. (Stelling et al. 2002), the network robustness of microbial metabolism has been studied computationally using elementary mode analysis. The relative occurrence of an enzymatic reaction serves as a qualitative assessment of the importance or even essentiality of the reaction. The rule is that a reaction with significant contributions can be predicated by counting the number of elementary modes retained when the reaction is knocked out. Stelling et al. concluded that the less elementary modes obtained, the more critical the reaction is and therefore the less robustness of the network in response to the changes.

In our study, we based the analysis of network robustness on a single knockout approach proposed by Wilhelm et al. (Wilhelm et al. 2004). They argued that network robustness is not identical with redundancy. This is particularly true when there are parallel routes to the same reaction product.

The basic idea of the approach (Wilhelm et al. 2004) is to use the ratio Z_i/Z to characterize the network robustness in response to the knockout of one enzyme E_i, where Z and Z_i stand for the number of elementary modes in the unperturbed network and the number remaining after knockout of the enzyme. In the extreme cases, when no elementary modes are left, the ratio is 0, indicating no robustness and when all elementary modes are left, the ratio is 1, indicating complete robustness of the network to the enzyme knockout. The value of this ratio is varied between 0 and 1 when only some elementary modes remained. Calculating the arithmetic mean of all these numbers that reflect the network robustness in response to individual enzyme knockout quantifies the overall robustness of the entire network, expressed as $R=\text{sum}(Z_i)/(r \times Z)$, where r denotes the total number of reactions in the network. The value of the overall robustness again varies between 0 and 1.

The structural topologies **ST1** and **ST2** have overall network robustness $R_{sg1}=0.6894$ and $R_{sg2}=0.6742$, respectively. The values imply that both topologies have around two-thirds of the pathways conserved during single-gene dele-

tion. Different robustness values are stemmed from the elementary modes EM1 and EM6. Knockout of the reaction enzyme carrying flux v_{12} causes the two elementary modes to disappear in **ST2**, but both are still operative in **ST1**. This illustrates the point that network topologies with the same number of elementary modes can give different measures of system robustness.

It is of interest to extend the analysis of system robustness of the trypanothione pathway to multiple knockouts. The approach of handling double-enzyme knockouts has been presented in work by Behre et al. (Behre et al. 2008). This approach first calculates the number of elementary modes remaining after the knockout of each pair of any two genes. The sum of the numbers is divided by the product of the number of pairs and the number of modes from the unperturbed pathway. The structural topologies **ST1** and **ST2** have overall robustness R_{dg1}=0.5303 and R_{dg2}= 0.5209 to double-gene deletion. This means that after knockout of two enzymes, half of the pathways in both topologies are still present.

It should be noted that the network robustness measured with the approaches by Wilhelm et al. and Behre et al. neglects new metabolic functions that could be created due to the changes in the structural topology, and only the capabilities to preserve existing metabolic functions are accounted for.

The measure of robustness can be used to evaluate the appropriateness of alternative network topologies. Morohashi et al. (Morohashi et al. 2002) stated that the robustness value of a network is, to some extent, an indicator of its plausibility, in that when cellular processes are preserved in cell mutants, they are likely to be robust against variability. In our study, both gene-knockout experiments indicate that the trypanothione pathway with structural topology **ST1** has only a marginally stronger robustness than that with structural topology **ST2**. As such, these results are inadequate to select between the two topologies.

In spite of minor numerical difference in network robustness, biologically, the structural topology **ST1** is preferred and supported by current experimental evidence. Krauth-Siegel et al. (Krauth-Siegel et al. 2005) state that trypanothione, in addition to converting into TS2, is involved in the synthesis of DNA precursors and in the detoxification of metals and drugs. However, regarding **ST2**, TS2 consumed in other cellular functions could not be interpreted biologically. The disulfide group of the metabolite TS2, which exists only in low concentrations, is known to be quickly reduced to trypanothione (Krauth-Siegel et al. 1987). This leads to the amount of TS2 available for other metabolic functions being very

limited. The assumption that TS2 may participate in cellular functions other than trypanothione regeneration is therefore not biologically reasonable.

In view of the preceding, the structural topology **ST1** will be discussed with further analysis. The mass-balance model and the stoichiometric matrix of the trypanothione metabolic pathway expressed by structural topology **ST1** is given in Equation (B.1) and matrix (B.2) in Appendix B.2.

Drug-target Identification

As stated in Chapter 1, one objective of studying the trypanothione metabolic pathway is to seek optimal anti-parasitic strategies that are efficient in terms of the depletion of intracellular trypanothione metabolite. The reaction steps involved in the pathway of trypanothione biosynthesis automatically become the major concerns of chemotherapeutic research.

An elementary mode can be viewed as a biological function of metabolic pathways. EM1 and EM6, identified from the topology **ST1**, represent a biosynthetic route that transforms some substrates into certain product(s). The elementary modes display two net reactions as follows, which differ in the exogenous metabolites employed, namely methionine (Met_{ext}) and cysteine (Cys_{ext}),

$$Met_{ext} + Arg_{ext} + Glu_{ext} + Gly_{ext} \longrightarrow tsh_{out} \tag{3.15}$$

$$Cys_{ext} + Arg_{ext} + Glu_{ext} + Gly_{ext} \longrightarrow tsh_{out} \tag{3.16}$$

The presence of exogenous metabolites in both elementary modes, including exogenous arginine (Arg_{ext}), glutamate (Glu_{ext}) and glycine (Gly_{ext}), demonstrates their essential role in trypanothione biosynthesis. The elementary mode analysis reveals the enzymes and the corresponding reactions that could be used as optimal chemotherapeutic targets.

Computationally, once the mass-balance model is defined, metabolic flux optimization can be employed to determine optimal flux distributions by maximizing or minimizing fluxes through particular cellular reactions with respect to some objective functions. Common choices to study the optimal internal fluxes against certain physiochemical constraints that describe the potential behaviour of an organism include biomass production, energy balance and flux limitations (Kauffman et al. 2003).

3.5 Metabolic Flux Analysis

Metabolic flux analysis (MFA) is the calculation and analysis of the flux distribution of the entire biochemical reaction network (Stephanopoulos et al. 1998). The theory of MFA relies solely on the known stoichiometry for all major intracellular reactions of a biochemical network. Given a metabolic pathway and sufficient flux measurements, MFA allows the reaction rates in the pathway to be determined. It is however assumed that the system in question is in or near a steady state, i.e. concentrations remain relatively constant over the course of the experiment (Wiechert 2001).

Calculation of steady-state flux distributions for metabolic pathways is not an easy task, not only because input fluxes are only partly known; but also because the structural topology may make MFA difficult to solve, due to the presence of bidirectional reaction steps or metabolic cycles contained in the pathway (Wiechert 2001). Based on the elementary mode analysis reported in Table 3.4, the trypanothione metabolic pathway under analysis is no exception, as illustrated by EM4: trypanothione regeneration. One immediate consequence of this situation is that it gives rise to multiple steady-state solutions satisfying the stoichiometric constraints of the metabolic pathway.

In this section, we apply the rationale of metabolic balancing to estimate steady-state flux distributions of the trypanothione metabolic pathway, and to gain insights into the critical properties of the pathway.

3.5.1 Steady-state Flux Estimation

Optimization of flux distributions is built on the assumption that organisms will reach a steady state that satisfies physiological constraints under given environmental conditions. As knowledge about the organisms is often only partly known, multiple steady states (and thus multiple sets of flux distribution) are possible. Optimization is usually required to identify a particular flux distribution that satisfies specific metabolic constraints, given biologically reasonable bounds on minimum and maximum values of unknown fluxes. This approach has been employed to study and predict optimal flux patterns and mutant phenotypes (Edwards and Palsson 2000b, Edwards and Palsson 2000a) through maximizing the growth rate.

For the steady-state simulation, rate constants of the external fluxes that are the inputs of the pathway have to be defined explicitly, from which the

corresponding internal fluxes can be calculated. In our study, absolute values of the external fluxes are only partially known, with 17 unknown flux variables constrained by 14 balance equations. This makes the computation of the steady-state solution(s) a difficult task. In this case linear programming can be used to determine intracellular flux distributions, provided that a suitable objective function can be specified (Stephanopoulos et al. 1998, Lee et al. 1999).

Uptake rates of substrates and secretion rates of metabolites are important inputs for the calculation of metabolic fluxes. In our analysis, the only experimental data used for the flux calculations are the external fluxes of exogenous arginine, methionine, cysteine and glucose, denoted by b_{arg}, b_{met} and b_{cys} and b_{glc}, respectively. The fluxes of glutamate (b_{glu}) and glycine (b_{gly}) are not known. Endogenous cysteine, the major byproduct of trans-methylation reactions, was found to be rapidly excreted into environment by trypanosomes (Bacchi et al. 1995), which is symbolized as b_{eff}. The cysteine uptake and excretion are described as two individual reactions in steady-state flux calculations due to the different metabolic functions they participate in.

The external fluxes b_{met} and b_{cys} and b_{glc} in *T. brucei* are reported as 28 $nmol\ min^{-1}\ (10^8\ cells)^{-1}$ (Hasne and Barrett 2000), 3 $nmol\ min^{-1}\ (10^7\ cells)^{-1}$ (Duszenko et al. 1985) and 0.109 $\mu mol\ min^{-1}\ (mg\ cell\ protein)^{-1}$ (Haanstra et al. 2008). Exogenous arginine transport in *T. brucei* was measured to be 0.05195 $pmol\ min^{-1}\ (10^6\ cells)^{-1}$ for the transporter with higher affinity (unpublished data from Mike Barrett).

By way of comparison, arginine transport was also measured in *L. donovani*, which is a closely related parasitic organism. Values reported include 1.66 $pmol\ min^{-1}\ (10^6\ cells)^{-1}$ (Kandpal et al. 1995), and a recent measurement at 3.1 $pmol\ min\ (10^6\ cells)^{-1}$ (Darlyuk et al. 2009). It is noteworthy that arginine transport observed in *L. donovani* is at least 30 fold of the number measured in *T. brucei*.

Arginine assimilation flux is calculated based on a volume of 35 μL per 10^9 cells of *L. major* promastigotes (Turnock and Ferguson 2007), which is used as an approximation to the cell volume of *L. donovani*. Variation in cell volume of different parasitic organisms is not reckoned to be a factor giving rise to model uncertainty in the interpretation and prediction of biological phenomenon; the difference appears to be negligible for parasitic protozoa (Turnock and Ferguson 2007, Quesne and Fairlamb 1996).

All intracellular fluxes are defined with units of μmol per min per mg of

cell protein. The measured external fluxes expressed per number of cells can be transformed into the standard units of per mg of cell protein. Unfortunately, no information is provided on how many transporters are contained in *T. brucei* for assimilation of exogenous metabolites. It has been reported that one trypanosome has a volume of 58 μm^3 and the protein concentration of a *T. brucei* cell amounts to 175 mg/ml (Opperdoes et al. 1984). Accordingly, 10^8 cells gives a cell volume of 5.8 μL.

We assume that the transport enzymes present in the cell membrane account for 10% of the cell volume, thus resulting in the designated protein concentration as 17.5 mg/ml. The external fluxes of exogenous arginine, methionine and cysteine transport in *T. brucei* are thus approximated as 5.118e-5, 0.276 and 0.3 μmol per minute per mg cell protein, respectively. These data have been considered during metabolic flux estimation of the trypanothione model.

As stated before, it is impossible to determine a unique solution for underdetermined systems without introducing additional constraints. One possibility is to specify an objective function expressed as a linear function of intracellular fluxes. To this end, we introduce the biomass yield, which enables the capabilities of the cell to support optimum growth[1] under various conditions. The mathematical representation of the objective function is formulated as a linear maximization problem as follows:

$$\text{maximize} \quad \text{Biomass} = \sum_{\text{all i}} c_i \cdot v_i \tag{3.17}$$

$$\text{subject to } \frac{dX}{dt} = S \cdot \boldsymbol{v} \cdot (X) = 0 \tag{3.18}$$

where v_i represents the reaction flux converting a precursor to a biomass component and c_i denotes the ratio of the growth precursor required per gram of biomass. When there is more than one precursor needed, the biomass component is expressed as a weighted sum of the reactions that synthesize the growth precursors.

We want to choose objective functions with biological relevance that can be readily related to experimental observations when they are available. In the model, we choose the biomass yield as an optimization function to represent cell growth, which takes trypanothione as a precusor. The growth flux (v_{gr}) is modelled as a single hypothetical reaction that converts trypanothione into

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[1]Note that growth is defined in the context of the pathway under consideration.

biomass. As trypanothione is assumed to be the only metabolite precursor for biomass yield, the weight term is set to 1.

In terms of the structural topology, the additional branch labelled as **ST1** in Figure 3.3 can be replaced with this hypothetical reaction without influencing the results of the analysis on network gaps discussed in Section 3.4. The mass-balance model specific to the task of flux-distribution estimation is detailed in Equation (B.1) in Appendix B.2, which identifies the six elementary modes shown in Figure 3.4.

Once properly formulated, the optimization problem is easily solved using standard algorithms. We employed the LP solver linprog from the Matlab Optimization Toolbox to estimate the unknown fluxes at steady state. Computationally, as by default linprog solves the minimization problem, maximizing the growth rate defined as an outgoing flux from trypanothione is then transformed to minimizing the element of the corresponding column of the stoichiometric matrix where the outgoing flux is specified.

To compute physiologically feasible fluxes, we set the lower bounds of unknown fluxes by assuming a directionality for each flux, thus allowing only non-negative fluxes. The upper bound of growth rate was set to unity, which then effectively gives the relative ratios of the reaction fluxes.

The solution of the LP problem, using the objective function of maximum biomass yield, determines flux distributions specifying the fluxes of all internal reactions and unknown exchanges, as shown in Figure 3.5.

The figure indicates two major peaks at reactions 10, 11 and 18, and intense peaks at reactions 3 and 5. The peaks at reactions 3, 5, 10 correspond to the production of AdoMet, Cysteine and trypanothione, which are explained by their high requirement, since they are involved not only in the trypanothione biosynthetic sub-pathway but also in the trans-sulphuration sub-pathway. Reactions 16 and 17 represent the measured transport of exogenous methionine and cysteine across the cell membrane. The largest flux is seen for the excretion of endogenous cysteine, which is the only output of the trypanothione model under consideration.

The products of reactions 1, 2, 4, 6–9, 19 and 20 are ornithine, putrescine, dAdoMet, spermidine, methionine, gGluCys and glutathione. It should be noted that, in the unperturbed state, the amounts of the metabolites yielded are very small, compared with the numbers computed for other reactions.

There are eight enzyme subsets (ES) identified from the stoichiometric matrix
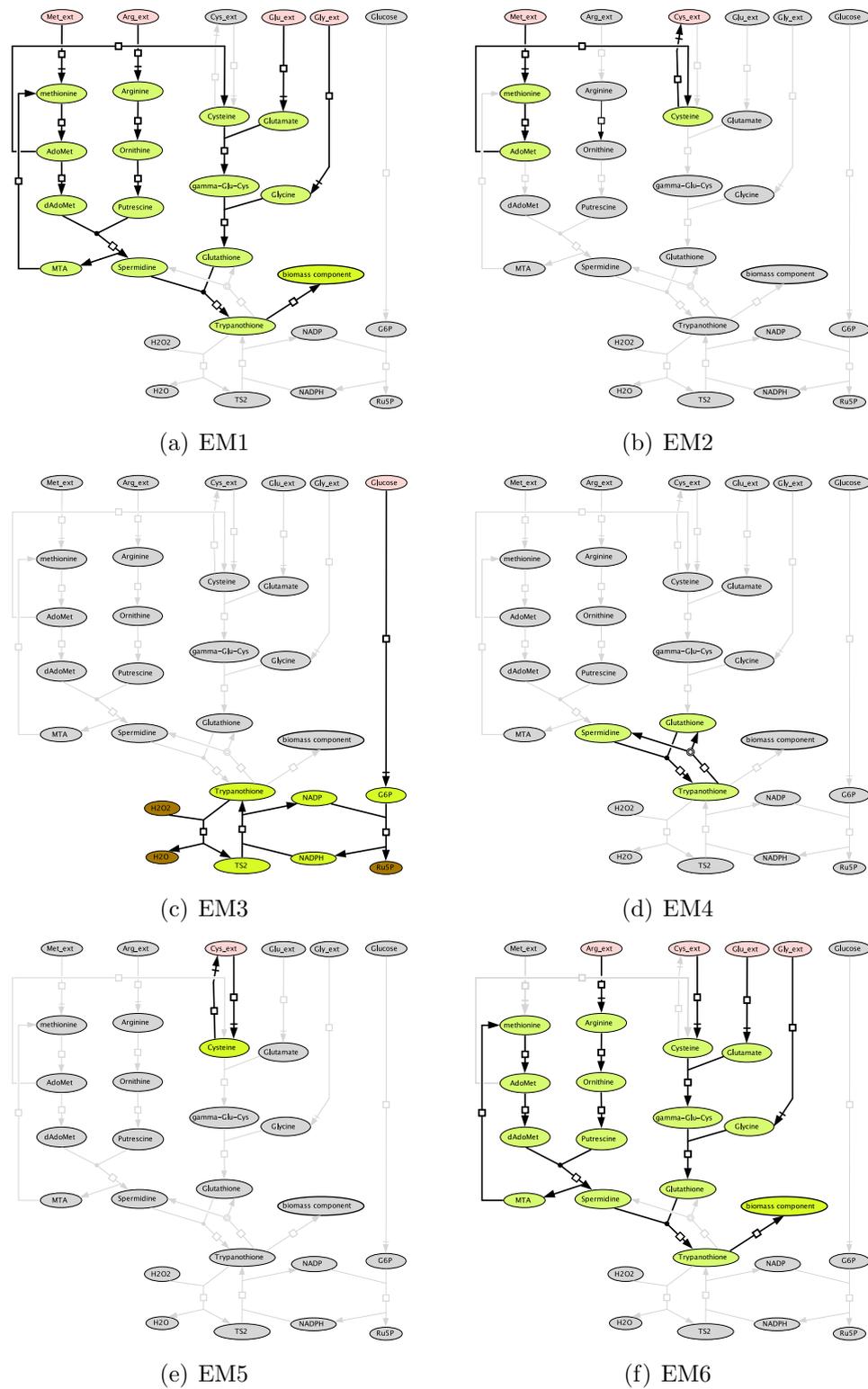
Figure 3.4: Six elementary modes of the 'gap-free' trypanothione pathway. In the pathway for LP optimization, reaction v_{tshout} is replaced with growth rate v_{gr} that relates to the biomass component. EM1 & EM6–trypanothione biosynthetic sub-pathway; EM2–trans-sulphuration sub-pathway; EM3–trypanothione redox cycle; EM4–trypanothione regeneration; EM5–cysteine transport.
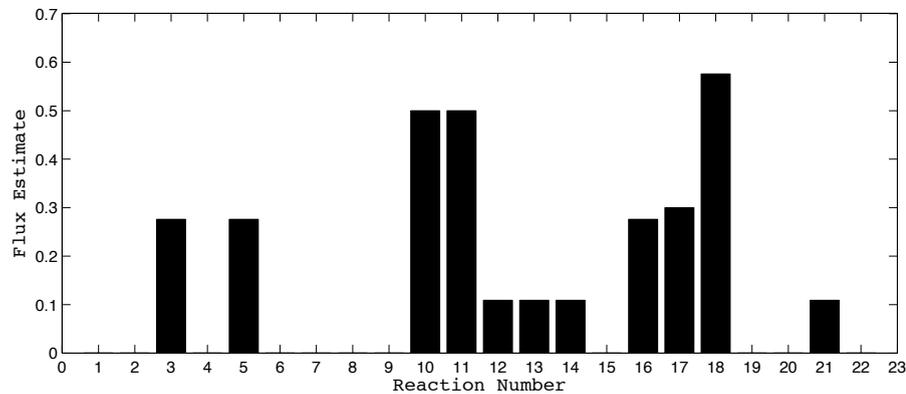
Figure 3.5: In unperturbed state, absolute values of individual reactions are estimated using linear optimization by stating an objective of maximum biomass yield for the model of trypanothione metabolism. Reaction 1–21 stand for the internal and exchange fluxes of the model as shown in Table 3.1. Reaction 22 indicates the trypanosomal growth rate.

(B.2) in Appendix B.2. Three non-trivial subsets are reported in Table 3.5.

Index	Reaction Number
ES1	1, 2, 4, 6, 7, 8, 9, 15, 19, 20, 22
ES2	5, 16
ES3	12, 13, 14, 21

Table 3.5: Enzyme subsets of the trypanothione pathway depicted in **ST1**.

As defined in Section 3.3.4, enzyme subsets are groups of enzymes that have constant flux ratios whenever the system is in steady state. This implies that when one reaction flux is known, the remaining fluxes contained in the same set can be deduced automatically. ES1 in Table 3.5 shows that the measured transport of exogenous arginine (5.118e-5 $nmol\ min^{-1}\ (mg\ cell\ protein)^{-1}$) determines the small values estimated for the fluxes going through reactions 1, 2, 4, 6–9, 19 and 20, resulting in a low rate of cell growth.

In the next section, we investigate the impact of external fluxes on the estimation of the steady-state flux distribution, which provides further insights into the regulation pattern of the trypanothione metabolism.

3.5.2 Sensitivity Analysis

In this section, we performed sensitivity analysis of the steady-state flux distributions induced by variations in individual measured fluxes. This case applies to the situation where a system component has not been experimentally measured or a large uncertainty exists for its value. All the internal flux variables are examined with respect to a 50% increase of the known transport fluxes for exogenous arginine, methionine, cysteine and glucose, respectively. When one external flux is under perturbation for the sensitivity analysis, the other fluxes are maintained at their measured values. Further work can be carried out to study variations in the flux estimates caused by simultaneous changes in the known input fluxes.

Steady-state flux estimation of the trypanothoine pathway by LP optimization is displayed in Figure 3.6, when external fluxes of exogenous arginine and methionine are perturbed by 50% of the measured values. It should be noted that because of the small values reported for arginine transport, the impact of variations is difficult to observe by inspecting the absolute values of the fluxes. The inset in Figure 3.6(a) shows the ratio of the perturbed and unperturbed fluxes, which gives a better view of the changes in the flux distribution. It shows that fluxes through reactions 1, 2, 4, 6–9, 19 and 20 are increased by up to 50% of the values predicted for the unperturbed state. This agrees with ES1 in Table 3.5, which shows that, in all steady-state conditions, reaction fluxes contained in the same subset always present with the same fixed flux proportions and the enzymes catalyzing the reactions are likely to be regulated with a similar pattern.

Both Figure 3.6(b) and the inset indicate that variations in methionine transport only have an influence on the trans-sulphuration sub-pathway, as illustrated by EM2 in Figure 3.4(b). Figure 3.7 shows that variations in the transport of exogenous cysteine and glucose affect the internal fluxes going through reaction 18 and reactions 12–14, respectively. As expected, glucose transport influences the enzymatic reactions contained in ES3 in Table 3.5, which constitutes the trypanothione redox cycle as shown in Figure 3.4(c).

It should be noted that only the perturbation of arginine transport was observed to affect the growth rate of the trypanothione pathway, increasing growth by up to 50% of the estimated value from the unperturbed state. The growth rate remains at the value estimated for the unperturbed state when the perturbation analysis is applied to other measured transport fluxes.

It is surprising that an increase in cysteine transport does not affect the downstream reactions but is recycled back into the medium. This differs from the experimental observations given by Duszenko et al. (Duszenko et al. 1985), however due to the lack of more recent data no solid conclusions can be drawn. A possible explanation for our observation is that cysteine excretion is indirectly regulated by AdoMet concentration, which is not considered in the sensitivity analysis of cysteine uptake. As a result of this, further constraints would be necessary in order to correctly model this relationship.

3.5.3 Elementary Flux Decomposition

In designing biological experiments, it is useful to know which system components have the greatest effect on the processes of interest. To this end, elementary mode utilization serves as a metric that can indicate important metabolic processes. It can also indicate the effect the changes in metabolic processes can have on the system (Schwartz and Kanehisa 2006).

By definition, any flux pattern can be described as a superposition of elementary modes with non-negative coefficients. However, this distribution is generally not unique. As previously proposed by Schwartz and Kanehisa (Schwartz and Kanehisa 2005), an optimization-based approach is required to study the optimal fluxes decomposed into a set of elementary modes. In this section, we apply this approach to the structural topology **ST1** of the trypanothione pathway.

Based on the description by Schwartz and Kanehisa (Schwartz and Kanehisa 2005), we perform flux decomposition and investigate how the elementary modes contribute to the construction of physiological steady states. For a steady-state flux vector Υ and a set of elementary modes, e_1, e_2, ... e_m, the method returns a set of non-negative values α_1, α_2, ... α_m that minimizes the cost function C_f in the format

$$C_f = \sum_{i=1}^{m} \alpha_i^2 \tag{3.19}$$

$$\text{subject to } \Upsilon = \sum_{i=1}^{m} \alpha_i \cdot e_i \tag{3.20}$$

$$\text{where } 0 \leq \alpha_i \leq 1 \text{ for } i = 1, \dots, m \tag{3.21}$$

In our experiment, Υ is the flux distribution solved from LP optimization in the unperturbed state, which is detailed in Table 3.6. Decomposition of the
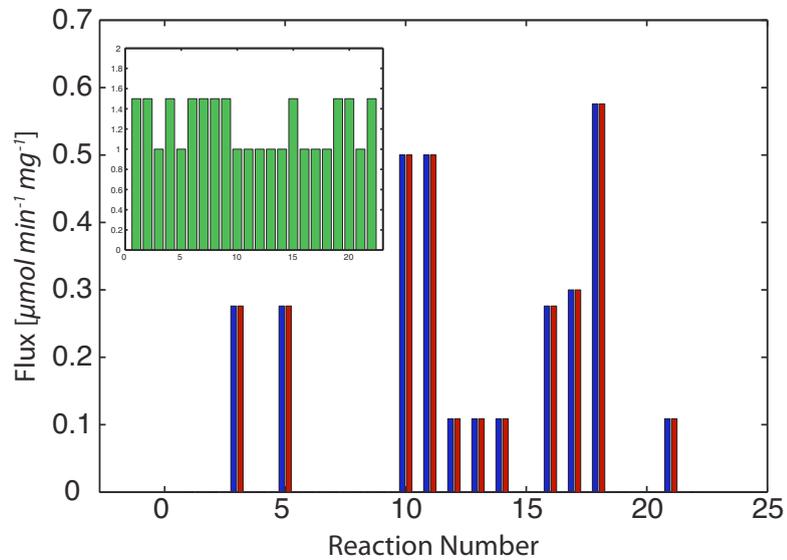
(a)
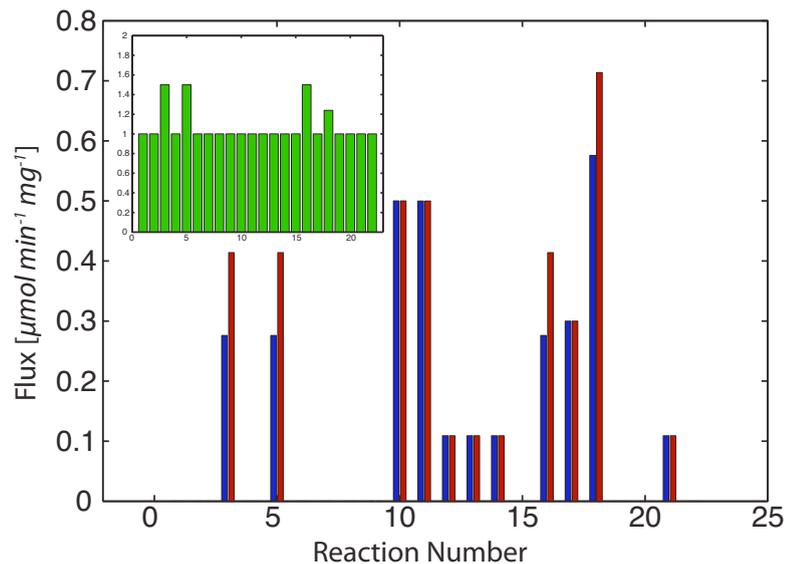
(b)

Figure 3.6: Sensitivity analysis of the unknown internal and exchange fluxes with respect to perturbations in (a) arginine transport and (b) methionine transport. Insets in both figures show relative changes in the estimated fluxes from both sensitivity analyses. The steady-state flux distributions in the perturbed states were estimated using linear optimization, with the maximum biomass yield as an objective and seeking the optimal value within the stoichiometrically defined domain. In both figures, blue bars and red bars represent absolute flux estimates under the unperturbed and corresponding perturbed conditions, respectively.
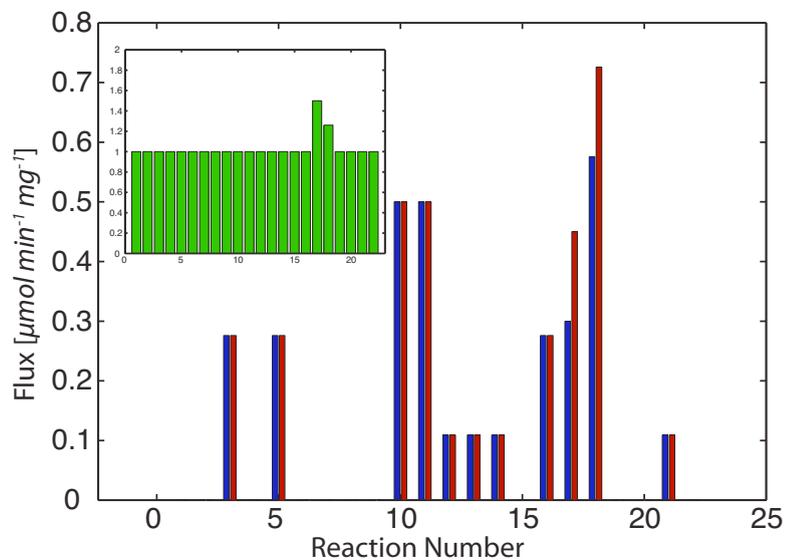
(c)
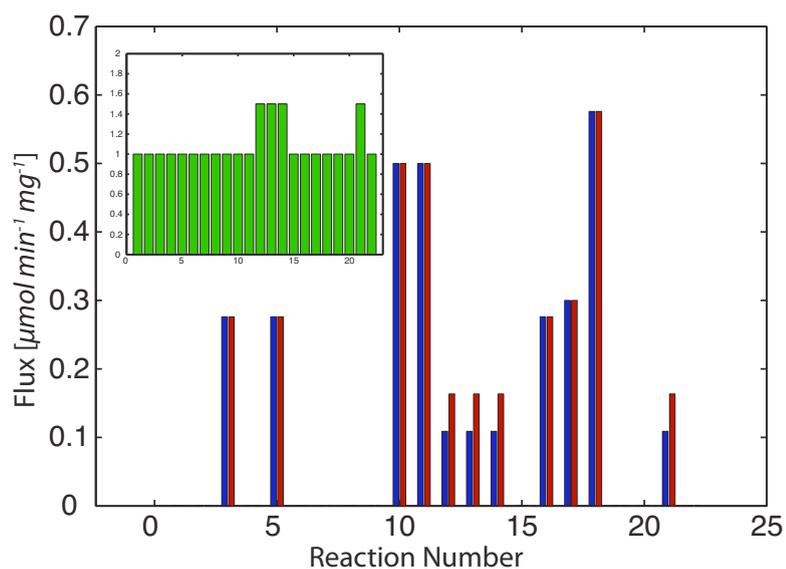
(d)

Figure 3.7: Sensitivity analysis of the unknown internal and exchange fluxes with respect to perturbation in (c) cysteine transport and (d) glucose transport. Insets in both figures show relative changes in the estimated fluxes from both sensitivity analyses. The steady-state flux distributions in the perturbed states were estimated using linear optimization, with the maximum biomass yield as an objective and seeking the optimal value within the stoichiometrically defined domain. In both figures, blue bars and red bars represent absolute flux estimates under the unperturbed and corresponding perturbed conditions, respectively.

flux distribution assigned the highest flux to EM4 (elementary mode flux value of 0.5). The smallest values were assigned to EM1 (elementary mode flux value of 1e-5) and EM6 (elementary mode flux value of 5e-5), which represent the biosynthesis of trypanothione and therefore the trypanosomal growth.

Steady-state Fluxes				
v_1=5.118e-5	v_2=5.118e-5	v_3=0.276	v_4 =5.118e-5	v_5=0.276
v_6=5.118e-5	v_7=5.118e-5	v_8=1.024e-4	v_9=1.024e-4	v_{10}=0.5
v_{11}=0.5	v_{12}=0.109	v_{13}=0.109	v_{14}=0.109	b_{arg}=5.118e-5
b_{met}=0.276	b_{cys}=0.3	b_{eff}=0.576	b_{glu}=1.024e-4	b_{gly}=1.024e-4
b_{glc}= 0.109	v_{gr}=5.118e-5			

Table 3.6: Steady-state flux distribution predicted by LP optimization.

We observed that the elementary mode flux values calculated with the above approach are considerably dependent on the 'measured' flux distribution to decompose. Decomposition of the flux distribution estimated in the perturbed condition of arginine transport gave a totally different set of elementary mode flux values for each mode, with the value assigned for EM6 almost 50% higher than that for EM1. As a consequence of this limitation, we employed a more valid method of control-flux analysis to characterize the biological significance of each reaction in supporting trypanosomal growth. Details are given at the end of Section 3.5.4

3.5.4 *In silico* Gene Deletions

One potential application of structural modelling is to analyze and predict cell growth in mutants with single gene deletions.

In silico gene deletions were carried out on the trypanothione mass-balance model using the maximum biomass yield as the objective function (see Appendix B.3). The transport data of exogenous metabolites arginine, methionine, cysteine and glucose, reported in Section 3.5.1, have been considered during the gene deletion study. Each of the 21 genes was systematically deleted from the mass-balance model, one at a time, and the resulting changes in the metabolic capabilities supporting biomass synthesis, and in consequence, optimum growth, were examined for each *in silico* mutant network.

Gene deletions in the context of computational optimization are in fact similar to studies of enzyme knockouts in the corresponding biological experiments.

To simulate a gene deletion, the flux through the enzymatic reaction was restricted to zero. The ratio Z_{mutant}/Z characterizes the systemic effect of a gene deletion on optimum growth and was calculated for deletions of each individual gene. In this measure, Z_{mutant} and Z represent the objective function value in the perturbed and 'wild-type' conditions of the pathway.

Gene essentiality is determined according to the rule that if the deletion of a gene does not significantly alter the maximum biomass yield, then it is classified as non-essential. The results for mutants of the trypanothione pathway are shown in Figure 3.8. The results were generated in a simulated environment with arginine, methionine, cysteine and glucose as the input sources, with the transport fluxes fixed at the measured values. The essential gene products identified were located on the trypanothione biosynthetic pathway with the transport of exogenous arginine, glutamate and glycine as the inputs of the pathway. The remaining genes in the pathway could be removed and trypanosome *in silico* still maintained the potential to support optimum growth. The *in silico* gene deletion results suggest that a number of the gene products (9 out of 21 genes) can be removed without eliminating the metabolic capability of trypanosomes to support optimum growth under the conditions considered.

Consistency with Experimental Evidence

The study of single gene deletions was evaluated in terms of the consistency between *in silico* predictions and experimental evidences of known mutants. ODC and AdoMetDC mutants (Willert and Phillips 2008, Roberts et al. 2002), SpdS (Taylor et al. 2008), ARG (Roberts et al. 2004), MetRcy (Riscoe et al. 1989), gGCS (Huynh et al. 2003) and TryS (Comini et al. 2004) have been verified to be essential genes and TPx (Schmidt and Krauth-Siegel 2003) a non-essential gene for optimum cell growth. There is no biological evidence given for the enzymes responsible for glutamate and glycine transport; *in silico* gene deletion results regarding their physiological significance still have to be validated by experimental data.

Even though the above analysis using the maximum biomass yield as an objective function returned consistent predictions, there were still genes that were mistakenly classified non-essential for optimum cell growth, for instance, gene product TR.

Trypanothione plays a critical role in the defense against damage by oxidants (e.g. hydrogen peroxide H_2O_2). Evidently, TR is an essential enzyme to
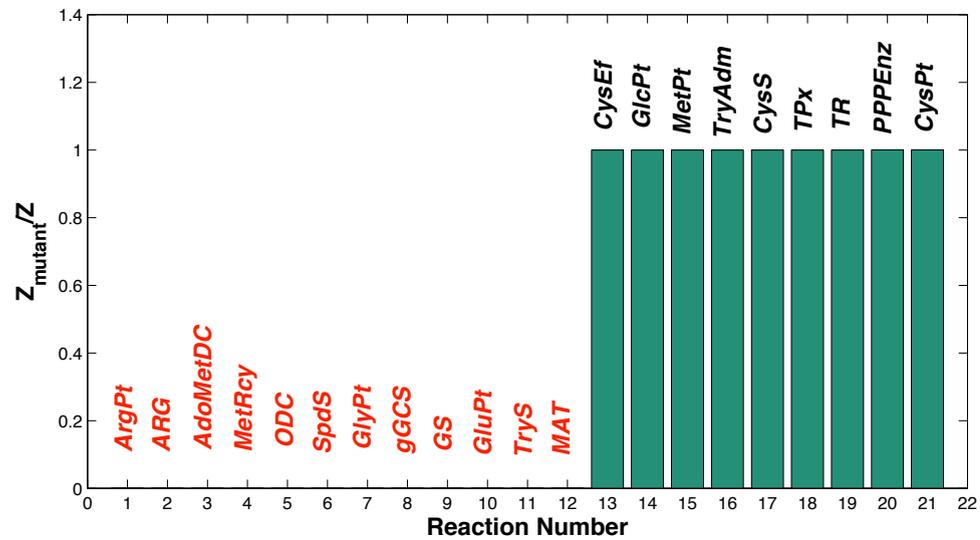
Figure 3.8: LP optimization for maximum biomass yield (optimum growth); maximum yields for biomass component for all possible single gene deletions in the trypanothione metabolic pathway. The optimal value of the mutant objective function (Z_{mutant}) is compared with the 'wild-type' objective function (Z), where Z is defined in Equation (3.17). The green bars that have a value of 1 for the ratio Z_{mutant}/Z represent gene deletions that maintain the maximum biomass yield at the same level as the *in silico* wild type. The bars denoted with the reaction names in red indicate gene deletions that reduced the maximum biomass yield to zero.

support trypanosomal growth. Krieger et al. (Krieger et al. 2000) showed that Trypanosomes that lack TR are vulnerable to oxidative stress and will slowly die. Regeneration of TSH from TS2 is not the rate-limiting step in the metabolism of H_2O_2 (Kelly et al. 1993), and the addition of putrescine did not restore cell growth, which indicates that the cause was not a shortage of polyamines (and consequently trypanothione) (Krauth-Siegel and Inhoff 2003).

Following an in-depth literature review, H_2O_2 production was postulated as a second objective function to tackle the deficiency of the existing function (maximum biomass yield) in coping with cell death that may be caused by H_2O_2 toxicity. Note that maximizing H_2O_2 production so as to kill the parasite was not the purpose of the linear optimization, rather the objective was minimization of the residual between the estimated rate and the measured rate, in order to simulate a realistic growing environment. In other words, the gene-deletion study at this stage aimed at analyzing the metabolic capability of trypanosomes to support optimum growth when H_2O_2 is modelled as a variable metabolite

and its production rate, $r_{H_2O_2}$, is fixed at the nominal value. A mathematical representation of the linear optimization is written in the form

$$\text{minimize } R_e = |r_{H_2O_2} - \tilde{r}_{H_2O_2}| \tag{3.22}$$

$$\text{subject to } \frac{dX}{dt} = S \cdot v \cdot (X) = 0 \tag{3.23}$$

where R_e stands for the residual error between the calculated production rate $\tilde{r}_{H_2O_2}$ and the measured value $r_{H_2O_2}$. A rate of 0.7 $\mu mol\ min^{-1}\ (mg\ cell\ protein)^{-1}$ (Henderson et al. 1987) was applied for H_2O_2 production in *T. brucei*. The mass-balance model used for calculating minimum R_e is detailed in Appendix B.4.

Equation (3.22) is a linear objective function that is referred to as a linear optimization problem, subject to the steady-state constraint expressed in Equation (3.23). The flux through the H_2O_2 production reaction is expressed in units of $\mu mol\ min^{-1}\ (mg\ cell\ protein)^{-1}$. Information on gene essentiality was obtained by minimizing the residual error R_e in the objective function. If the deletion of a gene results in a minimum residual error, then it is classified as non-essential.

The results identified from *in silico* knockout mutants are shown in Figure 3.9. As expected, TPx, TR, PPPEnz and GlcPt were classified as essential gene products, which emphasized their biological significance in the defense against oxidative stress. The *in silico* predictions agree with the experimental observation that turnover rate of TS2 mediated by TR may be the most important factor in coping with oxidative stress (Krauth-Siegel and Inhoff 2003), and enzymes (PPPEnz & GlcPt) involved in the Pentose Phosphate pathway play an important role in protection against activated oxygen species (Duffieux et al. 2000). Also, the enzymatic reaction of H_2O_2 metabolism by TSH under the catalysis by TPx is a major route for H_2O_2 consumption (Penketh and Klein 1986).

We conclude that the performance of gene-deletion studies depends critically on the definition of objective functions. In our study, biomass yield was formulated as one factor that characterizes trypanosomal growth, however this failed to reproduce some experimental observations. The other controlling factor, H_2O_2 detoxification, was then incorporated as a second objective function. Optimization of this second objective function can be understood as minimizing the difference between the computed and experimentally reported values for H_2O_2 production. With both objective functions, the *in silico* predictions on
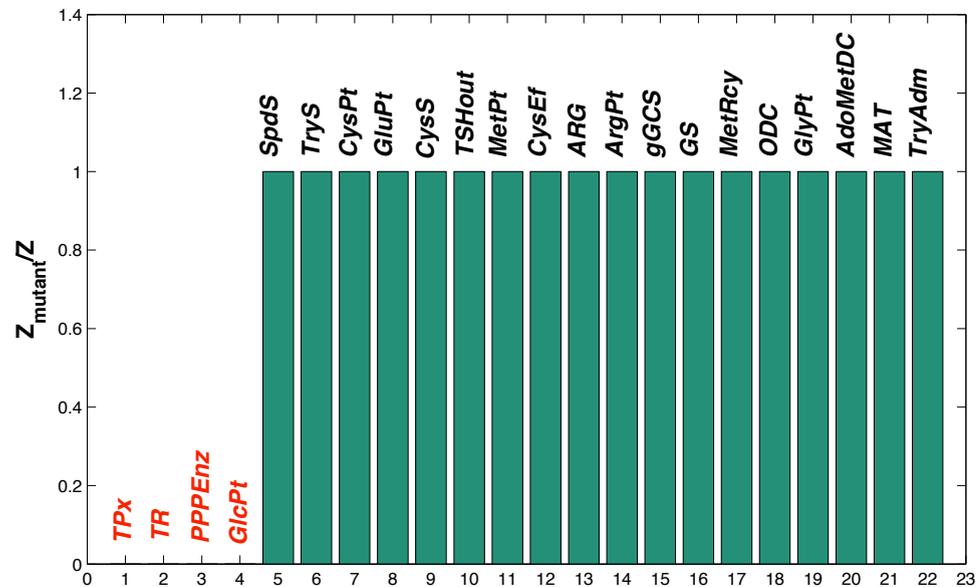
Figure 3.9: LP optimization for minimum residual error with respect to H_2O_2 production; residual error is between the calculated and measured H_2O_2 rate for all possible single gene deletions in the trypanothione metabolic pathway. The optimal value of the mutant objective function (Z_{mutant}) is compared with the 'wild-type' objective function (Z), where Z is defined in Equation (3.22). The green bars that have a value of 1 for the ratio Z_{mutant}/Z represent gene deletions that had the minimum residual error, which returned a calculated H_2O_2 production rate with the same value as the *in silico* wild type. The bars denoted with the reaction names in red indicate gene deletions that resulted in a calculated production rate of zero, giving rise to large residual errors.

gene essentiality by means of linear optimization provided good predictions of mutant phenotypes.

In spite of good consistency with available biological evidence, the approach failed on the identification of the enzyme responsible for cysteine assimilation. The incorrect classification of gene product CysPt (exogenous cysteine uptake) may be the result of multiple routes leading to cysteine production via either *de novo* biosynthetic pathway or exogenous uptake. In the case of single-gene knockouts, the inhibitory effect on one route will be compensated for by the other. As pointed out by Stelling et al. (Stelling et al. 2002), when pathway redundancy is present in the reaction network, qualitative predictions of gene essentiality may be problematic.

We therefore employ the method of control-coefficient analysis to quantita-

tively characterize the significance of individual reactions in supporting certain metabolic functions. This method can overcome the problem caused by pathway redundancy in the reaction network under study.

Control-effective Coefficients

Stelling et al. (Stelling et al. 2002) defined control-effective fluxes as representing "the importance of each reaction for efficient and flexible operation of the entire network". This method is dependent on the elementary modes and no optimization is needed.

Six elementary modes were derived from the mass-balance model, as shown in Figure 3.4. We investigate the efficiency of each elementary mode with respect to the objective functions of both maximum biomass yield and minimum residual error for H_2O_2 production. The results are presented in Figure 3.10, where the control-effective fluxes calculated for all the reactions fall into the range 0 to 1 (these quantities are dimensionless as they are coefficients).
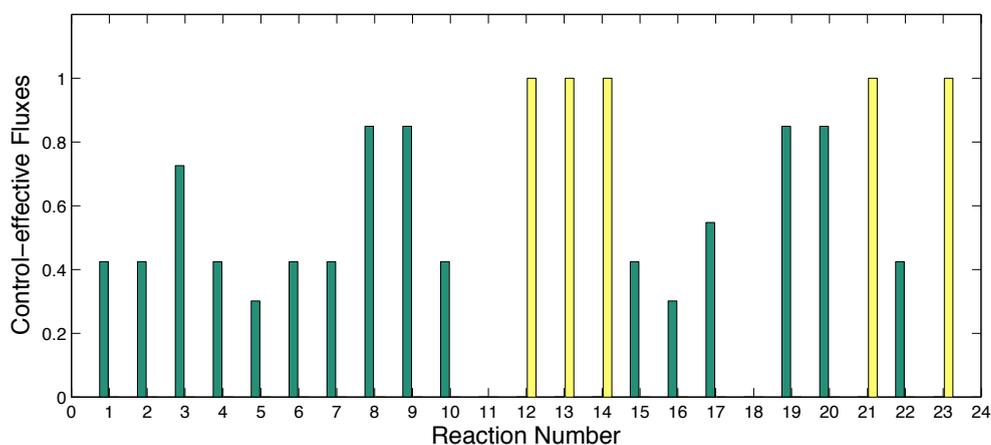
Figure 3.10: Control-effective fluxes of individual reactions of the trypanothione pathway; the x-axis indicates the reaction numbers in the mass-balance model, corresponding to reactions 1–21 listed in Table 3.1. Reactions 22 and 23 represent trypanothione consumption (υ_{tshout}) and H_2O_2 production ($r_{H_2O_2}$), detailed in Appendix B.2 and Appendix B.4. The green bars and yellow bars indicate the significance of individual reactions in supporting the objective of maximum biomass yield and minimum residual error concerning H_2O_2, respectively.

The *in silico* analysis indicates that reactions 11 and 18, trypanothione hydrolysis and cysteine excretion, are not essential for either objective. As expected, reactions located on trypanothione redox cycle and pentose phosphate

pathway are vital steps for H_2O_2 detoxification with the maximum control-effective flux values and the remaining reactions support maximum biomass yield. Reactions 8, 9, 19 and 20, which constitute the glutathione biosynthetic sub-pathway, were calculated to have the largest values, and are therefore the most pertinent for consideration as drug targets.

Reactions 5 and 16 (on the trans-sulfuration pathway) were the least relevant to optimum growth. It makes sense that depletion of the intracellular cysteine caused by the removal of this path can be compensated for by the assimilation of exogenous cysteine. It is noteworthy that with the method of control-effective flux analysis, reaction 17, cysteine uptake, was determined to be essential to the objective considered.

The method of control-effective flux analysis shows good consistency with the results derived from the *in silico* study of gene knockouts. The method successfully identified that non-negligible cysteine uptake is necessary for trypanothione biosynthesis, which corrected the prediction from gene deletions in the last section. The results demonstrate that, in the presence of pathway redundancy, the method of control-effective flux analysis is a more promising approach for the understanding of biological essentiality of individual reactions in support of certain metabolic functions.

3.6   Summary

While the structural modelling approach employed in this chapter is typically used for large-scale genomic models, the trypanothione metabolic pathway considered here is still complete and able to provide useful insights, even though it is relatively small in comparison. More importantly, as stated by Raman et al. (Raman et al. 2005), when only a single pathway is considered objective functions that are relevant to the problem in question can be more easily defined and given biological context.

Despite kinetic modelling being a more powerful modelling approach, structural analysis provides a more straightforward approach to the problem. It is computationally simpler but can still make accurate predictions that agree with experimental results. For predicting essentiality from gene knockouts, there is very little advantage in adopting the more complex kinetic modelling approach. Parameter estimation is also a complex problem for dynamic modelling when data is missing. However parameter estimation is not necessary for the structural

modelling approach, resulting in a more soluble problem with this approach.

Finally, the structural model can provide a guide for kinetic model construction and is useful for verifying consistency between the two modelling approaches in representing the pathway.

3.6.1   Related Work

The well-known application of mass-balance metabolic models is of the prediction of mutant-growth phenotypes. Additionally, as stated by Breitling et al. (Breitling et al. 2008), wrong predictions can also guide the iterative model improvement. As in many instances, a complete metabolic map is often hard to reconstruct, where the lack of experimental information or missing active reactions cause some functionalities to be excluded from the network (Herrgard et al. 2006, Kumar et al. 2007). The use of a model-driven evaluation of gene-deletion phenotypes in systematically tuning the model and hence improving our understanding of the biological system is demonstrated with the fully compartmentalized genome-scale metabolic model of *Saccharomyces cerevisiae* (Duarte et al. 2004).

The construction of metabolic pathways is largely dependent on the availability of biological evidence. It is demonstrated by the work of Nogales et al. (Nogales et al. 2008) where a genome-scale reconstruction of *P. putida* KT2440's metabolism was reliant on the integration of high-throughout genomic, biochemical and physiological information.

Herrgard et al. (Herrgard et al. 2006) introduced a method for identifying the modifications to be made for a genome-scale metabolic network in order to minimize the discrepancy between the predicted and observed fluxes. The selection of reactions to be added to or removed from the model is decided by a two-level optimization procedure, where the outer optimization problem searches through a set of reactions to include in or exlude from the model and the inner optimization problem produces a flux distribution as a solution to a flux estimation problem given a particular structure. One assumption imposed on their approach is that only a small fraction of the reactions in the model can be excluded from or included in the model, and for the purpose, some of the flux databases were used to train the model.

Kumar et al. (Kumar et al. 2007) developed an optimization based procedure to identify network gaps, which is demonstrated on the genome-scale

reconstruction of *Escherichia coli* and *Saccharomyces cerevisiae*. The authors postulated three types of modifications (detailed later in the text) to apply for model reconstructions. With the optimization procedure, the 'gap-free' model can be iteratively adjusted by comparing *in silico* predictions of growth phenotypes with *in vivo* experimental observations.

Reed et al. (Reed et al. 2006) used an optimization-based algorithm to identify the minimal number of metabolic reactions and transport reactions that needed to be added to the reconstructed network so that the computed cellular growth can agree with the experimental observations. A limitation to the computational approach implemented by the authors is that only the reactions stored in the database were examined as missing reactions for model reconstructions.

However, as a fundamental premise common to all the above related work, detection of potential sources of model mis-predictions can only be enabled when enough biological measurements, for example, *in vivo* growth rate, exchange flux (substrate uptake and byproduct secretion rate) and intracellular flux data, is available. In the work by Herrgard et al., the exchange fluxes have to be set explicitly to their measured values in order for at least one of the intracellular fluxes to be bounded for the inner optimization problem. Computational approaches proposed in the work by Kumar et al. and Reed et al. also took biological databases as essential sources where information regarding missing reactions can be obtained.

3.6.2   Conclusions and Discussion

Obtaining the metabolic information required to formulate the mass-balance model is relatively straightforward in comparison to constructing dynamic models where kinetics of individual rate equations, which are non-linear functions of parameters and metabolite concentrations, must be known. Structural modelling eliminates this requirement by treating the metabolic reaction fluxes as the unknown quantities that need to be determined. This approach expresses each balance equation as linear function of reaction rates.

Mass-balance models are useful in order to obtain a pathway-based perspective of metabolic functionality and phenotypes (Schilling et al. 2000). Studies relying on the stoichiometry of metabolic reaction systems have demonstrated that the underlying network topology limits the possible overall behaviour. A number of physiologically important results can be derived solely from the well-

known structure without knowledge of kinetic mechanisms and parameters.

We applied structural modelling approach to the trypanothione pathway in *T. brucei*, based on the concept of elementary modes, to understand trypanothione metabolism. Stelling et al. (Stelling et al. 2002) summarized that systems biology analysis centered on the elementary modes can assist in explanation of the relationship between network topology and metabolic functions, which can also facilitate the fundamental design of living cells.

We designed a computational investigation based on elementary mode analysis to detect network gaps in the structural topology of the trypanothione pathway. This refers to the situation where some metabolites or reactions are biologically important but are not used in metabolism. In the work by Kumar et al. (Kumar et al. 2007), network gaps in metabolic reconstructions can be 'filled up' by checking whether 1) reversing the directionality of existing reactions in the model, 2) adding new reactions from a multi-species database or finally 3) allowing for the direct importation (uptake and excretion) of the problem metabolite can restore flow into the metabolites.

The first two possibilities are not explored for the trypanothione pathway for the following reasons: (for the first) in the mass-balance model only the net intracellular fluxes are considered, which is independent of the reaction directionality and (for the latter) relevant biological information from *T. brucei* available for the addition of new reactions to the pathway is minimum. The third possibility is not applicable to the trypanothione metabolic pathway. In addition to the uptake/excretion fluxes that have already been incorporated, *T. brucei* is not capable of or obvious for the existing intracellular metabolites to be transported across the cell membrane.

Elementary mode decomposition allows a list of the metabolic functions that are supported by the elementary modes to be obtained, which gives an insight into the functional diversity of the network (Heinrich and Schuster 1996). This approach to studying the topological properties is useful in identifying essential routes in biochemical systems, for example, the purine metabolic pathway (Oancea 2003). The concept of elementary modes has been demonstrated as an established tool for studying system properties, namely to analyze network robustness and to investigate the importance of each reaction for effective and flexible operation of the entire network. System robustness was examined for the identified structural topologies by computing the elementary modes after successively simulating the knockout of each reaction. The robustness values computed

for the alternative network topologies ($ST1$ and $ST2$) were only marginally different and hence cannot be distinguished from each other. Nevertheless, the network topology ($ST1$) has biological support and is favored in our study.

Metabolic flux analysis delivers a metabolic flux map showing calculations of the steady state flux through each biochemical reaction in the network. The under-determined trypanothione metabolic pathway was solved for optimal steady state flux distributions with respect to an optimization criteria within the stoichiometrically defined domain, following which, the system translates into a LP problem (Varma and Palsson 1994). By means of this analysis method, the understanding of metabolic fluxes in response to external and genetic perturbations can be facilitated.

Metabolic fluxes in the trypanothione model determined via linear optimization revealed that the overall activity of metabolism is dominated by some high-flux reactions, while most other reactions have low fluxes. Particularly, arginine uptake has a determining role on trypanosomal growth. Metabolic flux analysis can also be employed to predict how perturbation of fluxes influence the extent of allowable states in the trypanothione pathway. The consequences of changing measured transport fluxes were also examined for all the unknown internal and exchange fluxes as well as the trypanosomal growth through a sensitivity analysis. The results indicate that most flux variables respond to variations in the measured transport fluxes in a local manner.

The capability of the trypanothione metabolic pathway to support cell growth through an *in silico* study of gene deletions with respect to different objectives was investigated. The consistency of two different objective functions with *in vivo* single-gene deletion mutants was examined in a qualitative base by observing the resulting optimization. We have found that the gene-deletion study of the trypanothione pathway can make predictions in a good agreement with experimental mutant phenotypes when both objective functions were applied (biomass yield and H_2O_2 residual). The importance of objective function definitions in constrained optimization problems has been evaluated by Burgard and Maranas (Burgard and Maranas 2003). In this chapter, the impact of objective function definitions on the performance of *in silico* gene deletion studies was also discussed.

In summary, a valid investigation relies on the network topology of interactions being correct. The consistency of *in silico* predicted and *in vivo* experimental mutant phenotypes indicates that the mass-balance model of the

trypanothione metabolic pathway is likely to be correct. Elucidation of the topological properties and flux distributions in the pathway provides an important and initial step toward the understanding of the regulation of trypanothione metabolism.

However, the time-dependent properties and regulatory principles of the pathway can only be examined with quantitative simulations when kinetic data becomes available. Thus, a structure-based modelling strategy is useful in combination with dynamic simulation of the non-linear differential equations of the patjway. The first kinetic model of the trypanothione metabolic pathway in *T. brucei* is given in Chapter 4, where the results of structural modelling guide the process of model simplification. This kinetic model will be strategically evaluated and refined with the proposed methodological framework in Chapter 7, where the steady-state flux distributions predicted by structural and kinetic modelling are compared.

Chapter 4

Kinetic Modelling of Trypanothione Metabolism in Bloodstream-form *Trypanosoma brucei*

In this chapter we develop a mathematical model of trypanothione metabolism in bloodstream-form *Trypanosoma brucei*.

4.1 Overview

The first basic kinetic model of trypanothione metabolism in *T. brucei* is described. The model is formulated based on an extensive literature search on the physical knowledge of the cell functions and available quantitative information about the system components and their interactions. The model is formulated with ODEs and applies Michaelis-Menten kinetics for the rate description of enzyme-mediated reactions.

The mechanistic kinetic model of the trypanothione metabolic pathway provides an integrated representation of the underlying biological processes that can aid the understanding of the trypanothione metabolism at the system level. Since mathematical models are manipulable, basic principles of metabolic regulations of the trypanothione pathway can be evaluated *in silico*. Hopes are that, by means of this model, successful therapeutic strategies for trypanosomal infection could be developed.

4.2 Computational Modelling of Trypanothione Metabolism

Trypanothione is a unique and essential compound for trypanosomatid parasitic protozoans. Despite its pivotal role in the survival of *Trypanosoma brucei*, the underlying physiological process is not comprehensively understood. This obstacle mainly arises from the complex interconnections among the system components and their reactions, which constitute a multitude of pathways integral to the trypanothione metabolism.

The precise knowledge of all involved enzymatic rate equations and associated parameter values is a prerequisite in the construction of kinetic models. Great efforts have been made to extract kinetic constants from bibliographic sources for both metabolite and enzyme levels in *T. brucei*. Information concerning the enzymes and metabolites is only partially known, and parameter values from different sources are often conflicting due to different cell lines or experimental conditions used in quantitative measurements. In particular, kinetic information is often determined for enzymes in isolated and purified forms, which may preclude the regulatory factors controlling the enzyme activity from consideration. However, there is little guidance available regarding how much parameter variability from the literature influences model simulation results.

Missing model parameters can be inferred from biological observations. Ranges for the unmeasured model parameters are derived empirically, guided by available biological information about the system. The intended use of the model is to understand the physiological operation of trypanothione metabolism and to elucidate the mechanisms of action of proven anti-parasitic agents. The kinetic model will be strategically evaluated and refined with the proposed methodological framework in Chapter 7.

4.3 Mathematical Model Description

van Reil (van Riel 2006) argued that many attempts of computational modelling pursue realistic large-scale complex models, but very often simplified models are feasible and at least as valuable in understanding the essential and qualitative features of biological systems.

In order to make the computational modelling of the trypanothione metabolic pathway more soluble, the following simplifications have been made:

1. *Intracellular glutamate and glycine are regarded as constant metabolites.* Biological knowledge about the assimilation of exogenous glutamate and glycine in *T. brucei* is limited and no experimental data is available for the kinetics of the corresponding enzyme transporters. On the contrary, intracellular concentrations of glutamate and glycine have been measured experimentally, reported at 3000 μM and 4000 μM, respectively (measured by the group of Mike Barrett at University of Glasgow). Elimination of uptake reactions of the exogenous metabolites reduces the available degrees of freedom of the model in simulation and as the dynamics of endogenous glutamate and glycine are unknown, treating the intracellular metabolite concentrations as constant will provide us with a feasible initial model with manageable complexity. In future use of the model, the impact of exogenous glutamate and glycine on trypanothione biosynthesis can be assessed by examining the influence of variations in intracellular concentrations of the metabolites.

2. *Consideration of the trans-sulphuration sub-pathway was limited to the first step only.* The pathway enzymes in sequence (Figure 3.1 in Chapter 3) including Methyltransferase, AdoHcy Hydrolyase, Cystathionine Syntase and Cystathionase are at best partly known. Analysis in Chapter 3 indicated that the trans-sulphuration sub-pathway was not critical to trypanothione biosynthesis and oxidative stress defense. In accordance with the principle that the intended use of the model should drive the modelling process, consideration of the trans-sulphuration sub-pathway was limited to the first step, which is concerned with converting AdoMet into AdoHcy under the catalysis of Methyltransferase. AdoHcy is defined as a constant metabolite since under normal conditions AdoHcy has to be removed rapidly due to its toxicity when accumulated (Bacchi et al. 1995) and in the presence of DFMO, no significant changes in the intracellular level of AdoHcy were observed after 36 hours of drug inhibition (Yarlett and Bacchi 1988).

3. *Cysteine excretion was eliminated.* Analysis in Chapter 3 indicated that exogenous cysteine was involved into two elementary modes fulfilling distinct metabolic functions and after the previous step of this simplification process, exogenous cysteine was found to participate into the mode of cysteine transport only. Under normal conditions, an increase in the branch

of endogenous cysteine excretion will always be cancelled by the opposite effect exerted by the other branch. Therefore, the mode of cysteine transport was not incorporated into the modelling process, and by consequence, the reaction branches involved in this elementary mode were removed from the model. Future work on the model could evaluate the biological role of cysteine excretion by varying the cysteine uptake activity and observing the effect on trypanothoine synthesis.

4. *Trypanothione participation in other cellular functions was eliminated.* In Chapter 3, trypanothione involved in the synthesis of DNA precursors and detoxification of metals and drugs was predicted to be necessary. Even though this prediction could be interpreted with the current biological evidence, it is however achieved with specific constraints made on missing reactions. No quantitative experimental data is available for the enzyme(s) catalyzing this reaction and its biological function in the regulation of trypanothione metabolism is unclear. Only verified enzymes and metabolites pertinent to the trypanothione metabolism are taken into account. This reaction is therefore eliminated from the network topology of the kinetic model.

As a consequence of the simplification process, the schematic representation of the trypanothione metabolic pathway given in Figure 3.1 (in Chapter 3) is reduced to the diagram depicted in Figure 4.1 for the kinetic modelling. It should be noted that due to the discussions in Section 3.5.4 in Chapter 4, H_2O_2 is modelled as a variable metabolite. This diagram presents us a complex system with metabolites participating in multiple reactions; this is complicated by the features associated with the system including homeostasis, feedback loops, recycling path, and enzyme bifunctionality.

We focus on continuous, deterministic descriptions of the dynamics by applying non-linear ordinary differential equations (ODE). The rate of enzyme mediated reactions for many enzymes are described with Michaelis-Menten kinetics. When the enzyme kinetic model involves regulators, the standard Michaelis-Menten kinetics is modified in accordance with the following rules.

Types of Regulation

An increase in the concentration of inhibitors will cause a decrease in the rate of production of product, which is in contrast to activators where an increase

Figure 4.1: Graphical representation of trypanothione metabolism for kinetic modelling.

in the concentration will give rise to a concomitant increase in the enzyme activity. There are three types of inhibitors, called *competitive*, *noncompetitive* and *uncompetitive*. These inhibitors represent a similar pattern in regulating enzyme kinetics of chemical reactions. For a simple reaction $S + E \longrightarrow P$, the Michaelis-Menten kinetic scheme can be formulated correspondingly. As uncompetitive inhibitors are not common and are difficult to design (Eisenthal and Cornish-Bowden 1998), the trypanothione metabolic pathway is limited to the following modes of regulation.

- **Competitive Inhibition**

$$\text{Rate} = \frac{k_{cat} \cdot E \cdot \dfrac{S}{K_m}}{1 + \dfrac{S}{K_m} + \dfrac{I}{K_i}}$$

- **Noncompetitive Inhibition**

$$\text{Rate} = \frac{k_{cat} \cdot E}{\left(1 + \dfrac{I}{K_I}\right)^{\gamma_i}} \cdot \frac{S}{K_m + S}$$

- **Substrate Inhibition**

$$\text{Rate} = \frac{k_{cat} \cdot E}{1 + \dfrac{K_m}{S} + \dfrac{S}{K_{si}}}$$

- **Product Activation**

$$\text{Rate} = \frac{k_{cat} \cdot E}{\left(1 + \dfrac{K_a}{A}\right)^{\gamma_a}} \cdot \frac{S}{K_m + S}$$

In the above schemes, k_{cat} represents the turnover number, expressed as number of substrate molecules turned into product per enzyme site per time unit. E and K_m are the total enzyme concentration and the half-saturation constant. K_i and K_a stand for the inhibitory and stimulating effect chemical compounds I and A exert on the enzyme activity, respectively. A regulatory term can be regarded as the concentration of chemical compounds divided by activation or inhibition constant. Apart from the scheme of substrate inhibition,

when the regulatory terms are set to zero, the above rate equations give identical kinetics to the standard Michaelis Menten. The parameters γ_i and γ_a represent the strength of inhibition and activation, respectively.

Structural analysis in Chapter 3 implies that the trypanothione metabolism in *T. brucei* is composed of two fundamental building blocks, including trypanothione biosynthesis and trypanothione redox system. These two building blocks appear to be spatially isolated in the network topology. This shows an agreement with observations in the study by Ravasz et al. (Ravasz et al. 2002) that cellular metabolism is best represented with a hierarchical and modular network. The hierarchical layout of the trypanothione pathway in *T. brucei* provides a natural breakdown of metabolism into relevant sub-networks. Further discussion on the subject is given in Section 7.8 in Chapter 7.

In the rest of this chapter, we summarize the model description for individual sub-networks responsible for the trypanothione biosynthesis and the trypanothione redox system. Trypanothione biosynthesis is achieved through integration of two elementary sub-pathways carrying out specific cellular functions, namely the polyamine biosynthetic sub-pathway and the glutathione biosynthetic sub-pathway. The trypanothione redox system is comprised of two metabolic processes, including the trypanothione oxidation and regeneration and the formation of NADPH and pentoses.

4.3.1   Polyamine Biosynthesis

Polyamines are ubiquitous cellular components essential for cell growth and division. In mammalian cells, the most common polyamines are putrescine, spermidine and spermine. ODC catalyses the initial step in the pathway leading to putrescine production from ornithine. In the presence of the aminopropyl donor decarboxylated AdoMet (dAdoMet), spermidine and spermine are synthesized from putrescine under the catalysis of spermidine synthase and spermine synthase respectively. AdoMetDC catalyses the conversion of S-adenosylmethionine (AdoMet) to the methyl donor dAdoMet.

ODC and AdoMetDC are rate-limiting enzymes that can be rapidly induced by various growth stimuli. A comprehensive review on *T. brucei* ODC and AdoMetDC can be found in (Persson 2007).

One primary use of polyamines in bloodstream-form trypanosomes lies in the formation of the conjugate GspdSH, which is an essential substrate for

trypanothione biosynthesis. It has been shown that trypanosomes depend on spermidine for growth and survival, which ceases when spermidine level drops below a certain level (Heby et al. 2007). There is therefore considerable therapeutic potential in compounds that disrupt polyamine biosynthesis (Wallace and Fraser 2004, Marton and Pegg 1995, Bacchi and Yarlett 2002, Kaiser et al. 2003). The antiparasitic drug, DFMO, for example, functions in a way that irreversibly interacts with ODC to reduce polyamine levels.

There is a dependence between levels of polyamines and viability and growth of parasites. In the parasitic organism *L. donovani*, polyamine supplementation can rescue intracellular pools of $\Delta adometdc$ (Roberts et al. 2002), Δodc (Jiang et al. 1999) and $\Delta spdsyn$ (Roberts et al. 2001)[1]. Unexpectedly, overexpression of the enzymes did not proportionally increase polyamine content (Roberts et al. 2007). It was found that lack of spermine (Kaur et al. 1986) and/or an increase in spermine pool (Ariyanayagam and Fairlamb 2001, Fairlamb et al. 1987, Bellofatto et al. 1987) did not affect trypanosomal growth rate. We therefore excluded spermine production from the model owing to its negligible role in the regulation of trypanothione metabolism.

Polyamine metabolism in mammalian cells has been studied using mathematical modelling (Rodriguez-Caso et al. 2006). The major differences between polyamine metabolism in mammals and trypanosomes lie in the specificity of metabolites and enzymes as well as the associated regulation patterns. The model of polyamine biosynthesis in *T. brucei* considers the enzyme activities and polyamine concentrations extracted from experimental studies. *T. brucei* lackes the capacity to assimilate exogenous performed radioactive putrescine and spermidine (Bacchi et al. 1980, Taylor et al. 2008). The polyamine biosynthetic sub-pathway takes exogenous methionine and arginine as inputs for the production of downstream metabolites. We also incorporate the enzymatic reaction converting AdoMet in S-adenosyl-L-homocysteine (AdoHcy) under the catalysis of methyltransferases due to its potential role in regulating AdoMet dynamics.

The polyamine biosynthetic sub-pathway is described mathematically by a set of rate equations for each of the *T. brucei* enzymes.

ODC Ornithine, putrescine and spermidine are metabolites common to both mammalian cells and trypanosome parasites. The major difference between enzyme ODC in the two families of organisms lies in their turnover rates (Persson

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[1]Δ stands for the enzyme knockout strains of parasites.

et al. 2003). ODC has an extremely short intracellular half-life in mammals, reportedly 15 min to 1 hr, which is in contrast to the more stable protein in *T. brucei* with a turnover rate greater than 6 hrs. As indicated by Ghoda et al. (Ghoda et al. 1990), the stability of *T. brucei* ODC results from the lack of the L-terminal PEST sequence (proline, glutamate, serine and threonine) necessary for rapid degradation and polyamine-dependent regulation.

Rapid turnover allows the enzyme to respond to regulatory stimuli promptly. Hayashi and Murakami (Hayashi and Murakami 1995) reported that exogenously added polyamines accelerated repression and destabilization of ODC activity in mammalian cells. On the contrary, *T. brucei* ODC activity in the procyclic form is unaffected by putrescine and spermidine, which indicates that the mechanism for polyamine-mediated regulation may be absent (Hua et al. 1995). The same argument with regard to the unresponsiveness of *T. brucei* ODC to polyamines was also made by Ghoda et al. (Ghoda et al. 1992). A conflicting experimental observation, however, reported by Osterman et al. (Osterman et al. 1994), which indicated that putrescine inhibited ODC with an inactivation coefficient equal to 350 ± 43 μM. The experimental work performed by Osterman et al. is considered as a relevant data source to our study for its known validity.

A mathematical rate expression for ODC is modelled in the form

$$V_{ODC} = k_{cat}^{ODC} \cdot [ODC] \cdot \frac{\dfrac{[Orn]}{K_{mOrn}^{ODC}}}{1 + \dfrac{[Orn]}{K_{mOrn}^{ODC}} + \dfrac{[Put]}{K_{iP}^{ODC}}} \tag{4.1}$$

where k_{cat}^{ODC} is the turnover number, and K_{mOrn}^{ODC} stands for the half-saturation constant of the enzyme by ornithine (Orn). K_{iP}^{ODC} is the constant of competitive inhibition by putrescine (Put). Square brackets $[\,\cdot\,]$ stand for intracellular concentrations of the corresponding metabolites. The same notation scheme is applied to other equations below.

In the presence of DFMO, ODC is irreversibly deactivated. A modified expression of the dynamic velocity of ODC is written below:

$$V_{ODC}^{DFMO} = k_{cat}^{ODC} \cdot \frac{[ODC]}{1 + \dfrac{[DFMO]}{K_{iDFMO}^{ODC}}} \cdot \frac{\dfrac{[Orn]}{K_{mOrn}^{ODC}}}{1 + \dfrac{[Orn]}{K_{mOrn}^{ODC}} + \dfrac{[Put]}{K_{iP}^{ODC}}} \tag{4.2}$$

where K_{iDFMO}^{ODC} represents the inhibition constant from the inhibitor DFMO.

As ODC activity responds to DFMO in a time and concentration dependent manner, the dynamics of intracellular DFMO must be known. *T. brucei* ODC has a higher K_{iDFMO}^{ODC} value of 130 μM for DFMO than that of the mammalian enzyme (39 μM) (Bitonti et al. 1985). Phillips et al. (Phillips et al. 1988) reported an even higher value on K_{iDFMO}^{ODC} around 160±60 μM. A differential equation for the transient DFMO concentration is expressed as follows

$$\frac{d[DFMO]}{dt} = k_s^{DFMO} - k_d^{ODC} \cdot [DFMO] \cdot V_{max}^{ODC}$$

where k_s^{DFMO} represents the uptake parameter of the inhibitor and V_{max}^{ODC} is the maximum velocity of the enzyme at the normal condition.

However, the DFMO uptake kinetics have not been rigorously characterised due to a few of factors involved in the process; drug transport and intracellular distribution, for instance. Procyclic-form *T. brucei* was reported to take up DFMO at a linear rate for the initial 60 mins (Phillips and Wang 1987). However, for simulation of the dynamics of drug-enzyme interactions over 4 days (the time span considered in model validation), not enough information is available for an unambiguous characterization of the kinetics of DFMO uptake over the prolonged time duration.

Despite the absence of a quantitative description, the DFMO-induced suppression of ODC activity is well understood in a qualitative sense. ODC activity was found to be completely blocked after a certain period of treatment with DFMO, which caused the amount of putrescine to drop below the experimentally detectable level (Bacchi et al. 1983, Fairlamb et al. 1987, Bellofatto et al. 1987).

In view of the qualitative knowledge, ODC activity in response to DFMO is modelled with an exponential decay function below that reflects the time-dependent pattern of the drug inhibition, expressed as

$$V_{max}^{ODC} \cdot e^{-\lambda \cdot t} \tag{4.3}$$

where λ stands for the decay rate of ODC under DFMO treatment, and can be obtained by simple curve fitting, resulting in a value 0.007.

Table 4.1 contains parameter values for *T. brucei* ODC.

AdoMetDC AdoMetDC is an essential enzyme for the formation of dAdoMet, the methyl donor for the biosynthesis of spermidine from putrescine. As is the

Parameters	Description	Value/Ranges	Source
V_{max}^{ODC}	maximum velocity	45 μmol/min/mg	(Phillips et al. 1988)
MW	molecular weight	45000	(Phillips et al. 1987)
k_{cat}^{ODC}	turnover rate	444$\pm$4.8 min^{-1}	(Osterman et al. 1994)
K_{mOrn}^{ODC}	half-saturation constant	160 μM or	(Bitonti et al. 1985)
		280$\pm$30 μM	(Phillips et al. 1988)
K_{iP}^{ODC}	inhibition constant	350$\pm$43 μM	(Osterman et al. 1994)
K_{iDFMO}^{ODC}	DFMO inhibition	220$\pm$70 μM	(Phillips et al. 1988)
λ	decay rate	0.007	*Analytical Solution*

Table 4.1: Parameter values for *T. brucei* ODC.

case for *T. brucei* ODC, *T. brucei* AdoMetDC is a stable enzyme and has a lower turnover rate than that in mammalian cells. AdoMetDC is a regulatory enzyme within the polyamine biosynthetic sub-pathway. Regulatory enzymes can respond to chemical signals with an increase or decrease in their activities. Metabolic pathways are regulated by controlling the activity of one or more enzymatic steps along the path. Allosteric control refers to allosteric enzymes that have distinct binding sites for effector molecules which control their reaction rates.

AdoMetDC is regulated by an allosteric mechanism with 'prozyme', an enzymatically inactive close homologue of AdoMetDC iteself. Prozyme induces a conformational change from an inactive structure to an active one; the binding of AdoMetDC with prozyme is postulated to enable a dynamical control on metabolic fluxes through the polyamine pathway (Willert et al. 2007). A similar mechanism of allosteric regulation was also found for *Trypanosoma cruzi* AdoMetDC (Beswick et al. 2006).

Willert et al. (Willert et al. 2007) discovered a regulatory mechanism in *T. brucei* that neither AdoMetDC or prozyme *per se* is sufficiently active to prompt normal cell growth, and only the complex of AdoMetDC|prozyme can maintain the physiological level of spermidine. Willert et al. drew a conclusion that putrescine stimulates the activity of AdoMetDC but has no effect on the AdoMetDC|prozyme heterodimer.

Recent work by Willert and Phillips (Willert and Phillips 2008) has extended the subject to examining the influence of AdoMetDC RNAi inhibition

and prozyme knockout on polyamine synthesis and parasite growth. In accordance with the study by Willert and Phillips, we elaborate an enzyme-ligand binding reaction describing the transition of the free inactive conformer (E') into a ligand-occupied active conformer (E^L), which complies with the formula of the total enzyme concentration $E^T = E^O + E^L$, where L stands for ligand.

$$E^O + L \rightleftharpoons E^O L \qquad (4.4)$$

Prozyme regulates *T. brucei* AdoMetDC by altering the value of k_{cat}, giving rise to the so-called 'V-system', which is in contrast to 'K-system' that alters the affinity for substance (Birnbaumer et al. 1980). Here we model the ligand-enzyme binding reaction as a one-step conformation system, with the assumption that the ligand can rapidly interact with the enzyme. This assumption makes sense given the observation that prozyme concentration is not comparable to the enzyme concentration of AdoMetDC (Willert et al. 2007), giving rise to the reaction occurring at a rapid-equilibrating rate following the linear mass action kinetics (i.e. [E]≫[S]). Because of the limited amount of prozyme in parasites, AdoMetDC is present in the cell in both ligand-occupied form and free form. Accordingly, we express the velocity equation of the total AdoMetDC as a superposition of two terms stemming from individual form of the enzyme. The representation of regulatory capabilities in summation of distinct states has been verified for allosteric enzymes in (Birnbaumer et al. 1980).

AdoMet decarboxylase (AdoMetDC) was reported to be strongly or weakly activated by putrescine (Bitonti et al. 1986, Tekwani et al. 1992, Willert et al. 2007). Inspection of the details of the experiments that led to these opposing conclusions showed that different basal levels of putrescine were used by the different groups and this possibly explains the apparent contradiction. Bitonti et al. and Willert et al. (Bitonti et al. 1986, Willert et al. 2007) both used crude extracts rich in putrescine. Tekwani et al. (Tekwani et al. 1992) however used dialyzed extract without putrescine. In the study of Tekwani et al., addition of 500 μM putrescine gave a 10 fold jump in AdoMetDC activity, which plateaued at 12 fold enhanced activity with higher concentrations. *In vivo* putrescine content was reported at 517 μM (Fairlamb et al. 1987) or 678 μM (Willert and Phillips 2008), suggesting that the 'strong' putrescine activation of AdoMetDC observed in the study by Tekwani et al. would not be achieved in *in vivo* environment. Observations in the works by Bitonti et al. and Willert et al.

are therefore considered as relevant data sources to our study.

A mathematical rate expression of the total AdoMetDC is shown below:

$$V_{AdoMetDC^T} = V_{AdoMetDC^L} + V_{AdoMetDC^O} \tag{4.5}$$

where $V_{AdoMetDC^L}$ and $V_{AdoMetDC^O}$ stand for the velocity contributed by the ligand-occupied and free form of the enzyme, modelled as follows

$$V_{AdoMetDC^L} = k_{cat}^{AdoPro} \cdot [AdoMetDC^L] \cdot \frac{\dfrac{[AdoMet]}{K_{mAdoMet}^{AdoPro}}}{1 + \dfrac{[AdoMet]}{K_{mAdoMet}^{AdoPro}}} \tag{4.6}$$

$$V_{AdoMetDC^O} = k_{cat}^{AdoMetDC^O} \cdot [AdoMetDC^O] \cdot \tag{4.7}$$

$$\frac{\dfrac{[AdoMet]}{K_{mAdoMet}^{AdoMetDC^O}}}{1 + \dfrac{[AdoMet]}{K_{mAdoMet}^{AdoMetDC^O}} + \dfrac{[dAdoMet]}{K_{idAdoMet}^{AdoMetDC^O}} + \dfrac{[K_{aPut}^{AdoMetDC^O}]}{[Put]}}$$

where $k_{cat}^{AdoMetDC^O}$, $K_{mAdoMet}^{AdoMetDC^O}$ and k_{cat}^{AdoPro}, $K_{mAdoMet}^{AdoPro}$ stand for the turnover rate and half-saturation constant to substrate AdoMet of the free ($AdoMetDC^O$) and ligand-occupied ($AdoMetDC^L$) form, respectively. Concentration of the free-form AdoMetDC can be calculated by reducing the total enzyme concentration by the ligand-binding portion

$$[AdoMetDC^O] = [AdoMetDC^T] - [AdoMetDC^L]$$

$K_{aPut}^{AdoMetDC^O}$ and $K_{idAdoMet}^{AdoMetDC^O}$ describe the activation and competitive inhibition exercised by putrescine and dAdoMet. No numbers have been reported for either parameter in *T. brucei*. We derived the constant $K_{aPut}^{AdoMetDC^O}$ from (Bitonti et al. 1986) that gave a value of 1.5 μM. *T. brucei* AdoMetDC was thought to be insensitive to dAdoMet, which is in contrast to the strong inhibition exerted by dAdoMet on the enzyme in many other species (Pegg and Jacobs 1983). We apply a range between 1 to 1000 μM to parameter $K_{idAdoMet}^{AdoMetDC}$ as the quantitative description of *T. brucei* AdoMetDC in response to product dAdoMet. The parameter boundaries are wide enough to cover the possibilities of both weak and strong product inhibition. The parameter estimates from *in silico* simulations can be used to qualitatively assess the biological observations.

The formation and dissociation of complex AdoMetDC|Prozyme are de-

scribed as an irreversible one-step binding model and a linear decay response, as shown below

$$R_b^{AdoMetDC^L} = k_b^{AdoMetDC^L} \cdot [AdoMetDC^T] \cdot [Prozyme] \qquad (4.8)$$

$$R_d^{AdoMetDC^L} = k_d^{AdoMetDC^L} \cdot [AdoMetDC^L] \qquad (4.9)$$

where $k_b^{AdoMetDC^L}$ and $k_d^{AdoMetDC^L}$ stand for the binding and dissociation rate of the ligand-enzyme interaction. The complex was formed at high affinity with the dissociation constant smaller than 0.5 μM (Willert et al. 2007); this gives the functional relation as $k_d^{AdoMetDC^L} = 0.5 * k_b^{AdoMetDC^L}$ to eliminate one unknown parameter from the model.

According to the recent experimental observations (Willert and Phillips 2008), an induction of *T. brucei* ODC activity was detected when AdoMetDC or prozyme was inhibited or knocked out. Inhibition of polyamine contents on ODC activity that has been demonstrated in mammalian cells was not applicable to *T. brucei*. Willert and Phillips hypothesized that the de-repression of ODC protein expression could be ascribed to the reduced inhibition by AdoMetDC activity, since when AdoMetDC protein expression is diminished, less ODC mRNA strands would be consumed by AdoMetDC, thus more ODC mRNA could be translated. Quantitatively, AdoMetDC knockdown induced gene expression of ODC and prozyme up to an average of 10 to 25 fold the controlled level; exogenous spermidine (100 μM) weakened the stimulating strength but still enabled 5 to 10 fold increases in the enzyme activity. Knockout of prozyme also boosted ODC expression by 4-5 fold.

To reflect this observation, enzyme ODC is modelled as a variable dependent on the expression level of AdoMetDC. Equations (4.10) and (4.11) describe the synthesis term of Prozyme and ODC, respectively, with the expression level of AdoMetDC and prozyme included in the lower part of the formulas to illustrate the regulatory effect they exercise.

$$R_s^{Prozyme} = \left(\frac{k_s^{prozyme}}{1 + [AdoMetDC^T]} \right) \cdot \left(1 + \frac{1}{[Spd]} \right) \qquad (4.10)$$

$$R_s^{ODC} = \left(\frac{k_s^{odc}}{1 + k_{eq}([AdoMetDC^T] + [Prozyme])} \right) \cdot \qquad (4.11)$$

$$\left(1 + \frac{1}{[Spd]} \right)$$

$$R_d^{ODC} = k_d^{ODC} \cdot [ODC] \qquad (4.12)$$

where $k_s^{prozyme}$ and k_s^{odc} represent the production coefficients of prozyme and ODC, respectively. k_{eq} acts as the scaling factor tackling distinct intracellular amounts of AdoMetDC and prozyme. k_d^{ODC} is the degradation constant of ODC.

AdoMetDC is a good therapeutic target in *T. brucei* (Wallace 1996). AbeAdo[2], an enzyme-activated inhibitor of AdoMetDC, was more potent than DFMO in curing *T. brucei* infections (Marton and Pegg 1995). In mammalian cells, dAdoMet does not accumulate under normal conditions because of its rapid utilization in spermidine synthesis. However, DFMO-treated cells display a striking accumulation of this metabolite due to the lack of putrescine for aminopropyltransferase reactions and the elevation of AdoMetDC activity.

In mammalian cells, AdoMetDC activity was detected to elevate threefold over the normal value within 12 hrs of DFMO addition and then decreased slightly, with AdoMet staying relatively constant (Mamont et al. 1982). This is in contrast to drug-treated *T. brucei* where the levels of AdoMet and dAdoMet were significantly increased (Fairlamb et al. 1987) concomitant with an approximately 30-70 percent decrease in AdoMetDC activity (Bacchi et al. 1983). Experiments on AdoMetDC down-regulation also revealed a decrease in AdoMet use for polyamine synthesis and an increase in available AdoMet for tryansmethylation reactions (Selzer et al. 1996).

Table 4.2 contains parameter values for *T. brucei* AdoMetDC.

MAT: Methionine Adenosyltransferase   In trypanosomes, MAT catalyzes the synthesis of AdoMet from methionine in the presence of ATP. AdoMet plays an important role in a variety of cellular functions, such as methylation, sulphuration and polyamine. Polyamines are not inhibitory to the enzyme, and AdoMet only exercises weak inhibition on the enzyme activity (Yarlett et al. 1993). Interference with *T. brucei* polyamine biosynthesis by DFMO resulted in a dramatic increase in the intracellular AdoMet and dAdoMet pools (Fairlamb et al. 1987, Bellofatto et al. 1987), and a two-fold increase in AdoMet synthetase activity *in vivo* (Yarlett et al. 1991). A mathematical rate expression of AdoMet synthetase is modelled in the form

$$V_{MAT} = \left(\frac{V_{max}^{Met} \cdot \frac{[Met]}{K_{mMet}^{MAT}}}{1 + \frac{[Met]}{K_{mMet}^{MAT}} + \frac{[AdoMet]}{K_{iAdoMet}^{MAT}}} \right) \cdot \left(\frac{V_{max}^{ATP} \cdot \left(\frac{[ATP]}{K_{mATP}^{MAT}} \right)^n}{1 + \left(\frac{[ATP]}{K_{mATP}^{MAT}} \right)^n} \right) \quad (4.13)$$

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[2]Systematic name: 5'-{[(Z)-4-Amino-2-butenyl]methylamino}-5'-deoxyadenosine

Parameters	Description	Value/Ranges	Source
$k_{cat}^{AdoMetDC^O}$	turnover rate	$0.078\pm0.024\ min^{-1}$	(Willert et al. 2007)
k_{cat}^{AdoPro}	turnover rate	$84\pm6\ min^{-1}$	(Willert et al. 2007)
$K_{mAdoMet}^{AdoPro}$	half-saturation constant	$110\pm20\ \mu$M	(Willert et al. 2007)
$K_{mAdoMet}^{AdoMetDC^O}$	half-saturation constant	$380\pm150\ \mu$M or $30\ \mu$M	(Willert et al. 2007) (Bitonti et al. 1986)
$K_{idAdoMet}^{AdoMetDC^O}$	inhibition constant	1-$1000\ \mu$M	*Modelling Assumption*
$K_{aPut}^{AdoMetDC^O}$	activation constant	$1.5\ \mu$M	*Analytical Solution*
k_b	binding constant	0–$10\ \mu$M/min	*Modelling Assumption*
$k_s^{prozyme}$	binding constant	0–$1\ \mu$M/min	*Modelling Assumption*
k_s^{odc}	synthesis constant	0–$1\ \mu$M/min	*Modelling Assumption*
k_d^{odc}	degradation constant	0–$0.1\ \mu$M/min	*Modelling Assumption*
k_{eq}	scaling constant	0–$1\ \mu$M/min	*Modelling Assumption*
$[AdoMetDC^T]$	enzyme concentration	1e-4–$1\ \mu$M	*Modelling Assumption*
$[AdoMetDC^O]$	enzyme concentration	1e-4–$1\ \mu$M	*Modelling Assumption*

Table 4.2: Parameter values for *T. brucei* AdoMetDC.

where kinetic parameters of the enzyme are substrate-concentration dependent; V_{max}^{Met}, V_{max}^{ATP} and K_{mMet}^{MAT}, K_{mATP}^{MAT} denote the maximum velocity and half-saturation constant for each of the two reactants, respectively. $K_{iAdoMet}^{MAT}$ stands for the competitive inhibition by AdoMet. n is the Hill rate of the enzyme activity with respect to co-substrate ATP.

The maximum velocity of MAT has never been reported in *T. brucei*, and the only measurement available for the enzyme was observed in parasitic organism *L. infantum* by Reguera et al. (Reguera et al. 2001)[3]. For this reason, the enzyme activity of MAT is considered as an unknown parameter during the *in silico* simulation of the kinetic model. We thus refined Equation (4.13) by using a single parameter, V_{max}^{MAT}, to represent the enzyme maximum velocity, which

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[3]MAT with methionine and ATP as substrates were measured to hold the maximum activity of 0.19 μmol/mg/min and 0.12 μmol/mg/min, respectively.

results in the rate equation below

$$V_{MAT} = V_{max}^{MAT} \cdot \left(\frac{\frac{[Met]}{K_{mMet}^{MAT}}}{1 + \frac{[Met]}{K_{mMet}^{MAT}} + \frac{[AdoMet]}{K_{iAdoMet}^{MAT}}} \right) \cdot \left(\frac{\left(\frac{[ATP]}{K_{mATP}^{MAT}}\right)^n}{1 + \left(\frac{[ATP]}{K_{mATP}^{MAT}}\right)^n} \right) \quad (4.14)$$

Table 4.3 contains parameter values for *T. brucei* MAT. In the model, we consider ATP as a constitutely supplied protein, since the knowledge concerning ATP generation and energy conversion with ADP is lacking. ATP concentration is fixed at its normal cellular value, approximately 4000 μM.

Parameters	Description	Value/Ranges	Source
V_{max}^{MAT}	maximum velocity	1e-3–20 μmol/mg/min	*Modelling Assumption*
K_{mMet}^{MAT}	half-saturation constant	20 μM w.r.t. [ATP]$\subset$10-250 μM 200 μM w.r.t. [ATP]$\subset$0.5-5 mM	(Yarlett et al. 1993)
K_{mATP}^{MAT}	half-saturation constant	53 μM w.r.t. [Met]$\subset$10-250 μM 1750 μM w.r.t. [Met]$\subset$500-5000 μM	(Yarlett et al. 1993)
$K_{iAdoMet}^{MAT}$	inhibition constant	240 μM	(Yarlett et al. 1993)
[ATP]	concentration in cytosol	4000 μM	*Normal Value*

Table 4.3: Parameter values for *T. brucei* MAT.

A common feature of MAT protein in parasitic protozoans lies in its sigmoidal kinetics for both methionine and ATP (Reguera et al. 2007), which display a concentration dependent pattern. It was evidenced in (Yarlett et al. 1993) that non-cooperativity was shown for low methionine and ATP concentrations, whilst positive cooperativity of the enzyme was realized at higher concentrations of ATP with a Hill rate equal to 2.0.

ARG: Arginase Arginase is a metalloenzyme that plays key roles in many pathways involving arginine catabolism and metabolism in cells. Comprehensive reviews on mammalian arginine metabolism are presented elsewhere (Wu and Sidney M. Morris 1998, Li et al. 2001, Sidney M. Morris 2002).

In parasitic protozoan, role of arginase is not limited to polyamine biosynthesis. It has been postulated to be pivotal in helping parasitic species *Leishmania* (Vincendeau et al. 2003) and *T. cruzi* (Peluffo et al. 2004) escape immune responses by modulating Nitric oxide (NO) production upon macrophage infection. NO is toxic to parasites, and is produced in macrophages by NO synthase from arginine. A correlation between arginine metabolism and the cell replication rate was proposed for *T. cruzi* (Pereira et al. 2002).

Vincendeau et al. (Vincendeau et al. 2003) argued that arginine concentration could play a critical role in determining NO toxicity to trypanosomes by macrophages, where the authors observed that an arginine content greater than 400 μM led to total parasite death. The same effect was obtained with addition of an arginase inhibitor, e.g. N^w-hydroxy-L-arginine, even for low concentrations of intracellular arginine (Iniesta et al. 2001).

Gobert et al. (Gobert et al. 2000) presented the only or one of the few experimental works to study the arginase up-regulation in macrophages from *T. brucei*. The authors demonstrated that arginase and NO synthase competed for substrate availability, and an early and fast induction of arginase activity could restore the death of *T. brucei* caused by NO synthase induction. Specifically, observations during the first 24 hours of trypanosomal infection displayed a linear and time dependent utilization of arginine in ornithine production with a percentage of consumption equal to 2.7 per hour.

Kropf et al. (Kropf et al. 2005) reported that arginine in *Leishmania* parasites can be converted to NO. The balance between the reactions consuming arginine – NO and ornithine production – appears to be determined by the requirement for parasite growth. When arginase, which catalyzes ornithine production, is inhibited or knocked out, arginine participation in NO production is enhanced to prevent the accumulation of intracellular arginine. Kropf et al. observed that when arginase is inhibited, parasite growth can be restored by the addition of ornithine from an external source. This supports the conclusion that arginase activity directly regulates parasite growth.

Arginine dynamics in response to DFMO have only been examined in procyclic-form *T. brucei* (Bellofatto et al. 1987) and have never been studied in the bloodstream form. We attempt to understand the arginine metabolism in bloodstream-form *T. brucei* by explicitly incorporating the reactions of exogenous arginine uptake and ornithine production into the model. Since little knowledge about *T. brucei* arginase can be found from the literature, we started with a standard

Michaelis-Menten kinetics as follows

$$V_{ARG} = V_{max}^{ARG} \cdot \frac{\dfrac{[Arg]}{K_{mArg}^{ARG}}}{1 + \dfrac{[Arg]}{K_{mArg}^{ARG}}} \tag{4.15}$$

where V_{max}^{ARG} and K_{mArg}^{ARG} stand for the maximum velocity and half-saturation constant to substrate arginine (Arg). Experimental measurements in *L. donovani* arginase (Silva et al. 2007) were used in our model, as a substantial amount of similarity exists between the two parasitic organisms.

Table 4.4 contains parameter values for *L. denovani* ARG.

Parameters	Description	Value/Ranges	Source
V_{max}^{ARG}	maximum velocity	144.9 μmol/mg/min	(Silva et al. 2007)
K_{mArg}^{ARG}	half-saturation constant	21500 μM	(Silva et al. 2007)

Table 4.4: Parameter values for *L. donovani* ARG.

SpdS: Spermidine Synthase Interference with polyamines has been considered as an efficient means of parasite destrubtion. Consequently, enzymes, responsible for polyamine biosynthesis have been the focus of anti-trypanosomal research. Compared with other enzymes (e.g. ODC and AdoMetDC) in the pathway, the biochemical properties of SpdS that mediates spermidine production are not well understood.

The only two studies available for *T. brucei* SpdS however return us again the fact that different groups frequently present conflicting observations on the enzyme activity. Taylor et al. (Taylor et al. 2008) detected a V_{max} of 0.0119±0.0014 μmol/min/mg, whereas Bitonti et al. (Bitonti et al. 1984) argued that the specific activity of SpdS in trypanosomes is comparable to the highest enzyme activity in mammalian tissues, which is reported to vary between 0.43 and 10.95 μmol/min/mg (Rodriguez-Caso et al. 2006).

As demonstrated by Taylor et al. (Taylor et al. 2008), *T. brucei* does not have an efficient mechanism for the uptake of exogenous spermidine, and relies on *de novo* synthesis to acquire this polyamine. Within 96 hours of switching down expression of spermidine synthase, there was a 33% decrease in the growth rate, with a reduction in *TbSpSyn* mRNA and a 60% decline in spermidine contents;

putrescine was found to drop to 40% of the normal value. The kinetic mechanism of the enzyme is modelled in equation (4.16).

dAdoMet is present in parasites with a low intracellular concentration (Yarlett et al. 1993). Methylthioadenosine (MTA) is the second product formed during the transfer of an aminopropyl group from dAdoMet to putrescine. MTA is not detectable in mammals because of its rapid degradation rate (Rodriguez-Caso et al. 2006). In *T. brucei*, MTA is rapidly consumed by MTA phosphorylase to adenine and 5'-methylthioribose-1-phosphate, where the latter is then converted back to methionine (Berger et al. 1996) (More details can be found later in this chapter). Owing to the rapid metabolization of MTA, the intracellular concentration of this compound is low (Peter S. Backlund et al. 1982). Spermidine, however, is present in millimolar concentrations. The spermidine-putrescine ratio of intracellular concentrations in *T. brucei* was given in the range of 2:1 (Taylor et al. 2008) or 19:1 (Bacchi et al. 1977) in different studies.

A mathematical rate expression for SpdS is modelled in the form

$$
V_{SpdS} \;=\; V_{max}^{SpdS} \cdot \frac{\dfrac{[dAdoMet]}{K_{mdAdoMet}^{SpdS}}}{1 + \dfrac{[dAdoMet]}{K_{mdAdoMet}^{SpdS}} + \dfrac{[MTA]}{K_{iMTA}^{SpdS}}} \cdot \frac{\dfrac{[Put]}{K_{mP}^{SpdS}}}{1 + \dfrac{[Put]}{K_{mP}^{SpdS}} + \dfrac{[Spd]}{K_{iD}^{SpdS}}} \quad (4.16)
$$

where V_{max}^{SpdS} is the maximum velocity of SpdS. $K_{mdAdoMet}^{SpdS}$ and K_{mP}^{SpdS} represent half-saturation constant to substrate dAdoMet and putrescine. K_{iD}^{SpdS} and K_{iMTA}^{SpdS} are the constants of competitive inhibition by spermidine (Spd) and MTA, respectively.

Table 4.5 contains parameter values for *T. brucei* SpdS.

Enzyme involved in methionine recycling from MTA   MTA, the byproduct of aminopropyl group transfer from dAdoMet, is recycled to methionine via a series of enzymatic steps in trypanosomes (Sufrin et al. 2008). The MTA recycling path has been illustrated in mammals (Peter S. Backlund and Smith 1981), and also observed in parasitic protozoa (Berger et al. 1996, Reguera et al. 2007). MTA is first converted to methylthioribose-1-phosphate by MTA phosphorylase; the latter product is then metabolized to keto-methylthiobutyrate, which is finally transaminated to methionine.

Methionine has a unique role in cellular growth, originating in its metabolic conversion to AdoMet, which then participates in several downstream pathways

Parameters	Description	Value/Ranges	Source
V_{max}^{SpdS}	maximum velocity	0.0119μmol/mg/min	(Taylor et al. 2008)
$K_{mdAdoMet}^{SpdS}$	half-saturation constant	0.09 ± 0.01 μM	(Taylor et al. 2008) (Bitonti et al. 1984)
K_{mP}^{SpdS}	half-saturation constant	205 ± 65 μM	(Taylor et al. 2008) (Bitonti et al. 1984)
K_{iD}^{SpdS}	inhibition constant	100 μM	(Taylor et al. 2008)
K_{iMTA}^{SpdS}	inhibition constant	1–1000 μM	*Modelling Assumption*

Table 4.5: Parameter values for *T. brucei* SpdS.

of polyamine biosynthesis and methylation reactions. A constant supply of methionine is imperative for cell proliferation, which requires adequate AdoMet in physiological functions. The amount of methionine in the environment is limited and *de novo* synthesis is energetically expensive (Riscoe et al. 1989). MTA recycling therefore seems to be an important source of this amino acid.

Because of the importance of MTA recycling in retaining cell viability, interference with methionine metabolism has been explored as a potential target for chemotherapy for African trypanosomiasis (Porter and Sufrin 1986, Bacchi et al. 1991, Riscoe et al. 1989, Sufrin et al. 1995).

It has been established that methionine is regenerated via a multistep metabolism of MTA, kinetics for which, however, are not available experimentally. Available quantitative descriptions for the recycling path limit to the half-saturation constant of MTA phosphorylase with respect to its substrate MTA. Since the enzyme has a broad substrate specificity (Ghoda et al. 1988), the *in vivo* maximum velocity is hard to obtain, but it is assumed to hold a very high value (Bacchi et al. 1991).

MTA degradation is important; its accumulation is potentially toxic to mammalian cells (Christa et al. 1988). MTA can be degraded through normal protein efflux or utilization in the recycling path. In our study, we consider methionine regeneration as the major route of MTA consumption, which is assumed to occur via a single-step reaction. 'MetRcy' is used to denote the enzyme catalyzing this reaction. Standard Michaelis-Menten kinetics are applied to describe the

enzyme kinetics, shown below:

$$V_{MetRcy} \;\; = \;\; V_{max}^{MetRcy} \cdot \frac{\dfrac{[MTA]}{K_{mMTA}^{MetRcy}}}{1 + \dfrac{[MTA]}{K_{mMTA}^{MetRcy}}} \tag{4.17}$$

where V_{max}^{MetRcy} and K_{mMTA}^{MetRcy} are the maximum velocity of the enzyme and half-saturation constant to substrate MTA.

Table 4.6 contains parameter values for *T. brucei* MetRcy catalyzing the methionine regeneration from MTA.

Parameters	Description	Value/Ranges	Source
V_{max}^{MetRcy}	maximum velocity	1–50 μmol/min/mg	*Modelling Assumption*
K_{mMTA}^{MetRcy}	half-saturation constant	1–1000 μM	*Modelling Assumption*

Table 4.6: Parameter values for the enzyme on the methionine recycling path in *T. brucei.*

Trans-methylation Processes AdoMet is the common substrate for both transmethylation processes and polyamine biosynthesis. The byproduct of AdoMet methyl donation is S-adenosylhomocysteine (AdoHcy). AdoHcy is then cleaved to salvage adenosine and homocysteine, where the latter is consumed by trans-sulfuration events to produce cystathionine and cysteine (Bacchi et al. 1995).

Enzymes of MAT, AdoHcy hydrolase, homocysteine methyltransferase and AdoMet transmethylases have been detected in cell-free extracts of *T. brucei* (Yarlett and Bacchi 1988). AdoHcy exerts a feedback inhibition on AdoMet in most methylation reactions (Chiang et al. 1996). In *T. brucei*, transmethylation reactions were found to be very sensitive to AdoHcy inhibition with an apparent K_I value of 12.9 μM (Yarlett et al. 1991).

By contrast, in mammalian cells, homocysteine is recycled to methionine via enzymatic catalysis by BHMT[4] or MTR[5] (Sufrin et al. 1995). Parasitic *T. cruzi* and *Leishmania* species lack the enzyme methionine synthase, which catalyses the methionine production from homocysteine (Reguera et al. 2007). However,

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[4]Systematic name: Betaine-homocysteine methyltransferase

[5]Systematic name: 5-methyltetrahydrofolate-homocysteine methyltransferase

debate remains as to whether homocysteine can be converted to methionine in *T. brucei*. It was speculated that whether homocysteine is remethylated to methionine or irreversibly committed to tran-ssulfuration, largely depends on AdoMet concentration (Yarlett and Bacchi 1988). A different argument by Goldberg et al. (Goldberg et al. 2000) suggested that, even though homocysteine remethylation may exist in *T. brucei*, as most trans-sulfuration metabolites are excreted from trypanosomes, any homocysteine recycled to methionine was not as significant as methionine regeneration from MTA. In the current stage of model construction, we consider the MTA recycling path as the unique source of methionine reproduction.

Yarlett and Bacchi (Yarlett and Bacchi 1988) observed that, in contrast to an enormous elevation of AdoMet level, AdoHcy remained undetectable in *T. brucei* after 12hrs and 36hrs of DFMO treatment . This could be explained with the high activity of cystathionine synthase in *T. brucei* that effectively converts AdoHcy into cystathionine and cysteine.

The ratio of AdoMet to AdoHcy, termed the 'Methylation index' indicates the transmethylation potential of a cell. Normal methylation index values were shown to range from 2:1 to 9:1 (Bacchi et al. 1995). The ratio increased almost 20 fold in DFMO-treated trypansomes compared to the ratio under normal conditions from 6.5 to 114 after 24 hours of DFMO treatment (Yarlett and Bacchi 1988). Under normal conditions, a decline of the ratio, resulting from either a reduction in AdoMet or an accumulation of AdoHcy, can induce the so-called hypermethylation status leading to cell death (Reguera et al. 2007).

Bloodstream-form trypanosomes undergo significant changes in cell methylation events in response to the intervention of polyamine synthesis. Under perturbed conditions, the levels of transmethylation byproducts, cystathionine and cysteine can be rapidly increased. In DFMO-treated trypanosomes, cysteine and cysthationine in the incubation medium, increased about 5-fold (Bacchi et al. 1995), while the intracellular pools did not vary significantly from the control cells. A substantial flow from AdoMet through AdoHcy was also observed, indicating that the rate of AdoMet utilization in trans-methylation processes under DFMO treatment may be several times higher than that in normal conditions.

In our study, the consideration of trans-methylation processes is limited to the first step describing the conversion of AdoMet into AdoHcy. The remaining reactions under the catalysis of a number of enzymes, including AdoHcy hy-

drolase, cystathionine syntase and cystathionase, are excluded from the model, partially because no or very limited kinetic information is available for the enzymes. More importantly, under the DFMO-induced condition, the intermediates of tran-smethylation processes were quickly excreted into the environment, and no significant changes were observed in intracellular concentrations of the metabolites (Bacchi et al. 1995, Yarlett and Bacchi 1988).

Since the kinetic modelling is primarily concerned with evaluating the trypanothione metabolism under the treatment of anti-trypanosomal drug DFMO, the trans-methylation intermediates that have no or negligible effects on the intended use of the model are not included. AdoHcy production is incorporated in the model for its potential role in adjusting the intracellular AdoMet concentration, where the latter is a critical metabolite for trypanothione biosynthesis. AdoHcy is regarded as a constant metabolite during the *in silico* simulation and the methylation index 2:1 is assumed for the ratio of AdoMet to AdoHcy at normal conditions (resulting in the constraint $[AdoHcy] = 0.5 \cdot [AdoMet]$) to approximate the relationship between the concentrations of the metabolites.

'AHS' is used to denote the enzyme catalyzing the AdoHcy production from AdoMet. The kinetic mechanism of the enzyme is modelled based on the standard Michaelis-Menten kinetics

$$V_{AHS} = V_{max}^{AHS} \cdot \frac{\frac{[AdoMet]}{K_{mAdoMet}^{AHS}}}{1 + \frac{[AdoMet]}{K_{mAdoMet}^{AHS}} + \frac{[AdoHcy]}{K_{iAdoHcy}^{AHS}}} \tag{4.18}$$

where V_{max}^{AHS} and $K_{mAdoMet}^{AHS}$ are the maximum velocity and half-saturation constant to substrate AdoMet. $K_{iAdoHcy}^{AHS}$ is the constant of competitive inhibition by AdoHcy.

Table 4.7 contains parameter values for *T. brucei* AHS catalyzing the chemical conversion from AdoMet to AdoHcy.

Methionine & Arginine Transport   The uptake rate V_{max}^{MetPt} of exogenous methionine into the cytosol of *T. brucei* was measured to be 8.9×10^{-7} mol/L/min at 26 μM, the human serum concentration of methionine (Goldberg et al. 2000). Different kinetics were reported by Hasne and Barrett (Hasne and Barrett 2000) as $V_{max}^{MetPt} = 28.8 \pm 0.1$ $nmol \cdot min^{-1} \cdot (10^8 \ cells)^{-1}$ and $K_m^{MetPt} = 32.8 \pm 3.4$ μM. In our study, the numbers from Hasne and Barrett are used for its known validity.

Parameters	Description	Value/Ranges	Source
V_{max}^{AHS}	maximum velocity	1e-3–50 μmol/min/mg	*Modelling Assumption*
$K_{mAdoMet}^{AHS}$	half-saturation constant	1–1000 μM	*Modelling Assumption*
$K_{iAdoHcy}^{AHS}$	inhibition constant	12.9 μM	(Yarlett et al. 1991)

Table 4.7: Parameter values for the enzyme of AdoHcy production in *T. brucei*.

Kinetics of exogenous methionine uptake are modelled as a standard Michaelis Menten, given below:

$$V_{MetPt} \;=\; V_{max}^{MetPt} \cdot \frac{\dfrac{[Met]_{exg}}{K_m^{MetPt}}}{1 + \dfrac{[Met]_{exg}}{K_m^{MetPt}}} \tag{4.19}$$

where $[Met]_{exg}$ is the exogenous methionine concentration (26 μM). V_{max}^{Met} and K_m^{MetPt} are the maximum velocity and the half-saturation constant with respect to the substrate.

To express the maximum velocity in $\mu mol\ min^{-1}\ (mg\ cell\ protein)^{-1}$, an assumption that transporter enzymes account for 10% of overall cell protein of trypanosome is applied (refer to Section 3.5.1 for detailes). This gave the maximum velocity of the transporter enzyme of exogenous methionine a value of 0.276 $\mu mol\ min^{-1}\ (mg\ cell\ protein)^{-1}$.

A biological experiment examining exogenous arginine uptake was designed for the task by the group of Mike Barrett. The experimental results showed that exogenous arginine was taken into *T. brucei* through two transporters with distinct affinities. Carriers were measured with a high capacity V_{maxH}^{ArgPt} and a low capability V_{maxL}^{ArgPt} upto 0.9 μmol/min/mg and 0.14 μmol/min/mg, respectively; half-saturation constants K_{mH}^{ArgPt}, 1130 μM and K_{mL}^{ArgPt}, 26 μM, were measured for each carrier. Exogenous arginine is considered as a constant supply into the system; the concentration is assumed to be 33 μM in plasma. In accordance with the experimental observations, arginine transport was expressed as the superposition of transport functions via both carriers as follows

$$V_{ArgPt} \;=\; V_H^{ArgPt} + V_L^{ArgPt} \tag{4.20}$$

where

$$V_H^{ArgPt} = V_{maxH}^{ArgPt} \cdot \frac{\dfrac{[Arg]_{exg}}{K_{mH}^{ArgPt}}}{1 + \dfrac{[Arg]_{exg}}{K_{mH}^{ArgPt}}} \tag{4.21}$$

$$V_L^{ArgPt} = V_{maxL}^{ArgPt} \cdot \frac{\dfrac{[Arg]_{exg}}{K_{mL}^{ArgPt}}}{1 + \dfrac{[Arg]_{exg}}{K_{mL}^{ArgPt}}} \tag{4.22}$$

The aforementioned biological experiment indicated that in *T. brucei* arginine has a much lower uptake capability compared to methionine. In Chapter 3 we highlighted that arginine transport observed in *L. donovani* is about 30 times higher than the number measured in *T. brucei*. Even at the level observed in *L. donovani*, arginine transport is still not comparable to methionine transport. Due to the large uncertainty in the numbers reported for arginine transport in *T. brucei*, we choose to treat them as unknown parameters. This results in the following kinetic equation, which replaces the preceding set of equations.

$$V_{ArgPt} = V_{max}^{ArgPt} \cdot \frac{\dfrac{[Arg]_{exg}}{K_m^{ArgPt}}}{1 + \dfrac{[Arg]_{exg}}{K_m^{ArgPt}}} \tag{4.23}$$

where $[Arg]_{exg}$ is the exogenous arginine concentration (30 μM). V_{max}^{ArgPt} and K_m^{ArgPt} are the maximum velocity and half-saturation constant with respect to exogenous arginine. They are expressed in μmol per min per mg of cell protein and μM, and constrained by the boundary conditions $0 < V_{max}^{ArgPt} \leq 1$ and $1 \leq K_m^{ArgPt} \leq 1000$, respectively. These unknown parameters will be estimated during the *in silico* simulation.

4.3.2 Glutathione Biosynthesis

Glutathione biosynthesis in the model starts from cysteine, to which bloodstream-form *T. brucei* is very sensitive. Cysteine in human serum has concentration of 13 μM and can be effectively incorporated into the cell with the maximum velocity and half-saturation constant at 0.3 $\mu mol \cdot min^{-1} \cdot (mg\ cell\ protein)^{-1}$ and 400 μM (Duszenko et al. 1985). Cysteine transport is modelled with the standard

Michaelis-Menten kinetic, as below:

$$V_{CysPt} = V_{max}^{CysPt} \cdot \frac{\frac{[Cys]_{exg}}{K_m^{CysPt}}}{1 + \frac{[Cys]_{exg}}{K_m^{CysPt}}} \tag{4.24}$$

where $[Cys]_{exg}$ stands for the concentration of exogenous cysteine. V_{max}^{CysPt} and K_m^{CysPt} are the maximum velocity and half-saturation constant with respect to exogenous cysteine.

Conversion of cysteine into glutathione occurs as a two-step enzymatic reaction. The first and rate-limiting step in the biosynthesis of glutathione is the ligation of glutamate (Glu) and cysteine (Cys), catalyzed by γ-glutamylcysteine synthase (gGCS); the product L-γ-glutamyl-L-cysteine (gGluCys) is then converted to glutathione by glutathione synthase (GS) through addition of glycine (Gly). Huynh et al. (Huynh et al. 2003) observed that gGCS is an essential enzyme for the growth of *T. brucei* cells, implying gGCS as a potential drug target.

Enzyme γGCS is modelled in the form:

$$V_{gGCS} = V_{max}^{gGCS} \cdot \frac{\frac{[Glu]}{K_{mGlu}^{gGCS}}}{1 + \frac{[Glu]}{K_{mGlu}^{gGCS}} + \frac{[GSH]}{K_{iGSH}^{gGCS}}} \cdot \frac{\frac{[Cys]}{K_{mCys}^{gGCS}}}{1 + \frac{[Cys]}{K_{mCys}^{gGCS}}} \cdot \frac{\frac{[ATP]}{K_{mATP}^{gGCS}}}{1 + \frac{[ATP]}{K_{mATP}^{gGCS}}} \tag{4.25}$$

where $[Glu]$ and $[Cys]$ represent the concentrations of the co-substrates of the enzyme. $[ATP]$ represents the concentration of a cofactor of the reaction, where the value is reported in Table 4.3. V_{max}^{gGCS} is the maximum velocity of the enzyme. K_{mGlu}^{gGCS}, K_{mCys}^{gGCS} and K_{mATP}^{gGCS} are the half-saturation constants for each reactant of the reaction. K_{iGSH}^{gGCS} stands for the feedback inhibition from product glutathione, which is competitive with respect to the co-substrate glutamate.

Table 4.8 contains parameters for *T. brucei* gGCS.

GS was only measured in *Plasmodium falciparum* (Meierjohann et al. 2002) and no information is available for the enzyme in *T. brucei*. In our analysis,

Parameters	Description	Value/Ranges	Source
V_{max}^{gGCS}	maximum velocity	7.6 μmol/min/mg	(Lueder and Phillips 1996)
K_{mGlu}^{gGCS}	half-saturation constant	240 μM	(Lueder and Phillips 1996)
K_{mCys}^{gGCS}	half-saturation constant	690 μM	(Lueder and Phillips 1996)
K_{mATP}^{gGCS}	half-saturation constant	70 μM	(Lueder and Phillips 1996)
K_{iGSH}^{gGCS}	feedback inhibition	1100 μM	(Lueder and Phillips 1996)
$[Glu]$	intracellular concentration	3000 μM	*Experimental Data from Mike Barrett*

Table 4.8: Parameter values for *T. brucei* γGCS.

kinetics of GS are modelled with the standard Michaelis-Menten law:

$$V_{GS} = V_{max}^{GS} \cdot \frac{\frac{[Gly]}{K_{mGly}^{GS}}}{1 + \frac{[Gly]}{K_{mGly}^{GS}}} \cdot \frac{\frac{[ATP]}{K_{mATP}^{GS}}}{1 + \frac{[ATP]}{K_{mATP}^{GS}}} \cdot \frac{\frac{[gGluCys]}{K_{mgGluCys}^{GS}}}{1 + \frac{[gGluCys]}{K_{mgGluCys}^{GS}}} \qquad (4.26)$$

where V_{max}^{GS} is the maximum velocity. K_{mGly}^{GS}, $K_{mgGluCys}^{GS}$ and K_{mATP}^{GS} represent the half-saturation constants of the enzyme with respect to the substrates glycine, gGluCys and ATP, respectively.

Table 4.9 contains parameters for *T. brucei* GS.

Parameters	Description	Value/Ranges	Source
V_{max}^{GS}	maximum velocity	1e-3–20 μmol/min/mg	*Modelling Assumption*
K_{mGly}^{GS}	half-saturation constant	1–1000 μM	*Modelling Assumption*
K_{mATP}^{GS}	half-saturation constant	1–1000 μM	*Modelling Assumption*
$K_{mgGluCys}^{GS}$	half-saturation constant	1–1000 μM	*Modelling Assumption*
$[Gly]$	intracellular concentration	4000 μM	*Experimental Data from Mike Barrett*

Table 4.9: Parameter values for *T. brucei* GS.

The Reversible Reaction of Trypanothione Production

The biosynthetic reaction of trypanothione consists of two steps. First, two molecules of spermidine are combined with glutathione to synthesize a glutathione-spermidine dithiol conjugate (GspdSH) that plays a central role in several detoxification processes, which is followed by the formation of trypanothione from the conjugate and one molecule of glutathione. The enzymes of trypanothione biosynthesis are potential targets for drug design.

TryS: Trypanothione Synthetase   Trypanothione synthetase has been the focus of anti-trypanosomal research, owing to not only its significant role in trypanosomal viability but also its capability in regulating the levels of GspdSH, spermidine and glutathione.

Recent work (Willert and Phillips 2008) discovered that, the absence of GspdSH or trypanothione led to trypanosome death. Willert and Phillips observed that trypanosome death followed an irreversible process. This implies that when the intracellular spermidine level is lower than a physiologically required level, the biosynthesis of GspdSH or trypanothione cannot be hardly fulfilled. The authors suggested that the cell did not build up GspdSH and trypanothione pools until spermidine level reached an appropriate steady-state set point. This is commonly referred to as the homeostasis response, a so-called ultrasensitive response to external signals.

Both a synthetase and amidase activity has been associated with glutathionyl-spermidine synthetase in *Escherichia coli* (Kwon et al. 1997, Bollinger et al. 1995), and trypanothione synthetase in *Leishmania* parasites (Fyfe et al. 2008), *Crithidia fasciculata* (Oza et al. 2002) and *T. brucei* (Oza et al. 2003). The conflicting activities of synthetase and amidase, which allow for a bidirectional response between the involved metabolites, may serve to modulate intracellular levels of the metabolites without additional protein synthesis or degradation of existing metabolites.

The regulation mechanism between synthetase and amidase is not yet precisely characterized in *T. brucei*. However, some qualitative properties of this enzyme can be predicted by studying other organisms. For example, under normal conditions, in *Escherichia coli*, the activity of the amidase site is about 40-fold lower than that of the synthetase site and the former prefers GspdSH as a substrate over trypanothoine for effective hydrolysis.

Fyfe et al. (Fyfe et al. 2008) observed that the structure of *L. major* trypan-

othione synthetase-amidase has two active sites, where each site binds to different molecules for specific functions. Due to the substantial amount of similarity shared by parasitic *Leishmania* and *Trypanosoma* species, it is assumed that there are no interactions between the active sites of *T. brucei* synthetase-amidase and the kinetic behaviour of the conflicting activities are modelled separately. Kinetic mechanisms of the synthetase catalyzing GspdSH and trypanothione production are given in equations (4.28) and (4.27), and the amidase kinetics responsible for the hydrolysis of trypanothione and GspdSH in equations (4.29) and (4.30), respectively. Parameters for trypanothione synthetase (TryS) and admidase (TryAdm) are contained in Table 4.10.

$$V_{TryS}^{gd} = \frac{K_{cat}^{TryS} \cdot [TryS]}{1 + \frac{K_{mGSH}^{TryS}}{[GSH]} + \frac{[GSH]}{K_{siGSH}^{TryS}}} \cdot \frac{\frac{[ATP]}{K_{mATP}^{TryS}}}{1 + \frac{[ATP]}{K_{mATP}^{TryS}}} \cdot \qquad (4.27)$$

$$\frac{\left(\frac{[Spd]}{K_{mD}^{TryS}}\right)^n}{1 + \left(\frac{[Spd]}{K_{mD}^{TryS}}\right)^n + \frac{[GspdSH]}{K_{mGspdSH}^{TryS}}}$$

$$V_{TryS}^{gg} = \frac{K_{cat}^{TryS} \cdot [TryS]}{1 + \frac{K_{mGSH}^{TryS}}{[GSH]} + \frac{[GSH]}{K_{siGSH}^{TryS}}} \cdot \frac{\frac{[GspdSH]}{K_{mGspdSH}^{TryS}}}{1 + \frac{[GspdSH]}{K_{mGspdSH}^{TryS}}} \cdot \frac{\frac{[ATP]}{K_{mATP}^{TryS}}}{1 + \frac{[ATP]}{K_{mATP}^{TryS}}} \quad (4.28)$$

$$V_{TryAdm}^{TSH} = V_{maxTSH}^{TryAdm} \cdot \frac{\frac{[TSH]}{K_{mTSH}^{TryAdm}}}{1 + \frac{[TSH]}{K_{mTSH}^{TryAdm}}} \qquad (4.29)$$

$$V_{TryAdm}^{GspdSH} = V_{maxGspdSH}^{TryAdm} \cdot \frac{\frac{[GspdSH]}{K_{mGspdSH}^{TryAdm}}}{1 + \frac{[GspdSH]}{K_{mGspdSH}^{TryAdm}}} \qquad (4.30)$$

Parameters	Description	Value/Ranges	Source
k_{cat}^{TryS}	turn-over rate	$2.9\pm0.4\ s^{-1}$	(Oza et al. 2003)
$K_{mGspdSH}^{TryS}$	half-saturation constant	$2.4\ \mu$M	(Oza et al. 2003)
K_{mGSH}^{TryS}	half-saturation constant	$56.2\ \mu$M	(Oza et al. 2003)
K_{mATP}^{TryS}	half-saturation constant	$7.1\ \mu$M	(Oza et al. 2003)
K_{mD}^{TryS}	half-saturation constant	$37.8\ \mu$M	(Oza et al. 2003)
V_{maxTSH}^{TryAdm}	maximum velocity	6.4e-3 μmol/min/mg	(Oza et al. 2003)
K_{mTSH}^{TryAdm}	half-saturation constant	1–$1000\ \mu$M	*Modelling Assumption*
$V_{maxGspdSH}^{TryAdm}$	maximum velocity	21e-3 μmol/min/mg	(Oza et al. 2003)
$K_{mGspdSH}^{TryAdm}$	half-saturation constant	1–$1000\ \mu$M	*Modelling Assumption*
K_{siGSH}^{TryS}	substrate inhibition	$36.5\ \mu$M	(Oza et al. 2003)
n	hill coefficient	1–3	*Modelling Assumption*
$[TryS]$	enzyme concentration	1e-5–0.5 μM	*Modelling Assumption*

Table 4.10: Parameter values for *T. brucei* TryS and TryAdm.

where k_{cat}^{TryS} is the turnover number. $K_{mGspdSH}^{TryS}$, K_{mGSH}^{TryS}, K_{mD}^{TryS} and K_{mATP}^{TryS} represent the half-saturation constants of TryS with respect to the substrates GspdSH, glutathione and spermidine and co-factor ATP, respectively. n represents the hill coefficient that substrate spermidine may exercise on the enzyme. K_{siGSH}^{TryS} is the substrate inhibition coefficient of glutathione. V_{maxTSH}^{TryAdm}, $V_{maxGspdSH}^{TryAdm}$ and K_{mTSH}^{TryAdm}, $K_{mGspdSH}^{TryAdm}$ stand for the maximum velocity and half-saturation constants of enzyme TryAdm with respect to the substrates TSH and GspdSH, respectively.

Table 4.10 contains parameters for *T. brucei* TryS and TryAdm.

4.3.3 Trypanothione Oxidation and Regeneration

In most eukaryotes, the glutathione peroxidase-glutathione reductase system protects cells against oxidative stress. In trypanosomes, the defense mechanism against oxidative stress is instead achieved by the cyclic action of trypanothione and the enzymes trypanothione peroxidase and trypanothione reductase.

TPx: Trypanothione Peroxidase   Oxidative stress, represented as the level of hydrogen peroxide (H_2O_2), is toxic to parasites, which is produced from molecular oxygen in the living environment of parasites. Under normal conditions, H_2O_2 is maintained at very low intracellular concentrations by various enzymes and molecular antioxidants. An intracellular concentration of 0.1 μM is assumed in our analysis. H_2O_2 can be consumed by either glutathione or trypanothione as substrate via both enzymatic and non-enzymatic reactions.

The non-enzymatic processes are excluded from the model, since the non-enzymatic reduction of H_2O_2 accounts for only a small proportion of the overall H_2O_2 consumption (Henderson et al. 1987). The major route of H_2O_2 consumption is through the fast response of trypanothione under the catalysis of trypanosome peroxidase, as shown in reaction (4.31). Trypanosomes can convert one molecule of H_2O_2 to two molecules of H_2O using trypanothione which is oxidized to form trypanothione disulfide (TS_2) in the process. TS_2 then rapidly reacts with NADPH to regenerate the intracellular pool of trypanothione. *T. brucei* was found to have a value of 1.3 $nmol \cdot min^{-1} \cdot (10^8 \ cells)^{-1}$ for trypanothione peroxidase when using H_2O_2 as an oxidant (Penketh and Klein 1986).

$$T(SH)_2 + H_2O_2 \longrightarrow TS_2 + 2H_2O : TPx \qquad (4.31)$$

The standard Michaelis-Menten kinetics are applied to TPx, shown below:

$$V_{TPx} \quad = \quad V_{max}^{TPx} \cdot \frac{\dfrac{[TSH]}{K_{mTSH}^{TPx}}}{1 + \dfrac{[TSH]}{K_{mTSH}^{TPx}}} \cdot \frac{\dfrac{[H_2O_2]}{K_{mH_2O_2}^{TPx}}}{1 + \dfrac{[H_2O_2]}{K_{mH_2O_2}^{TPx}}} \qquad (4.32)$$

where V_{max}^{TPx} is the maximum velocity of enzyme TPx. K_{mTSH}^{TPx} and $K_{mH_2O_2}^{TPx}$ are the half-saturation constants of TPx with respect to the substrates TSH and H_2O_2, respectively.

Trypanothione peroxidase has a very low half-saturation constant with re-

spect to substrate H_2O_2, and is assumed to have a value of 1 μM in our analysis (Henderson et al. 1987, Penketh and Klein 1986). The half-saturation constant K_{mTSH}^{TPx} is an unknown parameter. A range of 1–1000 (μM) is given to this parameter.

H_2O_2 production in *T. brucei* has been determined to fall somewhere in the range 0.2–0.7 $nmol \cdot min^{-1} \cdot (10^8 \ cells)^{-1}$ (Henderson et al. 1987, Penketh and Klein 1986). The dynamics of H_2O_2 are modelled in the form of the difference between the production rate and consumption rate as follows

$$\frac{d[H_2O_2]}{dt} = R_g^{H_2O_2} - V_{TPx} \tag{4.33}$$

where $R_g^{H_2O_2}$ represents H_2O_2 generation in bloodstream-form *T. brucei*.

TR: Trypanothione Reductase   Trypanothione reductase serves an important role in the regulation of the intracellular thiol-redox balance and in defense against oxidant.

It catalyses the conversion of TS_2 into trypanothione and trypanosomes with reduced TR levels were found to be very vulnerable to oxidative stress (Krieger et al. 2000, Khan 2007). Kinetics for TR are only available for *T. cruzi*; however these values are included in the model of *T. brucei* due to the similarity shared by the two organisms. The standard Michaelis-Menten law is applied to describe the kinetic mechanism of TR.

$$V_{TR} = V_{max}^{TR} \cdot \frac{\dfrac{[NADPH]}{K_{mNADPH}^{TR}}}{1 + \dfrac{[NADPH]}{K_{mNADPH}^{TR}}} \cdot \frac{\dfrac{[TS_2]}{K_{mTS_2}^{TR}}}{1 + \dfrac{[TS_2]}{K_{mTS_2}^{TR}}} \tag{4.34}$$

where V_{max}^{TR} is the maximum velocity and $K_{mTS_2}^{TR}$ and K_{mNADPH}^{TR} stand for the half-saturation constants with respect to the substrate TS_2 and coenzyme NADPH.

Table 4.11 contains parameters for *T. brucei* TR.

Contradicting Observations on Glutathione Reductase   The removal of H_2O_2 through the action of glutathione peroxidase and glutathione reductase in mammalian cells is replaced in *T. brucei* by trypanothione acting as a redox carrier. It is commonly recognized that *T. brucei* does not contain glutathione peroxidase, but a conflicting observation was reported regarding the presence of

Parameters	Description	Value/Ranges	Source
V_{max}^{TR}	maximum velocity	112.7 μmol/min/mg	(Krauth-Siegel et al. 1987)
$K_{mTS_2}^{TR}$	half-saturation constant	9 μM	(Krauth-Siegel et al. 1987)
K_{mNADPH}^{TR}	half-saturation constant	36 μM	(Krauth-Siegel et al. 1987)

Table 4.11: Parameter values for *T. brucei* TR. Note that the values are obtained from experiments on *T. cruzi*.

glutathione reductase (GR) in the organism. Krauth-Siegel and Comini (Krauth-Siegel and Comini 2008) observed that *T. brucei* lacks the necessary genes for glutathione reductase. However, Fairlamb and Cerami (Fairlamb et al. 1985) indicate that the activity of glutathione reductase could be readily measured in the presence of the co-factor trypanothione, and the enzyme-mediated reaction can be written as follows

$$\text{GSSG} + \text{NADPH} \longrightarrow 2\text{GSH} + \text{NADP} : \text{GR} \tag{4.35}$$
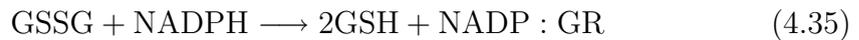
GSSG is toxic to cells and under normal conditions it does not accumulate because it reduces to glutathione. The rate equation of the enzyme GR is modelled below. The maximum velocity of GR was reported as 1.4 $nmol \cdot min^{-1} \cdot (10^8\ cells)^{-1}$ (Penketh and Klein 1986) or 4.5 $nmol \cdot min^{-1} \cdot (10^8\ cells)^{-1}$ (Fairlamb et al. 1985).

$$V_{GR} = V_{max}^{GR} \cdot \frac{\frac{[GSSG]}{K_{mGSSG}^{GR}}}{1 + \frac{[GSSG]}{K_{mGSSG}^{GSSG}}} \cdot \frac{\frac{[NADPH]}{K_{mNADPH}^{GR}}}{1 + \frac{[NADPH]}{K_{mNADPH}^{GR}}} \tag{4.36}$$

where V_{max}^{GR} is the maximum velocity and K_{mGSSG}^{GR} and K_{mNADPH}^{GR} stand for the half-saturation constants with respect to the substrate GSSG and co-enzyme NADPH.

In *T. brucei*, trypanothione is a spontaneous reductant of GSSG and thioredoxin (Krauth-Siegel et al. 2005). In the present kinetic model, the involvement of trypanothione in the consumption of GSSG and thioredoxin is neglected owing to its instantaneous occurrence. The enzymatic reaction in which trypanothione metabolizes H_2O_2 is taken as the only reaction that trypanothione participates

in as a substrate. Moreover, because of the contradicting observations reported for GR, the rate equation (4.36) is not taken into account in the modelling process. Further clarification is required regarding the presence of this enzyme and its associated kinetics.

4.3.4 Formation of NADPH and Pentoses

Glycolysis in bloodstream-form *T. brucei* cells provides a convenient context for studying the enzyme inhibitors to be used as anti-parasitic drugs (Eisenthal and Cornish-Bowden 1998) because of the significant differences in the organization of glycolysis in parasites and host cells. The model of trypanosomal glycolysis developed by Bakker et al. (Bakker et al. 1997) provides a good starting point for a systematic investigation of potential drug targets.

Glycolysis represents the sole pathway for glucose metabolism to provide cells with energy ATP, and bloodstream-form *T. brucei* depends completely on rapid glycolysis for its energy supply. Glucose metabolism is facilitated by the glucose transporter of *T. brucei*, which follows a reversible process. Intracellular glucose is converted into D-glucose 6-phosphate (G6P) under the catalysis of hexokinase (HKK), and consequently glycerol and ribose. The majority of intermediate compounds and enzymes of the glycolysis pathway are located in the glycosome of *T. brucei*.

The pentose phosphate pathway plays a crucial role in the metabolism of many parasitic protozoa and in the host-parasite relationship (Barrett et al. 2003). The pathway maintains intracellular pools of NADP and NADPH, where the latter is used as substrate in the protection of trypanosomes against oxidative stress. The cellular ratio of NADPH to NADP is an important means of regulating the metabolic flux through the pathway. When the NADPH/NADP ratio is high, a powerful inhibition of trypanosomal growth may occur.

The pentose phosphate pathway converts glucose 6-phosphate (G6P) to ribose 5-phosphate (Ru5P) via a sequence of enzymatic reactions, and the majority of the pathway intermediates are shown to be cytosolic in *T. brucei*. The enzyme hexokinase catalyzing the production of glycosomal G6P is reported to have an exclusively glycosomal location and *T. brucei* has not been evidenced to be able to synthesize G6P *de novo* in cytosol. This compartmentation constraint leads to a question of where the required metabolites of the pentose phosphate pathway come from, especially in the case of G6P. One theory is that a trans-

porter exists in the glycosomal membrane that facilitates the transfer of this intermediate between the glycosome and cytosol (Barrett 1997). However, no kinetic information describing this postulated membrane transporter in *T. brucei* has ever been reported. To enable the *in silico* simulation, we assume that G6P concentrations in the glycosome and the cytosol are equal, and thus the well-understood kinetics of glycosomal hexokinase can be applied to the present model.

The complete pentose phosphate pathway consumes G6P to produce ribose via a series of enzymatic reactions catalyzed by the enzymes Glucose-6-phosphate Dehydrogenase (G6PDH), 6-phosphogluconolactonase (6PGL), 6-phosphogluconate dehydrogenase (6PGDH), ribose 5-phosphate isomerase and ribokinase, leading to the intermediate products D-glucono-1,5-lactone 6-phosphate (6-PGL), 6-phospho-D-gluconate (6-PG), D-ribulose 5-phosphate (Ru5P), D-ribose 5-phosphate (R5P) and ribose, respectively. In the present kinetic model, the pentose phosphate pathway is revised in accordance with the intended use of the model. Two primary factors for trypanosomal growth – trypanothione biosynthesis and oxidant detoxification – are the focus of the study of trypanothione metabolism. Owing to the critical role of NADPH in the defense mechanism against oxidative stress, the consideration of the pathway is limited to the elementary steps that produce NADPH.

The pentose phosphate pathway can be separated into an oxidative branch and a non-oxidative branch. The oxidative branch of the pathway involves G6PDH, 6PGL and 6PGDH, which are essential for the protection of the parasite against oxidative stress. The reactions processing the exchange between NADP and NADPH are the reactions catalyzed by G6PDH and 6PGDH, given as follows

$$\text{G6P} + \text{NADP} \longrightarrow \text{6-PGL} + \text{NADPH} : \text{G6PDH} \qquad (4.37)$$

$$\text{6PG} + \text{NADP} \longrightarrow \text{Ru5P} + \text{NADPH} : \text{6PGDH} \qquad (4.38)$$

The intermediate reaction responsible for the production of 6PG (the reactant in the chemical reaction in equation (4.38)), occurs spontaneously compared to other enzymatic reactions in the pathway. 6PGL is a unstable compound and the necessity of 6PGL to catalyze this reaction in the cells is questionable (Duffieux et al. 2000). For this reason, a direct conversion from 6PGL into Ru5P is assumed. Combined with the aforementioned reactions for glucose transport

and glycosomal G6P production, the revised pentose phosphate pathway is represented by the following chemical reactions:

$$Glucose_{ext} \quad \longleftrightarrow \quad Glucose_g : \text{GlcPt} \tag{4.39}$$

$$Glucose_g + \text{ATP} \quad \longrightarrow \quad \text{G6P} + \text{ADP} : \text{HKK} \tag{4.40}$$

$$\text{G6P} + \text{NADP} \quad \longrightarrow \quad \text{6PG} + \text{NADPH} : \text{G6PDH} \tag{4.41}$$

$$\text{6PG} + \text{NADP} \quad \longrightarrow \quad \text{Ru5P} + \text{NADPH} : \text{6PGDH} \tag{4.42}$$

Kinetic mechanisms of the required enzymes catalyzing the above reactions are documented in the following paragraphs.

GlcPt: Glucose Transport   The kinetics of glucose transport are known in detail. We apply the kinetic equation defined by Haanstra et al. (Haanstra et al. 2008) to model the dynamics of glucose transport, shown below:

$$V_{GlcPt} = V_{max}^{GlcPt} \cdot \frac{[Glc]_{ext} - [Glc]_g}{K_m^{GlcPt} + [Glc]_{ext} + [Glc]_g + \alpha \cdot [Glc]_{ext} \dfrac{[Glc]_g}{K_m^{GlcPt}}} \tag{4.43}$$

where V_{max}^{GlcPt} is the maximum velocity of the forward glucose transport reaction. $[Glc]_{ext}$ and $[Glc]_g$ represent the concentrations of exogenous and glycosomal glucose. As glucose transport is modelled as a reversible reaction, K_m^{GlcPt} is thus the generalized half-saturation constant. α is the scaling factor adjusting the weight of the forward glucose uptake and the backward glucose efflux.

Table 4.12 contains parameters for *T. brucei* glucose transport.

Parameters	Description	Value/Ranges	Source
V_{max}^{GlcPt}	maximum velocity	0.109 μmol/min/mg	(Haanstra et al. 2008)
K_m^{GlcPt}	half-saturation constant	1000 μM	(Haanstra et al. 2008)
α	scaling factor	0.75 μM	(Haanstra et al. 2008)
$[Glc]_{ext}$	concentration in blood	5000 μM	(Haanstra et al. 2008)

Table 4.12: Parameter values for *T. brucei* glucose transport.

HXK: Hexokinase The kinetic equation of Hexokinase from the model of glycolysis designed by Haanstra et al. (Haanstra et al. 2008) is adopted, which is expressed as follows

$$V_{HKK} = V_{max}^{HXK} \cdot \frac{\dfrac{[Glc]_g}{K_{mGlc_g}^{HXK}} \cdot \dfrac{[ATP]_g}{K_{mATP}^{HXK}}}{\left(1 + \dfrac{[ATP]_g}{K_{mATP}^{HXK}} + \dfrac{[ADP]_g}{K_{mADP}^{HXK}}\right) \cdot \left(1 + \dfrac{[Glc]_g}{K_{mGlc_g}^{HXK}} + \dfrac{[G6P]}{K_{mG6P}^{HXK}}\right)} \quad (4.44)$$

where V_{max}^{HXK} is the maximum velocity. $K_{mGlc_g}^{HXK}$ and K_{mATP}^{HXK} are the half-saturation constants of the enzyme with respect to endogenous glucose and co-factor ATP in glycosome. K_{mADP}^{HXK} and K_{mG6P}^{HXK} represent the inhibition constant of the products ADP and G6P, respectively.

Table 4.13 contains parameters for *T. brucei* HXK.

Parameters	Description	Value/Ranges	Source
V_{max}^{HXK}	maximum velocity	1.93 μmol/min/mg	(Haanstra et al. 2008)
$K_{mGlc_g}^{HXK}$	half-saturation constant	100 μM	(Haanstra et al. 2008)
K_{mATP}^{HXK}	half-saturation constant	116 μM	(Haanstra et al. 2008)
K_{mADP}^{HXK}	inhibition constant	126 μM	(Haanstra et al. 2008)
K_{mG6P}^{HXK}	inhibition constant	12000 μM	(Haanstra et al. 2008)
$[ATP]_g$	concentration in glycosome	240.5 μM	(Haanstra et al. 2008)
$[ADP]_g$	concentration in glycosome	1519 μM	(Haanstra et al. 2008)

Table 4.13: Parameter values for *T. brucei* HXK.

G6PDH: Glucose-6-phosphate Dehydrogenase G6PDH is the first enzyme of the pentose phosphate pathway. The product of the enzyme-mediated reaction, G6P, is toxic to trypanosomes when it is accumulated. Several studies on *T. brucei* G6PDH however return us again to the fact that different groups frequently present conflicting observations of enzyme activity. Cronin et al. (Cronin et al. 1989) detected a maximum velocity of 0.0168±0.0057

μmol/min/mg and 0.0124$\pm$0.0038 μmol/min/mg for the bloodstream form and procyclic form of *T. brucei*, respectively, whereas Heise and Opperdoes (Heise and Opperdoes 1999) measured a maximum velocity of procyclic form *T. brucei* G6PDH of 14μmol/min/mg. Duffieux et al. (Duffieux et al. 2000) however reported a much higher value of 740 μmol/min/mg.

The maximum velocity of bloodstream-form *T. brucei* G6PDH is thus considered as an unknown parameter due to the conflicting data. The published *in vivo* experiments were performed in a realistic environment, where the potential G6P transport between the glycosome and the cytosol are naturally taken into account. However, in our kinetic model, no distinction was made between glycosomal G6P and cytosolic G6P. Treating the maximum velocity of G6PDH as an unknown parameter adds an extra degree of freedom to the system, which allows the *in silico* model to be solved computationally.

Compared to the wide range of values reported for the specific activity of G6PDH, there is less uncertainty about the value of half-saturation constants of the enzyme with respect to the involved substrates. The half-saturation constants are hence considered as known parameters, which are extracted from the published articles.

The Michaelis-Menten based kinetics are applied to model the dynamics of G6PDH, shown below:

$$V_{G6PDH} = V_{max}^{G6PDH} \cdot \frac{\frac{[G6P]}{K_{mG6P}^{G6PDH}}}{1 + \frac{[G6P]}{K_{mG6P}^{G6PDH}}} \cdot \frac{\frac{[NADP]}{K_{mNADP}^{G6PDH}}}{1 + \frac{[NADP]}{K_{mNADP}^{G6PDH}} + \frac{[NADPH]}{1 + \frac{[NADPH]}{K_{iNADPH}^{G6PDH}}}} \quad (4.45)$$

where V_{max}^{G6PDH} is the maximum velocity. K_{mG6P}^{G6PDH} and K_{mNADP}^{G6PDH} are the half-saturation constants of G6PDH with respect to the substrate G6P and co-enzyme NADP. K_{iNADPH}^{G6PDH} represents the inhibition constant of product NADPH on the enzyme.

Table 4.14 contains parameters for *T. brucei* G6PDH.

Igoillo-Esteve et al. (Igoillo-Esteve et al. 2007) hypothesized that under normal conditions, *T. cruzi* G6PDH is maintained in its reduced less-active form by NADPH. Under oxidative stress, in which the level of the reduced cofactor drops and other molecules like H_2O_2 are present, the enzyme is oxidized to attain its more active form. This process would constitute a fast response to increase NADPH level to counteract the oxidative stress.

Parameters	Description	Value/Ranges	Source
V_{max}^{G6PDH}	maximum velocity	1e-4–40 μmol/min/mg	*Modelling Assumption*
K_{mG6P}^{G6PDH}	half-saturation constant	138 μM	(Duffieux et al. 2000)
K_{mNADP}^{G6PDH}	half-saturation constant	35 μM	(Duffieux et al. 2000)
K_{iNADPH}^{G6PDH}	inhibition constant	1–1000 μM	*Modelling Assumption*

Table 4.14: Parameter values for *T. brucei* G6PDH.

We propose that *T. brucei* G6PDH activity may also be induced by oxidative stress due to the similarity shared by the two organisms. Accordingly, the rate equation (4.45) is modified with the last factor representing the activation of G6PDH by oxidative stress.

$$V_{G6PDH} = V_{max}^{G6PDH} \cdot \frac{\frac{[G6P]}{K_{mG6P}^{G6PDH}}}{1 + \frac{[G6P]}{K_{mG6P}^{G6PDH}}} \cdot \frac{\frac{[NADP]}{K_{mNADP}^{G6PDH}}}{1 + \frac{[NADP]}{K_{mNADP}^{G6PDH}} + \frac{[NADPH]}{1 + \frac{[NADPH]}{K_{iNADPH}^{G6PDH}}}} \cdot \frac{[H_2O_2] + K_a}{[H_2O_2]_{ss} + K_a}$$

(4.46)

where $[H_2O_2]_{ss}$ and $[H_2O_2]$ represent the concentration of H_2O_2 under steady-state and oxidative stress conditions. K_a is the activation constant. When $[H_2O_2] = [H_2O_2]_{ss}$ the factor is 1.

6PGDH: 6-phosphogluconate Dehydrogenase Hanau et al. (Hanau et al. 1996) performed detailed kinetic analysis of *T. brucei* 6PGDH. The authors observed that NADPH acts as a competitive inhibitor of NADP and Ru5P is a competitive inhibitor of 6PG. The maximum velocity of this enzyme has been measured experimentally by Hanau et al.; however, this parameter is treated as unknown in the present kinetic model for the same reasons as G6PDH.

The Michaelis-Menten based kinetics for 6PGDH dynamics are

$$V_{6PGDH} = V_{max}^{6PGDH} \cdot \frac{\frac{[6PG]}{K_{m6PG}^{6PGDH}}}{1 + \frac{[6PG]}{K_{m6PG}^{6PGDH}} + \frac{[Ru5P]}{K_{iRu5P}^{6PGDH}}} \cdot \frac{\frac{[NADP]}{K_{mNADP}^{6PGDH}}}{1 + \frac{[NADP]}{K_{mNADP}^{6PGDH}} + \frac{[NADPH]}{K_{iNADPH}^{6PGDH}}}$$

(4.47)

where V_{max}^{6PGDH} is the maximum velocity. K_{m6PG}^{6PGDH} and K_{mNADP}^{6PGDH} are the half-

saturation constants of 6PGDH with respect to the substrate 6PG and co-enzyme NADP. K_{iRu5P}^{6PGDH} and K_{iNADPH}^{6PGDH} represent the inhibition constants of the products of the enzyme, Ru5P and NADPH, respectively.

Table 4.15 contains parameters for *T. brucei* 6PGDH.

Parameters	Description	Value/Ranges	Source
V_{max}^{6PGDH}	maximum velocity	1e-4–40 μmol/min/mg	*Modelling Assumption*
k_{cat}^{6PGDH}	turnover number	27 s^{-1}	(Hanau et al. 1996)
K_{m6PG}^{6PGDH}	half-saturation constant	3.5 μM	(Hanau et al. 1996)
K_{mNADP}^{6PGDH}	half-saturation constant	1 μM	(Hanau et al. 1996)
K_{iRu5P}^{6PGDH}	inhibition constant	30 μM	(Hanau et al. 1996)
K_{iNADPH}^{6PGDH}	inhibition constant	0.6 μM	(Hanau et al. 1996)
$[Ru5P]$	intracellular concentration	20 μM	*Modelling Assumption*

Table 4.15: Parameter values for *T. brucei* 6PGDH.

4.4 The Initial Kinetic Model

Differential equations for the different metabolites and other time-dependent variables of the trypanothione metabolism are formulated in Table 4.16. There are 20 ODEs for variable metabolites derived from 23 catalytic mechanisms and 3 ODEs for time-dependent variables, derived from 3 regulatory mechanisms. The steady-state concentrations of the variable metabolites and time-dependent variables are given in Table 4.17.

To simulate a realistic environment for G6P production, a volume of 0.2451 μL $(mg\ cell\ protein)^{-1}$ is applied to the glycosome (V_g). The remaining intracellular and transport reactions are assumed to take place in the same cell volume.

Variables	Differential Equations
$[Met]$	$\frac{d[Met]}{dt} = V_{MetPt} - V_{MAT} + V_{MetRcy}$
$[AdoMet]$	$\frac{d[AdoMet]}{dt} = V_{MAT} - V_{AdoMetDC^T} - V_{AdoHcy}$
$[dAdoMet]$	$\frac{d[dAdoMet]}{dt} = V_{AdoMetDC^T} - V_{SpdS}$
$[Arg]$	$\frac{d[Arg]}{dt} = V_{ArgPt} - V_{ARG}$
$[Orn]$	$\frac{d[Orn]}{dt} = V_{ARG} - V_{ODC}$
$[Put]$	$\frac{d[Put]}{dt} = V_{ODC} - V_{SpdS}$
$[MTA]$	$\frac{d[MTA]}{dt} = V_{SpdS} - V_{MetRcy}$
$[Cys]$	$\frac{d[Cys]}{dt} = V_{CysPt} - V_{gGCS}$
$[gGluCys]$	$\frac{[gGluCys]}{dt} = V_{gGCS} - V_{GS}$
$[Spd]$	$\frac{d[Spd]}{dt} = V_{SpdS} - V_{TryS}^{gd} + V_{TryAdm}^{GspdSH}$
$[GspdSH]$	$\frac{d[GspdSH]}{dt} = V_{TryS}^{gd} - V_{TryS}^{gg} + V_{TryAdm}^{TSH} - V_{TryAdm}^{GspdSH}$
$[GSH]$	$\frac{d[GSH]}{dt} = V_{GS} - V_{TryS}^{gd} - V_{TryS}^{gg} + V_{TryAdm}^{GspdSH} + V_{TryAdm}^{TSH}$
$[TSH]$	$\frac{d[TSH]}{dt} = V_{TryS}^{gg} - V_{TryAdm}^{TSH} - V_{TPx} + V_{TR}$
$[TS2]$	$\frac{d[TS2]}{dt} = V_{TPx} - V_{TR}$
$[Glu]_{in}$	$\frac{d[Glu]_{in}}{dt} = V_{GlcPt} - V_{HXK}$
$[G6P]$	$\frac{d[G6P]}{dt} = (V_{HXK} - V_{G6PDH})/V_g$
$[6PG]$	$\frac{d[6PG]}{dt} = V_{G6PDH} - V_{6PGDH}$
$[NADP]$	$\frac{d[NADP]}{dt} = V_{TR} - V_{G6PDH} - V_{6PGDH}$
$[NADPH]$	$\frac{d[NADPH]}{dt} = V_{G6PDH} + V_{6PGDH} - V_{TR}$
$[H_2O_2]$	$\frac{d[H_2O_2]}{dt} = R_g^{H_2O_2} - V_{TPx}$
$[ODC]$	$\frac{d[ODC]}{dt} = R_s^{ODC} - R_d^{ODC}$
$[AdoMetDC^L]$	$\frac{d[AdoMetDC^L]}{dt} = R_b^{AdoMetDC^L} - R_d^{AdoMetDC^L}$
$[Prozyme]$	$\frac{d[Prozyme]}{dt} = R_s^{Prozyme} - R_b^{AdoMetDC^L}$

Table 4.16: Differential equations for the different metabolites and other time-dependent variables of the trypanothione metabolism.

Variables	Value/Ranges	Source
$[Orn]$	0–43 μM	(Fairlamb et al. 1987) (Bellofatto et al. 1987)
$[AdoMet]$	0–34 μM	(Fairlamb et al. 1987) (Bellofatto et al. 1987)
$[Met]$	3798 μM	Experimental Data from Mike Barrett
$[Arg]$	700$\pm$35 μM	Experimental Data from Mike Barrett
$[dAdoMet]$	9 μM	(Fairlamb et al. 1987)
$[Put]$	500–700 μM	(Fairlamb et al. 1987)
$[Spd]$	2000–14000 μM	(Fairlamb et al. 1987)
$[MTA]$	20 μM	Modelling Assumption
$[Cys]$	100-1000 μM	Modelling Assumption
$[GSH]$	234 μM	(Fairlamb et al. 1987)
$[gGluCys]$	100 μM	Modelling Assumption
$[GspdSH]$	48 μM	(Fairlamb et al. 1987)
$[TSH]$	340 μM	(Fairlamb et al. 1987)
$[TS_2]$	$0.1 \cdot [TSH]$	Analytical Solution
$[NADPH]$	150 μM	Experimental Data by Mike Barrett
$[Glc]_g$	0 μM	(Haanstra et al. 2008)
$[G6P]$	500 μM	(Haanstra et al. 2008)
$[NADP]$	150 μM	Experimental Data by Mike Barrett
$[6PG]$	20–500 μM	Modelling Assumption
H_2O_2	0.1 μM	Modelling Assumption
$[ODC]$	0.01–0.2 μM	Modelling Assumption
$[Prozyme]$	5e-6–5e-2 μM	Modelling Assumption
$[AdoMetDC^L]$	0–0.1 μM	Modelling Assumption

Table 4.17: Steady-state concentrations of the different metabolites and other time-dependent variables of the trypanothione metabolism.

4.5 Summary

In this chapter we outlined the development of the first kinetic model of trypanothione metabolism in bloodstream-form *T. brucei*. A critical review of the scientific papers relevant to the trypanothione metabolic pathway was presented.

We illustrated the model structure and enzyme dynamics on the basis of two individual sub-networks that fulfill specific metabolic functions, including the trypanothione biosynthetic sub-network and the trypanothione redox sub-network. Description of the mathematical model concentrated on potential regulation of trypanosomal enzymes in the context of the control of intracellular levels of key metabolites and trypanosomal growth. We also explained why biological measurements extracted from the literature have to be used with caution and some of the issues raised by conflicting observations in the context of the modelling procedure were discussed.

Overall, there are a large number of unknown parameters in the kinetic model of trypanothione metabolism and the experimental data available for solving the inverse problem is incomplete and uncertain. In Chapter 7 we propose a methodological framework to strategically evaluate and refine this kinetic model, where both problems of structure identification and parameter estimation are investigated. A grey-box mathematical model representation, which combines mechanistic (white-box) models and empirical (black-box) models, is applied to successfully model the polyamine biosynthetic sub-pathway.

Missing quantitative information about the metabolic system makes the *in silico* model simulation extremely challenging, in which case a reliable parameter estimation is necessary for identifying unknown parameters. The computational issues associated with solving the inverse problem of non-linear dynamic systems (via single-objective optimization) are discussed in Chapter 5.

Chapter 5

Parameter Estimation of Computational Models

In this chapter some of the basic theory associated with optimization is reviewed. A brief discussion of a global optimization approach, Particle Swarm optimization, and the derivation of the PSwarm algorithm is presented. The computational issues related to global optimization in studying the dynamic behaviour of biological systems are investigated and analysis techniques that can be employed to evaluate and refine the estimated solutions are discussed.

5.1   Introduction

Optimization in general deals with the minimization (or maximization) of an objective function that measures the quality of an estimated parameter set in satisfying a given criteria (e.g. minimization of residuals) for a model that describes, for example, the dynamics of a non-linear system. In the context of biological systems, the optimization frequently aims to match experimental results as closely as possible, however direct determination of *in vivo* parameters is difficult and often noisy. Given sufficient experimental measurements of system components, the goal is to minimize the objective function, normally written as a least-squares expression, by adjusting the model parameters. Parameter estimation problems, also known as inverse problems, belong to the family of system identification, where the model structure is assumed as given.

Traditionally, parameter estimation is tackled by solving the initial-value problems (Hemker 1972). Arbitrary guesses for the model initial condition and parameters are chosen, which are refined by comparing the model trajectory

obtained at each iteration of function evaluations with experimental data. The initial-value approach is efficient and can rapidly converge to the correct solution only if the initial guess is in the vicinity of the optimal solution (Bock 1982).

Another group of methods for parameter estimation uses a multiple-shooting approach (Childs and Osborne 1996). These methods are generally used to solve boundary-value problems. Multiple-shooting methods involve the superposition of initial-value solutions of the differential equations over short sub-intervals and enforcing continuity of the solution across interval boundaries. This consequently transforms the original system into a constrained, over-determined system that is parameterized by both kinetic parameters and the values of state variables at the boundaries of each sub-interval. The advantages of multiple-shooting approaches lie in the conceptual simplicity and the ability to make use of established solution methods for initial-value problems.

Both of these approaches, however, require a good selection of mesh (as an initial guess to parameter estimate) in order to avoid drifting far away from the solution trajectory or converging to a local optimum. When solving stiff boundary-value problems, the number of mesh points grows unacceptably large with an increase in the stiffness of the problem (Ascher and Petzold 1998).

In this thesis, the initial-value problem of time-dependent ODEs is considered. This chapter lays the foundation for the subjects of interest with regard to parameter estimation and model identifiability. The structure of this chapter is as follows. In Section 5.2, an introduction to optimization theory is given. In Section 5.3.1, the Particle Swarm global optimization approach is described. In Section 5.4, the performance of the optimization method in various scenarios is evaluated using the ERK signalling pathway as a case study. The identifiability analysis performed on the mathematical model of the signalling pathway is presented in Section 5.5.

5.2   Optimization Background

There are two important concepts associated with optimization, namely *search space* and *fitness landscape*. Exploring the search space refers to the process of searching for an optimal solution among a collection of candidate solutions. The optimum solution is often signified with a vector, where the length of the vector indicates the number of variables to be optimized. This also gives the dimensionality of the search space. Here we take a two parameter problem as

an example, forming a two-dimensional search space. Suppose each candidate solution can be assigned a real-valued fitness. In this case, the fitness landscape can be thought of as a three-dimensional plot in which the two parameters form a 2D mesh and the fitness at each point is plotted along the third axis. An example fitness landscape based on trigonometric functions is shown in Figure 5.1. Such plots are called landscapes because the plot of fitness values typically contains 'hills', 'peaks' and 'valleys'.
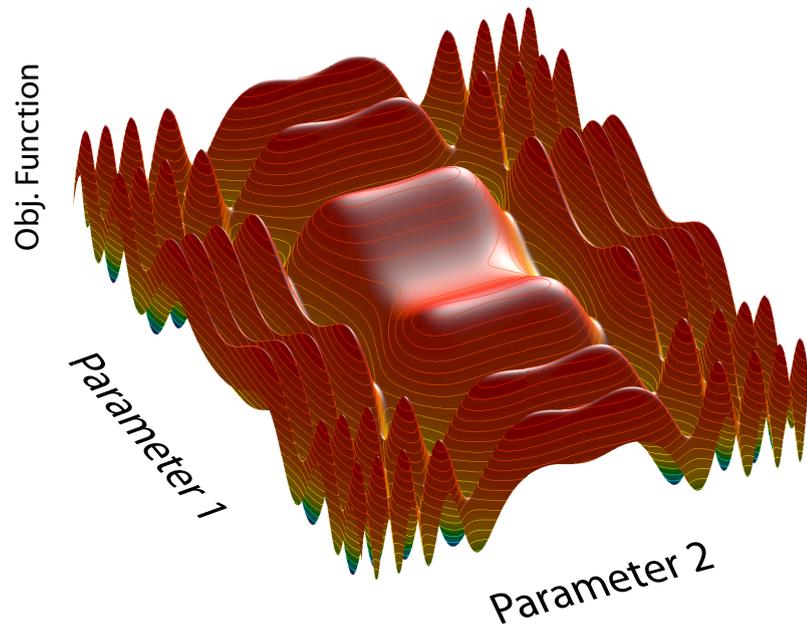
Figure 5.1: A simple fitness landscape for a two-parameter problem.

Optimization techniques can be seen as ways of moving solution candidates around on the landscape defined by the fitness function. The performance of the techniques is characterized by the rate of convergence and diversity of optimal solutions. A difficult but common problem is that the search stops at a local optimum, a problem termed premature convergence. A local optimum is not necessarily the highest point in the landscape, but is the highest point in the local vicinity and any small movement from it results in a reduction in fitness.

A number of computational approaches to parameter optimization have been employed for biological systems. Optimization methods are generally classified into either local or global categories based on their capability in solving multi-modal problems, where multiple local minima are expected. Global optimization methods, as the name indicates, attempt to seek the global minimum in the landscape. Local optimization methods, such as the Gauss-Newton method,

are however very likely to arrive at solutions of a local nature. A systematic multi-start method is often used in conjunction with local optimization methods in order for them to be able to tackle the problem of multi-modality. It is commonly known that global optimization methods can provide us with globally optimal solutions, and a combination of stochastic searching strategies and deterministic global methods have been shown to be able to enhance the efficiency of optimization approaches (Rodriguze-Fernandez et al. 2006).

Comparison of optimization methods is beyond the scope of the thesis, however. A comprehensive study investigating the performance of some local and global optimization methods is presented by Moles et al. (Moles et al. 2003), where the authors found that evolutionary strategies coupled with stochastic ranking technique might be the most competitive optimization algorithm; however, the computational cost of this algorithm can be very expensive when solving complex parameter estimation problems.

It would become very difficult to distinguish global and local methods when the available observed data is noisy and incomplete. One major issue associated with optimization is concerned with *Parameter Identifiability*, which aims to discern whether or not unknown model parameters can be uniquely determined from experimental data. Theoretically, there are two situations where parameters cannot be independently identified regardless of the optimization methods applied.

First, intrinsic constraints in the model formulation can cause parameters to be non-identifiable. Given a simple mathematical function x with respect to time t and parameters a and b:

$$x(t) = e^{(a-b)t} \cdot x_0$$

the time-dependent ODE model is derived as

$$\frac{dx}{dt} = a \cdot x - b \cdot x = (a - b) \cdot x$$

In this case, the parameters a and b are correlated with each other. The factor $(a - b)$ will be treated as a single parameter that will be tuned during the optimization process; a unique solution for parameters a and b cannot be obtained.

Second, the lack of information in the observation data causes the parameters to be non-identifiable. Given another mathematical function with respect to time

t and parameters a and b:

$$x(t) = e^{-bt} \cdot C + \frac{a}{b}$$

where C is a constant. The time-dependent ODE model is represented as

$$\frac{dx}{dt} = -b \cdot x + a$$

In this case, optimization with measurements at steady state leads to a and b not being independently solved for, since the ratio between the two parameters is the factor that will be adjusted by the optimizer. As shown in Figure 5.2, the steady-state level is decided by the ratio a/b, and the rate at which the variable x decays to steady state is controlled by the parameter b in the term e^{-bt} of the function. In this figure, the ratio a/b is fixed at 0.5 to illustrate that different values of a and b can yield the same steady state, but different transient behaviour. The two parameters are more likely to be independently identified when the transient data, rather than steady-state data, is used for optimization. Further investigation is necessary to examine if noise in experimental data will influence parameter identifiability.

5.2.1 Problem Statement

This work deals exclusively with Bound Constrained Optimization problems, where parameters to be determined are subject to certain constraints. Mathematically, the formulation is that of a non-linear programming problem with boundary conditions on the parameters:

$$\text{minimize } f(z) \text{ subject to } z \in \Omega$$

with

$$\Omega = \{z \in \mathbb{R}^n : l \leq z \leq u\},$$

where the inequalities $l \leq z \leq u$ are posed on each parameter to be solved for and $l \in (-\infty, \mathbb{R})^n$, $u \in (\mathbb{R}, +\infty)^n$, and $l \leq u$. In the context of optimization of biological systems, model parameters must typically be non-negative.

We consider deterministic, non-linear dynamic models of biochemical systems, described by ordinary differential equations (ODEs). A common statement
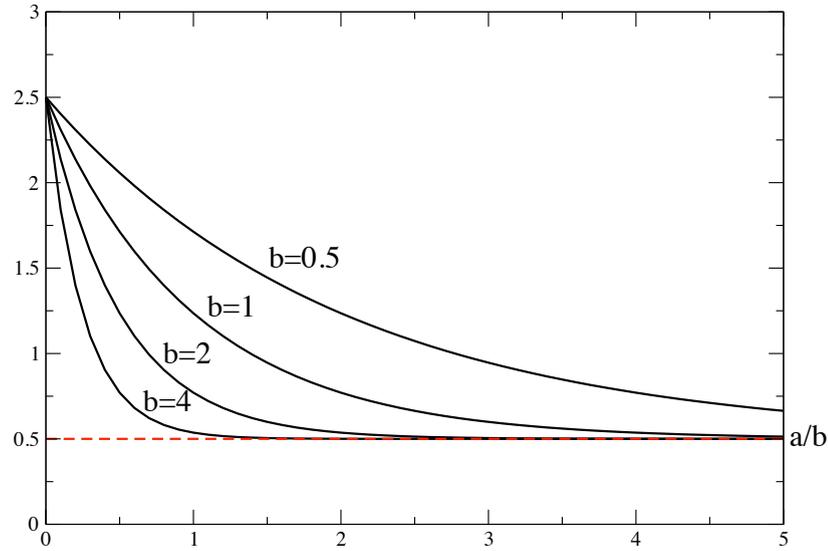
Figure 5.2: A simple decay function for an illustration of the non-identifiability problem. The red dotted line represents the steady-state level, labelled with a/b. The horizontal axis represents time and the vertical axis represents transient values of dependent variable x. The convergence of the function to the steady state is proportional to the increase in the value of parameter b.

of this type of problem is given below:

$$\dot{\mathbf{z}}(t, x) = \mathbf{f}(\mathbf{z}(t, x), \mathbf{p})$$
$$\mathbf{y}^M(t, x) = \mathbf{g}(\mathbf{z}(t, x), \mathbf{p})$$

where $\mathbf{z}$ is the vector of state variables describing the change over time of the system and $\mathbf{p}$ is the vector of model parameters. $\mathbf{f}$ specifies the model, and $\mathbf{y}^M$ is the vector of measured states. The measurement process $\mathbf{y}^M$ is modelled by an observational function $\mathbf{g}$. Often, the experimental data points $\mathbf{y}^D$ are corrupted by measurement noise. Given a set of time points $i = 1, \ldots, m$ for system components $j = 1, \ldots, n$, if we assume the noise to be Gaussian with known constant variance $\eta \sim N(0, \sigma_{ij}^2)$, then the experimental data can be expressed as:

$$\mathbf{y}^D(t_i, x_j) = \mathbf{y}^M(t_i, x_j) + \eta_{ij}$$

The aim is now to estimate the initial value $\mathbf{z}_0$ and the parameters $\mathbf{p}$ from

the observed data $\mathbf{y}^D(t_i, x_j)$ by minimizing the objective function, $\chi(\mathbf{p})$, which is defined in the form of a least-squares residual of model predictions ($\tilde{\mathbf{y}}$) and experimental measurements ($\mathbf{y}^D$), as follow:

$$\chi(\mathbf{p}) = \sum_{1 \leq i \leq m} \sum_{1 \leq j \leq n} \left(\frac{\mathbf{y}^D(t_i, x_j) - \tilde{\mathbf{y}}(t_i, x_j, \mathbf{p})}{\sigma_{ij}} \right)^2 \tag{5.1}$$

5.2.2 Objective Function

Formulating objective functions is not a simple task and typically a rather empirical process. The value of the objective value must ultimately give a measure of how well the optimization requirements are satisfied. An incorrect mathematical formulation of the objective function can have an influence on the estimated solutions when solving non-linear dynamic systems.

Objective functions in the form of least-squares residuals of experimental observations and model predictions (for each measured system component at each discrete time point available) is a technique commonly integrated with optimization methods in solving inverse problems. The simple nature of the least-squares structure should be used with caution however; as an inappropriate formulation may affect the searching algorithm, leading to incorrect solutions as a result.

Take a simple pathway for illustrative purposes. Suppose the output is described by a non-linear function $y = f(t, \mathbf{p})$, where variable y is dependent on time t and parameter $\mathbf{p}$. The objective function formulated using the standard least-squares structure is applied to resolve the unknown parameter vector $\mathbf{p}$ using steady-state observations. The predicted dynamics of variable y can be produced by three different sets of parameter estimates, each having approximately the same objective function value. Figure 5.3 clearly indicates that different sets of parameter estimates, $\hat{\mathbf{p}}$, can have the same least-squares residual between the reference (dotted red) and model simulation (black solid), giving rise to diverse dynamics for variable y. Only Figure 5.3(c) is consistent with the target, which is to model steady sate behaviour. This example shows that objective functions based on residual errors, i.e. a sum of least-squares residual, is not capable of tackling the estimation problem when the fitting is characterized by more than one feature. This subject is discussed in detail in Chapter 6, where a solving strategy to cope with multiple features appropriately is proposed.

(a) Model with linear dynamics

(b) Model with oscillatory dynamics

(c) Model with the steady-state property
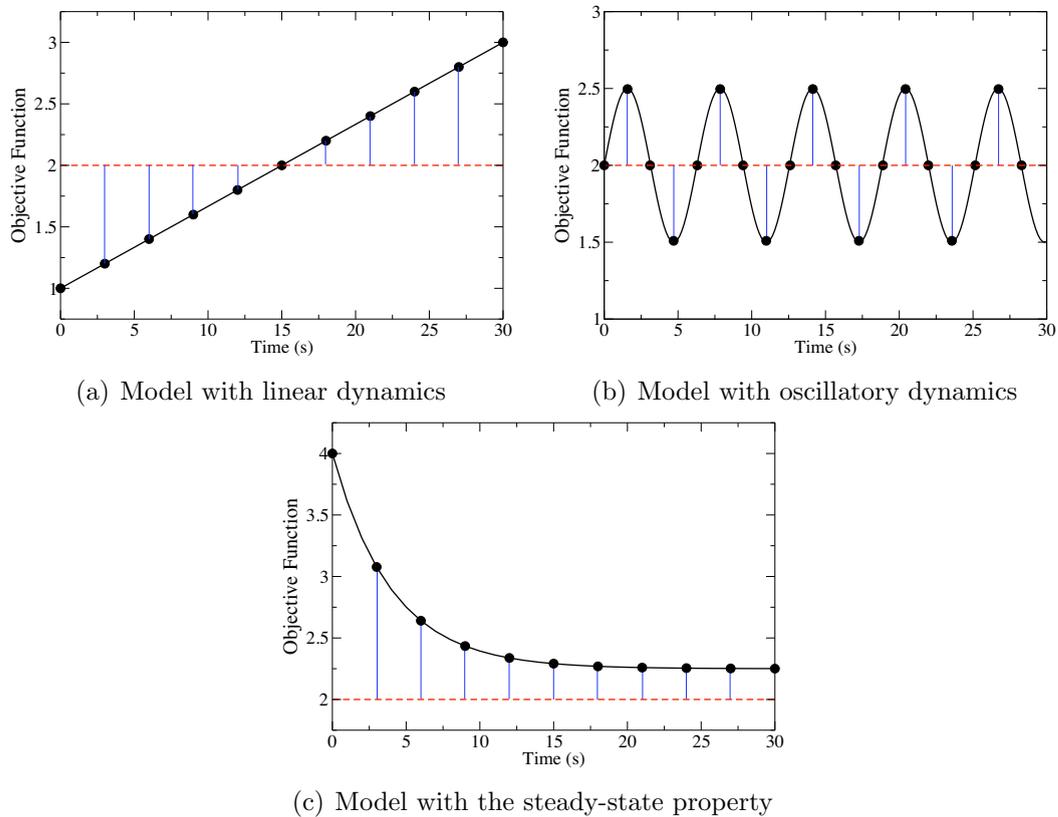
Figure 5.3: Diverse dynamics of variable y with respect to time, produced with different optimal parameter vectors. Vertical bars (blue) in each plot indicate the residual of the simulated (black solid with circles) and target data (red dotted) at each discrete time point. The sum of the residuals gives the overall value of the objective function. All the plots return approximately the same overall objective function value.

5.3 Partical Swarm Optimization

In the past few decades, many algorithms have been applied to solve optimization problems of dynamic systems. These include evolutionary algorithms (EAs) (Coello 2002), which are optimization algorithms that incorporate mechanisms that mirror biological evolutionary processes. They have proved popular as they require few assumptions about the problem and tend to have better global convergence properties.

Recently a modern heuristic algorithm–Particle Swarm Optimization (PSO) has been proposed (Kennedy and Eberhart 1995). PSO is a population-based optimization algorithm which was inspired by the social behaviour of fish schooling and birds flocking. Similar to other EAs, PSO can solve a variety of hard problems but with a faster convergence rate. The feature that it only requires

a few parameters to be tuned also makes the algorithm very attractive from the implementation point of view.

Copasi (Mendes 1993) is a popular software package for modelling, simulation and analysis of biochemical systems. It features many different algorithms for numerical optimization, ranging from those based on derivatives, to those inspired by nature, such as evolutionary algorithms. Recently, Copasi has been expanded with the PSO algorithm (Hoops et al. 2006) because of its very competitive performance in tackling the hard problem of high-dimensional parameter estimation.

The Bayesian approach (see Girolami 2008) is gaining increasing attention in this field, serving as a promising alternative; however, when the prior knowledge is scarce, the requirement for an appropriate prior distribution for all unknown parameters is very hard to satisfy. The approach can also be very computationally expensive when solving high-dimensional systems. Another drawback is the difficulty in making the Bayesian approach available within software packages – many of the fine-tuning tricks are problem specific and hence cannot be easily generalised. The theoretical sophistication causes the approach too subtle to be widely employed beyond the Bayes-educated community.

In light of its cost-effectiveness in tacking complex inverse problems, the PSO-based technique is investigated and employed in the thesis with an application to biological systems. A brief introduction on the basic PSO technique and the PSwarm algorithm is given in this section.

5.3.1 Basic PSO Technique

This description follows the presentation of the algorithm given by van den Bergh (van den Bergh 2001).

The PSO algorithm is based on a population of 'particles' that are initialised randomly and represents solutions of the optimization problem. Particles are associated with a velocity vector that guides the particle's movement through the search space and is dynamically adjusted according to the optimal position(s) of the particle throughout the previous generations.

Each particle is updated by two 'best' values termed *pbest* and *gbest*. The first value is the best position a particle has achieved so far – the so-called *personal best*, and the second value is the best particle position found among all particles – the *global best*. At each generation t, a stochastic combination

of the directions to the best position of the ith particle ($pbest_i^t$) and the best (among all) particle's position ($gbest^t$) is added to the previous velocity vector v_i^t associated with each particle x_i, allowing the search space to be effectively explored. Particles' velocities are updated by adding the new velocity vector v_i^{t+1} to the old position v_i^t according to the equations below:

$$
\begin{aligned}
v_i^{t+1} &= \omega \cdot v_i^t + \alpha_1 \cdot r_1 \cdot (pbest_i^t - x_i^t) + \alpha_2 \cdot r_2 \cdot (gbest^t - x_i^t) \\
x_i^{t+1} &= x_i^t + v_i^{t+1}
\end{aligned}
$$

where ω is called the inertia weight that was proposed by Shi and Eberhart (Shi and Eberhart 1998). Inertia weight is employed to control the impact of the previous history of velocities on the current velocity. α_1 and α_2 are two positive constants with values upto 2, called the *cognitive* and *social* parameter respectively. r_1 and r_2 are random numbers in the range 0 to 1.

There is evidence that has shown that PSO can outperform generic algorithm for difficult problem classes, primarily unconstrained global optimization problems (Vaz and Vicente 2007, Eberhart and Shi 1998, Parsopoulos and Vrahatis 2002b). Many successful applications of the PSO algorithm have been reported for solving engineering problems and numerous improvements to the algorithm have been proposed (Pant et al. 2008, Huang et al. 2005, Naka and Fukuyama 2001, Yoshida et al. 1999, van den Bergh and Engelbrecht 2004, Hu and Eberhart 2002a, Grosan et al. 2005, Higashi and Iba 2003, Krohling et al. 2004). However, very little work (Liu and Yokota 2005) has attempted to tackle biological problems with PSO. Refer to the thesis by van den Bergh (van den Bergh 2001) for an in-depth review of PSO-based variations.

Of all the modifications, the strategy of combining the PSO algorithm with the mutation mechanism often applied to EAs is the most prevalent technique. Mutation is an important component integral to genetic search. It has the potential to extend the effective area of the search space by introducing more diversity into the population (van den Bergh 2001). The mutation operator is triggered when a predefined threshold is satisfied that quantifies the current rate of convergence. A good choice of threshold is important to prevent the disruption of good candidates and the occurrence of a primitive random search.

One modern representative of the PSO-based variations is proposed by Ge et al. (Ge et al. 2007). The modification introduced an adaptive mutation mechanism to the standard PSO. At each generation, the prematurity state of

the algorithm is judged using the variance σ^2 and average fitness value $\bar{f}$ of all the particles according to the equations:

$$\tau^2 = \frac{\sigma_f^2}{\bar{f}^2}, \text{ where } \sigma_f^2 = \frac{1}{n}\sum_{i=1}^{n}(f_i - \bar{f})^2, \ \bar{f} = \frac{1}{n}\sum_{i=1}^{n}f_i$$

where n is the number of particles in the population and f_i is the fitness value of the ith particle. τ^2 represents the convergence degree of the population. If τ^2 is smaller than a given threshold (a predefined value) and the maximum number of iterations has not yet been reached, the algorithm is considered to have arrived at a premature convergence. When τ^2 is satisfied, inactive particles can be identified using the following inequality:

$$\frac{f_g - f_i}{\max[(f_g - f_j), (j = 1, \ldots, n)]} \leq \theta$$

where the difference in fitness values between the *gbest* particle, f_g, and the current particle, f_i, over the maximum difference in fitness values between the *gbest* particle and the rest of the population is compared with a predefined threshold, θ. In Ge et al.'s proposal, τ^2 and θ were given values of 0.005 and 0.01.

Next, Gaussian mutation is applied to the inactive particles, where each component in the inactive particle is perturbed using a Gaussian random disturbance with zero mean and constant variance. In our implementation, we randomly generated 20 samples with Gaussian mutation from each of the inactive particles. If no perturbed particle is better than the parent particle in terms of the fitness value, then the parent particle is retained, otherwise the best perturbed particle is used.

In the following section, we introduce the implementation of the core optimization algorithm that has been employed throughout the thesis.

5.3.2   PSwarm Algorithm

The PSwarm algorithm (Vaz and Vicente 2007) is a recently developed implementation of PSO. The algorithm is designed around a Search and Poll framework. The Search step is the key to the practical efficiency and takes advantage of the standard PSO procedure. The Poll step ensures robustness and explores around the positions of unsuccessful particles in the Search step by perturbing

the particle in all possible search directions. The Poll step is only involved when the Search step failed to find an improved solution (i.e. one that decreases the objective function value).

Generalized pattern search algorithms were defined and analyzed by Torczon (Torczon 1997). Their study dealt with derivative-free unconstrained optimization on continuously differentiable functions using the ideal spanning directions introduced by Lewis and Torczon (Lewis and Torczon 1996). The set of spanning directions D is the basic ingredient in the definition of the mesh, and is formed by the set of unit vectors and their negatives that are the basis for the problem space, as below:

$$D = [e_1, \ldots, e_n, -e_1, \ldots, -e_n]$$

PSwarm uses the spanning set to coordinate the search for variables. In the algorithm, $e_1, \ldots, e_n$ and $-e_1, \ldots, -e_n$ form two identity matrices (positive and negative) of order n representing the dimension of solution vector x.

Given a positive spanning set D and the current iterate k, two sets of points are defined: the mesh set M_k and the poll set P_k. The mesh is centered around the current iterate x_t and its fitness is parameterized through the mesh size parameter Δ_k as follows:

$$M_k = \{x_k + \Delta_k D z \ : \ z \in \mathcal{Z}_+\} \tag{5.2}$$

where $\mathcal{Z}_+$ is the set of nonnegative integers, which, in the algorithm, has the dimension equal to the dimension of solution vector x. The mesh set M_k is used to conduct the search step. If the poll step is activated, the function f is evaluated at the neighboring mesh points to check if a lower function value can be found. If either the search or poll step produces an improved mesh point, then the mesh size parameter is reset using the rule:

$$\Delta_{k+1} = \tau^{\omega_k} \Delta_k \tag{5.3}$$

where τ is a rational number ≥ 1 that remains constant over all iterations, and ω is an integer ≥ 0. If the poll step fails to find an improved mesh point, the mesh is reset by setting the mesh size parameter

$$\Delta_{k+1} = \tau^{\omega_k} \Delta_k \tag{5.4}$$

where τ is greater than one, as defined above and ω_k is an integer ≤ -1.

When no success is obtained at the search step, the poll step evaluates the function f at the point x_k in the poll set. The poll set P_k is composed of mesh points neighboring the current iterate x_k in the directions of the columns of D_k:

$$P_k = \{x_k + \Delta_k d \ : \ d \in D_k\} \tag{5.5}$$

A general procedure of pattern search framework is summarized in Algorithm 1, based on the description given by Audet and Dennis (Audet and Dennis 2000).

Algorithm 1 A general pattern search algorithm for unconstrained minmization (Audet and Dennis 2000).

1: **INITIALIZATION**:
2: Let $x_0 \in \mathbf{R}^n$ be such that $f(x_0)$ is finite. Let D be a positive spanning set, and let M_0 be the mesh defined by $\Delta_0 > 0$, and set $D_0 = D$. Set the iteration counter $k = 0$.
3: **SEARCH STEP**:
4: Evaluate f at a number of points in M_k. If an improved mesh point $\hat{x}_k$ is found for which $f(\hat{x}_k) < f(x_k)$, then set $x_{k+1} = \hat{x}_k$ and update $\Delta_{k+1} \geq \Delta_k$ according to rule in Equation (5.3). If the search step was successful, then go to step 7, otherwise invoke the poll step.
5: **POLL STEP**:
6: Evaluate f on the poll set defined in Equation (5.5). If an improved point is found for which $f(x_k + \Delta_k d) \leq f(x_k)$, then set $x_{k+1} = x_k + \Delta_k d$ and update $\Delta_{k+1} \geq \Delta_k$ according to rule in Equation (5.3). Otherwise, set $x_{k+1} = x_k$ and update $\Delta_{k+1} < \Delta_k$ according to rule in Equation (5.4).
7: Increment k by 1 and go back to the search step.

The algorithm uses the appealing 'barrier' strategy to handle the boundary constraints when solving unconstrained minimization problems, excluding any infeasible point from being used in the next iteration. The termination criteria include the satisfaction of the maximum number of iterations and number of function evaluations, and the specified tolerance threshold.

The PSwarm algorithm applies the standard PSO technique in the search step to enhance the capability of the pattern search method in solving multimodal problems, helping to prevent the population from being trapped at local optima. In the meantime, the poll step inherited from pattern search methods rigorously guarantees the convergence to stationary points from arbitrary starting points. PSwarm has been demonstrated to outperform several global optimization methods, giving a better convergence rate and an optimal set of pa-

rameter estimates and a Matlab implementation of the algorithm was obtained from Vaz and Vicente (Vaz and Vicente 2007).

One objective of the computational investigation is to compare the performance of the PSwarm algorithm and its variant with Gaussian mutation, termed PSwarmGM, when solving a complex biological problem – in this case, the ERK signalling pathway. The adaptive mutation mechanism proposed by Ge et al. (Ge et al. 2007) (introduced in Section 5.3.1) was adopted as the modification to the algorithm as previous results have shown it to have fast convergence and accurate parameterization in solving non-linear systems. The PSwarm implementation was modified to include this mutation mechanism by myself.

Crucial questions for model parameter estimation that arise in the process of computational modelling are concerned with

1. How capable an optimization algorithm is in tackling the problem of interest. This is reduced to testing the performance of an optimization method

   - when experimental measurements are not informative, and

   - when experimental measurements are contaminated with noise.

2. If unknown parameters are identifiable with a given model structure and how they are determined by the quality of experimental measurements.

To clarify the above questions, the ERK signalling pathway is considered as a case study. The computational study was conducted under a variety of conditions. The first question is investigated in Section 5.4, with both clean data sets and those corrupted with measurement noise, and data sets containing different levels of information content. The second question refers to the problem of model identifiability, which is discussed in Section 5.5.

5.4 Case Study: ERK Signalling Pathway

The ERK signalling system (also called the Ras/Raf1/MEK/ERK pathway) is "a ubiquitous pathway that conveys mitogenic and differentiation signals from the cell membrane to the nucleus" (Cho et al. 2003). Briefly, Raf1 (Raf-1* or activated Raf) is activated by binding to Ras proteins activated by external stimuli (e.g. growth factor). This then reacts with RKIP to form Raf1/RKIP, in turn activating ERK, which is responsible for gene expression of transcription factors. A schematic representation of the pathway is shown in Figure 5.4.
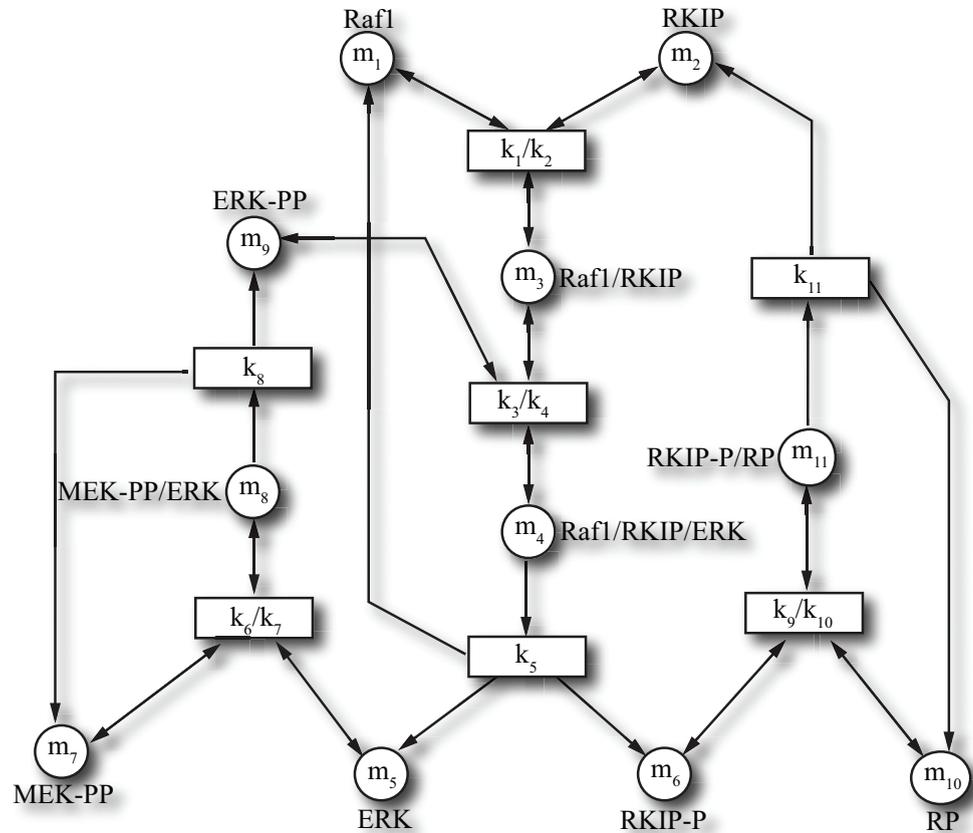
Figure 5.4: A schematic representation of ERK signalling pathway.

The cascade of Raf1-MEK-ERK regulates cellular proliferation, differentiation, and survival. Its malfunction is a common occurrence in human cancers, and due to this reason, there has been substantial scientific investigation into the functioning and structure of this pathway for the development of efficient therapies. The primary objective of our work consisted of studying the global optimization approach in solving the inverse problem of this interesting biological application. Information about the biological significance of the pathway in growth factor signaling and cancer can be obtained from the studies (Calder et al. 2006, Shankland et al. 2005, Schoeberl et al. 2002).

The ERK signalling pathway is selected as the case study of the computational investigation for its well-characterized mathematical model structure. Thus, parameter estimation uncertainties arising from an incorrect model structure can be eliminated. Since model parameters (e.g. reaction rates) are known, we can assess the accuracy and reliability of parameter estimation methods by calculating the deviation between estimated parameter sets and the reference

values. When the reference values of the parameters are not known, this measure is infeasible, as is the case for the parameter estimation of the polyamine biosynthetic sub-pathway in Chapter 7.

The computational model is presented in the form of non-linear ODEs based on mass-action kinetics for the production and decay of all the components involved in different chemical reactions. In Figure 5.4, nodes stand for system components labelled by proteins and edges indicate biochemical conversion between proteins with arrows denoting the reversibility of the conversion. Each node and reaction has an associated concentration and a rate constant, symbolized by m_i (where $i = 1, 2, \ldots, 11$) and k_j (where $j = 1, 2, \ldots, 11$), respectively. Rate constants are given in rectangles, where the pair k_n/k_{n+1} represents both the forward (k_n) and backward (k_{n+1}) rate of a particular reaction.

To understand the functionality of the ERK signalling pathway, a quantitative description of the system dynamics is necessary. The ODEs of the pathway are presented in Table 5.1.

Reaction Number	Kinetic Equations
1	$-k_1 \cdot m_1 \cdot m_2 + k_2 \cdot m_3 + k_5 \cdot m_4$
2	$-k_1 \cdot m_1 \cdot m_2 + k_2 \cdot m_3 + k_{11} \cdot m_{11}$
3	$k_1 \cdot m_1 \cdot m_2 - k_2 \cdot m_3 - k_3 \cdot m_3 \cdot m_9 + k_4 \cdot m_4$
4	$k_3 \cdot m_3 \cdot m_9 - k_4 \cdot m_4 - k_5 \cdot m_4$
5	$k_5 \cdot m_4 - k_6 \cdot m_5 \cdot m_7 + k_7 \cdot m_8$
6	$k_5 \cdot m_4 - k_9 \cdot m_6 \cdot m_{10} + k_{10} \cdot m_{11}$
7	$-k_6 \cdot m_5 \cdot m_7 + k_7 \cdot m_8 + k_8 \cdot m_8$
8	$k_6 \cdot m_5 \cdot m_7 - k_7 \cdot m_8 - k_8 \cdot m_8$
9	$-k_3 \cdot m_3 \cdot m_9 + k_4 \cdot m_4 + k_8 \cdot m_8$
10	$-k_9 \cdot m_6 \cdot m_{10} + k_{10} \cdot m_{11} + k_{11} \cdot m_{11}$
11	$k_9 \cdot m_6 \cdot m_{10} - k_{10} \cdot m_{11} - k_{11} \cdot m_{11}$

Table 5.1: An ODE-based computational model of ERK signalling pathway.

The performance of both standard PSwarm and PSwarmGM for the solution of the inverse problem of ERK signalling system is presented. As we are interested in the dynamic behaviour of the system and since the accuracy of parameter estimation depends on the actual parameter values, an exact knowledge of the actual parameters is required in order to assess the performance of the techniques.

To demonstrate the prospects of experimental design considerations, the following parameters k_i and initial concentrations of system components $m_i|_{t=0}$

have been chosen for the purpose of the presented simulation study.

$k_1 = 0.53, \ k_2 = 0.0072, \ k_3 = 0.625, \ k_4 = 0.00245, \ k_5 = 0.315, \ k_6 = 0.8,$

$k_7 = 0.0075, \ k_8 = 0.071, \ k_9 = 0.92, \ k_{10} = 0.00122, \ k_{11} = 0.87.$

$m_1|_{t=0} = m_2|_{t=0} = 2.5, \ m_3|_{t=0} = m_4|_{t=0} = m_5|_{t=0} = m_6|_{t=0} = 0,$

$m_7|_{t=0} = 2.5, \ m_8|_{t=0} = 0, \ m_9|_{t=0} = 2.5, \ m_{10}|_{t=0} = 3, \ m_{11}|_{t=0} = 0.$

The computational model of the ERK signalling pathway (Table 5.1) was then used to generate pseudo-experimental data from the above predefined parameters and initial concentrations of proteins, to which the estimation algorithm was applied. The time-dependent behaviour of the system components is shown in Figure 5.5. The time span of the computational simulation was 10 minutes and was solved with the *ode15s* solver in Matlab. This simulated data represents the exact results that are free of measurement noise.
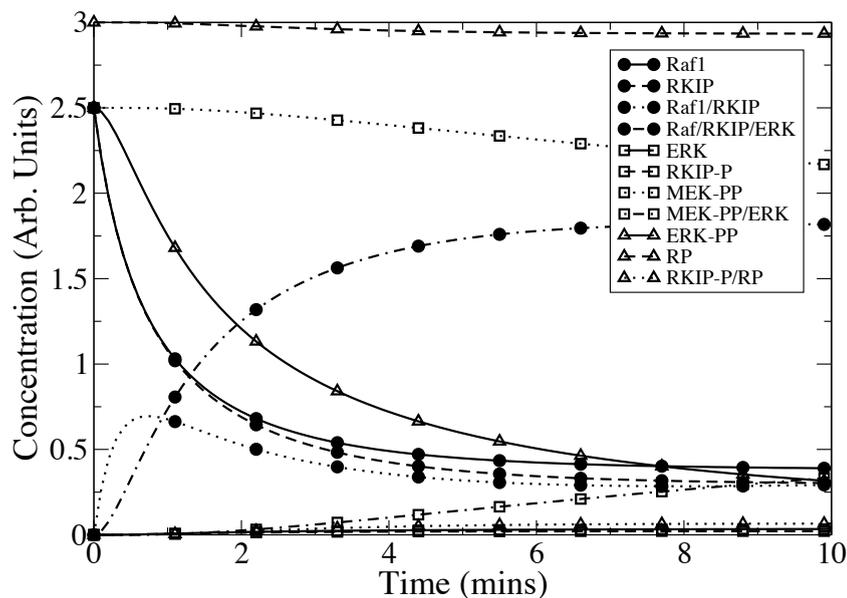
Figure 5.5: Simulation profiles of the dynamics of ERK signalling pathway computed with the reference values of model parameters and initial concentrations of system components.

5.4.1 Informative vs. Non-informative Measurements

This section is concerned with the element of the first question defined in Section 5.3.2 – what is the impact of experimental measurements on parameter estimation? Towards the end, we employed both estimation algorithms (PSwarm and PSwarmGM) to solve the inverse problem of the ERK signalling pathway by using data sets with different levels of information content.

Derivatives of the system dynamics over time are shown in Figure 5.6. It indicates that over the whole simulation interval 0–10 minutes, large changes in the concentrations of the system components occur within the first 5 minutes. No transient dynamics are observed in the later stage of simulation between 5 and 10 minutes, indicating that the system has reached a steady state.
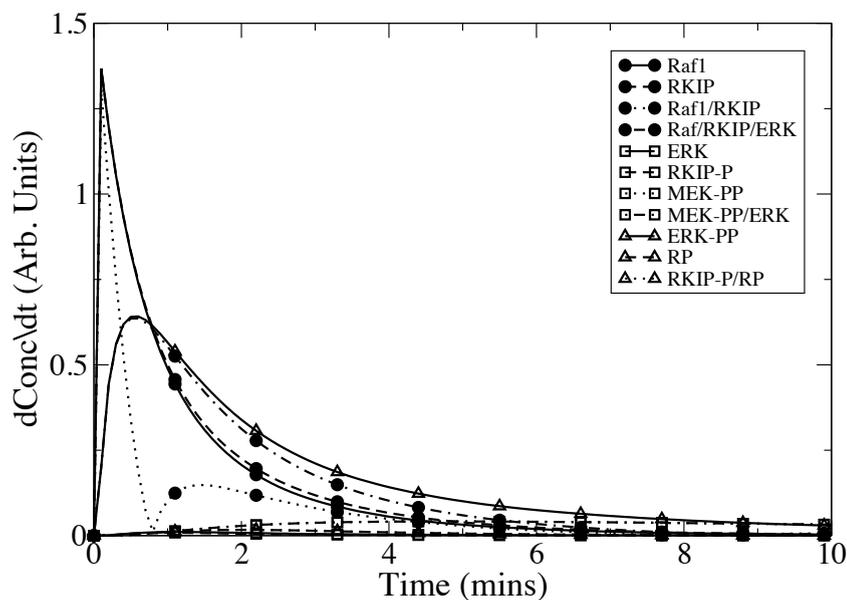
Figure 5.6: Derivatives of the concentrations of system components shown in Figure 5.5, with respect to time.

Solving the inverse problem of the ERK signalling pathway involves estimating 11 parameters. The objective function was formulated using the least-squares structure (see Equation (5.1)). The choice of the least-squares representation is suitable for the present study since the fitting is only characterized by the residual between model simulation and observed data. The estimation algorithms were evaluated when the simulated data over the three intervals 0–5 minutes,

5–10 minutes and 0–10 minutes were used as experimental measurements. The optimization methods of PSwarm and PSwarmGM were executed 100 times to solve the problem of interest. Each run started with a different initial population in the search space, and the objective function was evaluated every 30 seconds of the simulated time interval.

The convergence of the estimation algorithm PSwarm and PSwarmGM to minima from arbitrary starting points is evaluated by studying the properties of a sequence of global best solutions (*gbest*). Global convergence is ensured if the *gbest* solution at the current iteration is no worse than the *gbest* solution obtained from the previous iteration (Reyes-Sierra and Coello 2006). In order to determine the most favourable strategy, it is necessary to examine both final objective function value and the time taken to reach this value (convergence rate). As such, the strategy that reaches the lowest objective function value in the shortest time in the most useful.

The convergence curves of the algorithms are plotted in Figure 5.7, which describe the objective function values calculated by the *gbest* particle at each iteration versus computation time. The figure is scaled by applying a logarithm (base 10) to computation time and a double logarithm (base 10) to the objective function values in order to allow the convergence tendency to be better shown. The convergence characteristic was studied for both the best run (smallest objective function value) and worst run (largest objective function value) returned by the algorithm when the following data sets were used for the estimation. In this case, noise-free experimental measurements were considered.

- Data set 1 : pseudo-experimental data over time interval 0–5 minutes

- Data set 2 : pseudo-experimental data over time interval 5–10 minutes

- Data set 3 : pseudo-experimental data over time interval 0–10 minutes

For the best run, as shown in Figure 5.7(a), PSwarm presented a better convergence at most times for all the data sets. In particular, PSwarm outperforms PSwarmGM in terms of convergence rate with data set 2 (in blue). When data set 1 and 3 were used for estimating the parameters, PSwarm and PSwarmGM dominate the convergence rate in turn; however, in the later stages, PSwarm approaches the global minimum with a faster convergence speed. Similar convergence property is also observed for the worst run, as shown in Figure 5.7(b). During the entire course of parameter searching, PSwarm always

arrives at much better objective function value than PSwarmGM. The investigation demonstrates a good agreement with the finding by van den Bergh (van den Bergh 2001) that the mutation mechanism can slow down the rate of convergence of the PSO-based approach.

Figure 5.8 shows the estimated statistical distribution of the objective function values from PSwarm and PSwarmGM, using the three data sets mentioned above. It is evident that data set 1 results in a more accurate determination of the parameters than the other two data sets with both algorithms. In this case, no significant differences are detected between PSwarm and PSwarmGM. This suggests that parameter estimation with transient data considerably improves the performance of the optimization algorithms.

The density plots (Figure 5.8) computed with the last two data sets reveal an interesting result that might be surprising at first sight: parameter estimation with data set 3 using the whole-interval dynamics did not improve the optimization performance compared with the solutions using data set 2, which captures only a small range of the system dynamics. This may be due to the fact that when the observation data points are given equal weight in the objective function, adding more, less informative points (the steady-state points) reduces the information content of the data set 3.

Mean and standard deviations of the parameter estimates from 100 runs of PSwarm and PSwarmGM are reported for the three data sets, reported in Table 5.2 and Table 5.3. Both algorithms show better performance in recovering the underlying parameters from transient data over the range 0 to 5 minutes (data set 1). Additionally, with all data sets, PSwarmGM does not improve the accuracy of the estimated parameters of the ERK signalling pathway compared with PSwarm. In the former case, inaccurate mean values or large standard deviations were observed for all parameters.

5.4.2 Perfect vs. Noisy Measurments

In an attempt to investigate the effect of noise on parameter estimation, the following three scenarios are examined using both PSwarm and PSwarmGM.

- Scenario 1: perfect data without noise

- Scenario 2: noisy data with a normally distributed error of 5%

- Scenario 3: noisy data with a normally distributed error of 10%
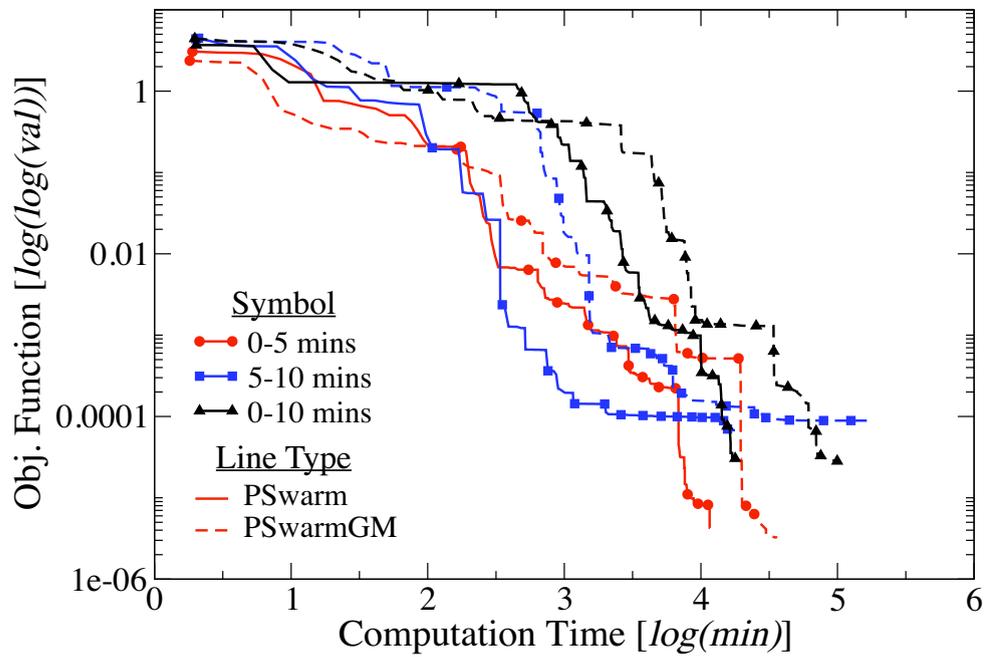
(a) Best run of the optimization algorithms
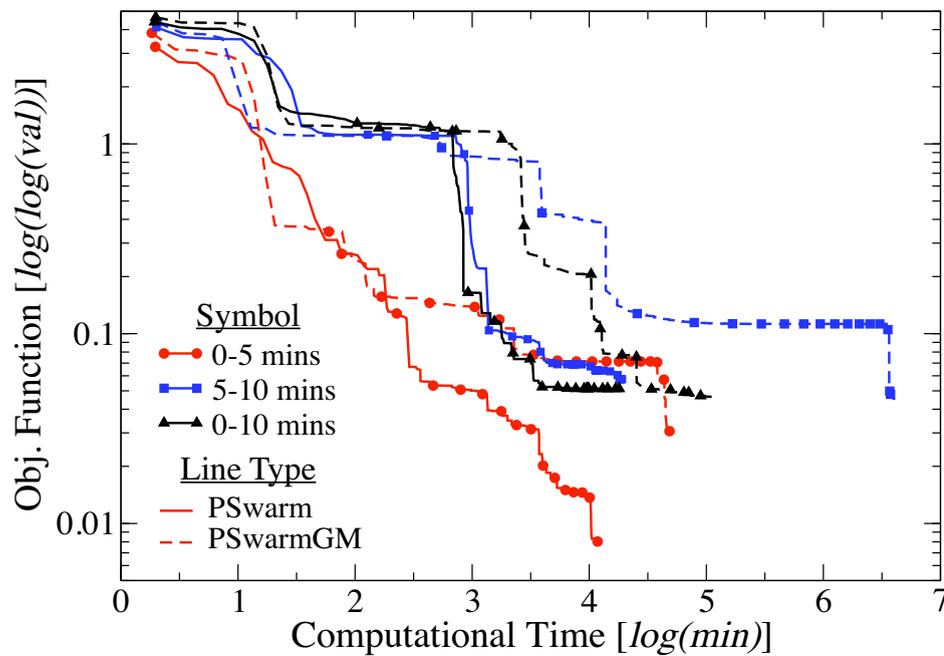
(b) Worst run of the optimization algorithms

Figure 5.7: Convergence curves (objective function versus computation time) of PSwarm (solid line) and PSwarmGM (dotted line) with all three data sets: red–data set 1; blue–data set 2; black–data set 3. PSwarm converges to the global minimum with better performance in all three cases.
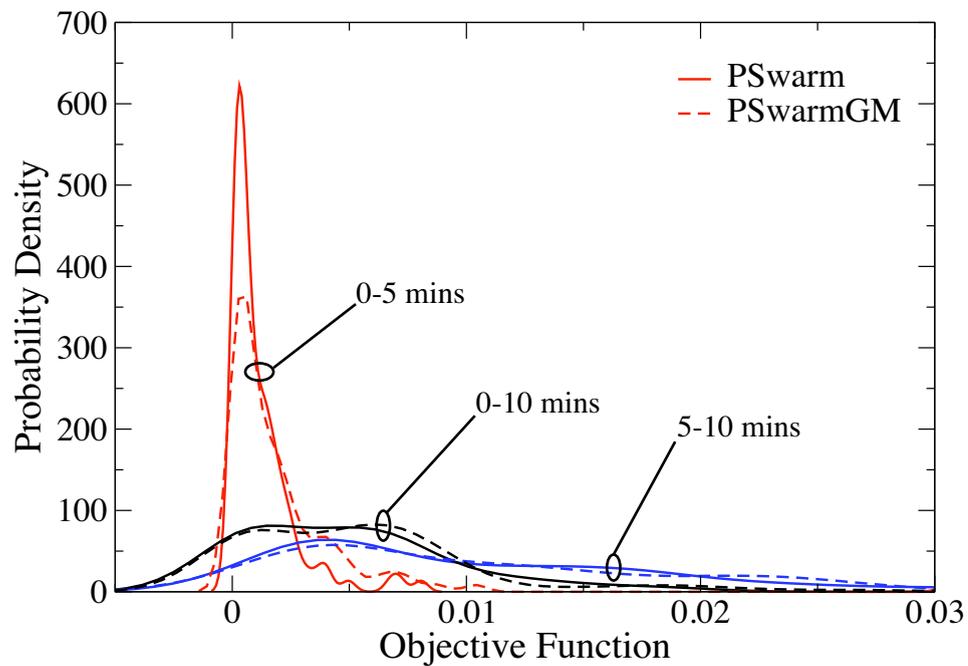
Figure 5.8: The estimated statistical distributions of the objective function values from PSwarm (solid line) and PSwarmGM (dotted line) using all data sets: red–data set 1; blue–data set 2; black–data set 3. Better performance from both algorithms is obtained when data set 1 was used.

The data sets employed in scenario 2 and 3 were generated by adding Gaussian white noise to the perfect pseudo-measurements. White noise satisfies the requirement that the errors are independent and identically distributed. We use a relative error ($\mathbf{r}$) of 5% and 10% of the measurements to define the standard deviation σ:

$$\hat{\mathbf{y}}_{(t_i)} = \mathbf{y}^o(t_i) \pm \sigma \tag{5.6}$$

where $\sigma = \mathbf{r} \cdot \mathbf{y}^o(t_i)$. $\mathbf{y}^o(t_i)$ and $\hat{\mathbf{y}}(t_i)$ stand for the pseudo-experimental and the noise-corrupted measurements at each discrete time point.

The uncertainty in the parameter estimates is analyzed for proteins *Raf1* and *Raf1/RKIP*, which have the largest changes in dynamics over time course (refer to Figure 5.6). Model predictions with the estimated parameters from scenario 2 and scenario 3 are comparable over the three time intervals (as defined in the last section) for both PSwarm and PSwarmGM. In this section, only the results from PSwarm, scenario 2 are discussed to avoid repetitive analysis.

The predictions of the protein concentrations are given in Figure 5.9. It shows that parameter estimation with PSwarm using 5% noisy measurements

Nominal Values	Data set 1	Data set 2	Data set 3
P1: 0.5300	0.5390±0.0069	0.7258±0.1754	0.5402±0.0082
P2: 0.0072	0.0265±0.0141	0.1212±0.1086	0.0312±0.0206
P3: 0.6250	0.6235±0.0083	0.6946±0.1651	0.6281±0.0070
P4: 0.00245	0.0046±0.0043	0.0125±0.0177	0.0070±0.0047
P5: 0.0315	0.0288±0.0026	0.0300±0.0064	0.0278±0.0035
P6: 0.8000	0.9316±0.0628	0.9308±0.0861	0.9381±0.0935
P7: 0.0075	0.0681±0.0574	0.0449±0.0395	0.0498±0.0410
P8: 0.0710	0.0247±0.0470	0.0616±0.0667	0.0306±0.0374
P9: 0.9200	0.9663±0.0485	0.9494±0.0830	0.8919±0.0902
P10: 0.00122	0.1500±0.0884	0.0834±0.1002	0.0608±0.0640
P11: 0.8700	0.7509±0.1101	0.8079±0.1495	0.7548±0.1094

Table 5.2: Mean and standard deviation values of the parameters estimated by PSwarm. Column 1 gives the nominal values of the parameters and columns 2 to 4 report the estimates from each data set.

Nominal Values	Data set 1	Data set 2	Data set 3
P1: 0.5300	0.5411±0.0096	0.7622±0.1756	0.5411±0.0087
P2: 0.0072	0.0302±0.0170	0.1410±0.1052	0.0321±0.0210
P3: 0.6250	0.6238±0.0101	0.6828±0.1678	0.6266±0.0090
P4: 0.00245	0.0052±0.0050	0.0121±0.0174	0.0065±0.0046
P5: 0.0315	0.0285±0.0030	0.0297±0.0062	0.0279±0.0035
P6: 0.8000	0.9465±0.0584	0.9340±0.0913	0.9382±0.0954
P7: 0.0075	0.0605±0.0576	0.0547±0.0405	0.0514±0.0407
P8: 0.0710	0.0261±0.0524	0.0576±0.0646	0.0309±0.0375
P9: 0.9200	0.9770±0.0426	0.9423±0.0879	0.9043±0.0848
P10: 0.00122	0.1832±0.1106	0.0814±0.0987	0.0727±0.0621
P11: 0.8700	0.7312±0.1331	0.8027±0.1429	0.7560±0.1098

Table 5.3: Mean and standard deviation values of the parameters estimated by PSwarmGM. Column 1 gives the nominal values of the parameters and columns 2 to 4 report the estimates from each data set.

over the range of 0–5 minutes and 0–10 minutes compare reasonably well with the predictions made using the nominal parameter values and those estimated using the noise-free measurements; the latter is shown in Figure 5.10.

The predictions with measurements over 5–10 minutes are poor, which may be due to poor identifiability of the parameters for the reactions stabilizing the steady state. It is observed that a decrease in the information content of the
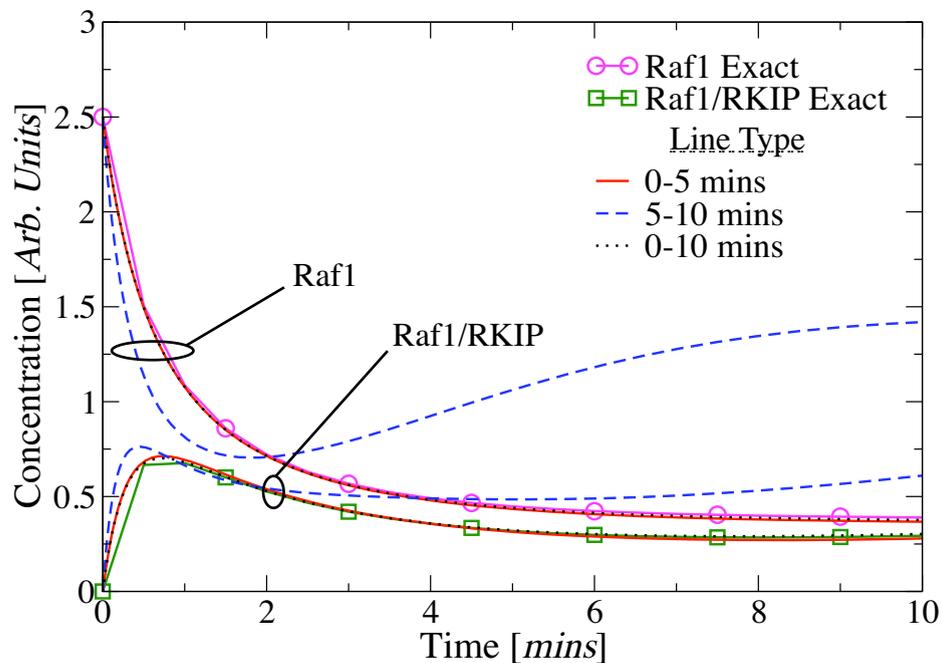
Figure 5.9: Model predictions of the protein concentrations of *Raf1* (open circle) and *Raf1/RKIP* (open square) with parameters estimated from PSwarm when 5% noise was added to the pseudo-experimental data.

measurements results in poor model predictions, and this is most prominent when measurement noise is present. An analysis of the information content of the data sets is presented in the next section.

5.5 Model Identifiability Analysis

There may exist several groups of functionally related parameters, which may consequently be difficult or impossible to determine unambiguously. Parameter values are estimated by fitting the model structure to experimental data, and parameters for which no unique solution exists are called *non-identifiable*. Non-identifiability originates from two sources, including structural non-identifiability and practical non-identifiability.

Structural non-identifiability is related to the model structure and independent of experimental measures. Such non-identifiability is referred to as an *a priori* identifiability analysis and in this case the model should be examined before computational procedures for parameter values. Practical non-identifiability is determined by the amount and quality of experimental measurements and is independent of structural identifiability. That is, a system may be confronted
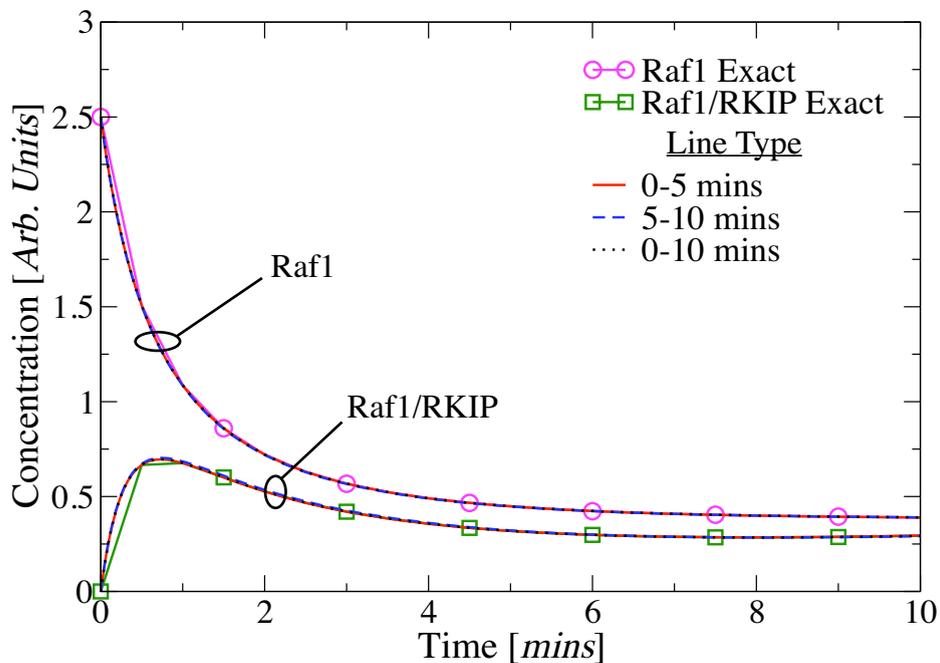
Figure 5.10: Model predictions of the protein concentrations of *Raf1* (open circle) and *Raf1/RKIP* (open square) with parameters estimated from PSwarm with the noise-free pseudo-experimental data.

with such identifiability difficulty even with a structurally identifiable model.

It is a necessary prerequisite for mathematical analysis of a parameterized model that it should be sufficiently identifiable. There are different analytical strategies that can be employed to investigate the model identifiability. In Appendix C, we detail the theoretical background of identifiability analysis and the numerical methods for computational calculation. An identifiability analysis of the ERK signalling pathway is conducted in this section. The main questions to be addressed are:

- if model parameters are identifiable with a given model structure, and

- how estimation accuracy is determined by the quality of experimental measurements

5.5.1 Structural Identifiability

The Taylor series and Ritt-Wu are two popular analytical approaches to prove structural identifiability. However, when it comes to solving complex non-linear problems, analytical approaches are not feasible. Muller (Muller 2002) presented

an overview of the relevant numerical methods. An estimation based method proposed by Muller et al. (Muller et al. 2002) achieved success in proving the structural identifiability of a non-linear model of anaerobic waste water treatment. The method investigates structural identifiability by calculating the condition number of covariance matrix of the estimates and observing the changes of the condition number in response to an increase in the number of data points. When structural non-identifiability is the case, the condition number tends towards infinity.

In this thesis, we employed the well-established numerical method of Jacquez and Greif (Jacquez and Greif 1985) to evaluate the structural identifiability of the parameters at the nominal parameter values. When the nominal values are hard to obtain, which is common in reality, the best set of parameter estimates can be used instead, since we assume that the best estimates at the convergence point are close to the true parameter values.

The correlation matrix of the ERK signalling pathway in Figure 5.11 was generated to evaluate (local) structural identifiability. The numerical method for calculating the correlation matrix was given in Appendix C.1. In this figure, certain pairs of parameters presented correlation coefficients very close to $+1$ or -1, indicating that some parameters cannot be identified from the data.
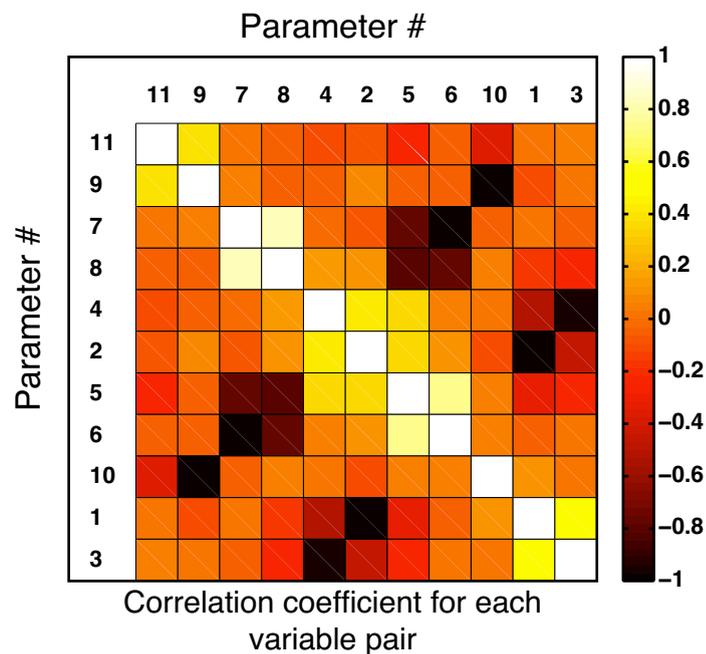
Figure 5.11: Correlation matrix for evaluating structural identifiability with the data set devoid of noise.

As an example, Figure 5.11 shows that pairs such as (k_6, k_7) and (k_9, k_{10}) have correlation values greater than 0.9, while pairs such as (k_1, k_7) and (k_3, k_9) have correlations less than 0.01. To further illustrate the identification difficulties, Figure 5.12 and Figure 5.13 present a fitness landscape of a two-parameterized situation for a pair of the highest correlated parameters (k_9, k_{10}) and lowest correlated parameters (k_6, k_{11}), respectively. The first case shows a very long valley along the diagonal of the parameters k_9 and k_{10}, reflecting the fact that there exist many combinations of the values which can give an equally low value of the objective function. The second case shows that, due to the lack of correlation, there is a unique pair of values for k_6 and k_{11} that corresponds to the minimum of the objective function.
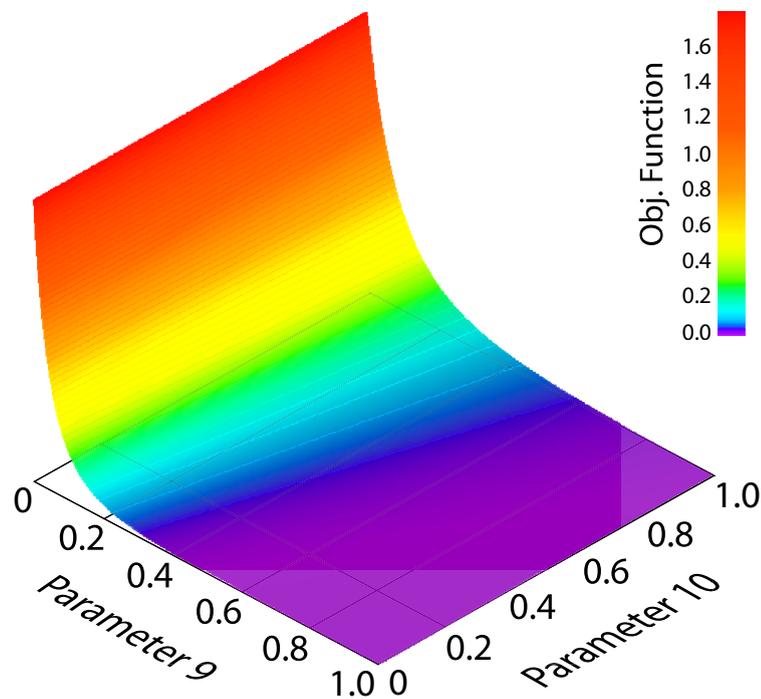
Figure 5.12: Objective function versus parameters k_9 and k_{10}, indicating the large parameter correlation.

In the context of optimization, a severe obstacle imposed by parameter correlation is that some of the parameters are unidentifiable or identifiable but poorly estimable. The latter refers to the situation where a small value of the objective function can be obtained but some of the estimated parameters always have a very large standard deviation. If this is the case, then the unidentifiable parameters would have to be fixed at measured values or at the best estimated values in order to make the model structurally identifiable. Alternatively, models can
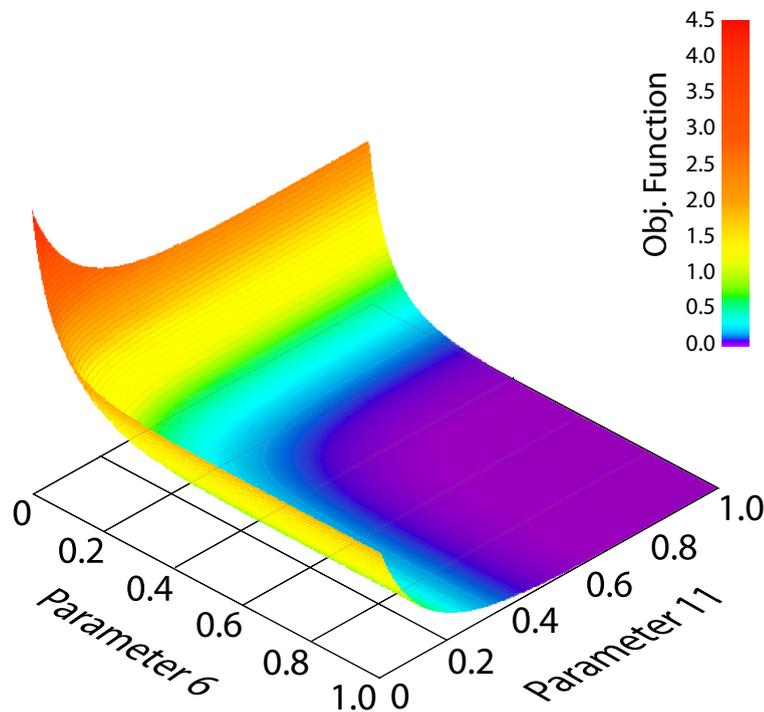
Figure 5.13: Objective function versus parameters k_6 and k_{11}, indicating a lack of identifiability.

be reduced further to remove unidentifiable parameters if necessary.

5.5.2   Practical Identifiability

Practical identifiability analysis arises due to limited amount and quality of experimental data. Practical non-identifiabilities manifest as functionally related parameters. The analysis examines the probability distribution of parameter estimates by fitting a model repeatedly to data containing a certain degree of noise and investigating the estimates.

The Hessian matrix, which contains the second derivatives of the objective function with respect to the parameter estimates at convergence, is a common means for testing the practical identifiability. The inverse of the Hessian matrix gives the variance-covariance matrix of the parameter estimates, which enables us to quantify the information content of an experimental data set (Faller et al. 2003). A basic procedure for constructing the FIM is presented in Appendix C.2.

The Hessian matrix is interesting in its own right. In gradient descent methods, the condition number of the Hessian gives an indication of the rate of convergence and the impact of floating point rounding errors on the minimum

objective function value (Thacker 1989). This number is defined as a ratio of its largest to smallest eigenvalues. When the ratio is close to unity, the matrix is well conditioned. However, in practice, the observable data is generally not rich enough to allow for reliable estimation of all parameters. This causes an ill-posed estimation problem, where a large uncertainty in the parameter estimates will result.

We computed the gradient of the objective function, with each column of the gradient corresponding to an individual system component, and calculated the Hessian matrix. The eigenvalues of the Hessian matrix are used to determine whether a particular set of data is sufficient for parameter estimation. We determined the information content of all the three sets of experimental measurements (noise free) over 0–5, 5–10 and 0–10 minutes by calculating the condition number of the Hessian matrix of the best fit estimated by each of the three data sets. All the three data sets returned a condition number close to zero, with the first data set (3.7973e-21) being slightly better than the others, and when 5% noise was added to the first data set, a condition number of 4.3305e-25 was obtained. The tests indicate that the practical identifiability can be improved by using transient and precise measurements.

The variance-covariance matrix is the key to parameter uncertainty analysis. The conventional approach dependent on the Hessian matrix is not allowed when there are non-identifiable parameters in the underlying model. Parameter non-identifiability results in the Hessian matrix being singular, which causes the probability distribution of the parameters to be asymmetric. It is fair to say that the usefulness of Hessian matrix based approach is limited to studying identifiable parameters only.

The subset of structurally identifiable parameters must be determined beforehand in order for the Hessian matrix approach to be useful. The technique of Singular Value Decomposition can be employed towards this end. Refer to Golub et al. (Golub et al. 1976) for technical details on the subject. Singular value decomposition identified a subset of structurally identifiable parameters ($k_1, \ldots k_6$) for the model under investigation, which gives a condition number 1.7395e-07 using the first measurement set. This shows that, after removing the rest of the parameters ($k_7, \ldots k_{11}$), the original ill-posed problem is transformed into a better conditioned problem.

5.6 Conclusions and Discussion

In this chapter, we investigated the problem of parameter estimation for dynamic biochemical systems. We gave an in-depth introduction to the theoretical background of optimization, and evaluated the usefulness of a global optimization approach – Particle Swarm optimization and the derived method – the PSwarm algorithm – in solving the inverse problem of non-linear systems. A careful analysis of an inverse problem related to the ERK signalling pathway was presented as an illustrative example, where an error-free model structure was assumed. The case study revealed the usefulness of PSwarm in tacking the problem of ill-conditioning and multi-modality in parameter estimation.

By definition, parameter estimation involves using observed data to estimate values of parameters based on statistical procedures. Parameter estimation problems with measurement sets containing different information content were analyzed. Noise in the measurements leads to noisy estimates of the parameters, resulting in poor model predictions with the estimated solutions. This is most prominent when using measurements providing the least information content.

We are aware of variations of standard PSO that are claimed to perform better. A modern, well-tested variant (Gaussian Mutation) was implemented and was also used in this study. In this case, we found that the Gaussian Mutation variant takes more iterations to converge than with standard PSwarm. Accordingly, standard PSwarm is chosen for the study of the trypanthione metabolic pathway in Chapter 7. We emphasize that the prediction of correct system dynamics is more important than the accuracy of the estimated parameters when evaluating the performance of the algorithms.

We conducted an analysis of model identifiability and examined the impact of prior structural knowledge and the quality of experimental measurements on the parameterization issue. Model identifiability analysis explains the challenges of estimating parameters for non-linear systems where the parameters are potentially highly correlated. It concluded that the ERK signalling pathway is neither structurally or practically identifiable, since there are pairs of parameters that are significantly correlated. Such analysis can guide the estimation problem by suggesting biological experiments for measuring parameters that are dependent on each other, or by removing parameters via model reformation for a better identifiability property.

The challenge of parameter estimation using experimental measurements

that suffer from a poor information content (i.e. steady-state data) is addressed in Chapter 6. We propose an approach that tackles this problem by coupling transient data, which is rich in information, with steady-state data in the calibration procedure. The proposed approach can be generalized to solve a variety of multi-objective optimization problems constrained by more than one condition, which is particularly useful for model validation of biological systems where the observed data for parameter estimation is measured in the perturbed condition of the system. The proposed approach is applied to solve the inverse problem of the polyamine biosynthetic sub-pathway of the trypanothione metabolic pathway in Chapter 7.

Chapter 6

MoPSwarm: The Proposed Approach for Perturbation-based Model Validation

In this chapter we propose a novel approach for applying a multi-objective optimization to validation of perturbation-based models of biological systems. We compare the results from the proposed approach with those from the conventional validation procedure.

6.1 The Challenge

It is necessary to be able to accurately and reliably determine values for system parameters in order to develop predictive models (van Riel 2006). This is especially important when the dynamic behaviour of a system is highly dependent on parameter values. Parameter estimation is therefore an important research area in Systems Biology.

Parameter estimation is a particularly complex task when the validation of a biological system can only be achieved by comparing simulation results with measurements from perturbation experiments. A common approach to model validation involves a two-step procedure to determine the vector of estimated parameters that can correctly describe a system's responses to perturbations. This involves fitting model parameters to experimental data generated by a reference cell type (wild type) and then testing this model on data generated by a variation (mutant). Parameter estimation is one building block of the model

validation procedure.

For the trypanothione metabolic pathway under investigation, the drug inhibitor DFMO, which degrades the activity of enzyme ODC, was added to the cell after the cell reached steady state. When solving the model of trypanothione metabolism, a decision vector $\mathbf{p}$ that can be considered as a potential solution for the unknown parameters should satisfy two constraints:

1. Simulation results of the model with $\mathbf{p}$ should be consistent with the observed steady state (wild type)

2. When the parameter of interest in $\mathbf{p}$ is perturbed with a certain percentage that constitutes the perturbed vector $\tilde{\mathbf{p}}$, simulation results of the model with $\tilde{\mathbf{p}}$ should be consistent with the measured perturbation data (mutant).

It is well-known that a major difficulty in estimating model parameters from the steady-state data (as the reference cell type) lies in the fact that a potentially very large number of different values can be obtained for the model parameters, showing equally good consistency with the experimental data. This frequently makes the common approach infeasible in practice. From the perspective of parameter estimation, this difficulty arises from the lack of information contained in the wild-type data used for this purpose, as discussed in Chapter 5. An intuitive way to solve the problem is to use informative data, for example, the transient concentrations of the system components, to optimize the parameters with respect to a mutant and then test potential solutions against the wild-type cell. Again, the challenge is still present regarding whether or not the decision vector can meet the steady-state constraint.

Existing implementations of multi-objective optimization with Particle Swarm (MOPSO) have never been applied to complex real-world applications, by which we mean the applications involving a large number of decision variables and dependent on differential equations to describe the system dynamics. Even the most up-to-date MOPSO implementation by Rabbani et al. (Rabbani et al. 2009) was initially designed for solving the project selection problem, formulated as a set of time-independent algebraic equations parameterized with binary (0 and 1) decision variables.

In this chapter, we thus propose a novel approach for the application of multi-objective optimization using Particle Swarm for the validation of perturbation-based models of biological systems. This implementation is inspired by the

single-objective optimizer, PSwarm, which is designed in a Search-and-Poll framework. With the proposed approach, the constraints arising from both the steady-state condition (wild type) and the perturbed condition (mutant) are handled simultaneously. In the following sections, the theoretical background of multi-objective optimization is introduced and our proposed approach is described and evaluated using the ERK signalling pathway as a case study. The pathway was studied in detail in Section 5.4 in Chapter 5.

6.2 Multi-objective Optimization

This section provides the necessary background for the work on multi-objective optimization, which is described later. Some of the basic concepts in multiobjective optimization are introduced and the visualization of approximate non-dominated solutions, as produced by the proposed multi-objective optimizer, is also presented. The theoretical background related to multi-objective optimization is explained based on descriptions given by Fonseca and Fleming (Fonseca and Fleming 1995) and Coello (Coello 2001).

Most problems in nature have several, possibly conflicting, objectives to be satisfied. Many of these problems are frequently treated as single-objective optimization problems by transforming all but one objective into constraints.

Multi-objective optimization attempts to find a solution to more than one objective function simultaneously. Specifically, the Multi-objective Optimization problem can be defined as the problem of finding a solution which would give values for all objective functions that are acceptable to the performance criteria (Osyczka 1985). Specifically, multi-objective optimization attempts to find a vector of decision variables $\boldsymbol{X}$ that optimizes a vector function $\mathbf{F}$

$$\boldsymbol{F}(\boldsymbol{X}) = [f_1(\boldsymbol{X}), f_2(\boldsymbol{X}), \ldots, f_k(\boldsymbol{X})]^T \qquad (6.1)$$

whose elements represent each individual objective function, $f_i(\mathbf{X})$.

Multi-objective optimization intends to seek good compromises (or trade-offs), which are *Pareto optimum*, rather than a single solution as in conventional global optimization. By definition, $\mathbf{X}^*$ is Pareto optimal if there exists no feasible vector of decision variables $\mathbf{X} \in \mathcal{R}$ which would improve one performance criteria without causing a simultaneous degradation of performance in at least one other criterion. More precisely, we have the following definition.

Definition 6.1 (Pareto Optimum)**:** *We say that a vector of decision variables* $\mathbf{X}^* \in \mathcal{R}$ *is Pareto optimal if there does not exist another* $\mathbf{X} \in \mathcal{R}$ *such that* $f_i(\mathbf{X}) \leq f_i(\mathbf{X}^*)$ *for all* $i \in 1, \ldots, k$ *and* $f_j(\mathbf{X}) < f_j(\mathbf{X}^*)$ *for at least one* $j \in 1, \ldots, k$. *Here* $\mathcal{R}$ *denotes the feasible region of the the problem, where all constraints are satisfied.*

The concept defines a set of solutions that is the so-called *Pareto optimum set*. The vectors $\mathbf{X}^*$ corresponding to the solutions included in the Pareto optimal set are referred to as *non-dominated* solutions[1]. The objective functions of non-dominated solutions in the Pareto optimal set form the *Pareto front*, which can be purely/partially convex, concave or discontinuous. Approximation of the Pareto front is sometimes referred to as *Pareto optimization*. If we plot the objective functions (for two objectives) of the Pareto-optimal solutions, then we can obtain a Pareto front as shown schematically in Figure 6.1.
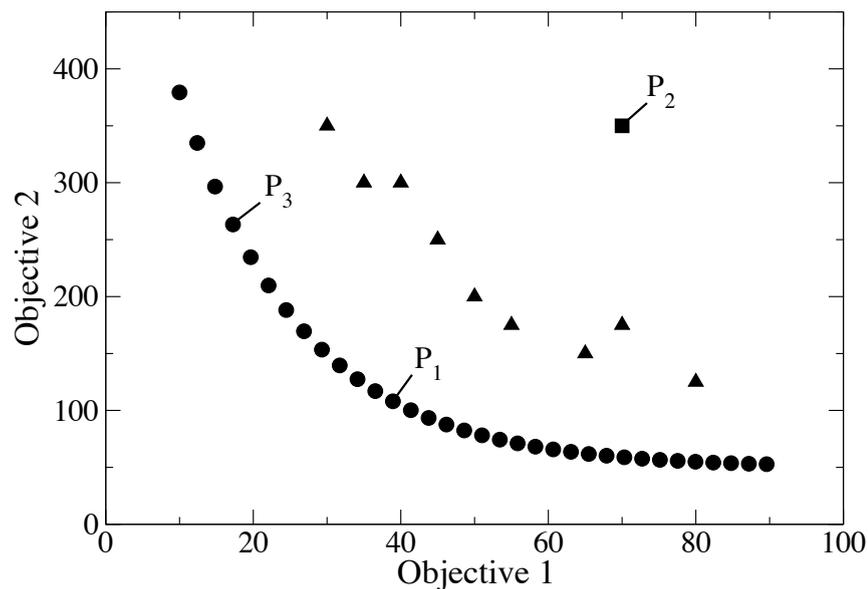
Figure 6.1: An ideal Pareto front for two objective functions is formed with filled circles in this figure. The rough curve in filled triangles can be understood as an intermediate front resolved during the course of search process. Test particles labelled with P_1, P_2 and P_3 illustrate different types of solutions. Refer to the text for a full explanation.

Figure 6.1 indicates that a solution lying on the Pareto-optimal frontier can-

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[1]We will clarify later how to interpret 'non-dominated solutions' in this context.

not improve one objective without sacrificing performance in the other. In a cost-effective scenario, no better performance can be possibly achieved without increasing cost. Given the constraints of the problem, no solutions can exist in the region below the front. All possible solutions located in the region above the front are defined as non-Pareto optimum indicated with filled triangles in Figure 6.1. As the search continues, the intermediate trade-off curve moves towards the true Pareto front in the possession of a smoother appearance. It is worth emphasizing that when solving real-world applications, no Pareto front can be as smooth as it is presented in Figure 6.1 (filled circles); a real example is demonstrated by Figure 6.3.

A different group of methods for solving multi-objective optimization problems do not incorporate directly the concept of Pareto optimum. A representative of non-Pareto approaches is the *Aggregation Function*, which combines all objectives into a single objective by using addition, multiplication or any other combination of arithmetical operators. Individual objective functions are frequently assigned a weighting value to distinguish the significance of different objectives. Non-Pareto approaches are simple and easy to implement but they are unable to produce certain segments of the Pareto front and are limited to handling only a few objectives. Linear combinations of weights are not applicable when the Pareto front is concave, regardless of the weights used (Fonseca and Fleming 1995).

Related work on aggregation functions by van Riel and Sontag (van Riel and Sontag 2006) studied the metabolic regulation in *Saccharomyces cerevisiae* when different nitrogen pulses are added to the cell at a number of time points. Model parameters were estimated via a constrained optimization method, where solutions are subject to constraint(s). The model was optimized by minimizing the difference between the experimental data and model output in a least-squares criterion subject to the constraint of steady-state condition implemented as the sum of squares. These two identification criteria are added together as the overall fitness function to be adjusted by the optimizer. van Riel and Sontag again raised the issue that it is difficult to define the relative importance of different objectives in a multi-objective criterion and the decision is always somewhat subjective.

6.2.1   Multi-objective Particle Swarm Optimization

Particle Swarm Optimization (PSO) is one of the newest techniques in the family of evolutionary algorithms, motivated by the simulation of social behaviour. PSO is particularly suitable for multi-objective optimization due to the high convergence rate of the algorithm. This is discussed in the context of single-objective optimization in Chapter 5. Due to the population-based nature of PSO, it appears to be fairly straightforward to transform the algorithm to solve multi-objective optimization problems using a 'Pareto ranking scheme' (Goldberg 1989). However, only a few works have been recently proposed for MOPSO.

The application of evolutionary algorithms into multi-objective optimization has been under investigation since the mid 1980s, and is largely centered on Genetic Algorithms (Fonseca and Fleming 1993, da Fonseca 1995, Deb et al. 2000). For example, da Fonseca (da Fonseca 1995) has applied the multi-objective genetic algorithm to a subset selection problem. The selection criteria constrained by the objectives of residual variance and mean-square prediction error are used in the selection of appropriate non-linear terms for polynomial models of a pilot scale liquid-level system. In their study, a large number of 1000 input-output pairs are employed for the optimization process.

Evolutionary algorithms deal with a set of possible solutions simultaneously. This is particularly suitable for coping with multi-objective optimization problems, where the search of the Pareto optimal set may be achieved in a single run of the algorithm. Evolutionary algorithms are also less susceptible to the shape or continuity of the Pareto front (Coello 2001). This corresponds to the problem of concavity and discontinuity when solving optimal vectors over a high-dimentional search space.

Fieldsend (Fieldsend 2004) compared the proposed techniques for MOPSO (Hu and Eberhart 2002b, Parsopoulos and Vrahatis 2002a, Coello and Lechuga 2002, Fieldsend and Singh 2002, Mostaghim and Teich 2003). These methods mainly differ in the two aspects, namely: the use of an external 'repository' for non-dominated solutions, which is retrieved by every particle after each flight cycle; and the selection of personal best *(pbest)* and global best *(gbest)* particles for each particle in the population. In Fieldsend's review, the impact of selection strategies for particles on the convergence rate and diversity of solutions is emphasized when solving a number of objectives simultaneously.

A recent and comprehensive survey on the state-of-the-art of multi-objective

optimization with PSO is also provided by Reyes-Sierra and Coello (Reyes-Sierra and Coello 2006). The key issues related to the extension of PSO to multi-objective optimization scheme are detailed in the survey, and include:

1. How to ensure the diversity in the solutions found in order to obtain a smooth and uniform distribution and to avoid convergence to a single solution;

2. How to determine, retain and update the non-dominated solutions during the search process;

3. How to select *leader* particles to guide the global search;

Reyes-Sierra and Coello underlined the fact that even though MOPSOs have been used in a few applications, they are almost never employed as a primary search engine in solving the real-world applications. This survey urges researchers from this domain to develop well-designed multi-objective optimizers in order to effectively solve real-world applications. The usefulness of MOPSOs should be examined with respect to the capability of the algorithm in producing reasonably good approximations of the Pareto front when applied to real-wold multi-objective optimization problems. For instance, applications that include a large number of decision parameters and time-dependent behaviour of system components is of interest. This is in contrast to the relatively simple algebraic equations for which MOPSOs were initially designed and tested with. The lack of relevant applications may explain why, despite a fast growth of this field, MOPSO techniques have not yet been widely used in practice.

In the following sections, we provide a detailed description of the proposed approach for solving complex multi-objective optimization problems. The performance of the approach is evaluated using the ERK signalling pathway as a case study.

6.2.2   Basic Concepts

In this section we define several mathematical terms used in this chapter in order to establish a common terminology.

Definition 6.2 (Weak Dominance)**:** *A decision vector* $\mathbf{x}^*$ *from a feasible region* $S \in \mathcal{R}$ *is said to weakly dominate a decision vector* $\mathbf{x} \in S$ *(denoted* $\mathbf{x}^* \preceq \mathbf{x}$*) iff: the*

decision vector $\mathbf{x}^*$ *is not worse than* $\mathbf{x}$ *in all objectives, stated as* $f_i(\mathbf{x}^*) \leq f_i(\mathbf{x})$ *for* $i = 1, \ldots, k$.

Definition 6.3 (Strong Dominance)**:** *A decision vector* $\mathbf{x}^*$ *from a feasible region* $S \in \mathcal{R}$ *is said to strongly dominate a decision vector* $\mathbf{x} \in S$ *(denoted* $\mathbf{x}^* \prec \mathbf{x}$*) iff: (i) the decision vector* $\mathbf{x}^*$ *is not worse than* $\mathbf{x}$ *in objectives where* $f_i(\mathbf{x}^*) \leq f_i(\mathbf{x})$ *for* $i = 1, \ldots, k$ *and (ii) the decision vector* $\mathbf{x}^*$ *is strictly better than* $\mathbf{x}$ *in at least one objective where* $f_i(\mathbf{x}^*) < f_i(\mathbf{x})$ *for* $i = 1, \ldots, k$.

Definition 6.4 (Fully Dominated Solutions)**:** *A decision vector* $\mathbf{y}$ *is termd a fully dominated solution with respect to a test vector* $\mathbf{x}$ *iff; the decision vector* $\mathbf{y}$ *is worse than* $\mathbf{x}$ *in all objectives, i.e.* $f_i(\mathbf{y}) > f_i(\mathbf{x})$ *for* $i = 1, \ldots, k$.

Definition 6.5 (Partially Dominated Solutions)**:** *A decision vector* $\mathbf{y}$ *is termed a partially dominated solution with respect to a test vector* $\mathbf{x}$ *iff; (i) the decision vector* $\mathbf{y}$ *is strictly better than* $\mathbf{x}$ *in at least one objective, i.e.* $f_i(\mathbf{y}) < f_i(\mathbf{x})$ *for* $i = 1, \ldots, k$ *and (ii) the decision vector* $\mathbf{y}$ *is no worse than twice of the objective value of* $\mathbf{x}$ *in all other objectives, i.e.* $f_i(\mathbf{y}) \leq 2 \times f_i(\mathbf{x})$ *for* $i = 1, \ldots, k$.[2]

Definition 6.6 (Non-dominated Solutions)**:** *A decision vector* $\mathbf{y}$ *is termed a non-dominated solution with respect to a test vector* $\mathbf{x}$ *iff; the decision vector* $\mathbf{y}$ *is strictly better than* $\mathbf{x}$ *in all objectives, i.e.* $f_i(\mathbf{y}) < f_i(\mathbf{x})$ *for* $i = 1, \ldots, k$.

It should be emphasized that different types of solutions (e.g. fully dominated, partially dominated and non-dominated solutions) must be defined with respect to a test particle. As illustrated by Figure 6.1, particle P_1 and P_3 are non-dominated solutions with respect to particle P_2, and conversely, P_2 is a fully dominated solution with respect to P_1 and P_3. Particle P_1 and P_3 are mutually partially-dominated solutions, as neither solution is superior to the other. Solutions lying on the true Pareto-optimal front should fully dominate all the other points 'inside' the front but partially dominate each other. In other words, no other solutions in the search space are superior to the Pareto-optimal solutions.

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[2]The choice of '$2 \times f_i(\mathbf{x})$' is arbitrary and can be changed. This is to avoid the selection of particularly bad particles.

6.3 Description of the Proposed Approach for Multi-objective Optimization

The currently proposed approach for multiple-objective optimization is based on the Search-and-Poll framework adopted in the single-objective optimizer PSwarm, which is a generalized pattern search algorithm – in the search step standard PSO is employed and the poll step is only activated when the search step failed.

Our proposal is based on the strategy of having an external repository (also termed as the archive in this chapter) in which every particle can deposit its flight experiences after each flight cycle[3]. The mechanism of crowding distance computation is incorporated in the multi-objective optimization with PSO (Raquel and Naval 2005) for the purpose of selecting the global best particle.

The crowding distance computation provides an estimate of the density of solutions surrounding a particular solution in the population (Deb et al. 2000). It estimates the size of the largest rectangle (or higher dimensional equivalent) enclosing the solution i without including any other solution in the population. The crowding distance of a particular solution takes the average distance of its two neighboring solutions, which helps distribute particles uniformly over the search space. This computation is carried out for each objective, and the final distance measure of a solution is computed by adding the individual crowding distance values calculated for each objective. The pseudo code of the mechanism (for an individual objective) is given in Algorithm 2, based on the description by Deb et al. (Deb et al. 2000).

One distinct feature of the proposed approach which we shall call 'MoPSwarm' is the employment of a *Poll* step in seeking non-dominated solutions. The Poll step is used in order to provide more opportunities to gain satisfactory convergence and ensure a good diversity. The problems we are interested in solving with MoPSwarm are Bound Constrained Optimization problems, the definition of which was given in Section 5.2.1 in Chapter 5.

In multi-objective optimization, a set of non-dominated solutions representing the best individuals found so far during the search process replaces the single global best particle in the single-objective optimization case. This leads to a de-

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[3]In practice, two external repositories are required, one stores the non-dominated solutions and the other stores the decision variables corresponding to non-dominated solutions. The repository of non-dominated solutions is manipulated through the searching process, and the other repository should be updated accordingly.

Algorithm 2 Crowding Distance Computation (Deb et al. 2000).

1: Get the number m of non-dominated solutions in the archive REP
2: Initialize distance value of each non-dominated solution $Distance_{REP(i)}$ to zero for all m stored solutions
3: **for** $i = 0$ to m **do**
4:     Sort the solutions in descending order of the objective value
5:     Set infinite crowding distance values to the lowest and highest objective function values $Distance_{REP(1)} = Distance_{REP(m)} = \infty$
6:     **for** $j = 2$ to $m - 1$ **do**
7:         Calculate the average distance of each particle solution using the formula $Distance_{REP(j)} = Distance_{REP(j)} + (Distance_{REP(j+1)} - Distance_{REP(j-1)})$
8:     **end for**
9: **end for**

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cision whether an external repository is used to store non-dominated solutions that are retrieved by every particle after each flight cycle.

MoPSwarm incorporates an external repository (REP) with a filtering process that encourages a uniform distribution of solutions along the Pareto front. No limit is imposed on the maximum number of solutions that REP can store. REP is initialized with the objective function values of the initial particles and the global best guide particle is then selected from a set of Pareto-optimal solutions via distinct selection strategies.

The proposed algorithm is summarized in Algorithm 3.

6.3.1 Local Best Selection

Choosing *pbest* and *gbest* particles to direct a swarm member's flight is not trivial in multi-objective optimization with PSO (Fieldsend 2004). In MoPSwarm, the selection criteria for *pbest* for individual particles follows the rule of *Strong Dominance*. At each iteration of the flying cycle, for each individual particle, the position that fully dominates the previous position is recorded. The Poll step is activated if the search for a local best guide fails at the Search step. In the Poll step, members in a finite set of trial points are examined in turn and selected in accordance with the rule of *Strong Dominance*. The pseudo code for the selection of local best guide is summarized in Algorithm 4.

Algorithm 3 MoPSwarm: A general algorithm for multiple-objective optimization

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1: Choose a population size s and termination criteria. Randomly initialize the initial particles in the population $\mathbf{x}^1(0), \ldots, \mathbf{x}^s(0)$ with initial local best positions $\mathbf{y}_{lb}^1(0), \ldots, \mathbf{y}_{lb}^s(0)$ and the initial velocities $\mathbf{v}^1(0), \ldots, \mathbf{v}^s(0)$.

2: Initialize the external repository REP with $f^1(\mathbf{z}), \ldots, f^k(\mathbf{z})$, where $\mathbf{z} \in \mathbf{x}^1(0), \ldots, \mathbf{x}^s(0)$ for k objectives

3: **while** Termination criteria are not met **do**

4:     Search for personal best positions, *pbest*, for individual particles in the population (defined in Section 6.3.1) using the Search-and-Poll framework

5:     Update the external repository REP (defined in Section 6.3.3) using the Search-and-Poll framework

6:     Refresh the external repository REP every n_r iterations (defined in Section 6.3.4)

7:     Select a global best particle, *gbest*, from REP to guide flying of each particle in the population (defined in Section 6.3.2).

8:     Update velocities of individual particles in the population with the formula $\mathbf{x}(t+1) = \mathbf{x}(t) + \mathbf{v}(t)$.

9: **end while**

6.3.2   Global Best Selection

As illustrated by Fieldsend (Fieldsend 2004), the selection strategies for *gbest* to guide the search of the next cycle fall into two categories, namely 'unrestricted' and 'restricted' groups. The former allows the selection of members freely from the external repository, whilst the latter restricts the selection of *gbest* using some form of distance measure.

Each method has it own advantages and disadvantages, and often one method compensates for another's deficiency. The unrestricted selection of *gbest* helps avoid 'clumping of particles' and prevents particles from becoming trapped within limited areas of the search space. However, this method will probably accept particles with 'bad' positions as the leading particle, thus requiring more computational time on average to converge. The restricted *gbest* selection may promote clumping of particles, but a good set of non-dominated solutions is more likely to be obtained at each iteration.

In our proposed approach, both strategies of 'unrestricted' and a geographically-based selection (Crowding Distance) strategy are examined. With the restricted selection strategy, a non-dominated solution from the top 10% least crowded area of the archive is randomly chosen as the global best guide. A different *gbest* is selected for each particle in the population.

Algorithm 4 Search for local best guide

1: **[Search Step]**
2: **for** $i = 1, \ldots, s$ do (for every particle i): **do**
3:     Compute $f(\mathbf{x}^i(t))$ for each objective $j \in 1, \ldots, k$
4:     **if** the decision vector $\mathbf{x}^i$ fully dominates $\mathbf{y}_{lb}^i$ **then**
5:         Set $\mathbf{y}_{lb}^i(t+1) = \mathbf{x}^i(t)$ (update the particle i local best position; search step successful)
6:         Reset the search direction for pattern search
7:     **end if**
8:     **[Poll Step]**: *Skip the poll step if the search step was successful.*
9:     Choose the spanning set D.
10:    **if** there exists $d(t) \in D$ such that $f(\mathbf{y}_{lb}^i(t) + \alpha^i(t)d(t))$ is a non-dominated solution with respect to $f(\mathbf{y}_{lb}^i(t))$ **then**
11:        Set $\mathbf{y}_{lb}^i(t+1) = \mathbf{y}_{lb}^i(t) + \alpha^i(t)d(t)$ (a new local best position found for the particle; poll step successful)
12:        Set $\alpha^i(t+1) = \phi(t)\alpha^i(t)$ (increase the mesh size parameter according to the rule in Equation (5.3))
13:    **else**
14:        Set $\mathbf{y}_{lb}^i(t+1) = \mathbf{y}_{lb}^i$ (No change in the local best position for all $d(t) \in D$; poll step unsuccessful)
15:        Set $\alpha^i(t+1) = \theta(t)\alpha^i(t)$ (decrease the mesh size parameter according to the rule in Equation (5.4))
16:    **end if**
17: **end for**

6.3.3 Updating the Archive

In the scheme of multi-objective optimization, the definition of 'superior' particles for the update of the archive REP is a critical consideration, which may have a major influence on the convergence of the algorithm and the diversity of the Pareto-optimal solutions. In our proposed approach, two different updating strategies are applied and the resulting optimization performance is assessed.

The two proposals are termed *Strong-Dominance* and *Weak-Dominance* updating strategies, which specify that during the search process

1. With the strong-dominance updating strategy, only the particles that are non-dominated by the stored solutions in the archive are inserted and the corresponding dominated solutions in the archive are deleted.

2. With the weak-dominance updating strategy, particles that are non-dominated or partially dominated by the stored solutions in the archive are inserted and only fully dominated stored solutions are deleted.

The Pseudo code of the *Weak-Dominance* updating strategy is summarized in Algorithm 5. The description in Algorithm 5 can also be used to refer to the *Strong-Dominance* updating strategy, where for the latter partially dominated solutions are not accepted in the Poll step (see statement 19 in the algorithm).

Algorithm 5 Weak-dominance updating strategy of the archive REP

1: **[Search Step]**
2: **for** $i = 1, \ldots, s$ do (for every particle i): **do**
3:     Compute $f(\mathbf{x}^i(t))$ for each objective $j \in 1, \ldots, k$
4:     **if** the current particle $f(\mathbf{x}^i(t))$ fully dominates the stored solution(s) in REP **then**
5:         Remove the dominated stored solutions from REP and insert $f(\mathbf{x}^i(t))$ into REP
6:         Retain the remaining stored solutions in REP; search step successful
7:     **end if**
8:     **if** the current particle $f(\mathbf{x}^i(t))$ partially dominates the stored solution(s) in REP **then**
9:         The current particle is retained for the Poll step
10:        No update is made to the archive REP; search step unsuccessful
11:    **end if**
12:    **if** the current particle $f(\mathbf{x}^i(t))$ is fully dominated by the stored solution(s) in REP **then**
13:        No update is made to the archive REP; search step unsuccessful
14:    **end if**
15: **end for**
16: **[Poll Step]**: *Skip the poll step if the search step was successful.*
17: Choose the spanning set D.
18: For $d^n(t)_{n=[1,\ldots,m]} \in D$ find $f(\mathbf{y}^i(t) + \alpha(t)\hat{d}(t)) \in \arg\min_{n\in[1,\ldots,m]} f(\mathbf{y}^i(t) + \alpha(t)d^n(t))$
19: **if** there exist $f(\mathbf{y}^i(t)+\alpha(t)\hat{d}(t))$ that fully dominates or partially dominates $f(\mathbf{y}^i(t))$ **then**
20:    Set $\mathbf{y}^i(t) = \mathbf{y}^i(t) + \alpha^i(t)\hat{d}(t)$ and $f(\mathbf{y}^i(t)) = f(\mathbf{y}^i(t) + \alpha(t)\hat{d}(t))$
21:    Remove the dominated solution(s) stored in the archive REP
22:    Insert the solution $f(\mathbf{y}^i(t))$ into the archive REP (update of the archive REP is done; poll step successful)
23:    Set $\alpha^i(t + 1) = \phi(t)\alpha^i(t)$ (increase the mesh size parameter according to the rule Equation (5.3)).
24: **else**
25:    No update is made to the archive REP; poll step unsuccessful
26:    Set $\alpha^i(t + 1) = \theta(t)\alpha^i(t)$ (decrease the mesh size parameter according to the rule in Equation (5.4)).
27: **end if**

6.3.4 Refreshing the Archive

In MoPSwarm, a check on the archive REP is performed every $n_r=10$ iterations[4] during the search process. The Pareto-optimal subset of the current archive is saved and all other (fully dominated) solutions are eliminated. The possibility that fully dominated solutions are inserted into the archive arises from the mechanism that we applied to the update of the archive, which is that the Poll step may accept potentially inferior solutions for the sake of diversity. This refresh step can help form a better Pareto-optimal front by eliminating the fully dominated solutions from the archive.

6.3.5 Termination Criteria

The maximum number of iterations is a commonly used stopping criterion for evolutionary algorithms. To ensure a proper termination in practice, in the proposed approach, we introduce a mechanism for deactivating particles when the mean magnitude of changing position of the particle within two neighboring iterations is smaller than the tolerance (1e-5).

Our experiences from experimental testing of MoPSwarm suggest that it is possible that under some conditions the expected value of the norm of the velocities vectors tends to zero for most of the particles after being through enough iterations of the search process. These particles will have less contribution to the global parameter search for their low search activity. Deactivated particles from the previous iteration will be removed from the population and won't be considered in the subsequent search process.

In the implementation, no particle deactivation is permitted in the first five iterations of the search cycle, which is designed to allow the particles with small initial velocity values to start moving. The algorithm terminates when there is only one particle remained in the population. The mechanism of particle deactivation can not only save the computational resources by removing the particles that don't show enough movement, but also promote a satisfactory convergence of other particles to the true Pareto front by preventing them from being trapped by the inactive particles.

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[4]Note that 10 interations is arbitrary and can be changed.

6.4 Case Study: ERK Signalling Pathway

To demonstrate the usefulness of MoPSwarm in solving the inverse problem of the ERK signalling pathway, a comparison of model validation via both the common approach and the proposed approach is carried out. The computational model of the pathway was given in Table 5.1 in Chapter 5.

Regardless of which approach is used, true perturbation data for the ERK signalling pathway has to be available beforehand. This can be the data describing the perturbation experiment applied to an arbitrary model parameter. To obtain a perturbation of the model, we applied a 90% knockdown of each parameter from its reference value, one at a time, and simulated the model with the mutated parameter set for perturbation data. The initial conditions and the reference parameter values for the model simulations are fixed at the numbers reported in Chapter 5. Each perturbation data set contains the transient concentrations of the system's components at 11 time points evenly spacing the time span between 0 and 100 minutes.

We observed that under these perturbed conditions some of the parameters have negligible effects on the change of concentrations of the system's components. To better demonstrate the prospects for experimental design considerations, only the perturbation data exhibiting an evident impact on the system's dynamics is retained. The perturbation data to be used as the 'true' data in the second step of model validation was generated with the parameter set where k_8 was mutated to 10% of the reference value, and the remaining parameters were unchanged.

The ODEs of the pathway used in the perturbed condition are shown in Table 6.1, where k_8^* is expressed as $k_8 \cdot \beta$. Here, β represents the perturbation applied to the parameter of interest (k_8).

In this *in silico* study, the initial condition of the pathway is considered as an unknown parameter so as to mimic a realistic situation, where prior knowledge about the system is at best partial. This consideration leads to 22 unknown parameters to be estimated in total. All unknown kinetic parameters were assumed to vary between 0 and 1. Values of the initial concentrations of the system's components are restricted to $\pm20\%$ of the given steady state to help the convergence of the algorithm from random positions in the search space.

Following the conventional approach, a set of parameters that can satisfy the steady-state constraint of the pathway was solved using the global optimizer

Reaction number	Kinetic equations
1	$-k_1 \cdot m_1 \cdot m_2 + k_2 \cdot m_3 + k_5 \cdot m_4$
2	$-k_1 \cdot m_1 \cdot m_2 + k_2 \cdot m_3 + k_{11} \cdot m_{11}$
3	$k_1 \cdot m_1 \cdot m_2 - k_2 \cdot m_3 - k_3 \cdot m_3 \cdot m_9 + k_4 \cdot m_4$
4	$k_3 \cdot m_3 \cdot m_9 - k_4 \cdot m_4 - k_5 \cdot m_4$
5	$k_5 \cdot m_4 - k_6 \cdot m_5 \cdot m_7 + k_7 \cdot m_8$
6	$k_5 \cdot m_4 - k_9 \cdot m_6 \cdot m_{10} + k_{10} \cdot m_{11}$
7	$-k_6 \cdot m_5 \cdot m_7 + k_7 \cdot m_8 + k_8^* \cdot m_8$
8	$k_6 \cdot m_5 \cdot m_7 - k_7 \cdot m_8 - k_8^* \cdot m_8$
9	$-k_3 \cdot m_3 \cdot m_9 + k_4 \cdot m_4 + k_8^* \cdot m_8$
10	$-k_9 \cdot m_6 \cdot m_{10} + k_{10} \cdot m_{11} + k_{11} \cdot m_{11}$
11	$k_9 \cdot m_6 \cdot m_{10} - k_{10} \cdot m_{11} - k_{11} \cdot m_{11}$

Table 6.1: The ODE-based computational model of ERK signalling pathway.

PSwarm for the simulation study. Two hundred runs of the steady-state optimization were executed in Matlab. Each run starts with a different initial population of particles and is computed over a simulated time span of 200 minutes. The set of parameters, $\mathbf{P}^*$, which gives the smallest value of the objective function, is retained. $\mathbf{P}^*$ is given below:

$$k_1 = 1, \ k_2 = 0.1880, \ k_3 = 0.8015, \ k_4 = 0.0437, \ k_5 = 1e - 6, \ k_6 = 0.9970,$$
$$k_7 = 0.1014, \ k_8 = 1e - 6, \ k_9 = 0.9758, \ k_{10} = 0.9903, \ k_{11} = 1e - 6.$$
$$m_1|_{t=0} = 0.3090, \ m_2|_{t=0} = 0.2320, \ m_3|_{t=0} = 0.6274, \ m_4|_{t=0} = 1.5646,$$
$$m_5|_{t=0} = 0.03099, \ m_6|_{t=0} = 0.01487, \ m_7|_{t=0} = 1.7822, \ m_8|_{t=0} = 0.7169,$$
$$m_9|_{t=0} = 0.1907, \ m_{10}|_{t=0} = 2.9364, \ m_{11}|_{t=0} = 0.0610.$$

To test if $\mathbf{P}^*$ is able to reproduce the true perturbation data, a 90% knockdown is applied to the estimated value of k_8 in $\mathbf{P}^*$, which forms the mutated parameter set, $\tilde{\mathbf{p}}^*$. The model dynamics under the perturbed condition are approximated with $\tilde{\mathbf{p}}^*$, where the initial condition for model simulation correspond to the final values of the steady-state model computed with $\mathbf{P}^*$.

The predicted perturbation profiles of Raf1/RKIP, Raf1/RKIP/ERK, MEK-PP and MEK-PP/ERK are shown in Figure 6.2. The other system components do not present dynamic changes in response to k_8 knockdown and are therefore omitted from the figure. This figure indicates that with the common approach, even the 'best' set of parameters estimated from the steady state failed to produce satisfactory results compared with the true perturbation data.
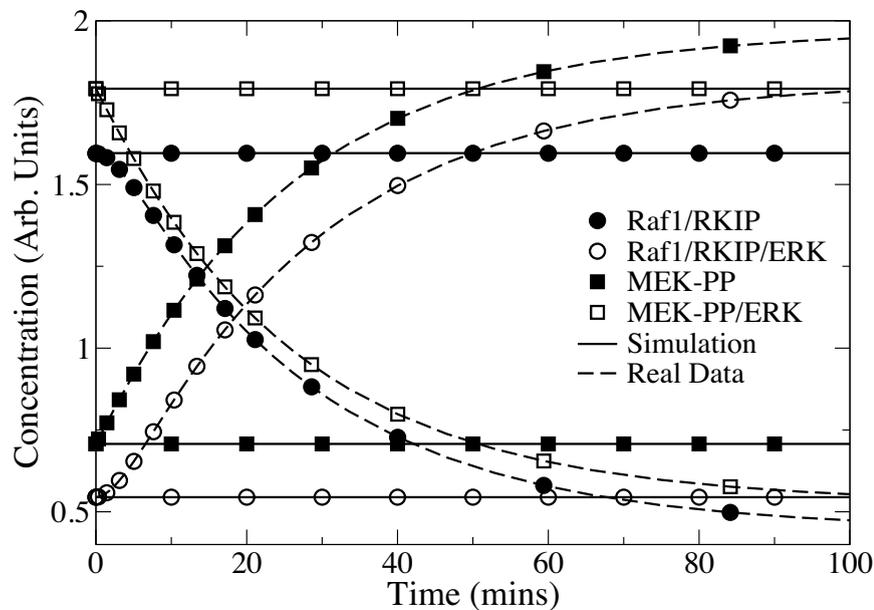
Figure 6.2: Comparison of the simulated perturbation profile (continuous lines) with the true perturbation data (dotted lines). Note that concentration profiles only match at t = 0.

6.5   A Comparative Study of MoPSwarm

In this section, we investigate the performance of several variants of MoPSwarm in solving the multi-objective optimization problem of ERK signalling pathway. The variants differ in the strategy used for selection of the global best guide and in whether the archive REP is refreshed. The weak-dominance updating strategy was adopted in all the MoPSwarm variants, which are summarized below

- *Variant 1*: The crowding distance computation is adopted and the archive is not refreshed.

- *Variant 2*: The unrestricted selection technique is adopted and the archive is refreshed every 10 iterations.

- *Variant 3*: The unrestricted selection technique is adopted and the archive is not refreshed.

- *Variant 4*: The crowding distance computation is adopted and the archive is refreshed every 10 iterations.

Solutions computed with the four variants of MoPSwarm are presented in Figure 6.3, and the corresponding Pareto front is convex. Note that the scales are different in each of these plots, indicating significant differences in the range of the objective function values. The maximum number of iterations was set to 100 and 20 particles were used in the initial population. Both objectives of the steady-state and perturbed condition are formulated using the penalized likelihood error criterion, in which the error increases exponentially as the distance between the estimated and observed value increases. The penalized likelihood error criterion is expressed as follows for time points $i = 1, \ldots, m$ and system components $j = 1, \ldots, n$:

$$\text{minimize } \varepsilon = \sum_{1 \leq i \leq m} \sum_{1 \leq j \leq n} \frac{1}{f_{ij}} \tag{6.2}$$

where f_{ij} is defined as a Normally distributed probability density function, with a mean of the experimental measurement $\mathbf{y}^D(t_i, x_j)$ and a standard deviation σ of 1. This is given as follows

$$f_{ij} = \frac{1}{\sqrt{2\pi\sigma^2}} \exp\left(-\frac{(\tilde{\mathbf{y}}(t_i, x_j, \mathbf{p}) - \mathbf{y}^D(t_i, x_j))^2}{2\sigma^2} \right) \tag{6.3}$$

where $\tilde{\mathbf{y}}(t_i, x_j, \mathbf{p})$ represents the model predictions. The aim is thus to minimize the error criterion ε for optimal model parameters $\mathbf{p}$, which corresponds to minimizing

$$\varepsilon = \sum_{1 \leq i \leq m} \sum_{1 \leq j \leq n} \exp(\tilde{\mathbf{y}}(t_i, x_j, \mathbf{p}) - \mathbf{y}^D(t_i, x_j))^2 \tag{6.4}$$

The multi-objective optimization problem under study contains a total of 23 unknown parameters, where the first 11 parameters, k_1 to k_{11}, are common to both objectives and the remaining parameters are specific to the individual objectives. Parameters k_{12} to k_{22} represent initial concentrations of the system components under the steady-state condition, and k_{23} represents the perturbation (β, varied between 0 and 1) applied to the parameter of interest. For example, if the estimated value of k_{23} is 0.8, then the prediction from MoPSwarm indicates that a 20% perturbation has to be applied in order for both objectives to be satisfied simultaneously. To ensure an objective comparison among the variants, the same population of initial particles was used.

The performance of different MoPSwarm variants is evaluated based on the following criteria: whether (1) a good distribution of the solutions found is at-
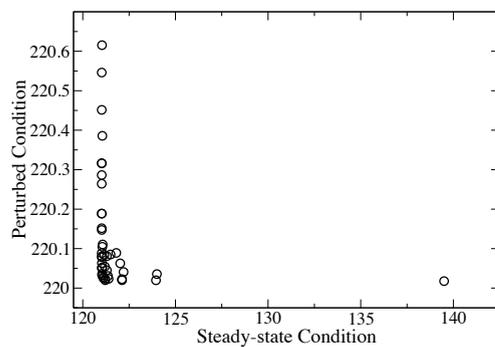
(a) Variant 1
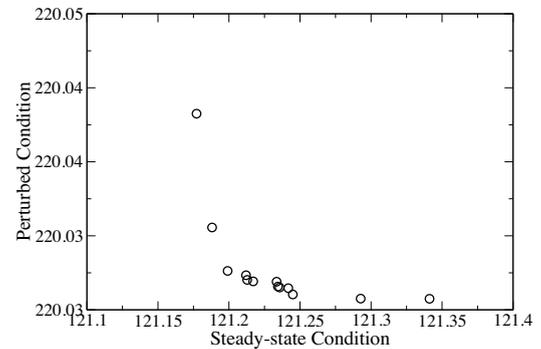
(b) Variant 2
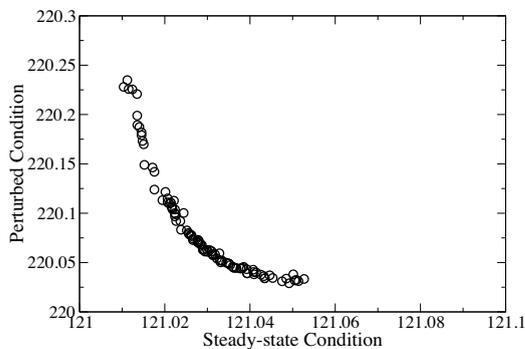
(c) Variant 3
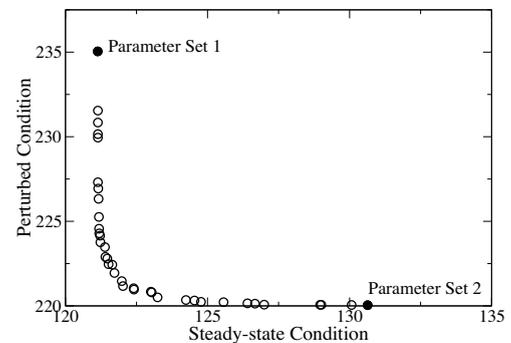
(d) Variant 4

Figure 6.3: Solutions of the multi-objective optimization problem from the different variants of MoPSwarm. The optimization problem of interest is constrained by two conditions: the steady-state condition (horizontal axis) and the perturbed condition (vertical axis). All the Pareto fronts resolved from the variants are convex. Note the different scales in the four figures.

tained and (2) a wide range of values is covered by the Pareto-optimal solutions found. In accordance with these criteria, *Variant 4* presents the best performance and is adopted for use in MoPSwarm. *Variant 3* is desirable in terms of the maximum number of trade-off solutions, but unfavorable in the spread of solutions. The solutions found with *Variant 3* are bounded in a small range, which is also seen in the results computed with *Variant 2*. *Variant 1* shows a better spread along the horizontal axis than the other two variants, but inferior to the distribution obtained when using *Variant 4*. The solutions found with *Variant 1* are also restricted to a small range of values for the objective representing the perturbed condition, and the majority are clustered in the corner nearest the

origin.

6.5.1   Archive Updating Strategies

Our *in silico* study also investigates how the choice of updating strategy for the archive REP affects the performance of the algorithm when solving the ERK signalling pathway. The strong-dominance updating strategy only allows non-dominated particles to be inserted into the archive, which differs from the weak-dominance updating strategy, where partially dominated particles are also accepted.

In all the four variants of MoPSwarm (In *Variant 1* to *Variant 4* defined above, the weak-dominance updating strategy was applied), the strong-dominance updating strategy is adopted instead and the performance of each variant is examined. Interestingly, all these variants converged to a single solution at the end of each run of the optimization (altogether there were 10 runs, each with a different population of initial particles). Thus adopting the strong-dominance updating strategy fails to maintain diversity in the non-dominated solutions. According to the criteria used to assess the performance of multi-objective algorithms, the weak-dominance updating strategy is preferable.

Figure 6.4 illustrates the potential problem with the strong-dominance updating strategy. If there is a non-dominated solution P_N (red filled square) found in the later stages of the flying cycle, the Pareto-optimal front (black filled circles) developed in the earlier stages will be completely eliminated. This makes it very difficult to re-form a well-distributed front again, rather the search engine is more inclined to move towards the origin as shown by the arrow in red.

Based on the experimental results, we summarize that in the multi-objective optimization scheme, the selection of global best guide and the update of external repository are two major problems that have to be addressed carefully. An appropriate selection mechanism is critical in guiding the search towards the Pareto-optimal set and achieving a well distributed trade-off front. We have found that it is a very challenging problem particularly where multi-optimization algorithms are proposed for solving real-world applications. Our *in silico* investigation (Figure 6.3) demonstrates that the Crowding Distance computation and the refresh step can facilitate the generation of a well-distributed and smooth Pareto-optimal front.
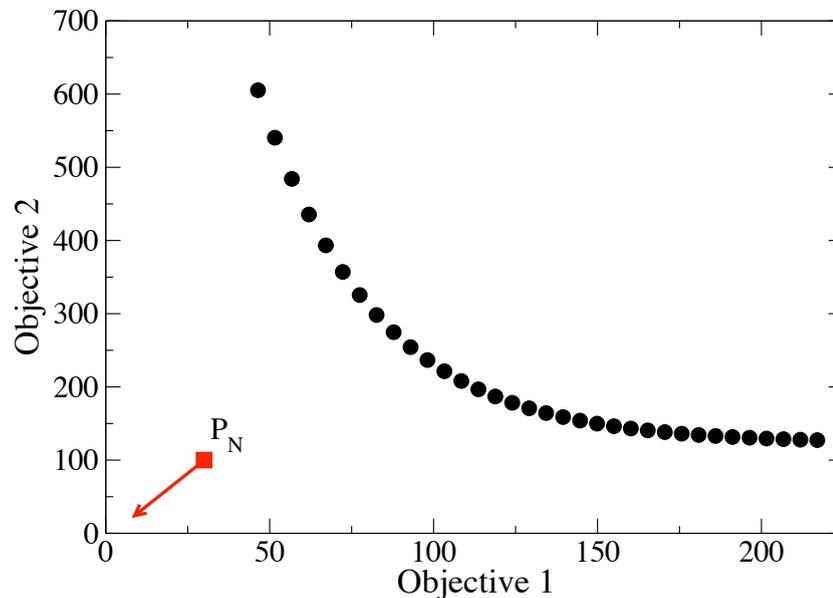
Figure 6.4: An illustration of the strong-dominance updating strategy. The solution P_N moves towards the origin indicated by red arrow.

6.5.2 Mutation Mechanism

Most of the existing implementations of multi-objective optimization with PSO apply some sort of mutation operator (Reyes-Sierra and Coello 2006). The mutation scheme adopted in the method designed by Coello et al. (Coello et al. 2004) is implemented in MoPSwarm (*Variant 4*) and compared with the 'vanilla' algorithm. The optimization results from the original MoPSwarm and MoPSwarm with mutation are shown in Figure 6.5. The results were obtained using 10 particles and 20 iterations.

Our *in silico* investigations indicate that the mutation operator resulted in inferior solutions, which is likely due to the additional disorder introduced into the system by the operator, making it more difficult/slower to converge. As explained in Figure 6.5, by the end of 20 iterations, MoPSwarm without the mutation operator produces slightly better results than the alternative. By the end of the search process (which stopped at 432 iterations due to single particle), MoPSwarm without mutation produced a good, well-distributed Pareto-optimal set, whereas MoPSwarm with mutation produced a solution set with much less diversity, with the results being essentially a small subset of those produced
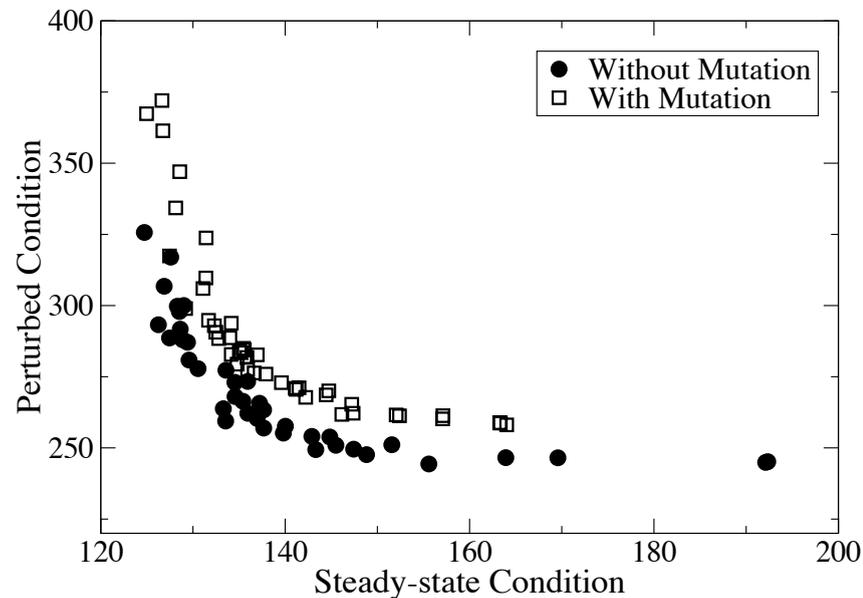
Figure 6.5: A comparison of the performance of MoPSwarm in the use of the mutation operator by the end of 20 iterations of the search process.

by MoPSwarm without mutation. The mutation operator tends to drag the search into a small and condensed area of the parameter space, giving rise to convergence to a small set of similar solutions.

Reyes-Sierra and Coello (Reyes-Sierra and Coello 2006) draw attention to the issue that the choice of a good mutation operator is a difficult task, and there are approaches that show good performance without using any kind of mutation operator. The incorporation of a mutation operator certainly deserves a more careful study, which is beyond the scope of the thesis, however. The mutation mechanism is excluded from our proposal according to the results from the *in silico* investigations on the issue.

6.5.3 Optimization Results with MoPSwarm

The predicted concentration profiles of Raf1/RKIP, Raf1/RKIP/ERK, MEK-PP and MEK-PP/ERK in both steady-state and perturbed conditions are generated with two particular sets of parameters estimated with *Variant 4* of MoPSwarm. These two sets of parameters are selected from the upper-left end and bottom-right end of the Pareto front, as shown in Figure 6.3(d).

Parameter Set 1:

$k_1 = 0.7885$, $k_2 = 0.0576$, $k_3 = 0.5600$, $k_4 = 0.0044$, $k_5 = 0.0218$,

$k_6 = 0.3934$, $k_7 = 0.0068$, $k_8 = 0.0482$, $k_9 = 0.7927$, $k_{10} = 0.0041$, $k_{11} = 0.7160$.

$m_1|_{t=0} = 0.4110$, $m_2|_{t=0} = 0.3200$, $m_3|_{t=0} = 0.5178$, $m_4|_{t=0} = 1.4004$,

$m_5|_{t=0} = 0.0391$, $m_6|_{t=0} = 0.0205$, $m_7|_{t=0} = 1.6726$, $m_8|_{t=0} = 0.8149$,

$m_9|_{t=0} = 0.1718$, $m_{10}|_{t=0} = 2.9295$, $m_{11}|_{t=0} = 0.0554$.

$\boldsymbol{\beta} = 0.2662$.

Parameter Set 2:

$k_1 = 0.6527$, $k_2 = 0.0106$, $k_3 = 0.5399$, $k_4 = 0.0039$, $k_5 = 0.0330$,

$k_6 = 0.3975$, $k_7 = 0.0010$, $k_8 = 0.0301$, $k_9 = 0.8044$, $k_{10} = 0.0026$, $k_{11} = 0.7328$.

$m_1|_{t=0} = 0.4115$, $m_2|_{t=0} = 0.3198$, $m_3|_{t=0} = 0.5081$, $m_4|_{t=0} = 1.3980$,

$m_5|_{t=0} = 0.0336$, $m_6|_{t=0} = 0.0201$, $m_7|_{t=0} = 1.8198$, $m_8|_{t=0} = 0.8063$,

$m_9|_{t=0} = 0.1767$, $m_{10}|_{t=0} = 2.8189$, $m_{11}|_{t=0} = 0.0549$.

$\boldsymbol{\beta} = 0.2588$.

Parameter Set 1 and **Parameter Set 2** are the solutions that have the minimum values for a single objective function across the Pareto-optimal set, that is, they have the best objective function values for the steady-state and perturbed conditions respectively. The steady-state and perturbed concentration profiles predicted with **Parameter Set 1** and **Parameter Set 2** are shown in Figure 6.6 & Figure 6.7 and Figure 6.8 & Figure 6.9, respectively. As indicated previously, only the four system components shown here present responses to knockdown of k_8, thus the other system components are omitted. It should be noted that this approach yielded good predictions of the dynamics of the other system components.

The steady-state concentration profiles of the pathway are simulated with the model given in Table 5.1 in Chapter 5. The perturbation profile of the pathway is simulated with the model given in Table 6.1, where the initial condition corresponds to the concentration level of the system components simulated for the steady-state condition. The true experimental data used for comparison against the simulation results in both conditions is produced with the reference and perturbed (with a 90% knockdown applied to k_8) parameter sets of the pathway, respectively.

Compared to the simulation results using the conventional approach (shown in Figure 6.2), predictions with our proposal are reasonably satisfactory for both objectives given the large number of unknown parameters. The simulation profiles shown in Figure 6.6 to Figure 6.9 indicate that with both parameter sets, the model predicts a steady-state value close to the target, and can also reproduce the measured dynamics of the system components, even though no exact match is found in the optimization of either objective.

The predicted values of the perturbation, β, vary between 0.1 and 0.3 along the Pareto front, which is comparable to the true perturbation value, $\beta = 0.1$. From a biological point of view, predictions of the perturbation between 0.1 and 0.3 indicate that similar effects on the system dynamics could be achieved by a lower degree of knockdown on the reaction enzyme of interest (i.e. a 70%–80% predicted perturbation produces a similar effect to the true perturbation of 90%). This observation is useful in a general sense in that although the activity of an enzyme can never be completely suppressed, enzymes that are easier to suppress are preferable as drug targets (Bakker et al. 2002). Therefore, one of the important applications of our proposal is to provide alternative solutions for perturbation experiments where modifying the rate reaction constants (or initial concentrations) is more economical and probable than designing a high-affinity compound inhibitor.

6.6   Discussion

Given that the use of mathematical models may have large consequences for drug development in case of model inadequacy or lack of precision, these models must go through a rigorous validation process before they can be adopted as predictive models. The use of a multi-objective optimization scheme is encouraged for reliable model validation. It appears to be a natural solution to the optimization problem that is constrained by more than one condition, particularly, in cases where, among all the unknown parameters, a subset of parameters is common to all the objectives and some of them are specific to certain objectives.

The proposed approach MoPSwarm is beneficial in tackling the systems where the components are time-dependent variables and the number of unknown parameters is large. MoPSwarm can successfully detect solutions exhibiting convex tradeoffs in the objective function space using a smaller number of population (20 initial particles) within a smaller number of generations (maximum
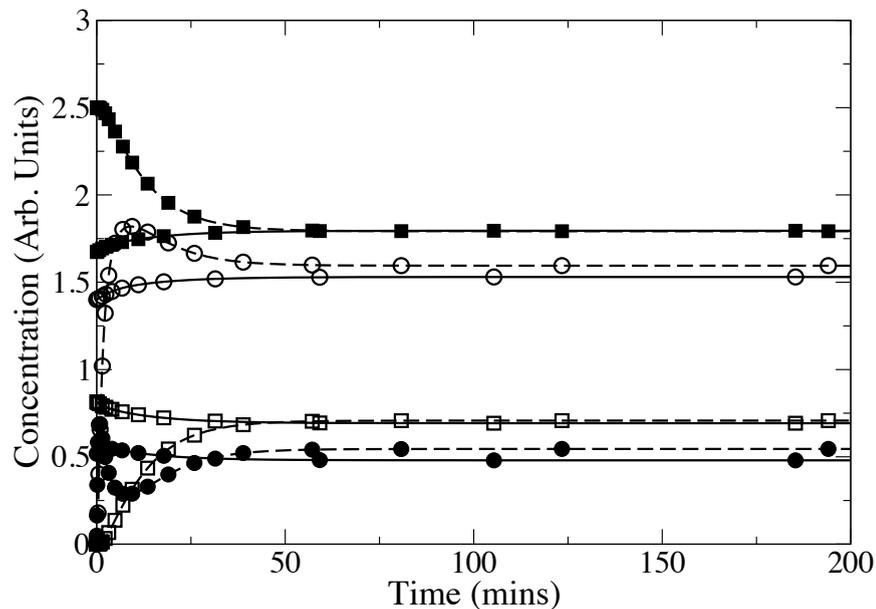
Figure 6.6: Comparison of the predicted concentration profile (continuous line) and the true steady-date data (segment line) for (*filled circle*): Raf1/RKIP, (*open circle*): Raf1/RKIP/ERK, (*filled square*): MEK-PP and (*open square*): MEK-PP/ERK. The predicted concentration profile is yielded with Parameter Set 1.

1000 generations). By applying the multi-objective optimization scheme, alternative solutions that are more practical and effective can be obtained for design of perturbation experiments. Compared to biological experiments, computational study is an economical strategy, which can help biologists design *in vivo* experiments in a more efficient way.

We presented a comparative study investigating some of the factors important to the performance of MoPSwarm in solving multi-objective optimization problems. We found that the choice of selection scheme for global best guide and the mechanism for updating the archive is critical for the Pareto-based approach, from the perspective of promoting an effective search towards the Pareto front and maintaining diversity in the population.

In summary, a multi-objective optimization scheme is advantageous over single-objective optimization in two major aspects.

First, in the context of single-objective optimization, a multi-criteria fitness value must be defined to aggregate individual fitness value, in order to perform
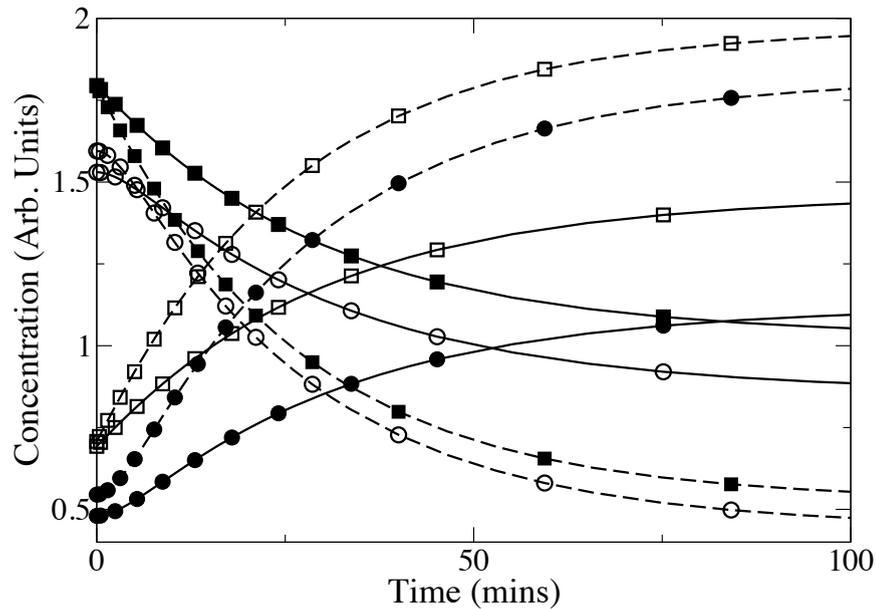
Figure 6.7: Comparison of the predicted concentration profile (continuous line) and the true perturbation data (segment line) for (*filled circle*): Raf1/RKIP, (*open circle*): Raf1/RKIP/ERK, (*filled square*): MEK-PP and (*open square*): MEK-PP/ERK. The predicted concentration profile is yielded with Parameter Set 1.

an optimization or search process with a single objective. This multi-criteria fitness value summarizes the degree of satisfaction of all the individual criteria. The danger of function aggregation lies in the fact that the objectives with large values may dominate the search process. This leads to the result that objectives with large function values could be satisfactorily optimized but the performance of objectives with small function values is always poor. One way to solve the problem is to weight the individual measures for each criteria and carefully combine them all together into a single measure. However, in our experience, it is not trivial to develop a good multi-criteria measure that aggregates the individual criteria properly, which may have a significant impact on the optimization performance.

Second, in the context of single-objective optimization, normally only one solution can be detected per optimization run. On the contrary, with the multi-objective optimizer, a set of Pareto-optimal solutions can be obtained with a single run of the algorithm. Considering all the objectives simultaneously but in
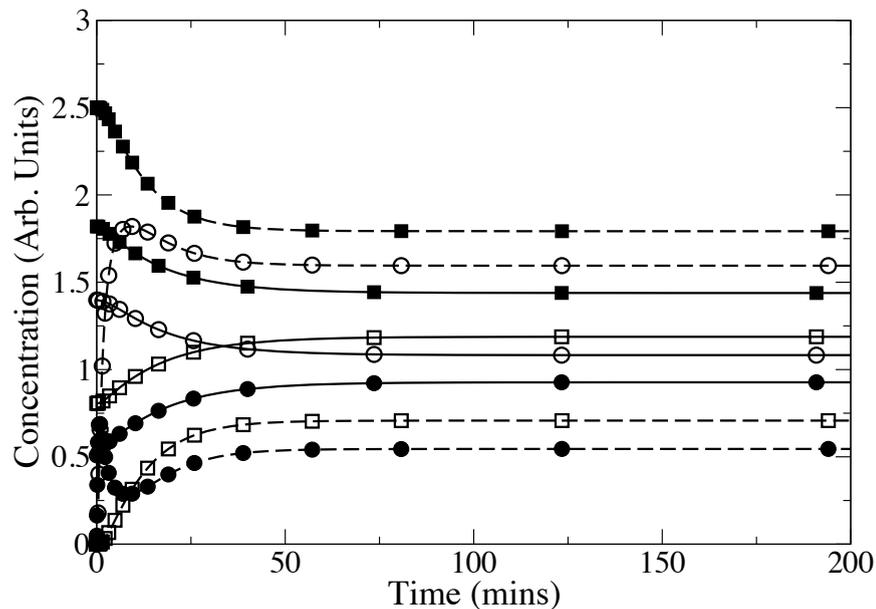
Figure 6.8: Comparison of the predicted concentration profile (continuous line) and the true steady-state data (segment line) for (*filled circle*): Raf1/RKIP, (*open circle*): Raf1/RKIP/ERK, (*filled square*): MEK-PP and (*open square*): MEK-PP/ERK. The predicted concentration profile is yielded with Parameter Set 2.

a separate form during the search process allows practitioners to favor certain objectives over the others in the selection of solutions. Given the Pareto-optimal front in Figure 6.3(d), biologists can choose the solution according to the importance ascribed to each objective. For example, if it is more important for the objective concerning the perturbed condition to be accurately optimized, then the solutions lying at the bottom of the front are favored, which are the ones that hold smaller values for this objective function.

Further statistical analysis of the Pareto-optimal solutions of multi-objective optimization problems can be carried out. Important information can be obtained from the Pareto-optimal front with regards to the reliability of the estimated parameters. This can be achieved by analyzing the variation of estimated values of the unknown parameters along the Pareto-optimal front. If the estimated values of one parameter vary widely, then it may indicate that either the available experimental data is not informative enough or the model contains intrinsic difficulties for estimating this parameter.
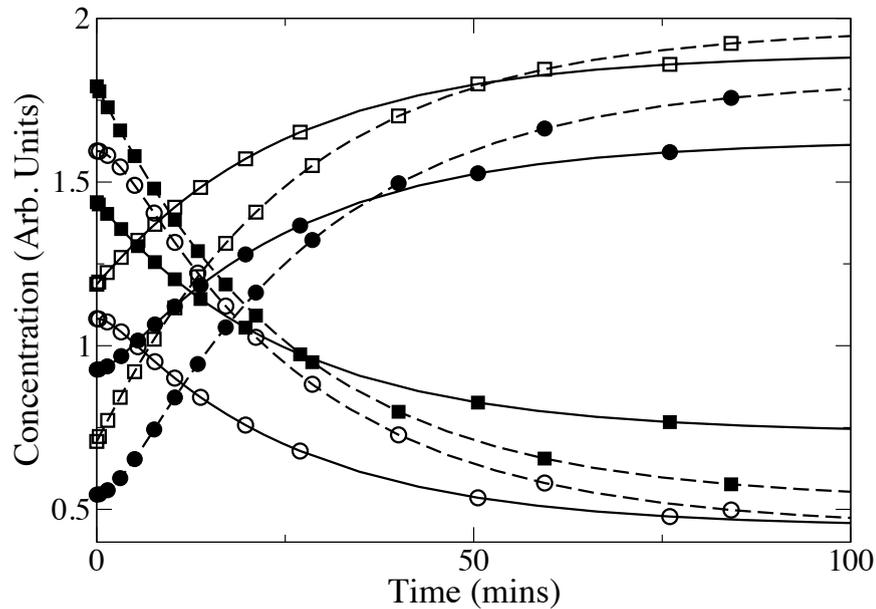
Figure 6.9: Comparison of the predicted concentration profile (continuous line) and the true perturbation data (segment line) for (*filled circle*): Raf1/RKIP, (*open circle*): Raf1/RKIP/ERK, (*filled square*): MEK-PP and (*open square*): MEK-PP/ERK. The predicted concentration profile is yielded with Parameter Set 2.

Application of the analysis (described above) to the solutions displayed in Figure 6.3(d) implies that it may be hard for parameters k_2 and k_7 to be accurately estimated with the given observed data, where the coefficient of variation (ratio of standard deviation over mean) of the estimated values of these parameters is large: up to 0.5. Large variations shown on k_2 and k_7 suggest that reactions involving these two parameters should ideally be further characterized or/and more biological experiments concerning the pathway (i.e. with a focus on the reactions containing k_2 and k_7) should be designed so as to obtain substantial measurements.

In Chapter 7, a schematic approach for system identification of the trypanothione metabolic pathway in *T. brucei* is presented. MoPSwarm (*Variant 4*) is applied to model validation of the polyamine biosynthetic sub-pathway.

Chapter 7

A Methodological Framework for Mathematical Modelling of the Trypanothione Metabolic pathway

In this chapter we describe the challenges of computational modelling of complex systems where prior knowledge about the system is incomplete and experimental data is sparse. We propose a methodological framework to address these challenges and illustrate the feasibility of the approach in guiding our work on the computational modelling of a real biological system – the trypanothione metabolic pathway in *Trypanosoma brucei*.

7.1   The Challenge

Sauro et al. (Sauro et al. 2006) stated that, owing to the inherent non-linearity of biological systems, the problem of system identification is extremely challenging. The authors drew attention to the biochemical network inverse problem when there are many variables for which there is no experimental data and also pointed out that few of the existing attempts have been applied to severely under-determined problems where only a limited amount of information can contribute to model construction.

Mechanistic modelling is a particularly useful tool in the study of new biological processes. However, despite its great value, the application of this type

of modelling of large-scale complex biological systems is not straightforward and sometimes impractical because it necessitates the consideration of many pathways for which detailed reactions and their kinetic parameters are not yet known. This is complicated by the fact that exact mechanisms accounting for the phenomenon under study are frequently elusive.

The experimental measurements of the trypanothione metabolic pathway are insufficient to achieve a complete mathematical description of the pathway. In addition, there are several challenges to constructing a suitable mechanistic mathematical model of the trypanothione pathway. First, there is uncertainty in the network topology describing chemical activities of the relevant system components. Second, the use of *in vivo* enzyme kinetics and regulatory data measured from different experimental settings also poses difficulties. It is necessary to explore both the model structure and missing parameters in order for the identification of an appropriate model.

There are many challenges that are imposed on conventional approaches to modelling, simulation and analysis by the complexity of biological pathways. This is further complicated by data sparsity, which leads to a requirement for novel approaches to cope with these challenges (van Riel 2006).

7.2 Goals and Objectives

As stated in Chapter 1, the intended use of the model has a critical impact on the approaches applied at different stages of the modelling procedure. There are two primary goals of our work on kinetic modelling of the trypanothione metabolic pathway as follows:

1. Given the mathematical formulation combined with the estimated parameters, the model can reproduce the observed biological phenomenon.

2. With a valid model, predictions of the system responses under different experimental conditions can be obtained and hypotheses regarding the biological pathway can be generated to be proved or disproved experimentally.

In order to achieve these goals, two categories of mathematical models are required for the pathway under consideration – a descriptive model and a predictive model. Different computational strategies are recommended to accomplish the specific objectives.

Descriptive models are concerned with the question of 'whether the model can reproduce the experimentally measured dynamics of the system'. One use of such models is to estimate the unknown kinetic parameters and the structural characters of the system *in silico*. Descriptive models are evaluated in terms of goodness of fit and finding a good fit involves simultaneously exploring the mathematical structure and unknown parameters. The compatibility of simulation results with experimental data provides evidence for the credibility of the model.

Predictive models are concerned with the question of 'does the model adequately describe the system of interest'. In other words, in addition to reproducing biological observations, models must be developed to allow predictions of system dynamics in response to environmental or genetic perturbations. Predictive models are usually validated using independent data that is not used for model building and parameter estimation. Good predictions of independent data can be used to assess the applicability of the model.

7.3 Our Approach

The challenge of kinetic modelling of complex biological systems is investigated, when prior knowledge about the system is incomplete and observed data is sparse. We develop a methodological framework to tackle the challenge and assess the usefulness of our proposal on the trypanothione metabolic pathway.

The schematic flowchart of the proposed methodological framework is illustrated in Figure 7.1. The major phases of this framework are *Initial Model Construction*, *Model Decomposition*, *Model Reconstruction* and *Model Validation*. The *Model Structure Exploration* step is employed in the phases of model decomposition and model reconstruction, where necessary and applicable. The proposed framework can be generalized to mathematical modelling of any nonlinear systems that are at best characterized with incomplete and sparse observation data.

One big strength of this framework is that it can be applied to tackle the system identification problem of different sub-systems in an independent manner. Once biological information is sufficient for the *in silico* investigation, these sub-systems can be further studied with regard to the correctness of mathematical model structures and unknown parameters can be estimated. After the remaining unexplored portion of the pathway is successfully resolved, the final
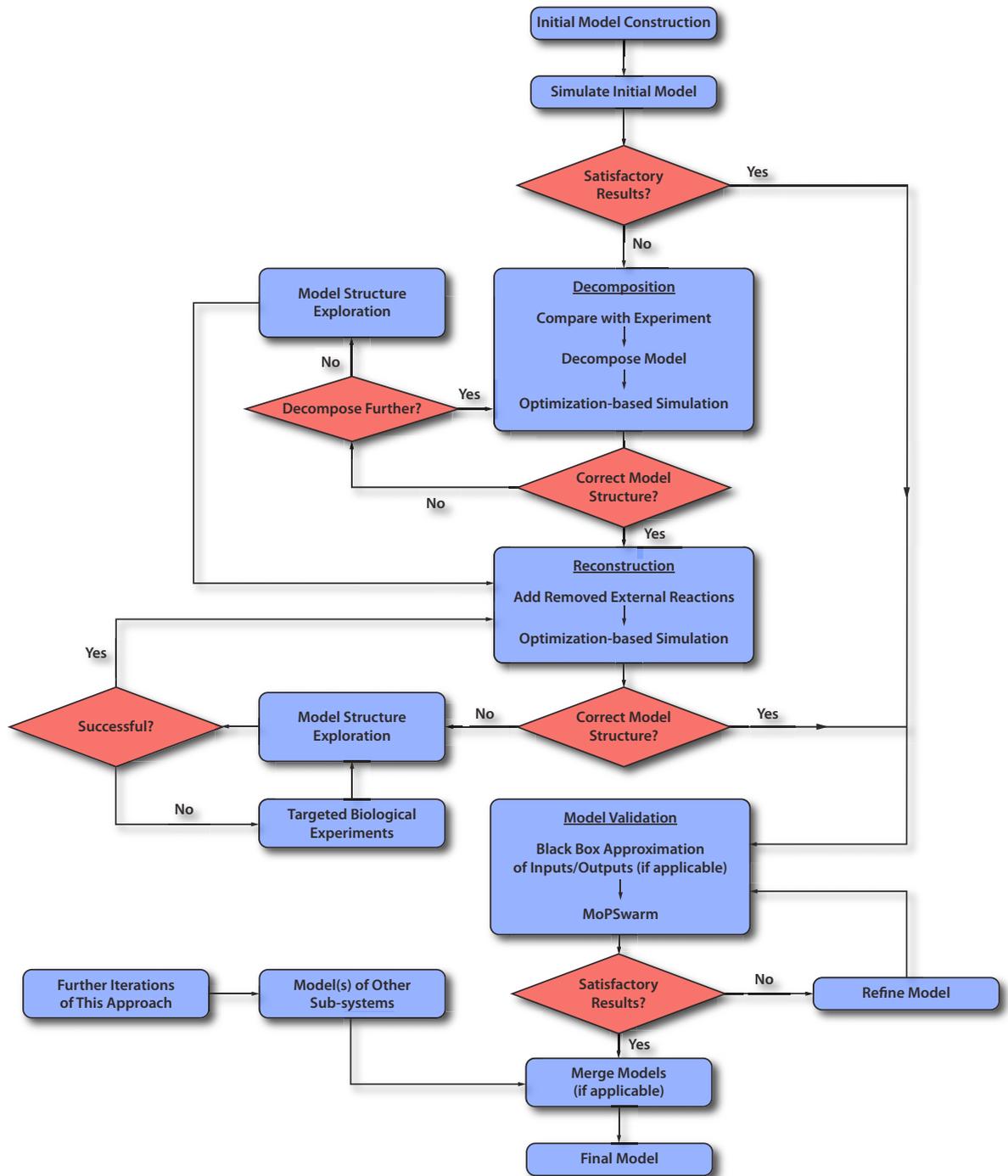
Figure 7.1: The schematic flowchart of the proposed methodological framework.

model of the trypanothione metabolic pathway can be developed by integrating the models describing individual sub-systems in the step of *Merge Model*. If the models are not directly connected, then black-box connectors are required to link the models and the involved parameters have to be estimated.

The phase of *Initial Model Construction* involves gleaning prior knowledge of the system from the literature, including identifying the network topology, the characters of enzymatic-reaction mechanisms and the experimentally measured initial states. The initial states, in the context of biological systems, refer to the initial concentrations of the system components and kinetic parameter values of the enzyme catalyzed reactions. The initial kinetic model of the trypanothione metabolic pathway was presented in Chapter 4.

The phase of *Model Decomposition* aims to decompose the initial system into functionally independent sub-systems, and study structural insufficiency via optimization-based simulation. Models of each sub-system are represented as closed systems, where the inputs and outputs of the sub-systems are known variables, approximated with the measured dynamics from biological experiments. The degree of decomposition is dependent on the correctness of mathematical model structures of the sub-systems and the availability of experimental measurements for system components. Choosing a model variable to initialize the decompositional process is not a trivial task. We take advantage of the structural modelling (see Chapter 3) as a strategy to determine the initial functional sub-system(s). In this phase, mathematical structures of the sub-systems are examined and the structures are rejected if no satisfactory fit with the experimental data can be obtained for any possible set in the parameter space. The consistency of the model behaviour is verified through a qualitative comparison with experimental data.

The phase of *Model Reconstruction* continues the investigation into the correctness of mathematical model structures, the appropriateness of which is examined via the same optimization procedure. Model reconstruction is an iterative procedure, which can be understood as the reverse of model decomposition, where enzymatic reactions may be added and/or removed from the sub-system. By the end of this phase, structurally-correct models can be identified that allow mechanistic insights into the system or sub-systems, and useful *in vivo* experiments can be suggested in order to enable structural exploration of the remaining portions of the system (if there are any) in the future.

Sub-system models that are obtained from the model decomposition and/or reconstruction phases may be structurally incorrect. Candidate models for these sub-system(s) are examined for appropriateness in the *Model Structure Exploration* step. Detailed biological information on structural constraints of the sub-system is required in order to design biologically meaningful model variants. The

structural constraints can be specified using the *network topology* (i.e. elementary reaction steps) and/or the *kinetic character* (i.e. regulatory mechanisms of enzymes or between enzymes). When it is feasible, the constraints are incorporated into the current mathematical structure of the sub-system to formulate new model structures and the appropriateness evaluated via optimization-based simulation. If a candidate mathematical structure reproduces experimental data sufficiently accurately, then according to Heinrich et al. (Heinrich et al. 1977), it is likely that this sub-system is correctly modelled.

The phase of *Model Validation* is concerned with proving that the model is an adequate representation of reality, which gives confidence that the model is useful not only for reproducing measured dynamics, but also consistently interpreting the underlying biological processes. The multi-objective optimization algorithm, MoPSwarm, proposed in Chapter 6 is applied in this phase. If only structurally-correct sub-system(s), rather than a complete kinetic model, are obtained from the previous phases, then a black-box approximation must be applied to the inputs and/or outputs of the sub-system(s) prior to the validation procedure in order to render predictive power. The applicability of the model with the parameter estimates solved using MoPSwarm is assessed by comparisons between the model predictions and independent data sets obtained from distinct states of the system. We term the data sets serving as the inputs to MoPSwarm algorithm as *Estimation Data* and the independent data sets for model assessment as *Validation Data*. Unsatisfactory results trigger the model refinement process, which may involve checking the reliability of validation data or examining the presence of regulatory mechanisms that may be missing from the model.

According to the conventional process of *in silico* simulation, the model should be solved as a whole, with all the parameters estimated simultaneously using the optimization method. The missing protein initial concentrations are treated as parameters to be estimated together with the unknown kinetic parameters. The initial kinetic model of the trypanothione metabolism proposed in Chapter 4 (see Section 4.4) consists of 23 ODEs derived from 23 catalytic mechanisms and 3 regulatory mechanisms. In this thesis, we focus on studying the trypanothione dynamics at the metabolic level. Towards the end, only ODEs for variable metabolites are taken into consideration, which results in a kinetic model consisting of 23 catalytic mechanisms with 80 kinetic parameters. There are 29 unknown kinetic parameters to be estimated with the optimization method and 3 unknown initial concentrations (intracellular AdoHcy, cys-

teine and G6P). According to Chapter 4, the initial concentration of AdoHcy can be approximated from the metabolite AdoMet with the equality constraint $[AdoHcy] = 0.2 \times [AdoMet]$.

Prior to computational simulation, models may be simplified by reducing the number of unknown parameters so as to improve the performance of system identification. Based on relevant qualitative knowledge, meaningful simplifications can be introduced on two unknown kinetic parameters as follows.

First, AdoMetDC is assumed to be present in the cell in two forms, namely a free form and a ligand-occupied form. The total concentration of AdoMetDC is therefore the superposition of free-form AdoMetDC and ligand-occupied AdoMetDC. We introduced a factor β to represent the percent of free-form AdoMetDC taking up the total enzyme concentration, thus the ligand-occupied form is expressed as $1 - \beta$ of the total concentration. Willert and Phillips (Willert and Phillips 2008) observed that the prozyme reacting with AdoMetDC is a limiting factor on the activity of AdoMetDC. This observation implies that the concentration of the ligand-occupied form accounts for a limited quantity of the total AdoMetDC concentration. β is assumed to vary between 0.5 and 1 in order to reflect the experimental observation and still allow the ligand-occupied AdoMetDC to change within a physiologically feasible range. In this way, the three unknown parameters are reduced to two unknown parameters – the total concentration of AdoMetDC and the ratio β. The mathematical rate expressions of the ligand-occupied and free form of the enzyme defined in Equation (4.6) and Equation (4.7) in Chapter 4 are modified to the following equations

$$V_{AdoMetDC^L} = k_{cat}^{AdoPro} \cdot [AdoMetDC^T] \cdot (1 - \boldsymbol{\beta}) \cdot \frac{\dfrac{[AdoMet]}{K_{mAdoMet}^{AdoPro}}}{1 + \dfrac{[AdoMet]}{K_{mAdoMet}^{AdoPro}}} \quad (7.1)$$

$$V_{AdoMetDC^O} = k_{cat}^{AdoMetDC^O} \cdot [AdoMetDC^T] \cdot \boldsymbol{\beta} \cdot \quad (7.2)$$
$$\frac{\dfrac{[AdoMet]}{K_{mAdoMet}^{AdoMetDC^O}}}{1 + \dfrac{[AdoMet]}{K_{mAdoMet}^{AdoMetDC^O}} + \dfrac{[dAdoMet]}{K_{idAdoMet}^{AdoMetDC^O}} + \dfrac{[K_{aPut}^{AdoMetDC^O}]}{[Put]}}$$

Second, a relation between the enzyme activities of trypanothione peroxidase, V_{max}^{TPx}, and H_2O_2 synthesis rate, R_{syn}, is approximated. The published values

for V_{max}^{TPx} and R_{syn} have been extracted from the literature, which are $1.3 \; nmol \cdot min^{-1} \cdot (10^8 \; cells)^{-1}$ and between 0.2–$0.7 \; nmol \cdot min^{-1} \cdot (10^8 \; cells)^{-1}$, as given in Chapter 4. We clarified that the standard unit of enzyme specificity is defined as $\mu mol \cdot min^{-1} \cdot (mg \; of \; cells)^{-1}$. However, both values are given in the literature in a different unit. To transform the values into the standard enzyme unit, the protein concentrations of the associated enzymes must be known. There are, unfortunately, difficult to measure *in vivo*. Instead, with the published values in units of number of cells, a ratio of V_{max}^{TPx} and R_{syn} can be derived, which falls in the range 1.8 to 6.5. In our study, a ratio of 6.5 is assumed to describe the relationship between the two parameters, thus removing one unknown parameter.

7.4 Initial Model Simulation

The experimental observations adopted for global optimization were obtained from the paper by Fairlamb et al. (Fairlamb et al. 1987). The reported data set describes the *in vivo* drug-induced dynamics of bloodstream-form *T. brucei* in response to DFMO. This is the only relevant data source that was obtainable for this project. A few of other types of data has been published (e.g. gene knockdown data), but they are comparatively insufficient to be used as the *Estimation Data* for the identification of model structure and unknown parameters.

The experimental measurements published by Fairlamb et al. are available for eight metabolites, namely AdoMet, dAdoMet, ornithine, putrescine, spermidine, glutathione, GspdSH and trypanothione, with data given for three time points (12 hrs, 36 hrs and 48 hrs). This results in a partial and sparse optimization problem, where the availability of observed data is given for only a portion of the total metabolites (8 out of 20 metabolites) and measured at a limited number of time points. This constraint poses a significant challenge to the optimization problem under study.

In order to apply the drug perturbation data reported by Fairlamb et al., a modification to the initial model has to be modified by incorporating the DFMO-induced factors. DFMO profoundly changes the metabolism of *T. brucei* by altering the enzyme activity of ODC, in consequence, influencing the polyamine synthesis and content. The steady-state protein levels of other enzymes, including SpdS, AdoMetDC, γ-GCS, prozyme, TryS and TR were unaffected by DFMO inhibition (Xiao et al. 2009).

According to Bacchi et al. (Bacchi et al. 1983), ODC activity was decreased by more than 99% within 12-hour treatment of DFMO. In our study, the generic form of enzyme activity is represented as the product of turnover number and the protein concentration

$$V_{max}^{ODC} = k_{cat}^{ODC} \cdot [ODC] \tag{7.3}$$

where k_{cat}^{ODC} remains fixed in all conditions but the enzyme concentration, $[ODC]$, may vary in some cases when the biological system is treated with an activator or inhibitor.

To describe the enzyme inhibition, time-dependent changes of the ODC concentration in response to DFMO have to be explicitly modelled. The inhibition is assumed to follow an exponential decay, expressed as

$$[ODC]_{ss} \cdot e^{-\lambda \cdot t} \tag{7.4}$$

where $[ODC]_{ss}$ is the steady-state ODC concentration. Parameter λ is solved for simple curve fitting using the qualitative description of the enzyme dynamics with addition of DFMO and takes a value of 0.007 in this instance. A detailed discussion of this drug-enzyme interaction was given in Section 4.3.1 in Chapter 4. The rate equation of the enzyme under normal conditions, which was defined in Equation 4.2, is modified to give the following:

$$V_{ODC} = k_{cat}^{ODC} \cdot [ODC]_{ss} \cdot e^{-\lambda \cdot t} \cdot \frac{\dfrac{[Orn]}{K_{mOrn}^{ODC}}}{1 + \dfrac{[Orn]}{K_{mOrn}^{ODC}} + \dfrac{[Put]}{K_{iP}^{ODC}}} \tag{7.5}$$

No boundary values for the unknown parameters can be directly extracted from the literature. In terms of optimization, there is a trade-off between small and large ranges within which the parameters may vary. Small ranges on unknown parameters lead to a narrow search space that will probably preclude potentially good solutions from consideration. On the other hand, a broad search space is more likely to lead to globally optimal solutions by thoroughly exploring the search space. However, in the latter case it is more likely that the optimization will move into a region of the search space where the ODE solver becomes numerically unstable. The lower and upper bounds imposed on the unknown parameters were assumed to be based on the modeller's experiences

and communications with the biologists. The boundary values are reported in Chapter 4 and are deemed appropriate for the current study. The possibility that the model is not soluble is thus less likely to stem from ill formation of the parameter search space.

In total, we carried out 300 optimization runs using the global optimization algorithm PSwarm. The algorithm parameters were set as follows: 50 initial particles in the population, 2000 maximum iteration number and 5000 maximum function evaluations. The simulations were performed for 48 hours and the model was solved using `ode15s` from Matlab.

The optimization method failed to produce a set of parameters that can reasonably reproduce the experimental data. Figure 7.2 shows the simulation results and experimental measurements for the metabolites critical to the regulation of trypanosomal growth, including spermidine, glutathione-spermidine conjugate and trypanothione. The simulation results were calculated with the estimated set of parameters having the smallest objective function value. In Figure 7.2(a), Figure 7.2(b) and Figure 7.2(c), the simulation results are constantly higher or lower than the observed data, representing distinct dynamic trends over the time course. Figure 7.2(d) shows interesting dynamics, where the simulation results are both higher and lower than the observed data at different time points.

The optimization problem under investigation is a non-linear, high dimensional problem. Simultaneously estimating all the unknown parameters in the pathway is computationally expensive and sometimes not possible. This is particularly true when the observed data is limited. The problem is further complicated by correlations between the unknown parameters. In the following sections, the proposed methodological framework is applied to guide the computational modelling of the trypanothione metabolic pathway. We aim at exploring the ill-defined kinetic mechanisms of the reactions, subsequently, inferring potentially suitable structures, and identifying the sub-system for which a sound mathematical model can be possibly obtained given the limited experimental data.

7.5 Model Decomposition

Cells can be seen as composed of a number of sub-systems. These sub-systems themselves can in turn be further broken down into sub-systems — for example,

(a) glutathione

(b) spermidine

(c) GspdSH

(d) trypanothione
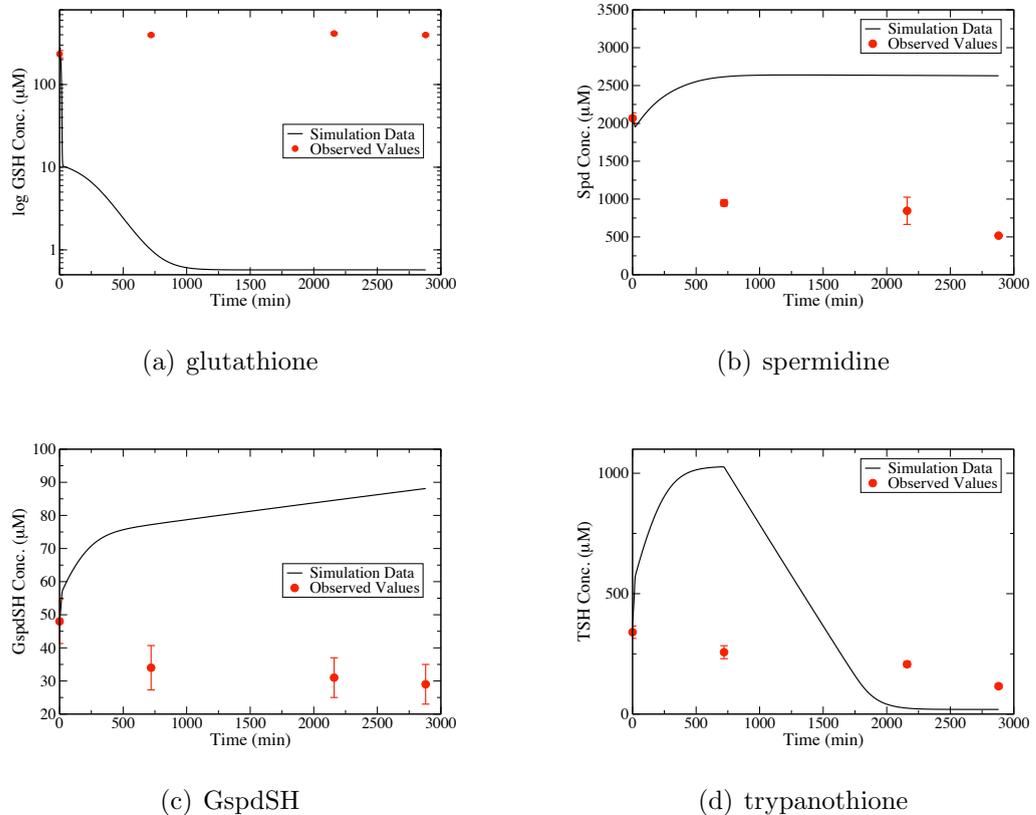
Figure 7.2: Simulation profiles compared to experimental data for the key metabolites when solving the model as a whole. Logarithmic axis is used for glutathione in order to better present the data. Error bars are presented where appropriate data was available in the original papers.

functionally independent modules. Biological systems can thus be seen as hierarchical systems. As stated in Chapter 1, a key problem is how to functionally modularize a system.

In this study, we propose a *decompositional* approach to break down the initial model, which is not well-characterized, into modules for the investigation of the correctness of mathematical model structures. Each module fulfills a certain metabolic function pertinent to the trypanothione metabolism. The boundaries of each module are formed by the model components for which there are concentration profiles with experimental data. We term these model components as *constant components*. Modules should be able to operate independently of the rest of the system. In the context of global optimization, parameters included in modules can be estimated separately from the remaining unknown parameters.

The proposed procedure for *Model Decomposition* is embodied in the follow-

ing basic steps:

> **Step 1:** If this is the first iteration, identify the initial point for model decomposition to derive initial modules and go to Step 3. Otherwise, go to Step 2.

> **Step 2:** Remove boundary components of the module derived from the previous step of model decomposition. The removal of boundary components involves deleting the associated chemical reactions. The formation of new boundaries should comply with the following conditions:

> > • If observed data is not available for the new boundary component after the first set of boundary components is removed, then the reactions associated with this component must also be deleted. This process may be continued for a number of iterations until the exterior components of the resulting module are *constant components*.

> > • If observed data is available for the new boundary component after the first set of boundary components is removed, then either the reaction(s) incoming to, or the reaction(s) outgoing from this component are deleted, depending on whether the component set participates in the module as an input or output.

> **Step 3:** apply global optimization to the module obtained from the first step. If the results are satisfactory when compared with experimental results, then this module is considered as an 'elementary' module (the module is assumed to be well-characterized) and no further decomposition is required.

> **Step 4:** (optionally) return to the first step if the optimization results are not satisfactory until the end point of decomposition is reached.

The proposed decompositional approach is an iterative process, the termination of which depends on the quality of the optimization results in describing the measured system dynamics. Modules derived using this approach should satisfy the following condition: boundary components of the module are treated as independent inputs and only the components within the module are variable components with dynamics dependent on time and the inputs. In order to apply the global optimization method to the modules, an additional constraint is imposed – observed data should be available for at least one variable component

contained in the module. The availability of observed data (e.g. coverage and adequacy) therefore also determines the degree of model decomposition.

In order to check whether the module's behaviour is consistent with experimental data, the behaviour is qualitatively compared based on the similarity of curves or on certain effects which the model can reproduce. This comparison method is defined by Heinrich et al. (Heinrich et al. 1977) as 'qualitative comparison'. The mathematical structure of the module is rejected if no satisfactory fit with the experimental data can be obtained.

Identifying the first component to initialise model decomposition is a non-trivial task, particularly for real systems, where the number of variable components involved is often large. It is noteworthy that in metabolic networks a potential relationship exists between topological modularity and the functional classification of different metabolites, for example in the metabolic network of *E. coli* (Ravasz et al. 2002).

We attempt to take advantage of the network hierarchy to guide the selection of starting point for the decompositional procedure. In Chapter 3, we conducted structural modelling of the trypanothione pathway. The results presented indicate that, from a topological perspective, trypanothione metabolism is organized in a hierarchical architecture. This provides a natural breakdown of the pathway into two large modules, responsible for two necessary components of trypanothione metabolism, namely trypanothione biosynthesis and oxidative stress defense.

However, the oxidative stress defense module cannot be processed for model decomposition. As stated previously, there must be at least one variable component contained in the module where observed data is available; however no components contained in this module were provided in the experimental measurements by Fairlamb et al..

There is however comparatively sufficient data to investigate the correctness of the mathematical model structure of the trypanothione biosynthesis module. Initially, the constant boundary components of this module are exogenous methionine, arginine, cysteine and intracellular trypanothione. Following optimization to assess the compatibility of the module with experimental data, the module will be further partitioned into smaller but more integrated sub-modules.

The time evolution of the intracellular trypanothione is approximated with a time-dependent polynomial. The use of different input functions to simulate the concentration profiles of the boundary components has been found to have

an impact on the accuracy of parameter estimation (Faller et al. 2003). With the four data points (including the initial concentration) measured by Fairlamb et al., a polynomial input function up to 4th order can be generated. In this stage, the error in parameter estimates is irrelevant, since the comparison of the module's behaviour to the experimental data is qualitative rather than a goodness-of-fit criterion, such as on the sum of least squares. A 3rd-order polynomial function is employed in this case, which is believed to be sufficient the qualitative comparison.

The first three stages of model decomposition of the trypanothione biosynthesis sub-system are shown in Figure 7.3.

Model Decomposition–Stage 1   Optimization of the trypanothione biosynthesis sub-system, which is shown Figure 7.3(A), failed to produce a satisfactory match with the experimental data. In particular, simulation results for glutathione, spermidine and GspdSH are no better than the results obtained by solving the entire model (shown in Figure 7.2).

According to the decompositional rules, the trypanothione biosynthesis sub-system is further decomposed by removing the boundary component, trypanothione, which involves deleting the incoming reactions of this metabolite. This process results in a new module with GspdSH and glutathione as the boundary components, shown in Figure 7.3(B). The results of this new module are however still not compatible with experimental data. This indicates that a further level of model decomposition is required in order to correct the module.

It is worth pointing out that setting glutathione as a known input results in the optimization method being unable to correctly tune the parameters involved in the upstream glutathione production reactions (sub-pathway in grey in Figure 7.3(B)). This involves the reactions catalyzed by exogenous cysteine transporter, gGCS and GS.

Model Decomposition–Stage 2   Removing the boundary component GspdSH and associated reactions from the module (Figure 7.3(B)) leads to two individual sub-pathways with spermidine and glutathione as the end products. As discussed above, the glutathione production sub-pathway cannot be examined for mathematical structure deficiency by parameter estimation because no observed data is available for the intermediate components included in this sub-pathway.

The spermidine biosynthesis module, shown in Figure 7.3(C), is suitable
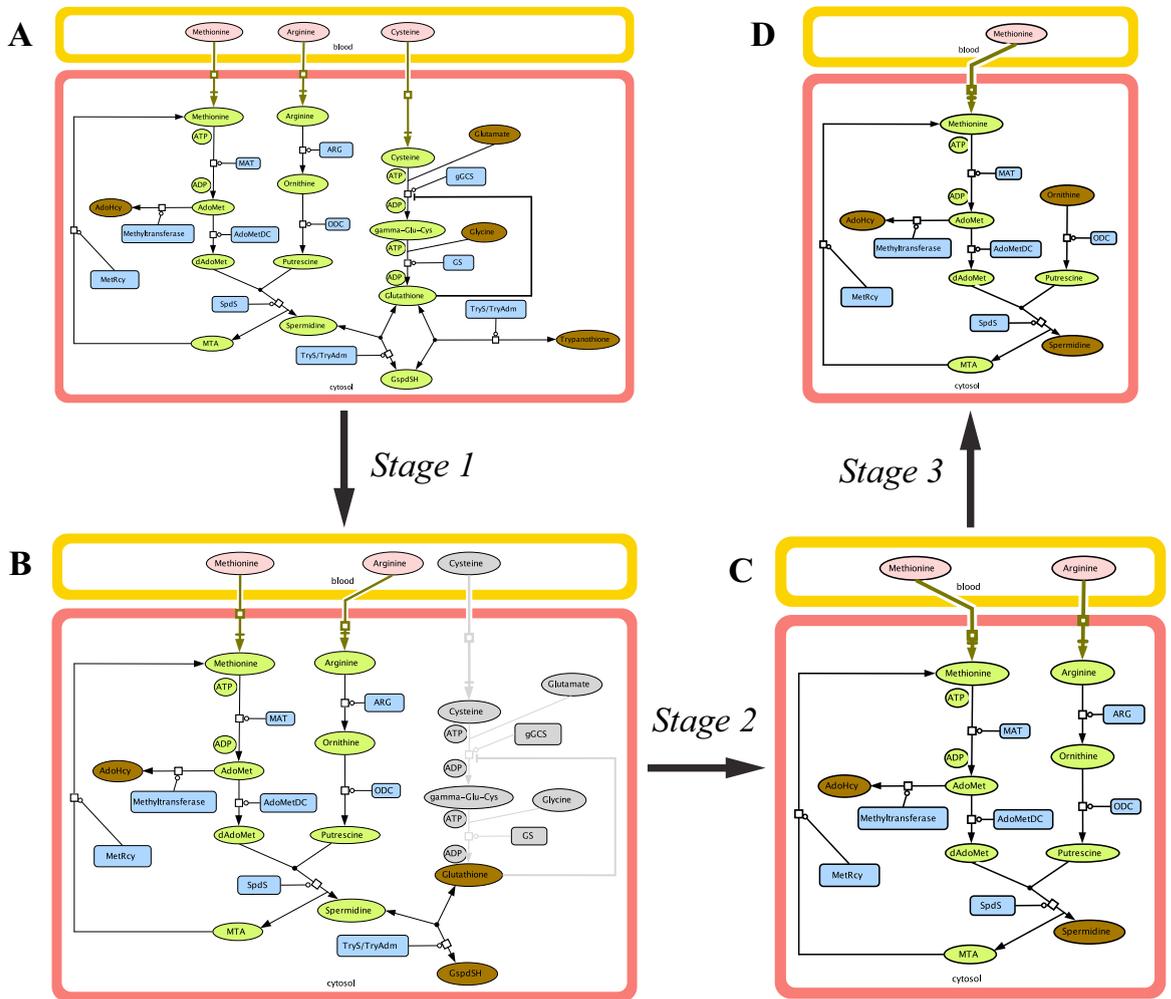
Figure 7.3: The first three stages of model decomposition of the trypanothione biosynthesis sub-system: (*top left*): overview of the trypanothione biosynthesis sub-network; (*bottom left*): reduced module of the sub-network via stage 1; (*bottom right*) reduced module of the sub-network via stage 2; (*top right*); reduced module of sub-network via stage 3. Boundary components of the three reduced modules are denoted in brown color: GspdSH and glutathione in *bottom left*; spermidine in *bottom right*; and spermidine and ornithine in *top right*.

for the investigation of the correctness of mathematical model structure. In this case, intracellular spermidine becomes the boundary component that is approximated with a 3rd-order polynomial function. The simulation results of the 'known' intermediate components (for which observed data is available) including AdoMet, dAdoMet, ornithine and putrescine are shown in Figure 7.4.

Inspection of Figure 7.4 shows that a moderate improvement in the opti-

(a) AdoMet

(b) dAdoMet

(c) ornithine

(d) putrescine
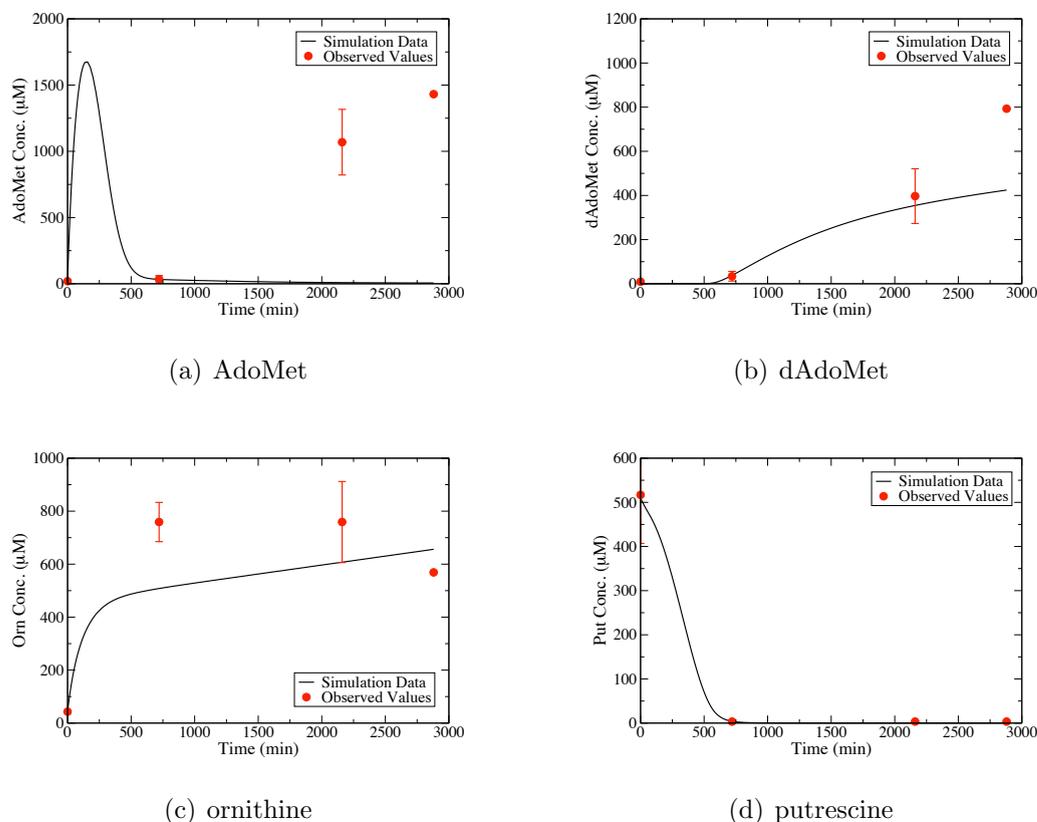
Figure 7.4: Simulation profiles compared to experimental data for the key metabolites obtained in Stage 2 of the model decomposition. Error bars are presented where appropriate data was available in the original papers.

mization performance in solving the spermidine biosynthesis module. Of the four intermediate metabolites, the match with experimental data for dAdoMet, ornithine and putrescine (Figure 7.4(b), Figure 7.4(c) and Figure 7.4(d)) is satisfactory given the sparse experimental data. The simulation results of AdoMet (Figure 7.4(a)) still contradict the target data in terms of the trend in the change of transient concentrations.

The simulation profile of arginine is shown in Figure 7.5. No values were reported by Fairlamb et al. for arginine concentrations in the DFMO inhibition experiment; however, its intracellular level is assumed not to significantly change during the course of drug inhibition (Alan H. Fairlamb, personal email, 28 August 2007). Different arginine dynamics were observed in the procyclic-form *T. brucei* by Bellofatto et al. (Bellofatto et al. 1987) (see Table A.2 in Appendix A), where arginine concentrations were increased up to four times of the steady-state level after 48 hours of DFMO treatment.
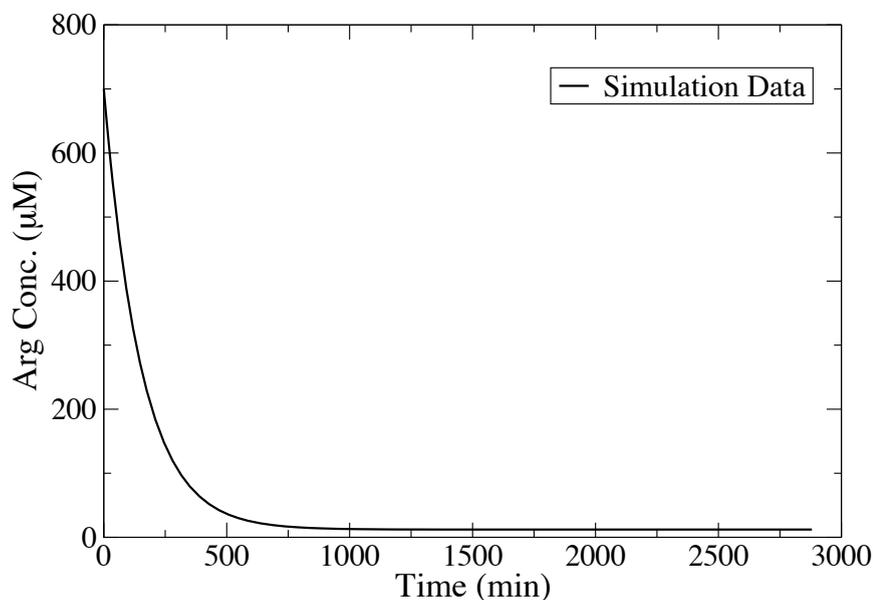
Figure 7.5: Simulation profiles for the arginine dynamics obtained in Stage 2 of the model decomposition. Arginine concentrations reduced to 90% of the steady-state level within 12 hours of DFMO treatment. No experimental data is available for arginine dynamics.

Regardless of which arginine dynamics are taken into account, simulation results of the mathematical model deviate from experimental measurements. As displayed in Figure 7.5, the model prediction for arginine concentrations are contradictory to both assumptions that the concentration increases approximately linearly or is unchanged during the course of DFMO treatment.

Model Decomposition–Stage 3   The unsatisfactory optimization results obtained from Stage 2 promote further decomposition in order to identify possible missing reactions or ill-defined kinetics of the spermidine biosynthesis module. Since the number of metabolites for which observed data is available is comparatively large in quantity (4 out of 7 metabolites have experimental measurements), the proposed *decompositional* approach allows several strategies to be developed for decomposition of this module.

We take advantage of the optimization results obtained in Stage 2 to guide the model decomposition in the current stage. Figure 7.4(a) and Figure 7.5 indicate that the dynamics predicted for AdoMet and arginine present differing

trends to the observed data. This implies that the reactions participating in the metabolism of AdoMet and arginine may be ill-characterized. As a consequence, the purpose of model decomposition in this stage is to examine the correctness of mathematical model descriptions of the related reactions. Two strategies can be developed in accordance with the focus of investigation centered on either AdoMet and arginine metabolism.

Strategy 1 with focus on AdoMet metabolism When the focus of investigation is concerned with AdoMet metabolism, the spermidine biosynthesis module (Figure 7.3(C)) is decomposed by removing the upstream reactions for ornithine production. Such decomposition leads to intracellular ornithine as the new boundary component of the derived module, shown in Figure 7.3(D). The concentration profile of intracellular ornithine is approximated with a 3rd-order polynomial function.

The simulation results of the intermediate components including AdoMet, dAdoMet and putrescine are shown in Figure 7.6.

It is clear that the simulation results for AdoMet, dAdoMet and putrescine do not differ considerably from those obtained in Stage 2 of the model decomposition. Apart from AdoMet (Figure 7.6(a)), the simulation results obtained for dAdoMet and putrescine (Figure 7.6(b) and Figure 7.6(c)) via global optimization are reasonably good, although not perfect. The overall optimization performance is satisfactory for solving the high-dimensional search space, given the limited and incomplete experimental data.

Simulation results in Figure 7.4(a) and Figure 7.6(a) show that almost no difference is observed in the AdoMet dynamics. The intracellular concentrations of AdoMet are estimated to remain constant over 12 to 48 hours after DFMO inhibition. This is in contrast to the experimental observations from Bacchi et al. (Bacchi et al. 1983) and Fairlamb et al. (Fairlamb et al. 1987) that report AdoMet concentrations increasing more than 50 fold 48 hours after DFMO treatment. However, more recent and reliable biological experiments (Xiao et al. 2009) support our model predictions that AdoMet concentrations do not significantly change in response to DFMO treatment. This is not shown in Figure 7.6 as Xiao et al. report only a qualitative comparison with their control experiment.

(a) AdoMet

(b) dAdoMet

(c) putrescine
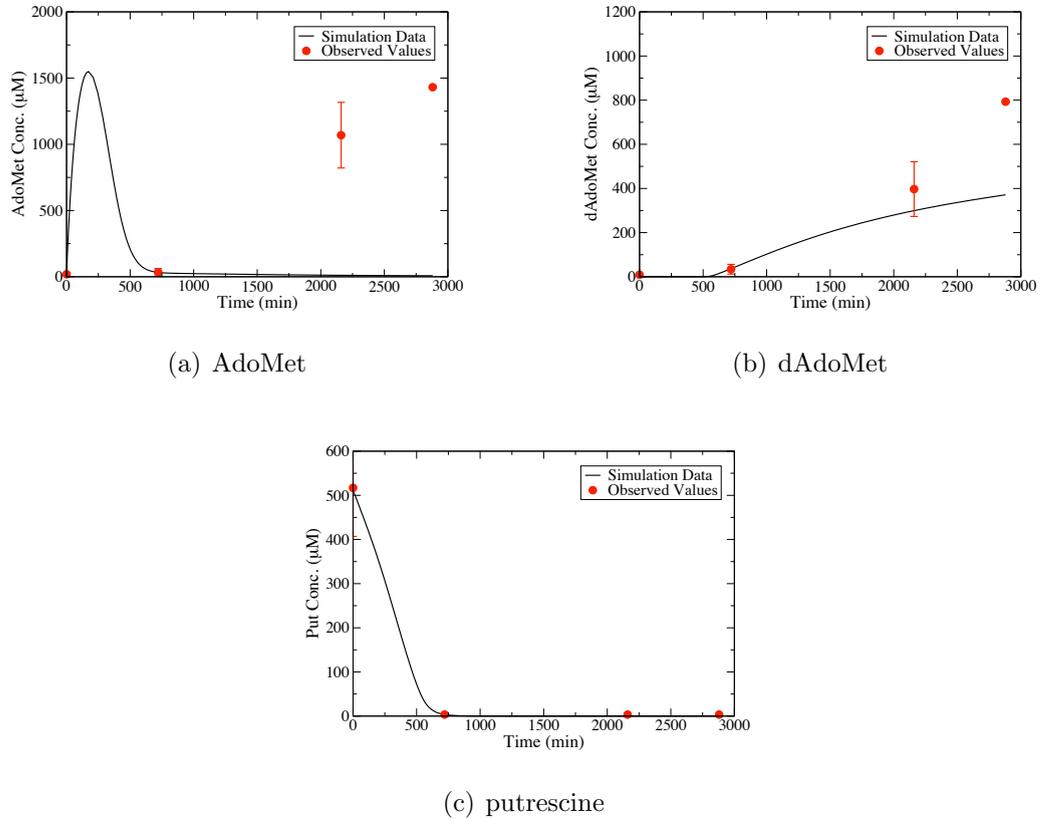
Figure 7.6: Simulation profiles compared to experimental data for the key metabolites obtained in Stage 3 of the model decomposition. Ornithine is an input. Error bars are presented where appropriate data was available in the original papers.

Strategy 2 with focus on arginine metabolism When the focus of investigation is concerned with arginine metabolism, the spermidine biosynthesis module is decomposed by removing the intracellular spermidine and by-product MTA. This leads to the deletion of methionine and the related reactions. AdoMet, dAdoMet and putrescine therefore become the new boundary components, as shown in Figure 7.7.

Figure 7.7 shows that the arginine and dAdoMet biosynthetic sub-pathways are topologically isolated and can be studied independently from each other, as no interactions between the two branches are indicated at the metabolic level. We are aware that changes in AdoMetDC activity have an impact on the gene expression level of ODC (Willert and Phillips 2008), and that ODC suppression may also have a concomitant influence on AdoMetDC activity, though this has not yet been evidenced.
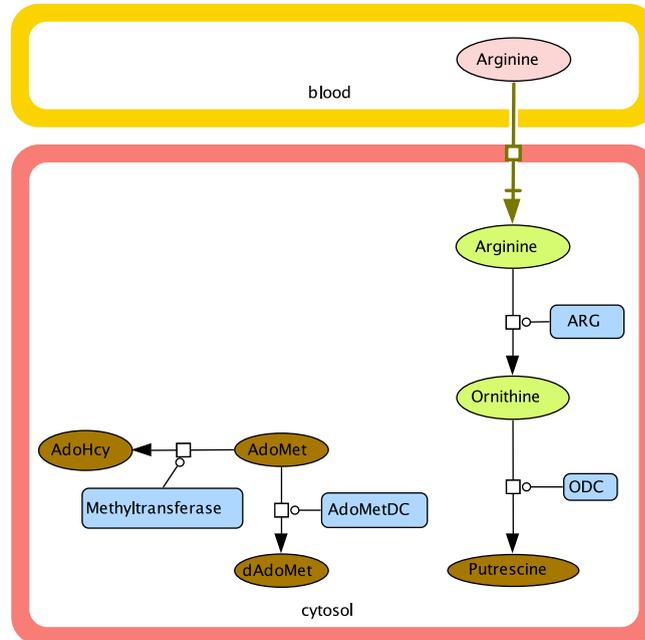
Figure 7.7: Results of decompositional Stage 3, arginine-centered strategy

The gene-level regulation between the enzyme activities of AdoMetDC and ODC is not considered in the phase of mode structure exploration, rather they are solved independently from each other during the optimization process. This consideration is consistent with the purpose of the current study, which aims to seek possible solution(s) for the unknown parameters within the defined search space so that model predictions are compatible with the measured dynamics of the system. Elucidating the underlying relation between the enzyme activities of AdoMetDC and ODC is not possible with the DFMO-induced data set that describes the changes in concentrations at the metabolic level, thus is not relevant to this study.

The module used for investigating the correctness of the model of arginine metabolism is the individual sub-pathway on the right side of Figure 7.7. This module takes putrescine as the boundary component and employs a 3rd-order polynomial function to approximate its concentration profile. No significant improvements were obtained for the simulation results of arginine dynamics. This implies that the enzymatic reactions concerning arginine dynamics may be incorrect, which suggests *in silico* exploration for appropriate model structures.

7.6 Model Structure Exploration

When one attempts to construct a novel model of a complex biological system, for which the prior knowledge is partial, the first and foremost thing to do is to examine the soundness of the mathematical model. The mathematical models considered in this thesis are defined as a set of differential equations, which associate the changes in concentration levels of system components with reaction rate equations. The reaction rate equations express the enzyme reaction rate as a function of the concentrations of other model components. The algebraic expression for each rate equation depends on the kinetics under consideration. In this thesis, we apply the law of Michaelis-Menten to model the biological system of interest.

The structure of mathematical models can be characterized with two types of structural constraints, including *network topology* and *kinetic character*. The former refers to the spatial layout of system components and the reversibility of the chemical conversion between them. The latter specifies the realization of interactions between system components, which is concerned with different manners by which a reaction mechanism may be regulated. Kinetic characters of standard Michaelis-menten and the derived forms featuring competitive inhibition, noncompetitive inhibition, substrate inhibition and product activation, are expressed in Section 4.3 in Chapter 4.

Model structure used to represent biological systems is often a key source of uncertainty, when there are alternative sets of assumptions for developing a model. Othmer (Othmer 1980) argued that, biologically, model structure is important as a constraint, for example, certain structures are not compatible with some dynamic behaviour fashions. Accurate model formulation can lower the uncertainty associated with model structure. *In silico* investigations aimed at identifying ill-characterized reactions and, subsequently, inferring potentially suitable structures are inevitable.

In biological systems, there is only a small amount of information that can be used to guide the design of candidate models. Very few studies take the time to test the appropriateness of model structures explicitly and show their effects on model predictions (Swameye et al. 2003). This is mainly because a lot of work is required and there is a lack of established methods to aid the design. Detailed biological information is also required in order to establish testable assumptions. In the absence of enough knowledge, system-specific decisions will have to be

made when designing variants of a mathematical model of the system.

As the trypanothione metabolic system is better known qualitatively than numerically, we assume that the model inappropriateness discovered in the phase of model decomposition most likely originates from the kinetic characters of the system reactions, and the network topology currently in use is acceptable. The modifications to kinetic mechanisms are reaction specific. In other words, changes made to the kinetic character of one reaction so that the time-series metabolite concentrations compare favorably with experimental data may not be applicable to other reations. As illustrated by Tyson et al. (Tyson et al. 2003), for a predefined stimuli-response relation, the model structure must comply with a certain pattern in order for the model to accurately mimic the measured dynamics. With additional or modified kinetic characters, a better model representation can be selected.

7.6.1 Identification of the Regulatory Link between ArgPt, ARG and ODC

The model decomposition study reveals that the enzyme kinetics employed in the arginine metabolism module may be ill defined (the individual sub-pathway on the right side of Figure 7.7), as discrepancies between the mathematical model and experimental observations are observed. There is no available biological knowledge about *T. brucei* that can be used for postulating underlying model structures. Instead, we refer to information about other organisms in the literature to aid the design of candidate models. The corresponding mathematical structure of the equations are evaluated with two different sets of experimental data by Bellofatto et al. (Bellofatto et al. 1987) and Fairlamb et al. (Fairlamb et al. 1987).

Early work indicated that, in mammalian cells, stimulation of ODC would cause a concomitant increase in the level of arginase expression (Cederbaum et al. 2003) or that ornithine may have an inhibitory effect on arginase (Selamnia et al. 1998). These two hypotheses indicate the same phenomenon that arginase activity may be regulated by the concentration of the enzyme ODC. We propose the following mathematical expression to reflect the postulated relation between

the two enzymes

$$V_{ARG} = \frac{V_{max}^{ARG}}{\left(1 + \frac{K_{aODC}^{ARG}}{[ODC]}\right)^{\gamma_1}} \cdot \frac{\frac{[Arg]}{K_{mArg}^{ARG}}}{1 + \frac{[Arg]}{K_{mArg}^{ARG}}} \tag{7.6}$$

where K_{aODC}^{ARG} (μM) represents the activation coefficient of the ODC concentration ([ODC]) on the enzyme activities of arginase; and γ_1 stands for the activation strength. These two parameters are assumed to vary between 0 and 10.

We performed an optimization-based simulation study on the arginine metabolism module to examine the presence of this regulatory relationship in *T. brucei*. The rate equations of the constituent enzymes, including arginine uptake, ODC and arginase, are expressed in Equation (4.23) and Equation (4.2) (in Chapter 4) and Equation (7.6), respectively. The simulation results for intracellular arginine and ornithine are shown in Figure 7.8. Both metabolites show a satisfactory match with the experimental data, where arginine dynamics observed in the procyclic-form *T. brucei* are assumed as the true experimental data.

(a) arginine   (b) ornithine
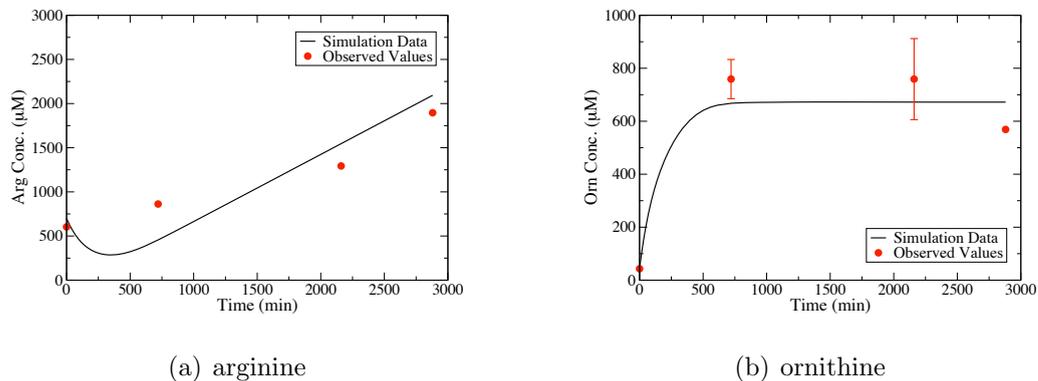
Figure 7.8: Simulation profiles compared to experimental data for the arginine and ornithine dynamics computed with the modified rate of change of the enzyme arginase. In this case, arginine dynamics observed in procyclic-form *T. brucei* is assumed as the true experimental data. Error bars are presented where appropriate data was available in the original papers.

However, a different mathematical model structure of the module is required in order to interpret the dynamics properly when constant arginine concentrations are assumed in the DFMO-induced experiment. Our modelling experience

suggests that a refined rate equation of the upstream reaction of endogenous arginine production – exogenous arginine uptake, has to be applied by incorporating the activatory regulation from the enzyme ODC as follows

$$V_{ArgPt} = \frac{V_{max}^{ArgPt}}{\left(1 + \frac{K_{aODC}^{ArgPt}}{[ODC]}\right)^{\gamma_2}} \cdot \frac{\frac{[Arg]_{exg}}{K_m^{ArgPt}}}{1 + \frac{[Arg]_{exg}}{K_m^{ArgPt}}} \tag{7.7}$$

where K_{aODC}^{ArgPt} (μM) represents the activation coefficient of the ODC concentration ($[ODC]$) on the enzyme activities of arginine transporter; and γ_2 stands for the activation strength. The two parameters are assumed to vary between 0 and 10.

We performed another simulation study on the arginine metabolism module, where the rate equations of ODC, arginine uptake and arginase are expressed in Equation (4.2) (in Chapter 4), Equation (7.7) and Equation (7.6), respectively. The simulation results of the intracellular arginine and ornithine are shown in Figure 7.9. Both metabolites can be correctly estimated in terms of both the transient changes and exact values of the intracellular concentrations.

(a) arginine   (b) ornithine
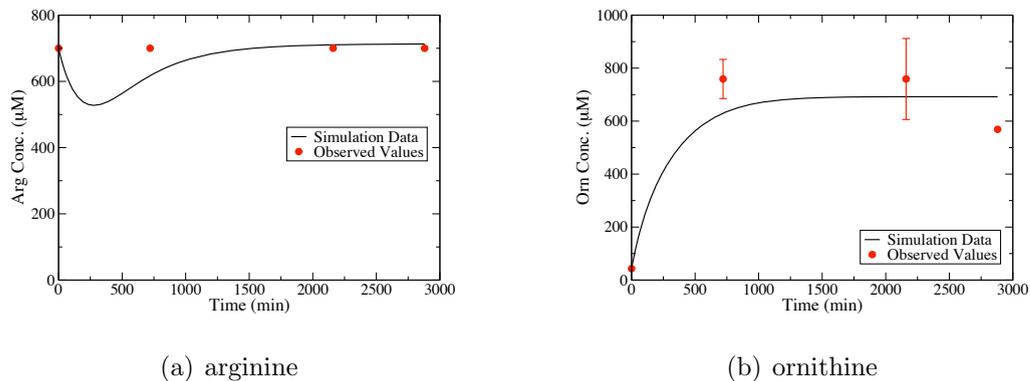
Figure 7.9: Simulation profiles compared to experimental data for the arginine and ornithine dynamics computed with the modified rate of change of the enzyme arginase and arginine transporter. In this case, constant arginine concentrations are assumed as the true experimental data. Error bars are presented where appropriate data was available in the original papers.

Applying the fitted module of arginine metabolism, we determined that the quantitative behaviour of the module is not compatible with both experimental data sets. By *in silico* investigation, we identified a regulatory link between the

arginine uptake, arginase and ODC enzymes as an essential feature of arginine metabolism. Biological experiments are necessary to experimentally verify this prediction in *T. brucei*, however. A recent biological experiment performed by Darlyuk et al. (Darlyuk et al. 2009) observed that in *L. donovani* exogenous arginine uptake increased significantly during cell starvation and was suppressed to avoid intracellular arginine accumulation (which is toxic to the cell growth) when polyamines are abundant or genetically ablated from the parasite, which supports the existence of this link in *T. brucei*.

7.7   Model Reconstruction

Briefly, our proposed approach attempts to identify the ill-defined reaction(s) by reducing the whole system into sub-systems for the examination of structure correctness via global optimization. In *Model Structure Exploration* phase, we discovered a potential regulatory link between arginase, arginine transporter and ODC, *which is postulated to be of biological importance in the regulation of intracellular polyamines contents.*

According to the decompositional rules, there are two situations where the phase of model decomposition terminates and the phase of model reconstruction begins. The first is, when in a certain stage of model decomposition, the module becomes 'elementary' and has deficiencies in the mathematical model structure. 'Elementary' refers to the situation where further decomposition of the module is not possible. The second is when the resulting module can produce a satisfactory match with experimental data for the components of interest.

The former restricts a detailed examination of the model structure correctness to a local area of the module. The phase of *Model Structure Exploration* is activated in this situation for scrutinizing only the reactions included in the resulting module for appropriate kinetic characters. The latter situation implies that the reactions removed from the previous stages of the decomposition procedure may be incorrectly described, and the kinetic mechanisms related should be examined for appropriate variants.

To continue the investigation of model structure deficiency, *Model Reconstruction* has to be performed in order to scrutinize the reactions in the rest of the mathematical model. This suggests a procedure where reactions that comprise the initial model but are excluded from the structurally-correct module are added back to the module following the same rules for deciding the bound-

ary components defined in the proposed *decompositional* approach. Thereby, model reconstruction, which is an iterative procedure, can be understood as the reverse of model decomposition. In this phase of the methodological framework, we intend to explore the ill-defined kinetic characters of the modules developed in each stage of the decomposition procedure via global optimization. The proposed mathematical model structure for arginine metabolism (discussed in Section 7.6.1) is applied throughout the *in silico* investigation.

Certainly, designing sound mechanisms to describe enzyme kinetics depends on how much biological knowledge is available for the reaction(s) in question. When lacking biological evidence, it is an intrinsic impossibility for ambiguously-defined reactions to be thoroughly studied. Therefore, the level to which the process of model decomposition or reconstruction is applied is in most cases determined by the richness of available biological information.

7.7.1   Experimental Results

We perform optimization-based simulation of the modules developed in all three stages of model decomposition. To maintain consistency in the use of experimental data, arginine dynamics assumed by Fairlamb et al. for the bloodstream-form *T. brucei* in response to DFMO inhibition are applied. Accordingly, Equation (4.2) (in Chapter 4), Equation (7.7) and Equation (7.6) proposed in Section 7.6.1 are employed to model the reactions relevant to arginine metabolism. Satisfactory simulation results were obtained for the fitted polyamine biosynthetic module in Figure 7.3(C), hence supporting our mathematical formulation of the reactions in the considered sub-system.

A satisfactory match with the experimental data was also obtained for the module in Figure 7.3(B), where GSH and GspdSH were boundary components. Solving the model of the trypanothione biosynthetic module shown in module in Figure 7.3(A) however resulted in simulation results not consistent to experimental data, particularly for the metabolites glutathione and GspdSH. In addition to the introduction of reactions associated with trypanothione synthesis, the major difference between the modules lies in the inclusion of the glutathione biosynthetic sub-pathway, where, for the former, glutathione is solved over time simultaneously with other variable components.

In the following section, we focus on evaluating the correctness of the mathematical equations that are currently applied to describe the kinetics of the

bi-functional enzyme trypanothione synthetase, which catalyzes the reactions associated with trypanothione synthesis (defined in Equations (4.27) to (4.30) in Chapter 4). Optimization-based simulation is performed on the entire model, where glutathione is defined as a known input, approximated with the experimentally measured dynamics.

Investigation on the kinetic character of trypanothione synthetase and amidase with DFMO-induced experimental data

Our literature research reveals two different GSH dynamics under the DFMO treatment. Specifically, GSH concentrations were observed to increase by almost 80% (Fairlamb et al. 1987) or reduce by 60% (Xiao et al. 2009) of the controlled level. The dynamics of other metabolites including spermidine, GspdSH and trypanothione did not differ significantly between the two experimental settings. The simulation results for putrescine, spermidine, GspdSH and trypanothione compared to the observed values from Fairlamb et al. are shown in Figure 7.10, where the glutathione dynamics are approximated using a 3rd-order polynomial function (fitted to the observations by Fairlamb et al.) and a first-order exponential function (fitted to the observations by Xiao et al.).

Figure 7.10 indicates that trajectories of the fitted model with different input functions for glutathione can closely reproduce the experimental data in a self-consistent manner. Model predictions for putrescine dynamics (Figure 7.10(a)) with two different inputs are almost identical in terms of both the trend and the estimated values of the concentration changes. Model predictions for the dynamics of spermidine (Figure 7.10(b)), GspdSH (Figure 7.10(c)) and trypanothione (Figure 7.10(d)) share certain similarities in the transient changes in concentration, even though they differ in exact values with respect to experimental data. Interestingly, concentration levels of spermidine and trypanothione are raised (with both inputs) by about 12% and 40%, respectively, of the initial values within 6 hours of drug addition, followed by a gradual decrease later on.

One explanation for our *in silico* observations that spermidine and trypanothione concentrations increase sharply is that *T. brucei* may attempt to restore proliferation in the early stage of trypanosomal infection via defense mechanisms, for example, the well-known antioxidant enzyme defense system based on trypanothione (Schirmer et al. 1987). Recently, the compensatory mechanism of arginase up-regulation by activated macrophages has been observed in *Leishmaina* (Kropf et al. 2005) and *T. cruzi* (Peluffo et al. 2004) to counteract parasite

(a) putrescine

(b) spermidine

(c) GspdSH

(d) trypanothione
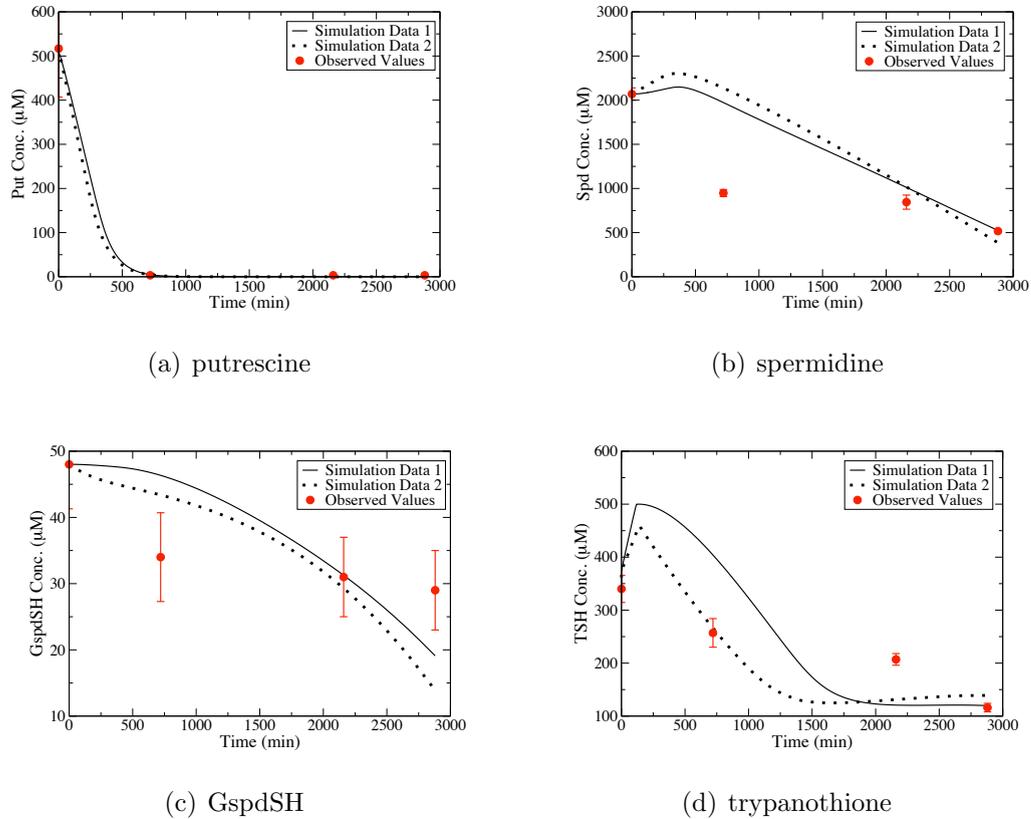
Figure 7.10: Simulation profiles compared to observed values (Fairlamb et al.) for the key metabolites when glutathione dynamics is approximated using different experimental data reported by Fairlamb et al. (continuous line) and Xiao et al. (dotted line). Error bars are presented where appropriate data was available in the original papers.

infection; however this mechanism has not been verified to exist in *T. brucei*. Further work is required in order to gain new insights on defense mechanisms in trypanosomes, and consequently to unravel the underlying causes.

A mathematical model is valid only if it can reproduce all existing data consistently, given that the data is relatively accurate and the sets are compatible with each other. To illustrate this principle, we continue the evaluation of the mathematical equations with another set of experimental data that is suitable for this investigation, namely the gene-knockdown profile of spermidine synthase measured by Xiao et al. (Appendix A.2). Since the dynamic behaviour of ornithine was not reported, intracellular putrescine combined with dAdoMet and glutathione are taken as the model inputs. All three inputs are approximated using 3rd-order polynomials.

In the RNAi knockdown experiment, spermidine synthase mRNA and protein were reduced up to 90% of the original value by day 2 after the RNAi induction. No correlated changes in the expression levels of other polyamine biosynthetic enzymes, trypanothione synthetase or trypanothione reductase were observed in the *in vivo* experiment. In accordance with biological information, the inhibition of enzyme SpdS is assumed to follow a time-dependent exponential decay that replaces the parameter V_{max}^{SpdS} in Equation 4.16, expressed as

$$V_{maxSS}^{SpdS} \cdot e^{-\lambda \cdot t} \tag{7.8}$$

where V_{maxSS}^{SpdS} stands for the maximum velocity of spermidine synthase under normal conditions. Parameter λ is solved by simple curve fitting using the qualitative description of the enzyme dynamics with addition of DFMO and takes a value of 0.0015 in this instance. The time span from 0 to 6 days is covered by the experiment.

The resulting time courses of the module for intracellular spermidine and GspdSH are shown in Figure 7.11. Both metabolites return a satisfactory match with the experimental data in terms of the transient changes in concentration, though it is not perfect with regard to the estimated concentration levels. Model prediction of the trypanothione dynamics reveals rather interesting behaviour, as shown in Figure 7.12. A high peak within the first 2 hours of DFMO treatment is observed and presented in more detail in the enlarged inset to discern the peak clearly.

Overall, in the phase of model reconstruction, the mathematical model structure of the polyamine biosynthetic module was evaluated to be correct in terms of the DFMO inhibition data. The entire model of trypanothione metabolism with glutathione as a known input can reproduce both experimental data sets in a consistent manner. However, it is not possible to assert that the mathematical equations currently in use to model the bi-functional enzyme trypanothione synthetase and the trypanothione redox system are definitely correct; the structure may be disproved later when more experimental observations about the system are available.

Prior to the step of *Model Structure Exploration*, which searches for model variants of the remaining sub-systems, targeted *in vivo* experiments are required to distinguish between the two conflicting observations on glutathione dynamics in response to DFMO inhibition and to explain the abrupt elevation of the

(a) spermidine
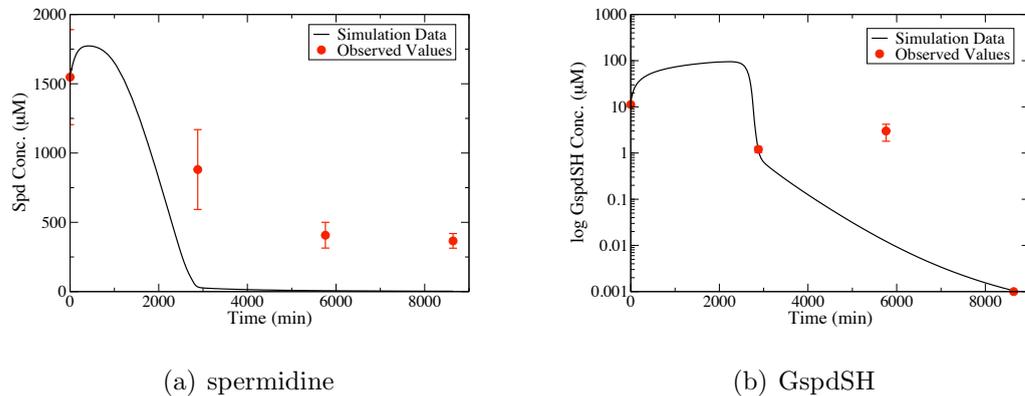
(b) GspdSH

Figure 7.11: Simulation profiles of spermidine and GspdSH compared to gene knockdown data by Xiao et al.. In the simulation plot of GspdSH, the data point measured after four days of RNAi knockdown of spermidine synthase seems to be corrupted with large noise level. Error bars are presented where appropriate data was available in the original papers.

metabolite concentration levels within the first 6 hours of DFMO treatment. Additionally, biological information about other organisms may suggest changes that can be used to further refine the model of *T. brucei*. As stated in Chapter 4 (Section 4.3.3), glutathione was found to enable a 15-fold activation of GspdSH hydrolysis (Fyfe et al. 2008) in *Escherichia coli*. This promotes an investigation on the potential stimulating effect that GspdSH exercises on amidase activity in *T. brucei*, which may give rise to a modification of the mathematical expression of the kinetic character of the enzyme.

The remarkable improvement obtained for the fitted model of trypanothione biosynthesis when glutathione dynamics are given as an input implies that the glutathione biosynthetic sub-pathway may be ill-characterized. This may be ascribed to the kinetic mechanisms of reactions related to glutathione production wrongly formulated or to some elementary reactions vital for the regulation of glutathione metabolism being missing from the current topological structure. We have explained that the kinetic character of the upstream reactions of glutathione production cannot be investigated by our approach, because dynamic responses of the intermediate metabolites of this sub-pathway (cysteine and gGluCys) in response to DFMO inhibition have never been measured *in vivo*, thus the optimization-based decompositional approach cannot be applied.

A recent hypothesis that the γ-glutamyl transpeptidase cycle is present in *T. brucei* (Mike Barrett, personal communication, March 2009) provides support
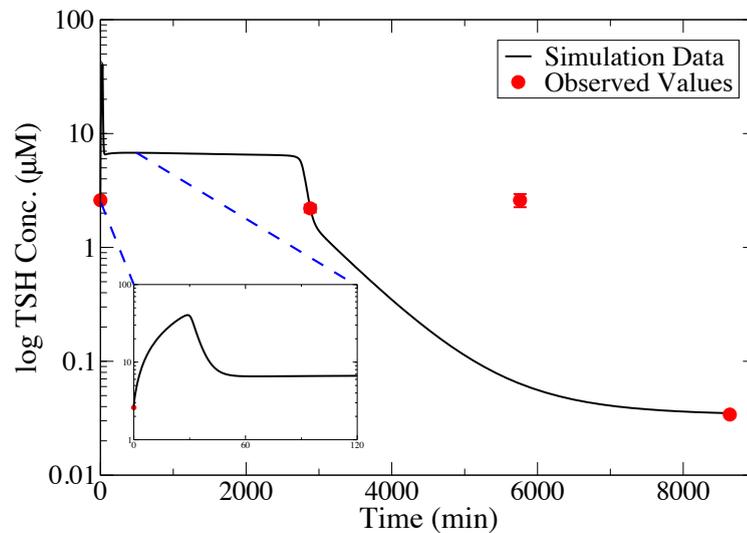
Figure 7.12: Simulation profiles of trypanothione compared to gene knockdown data from Xiao et al.. Inset in the figure refers to the enlarge version of the indicated dynamics of trypanothione over the first two hours of DFMO addition. Error bars are presented where appropriate data was available in the original papers.

for our postulation of the incompleteness of the network topology concerning glutathione biosynthesis. The γ-glutamyl transpeptidase cycle may have an important role in regulating glutathione dynamics, which accounts degradation of glutathione into cysteine, glycine and glutamate via intermediate compounds cysteinylglycine and (5-L-glutamyl)-L-amino acid, and regeneration of glutathione from the products. The γ-glutamyl transpeptidase cycle is supplementary to research in the area of glutathione metabolism and function (Vina et al. 1989). This concept is worth consideration for improved comprehension of glutathione metabolism in *T. brucei* when relevant biological evidence is substantial enough for the investigation. Model structure correctness of the glutathione-centered reactions can only be assessed when the glutathione dynamics are modelled. This involves incorporating the glutathione biosynthetic sub-pathway, the γ-glutamyl transpeptidase cycle and the GspdSH activatory function into a unified environment.

7.8 Remarks on Modelling Framework

In summary, we have demonstrated that the proposed methodological framework can be applied to systematically guide and refine the model construction procedure of biological systems when prior knowledge about the system is incomplete and the available experimental data is sparse. We illustrate that by means of optimization-based simulation, biological hypotheses describing different underlying processes can be formulated mathematically and evaluated by comparison to experimental data.

Defining a set of candidate models for complex biological systems is an important part of model development, but often underemphasized due to the amount of work required and the lack of established methods. Only a few studies have examined the impact of model structure on interpreting biological processes. The discrepancy between model predictions and experimental data emphasizes the necessity of investigating various structures for the mathematical models.

Swameye et al. (Swameye et al. 2003) compared models of the JAK-STAT signalling pathway with the assumption of a feed-forward cascade versus a model capturing the cycling capacity of STAT5 and identified that the cycling path is an essential feature of the JAK-STAT core module. The authors underlined the essentiality of detailed knowledge of biological pathways for establishing mechanistic models. Takors et al. (Takors et al. 1997) performed a simulation-based study to discriminate between 10 competing macrokinetic models, where the best suitable model was selected according to the discrimination criteria based on probability theory rather than the traditional sum of squares. The authors also highlighted the importance of the identification and discrimination of macrokinetic models in bioprocess development.

The works by the authors above have two features in common. First, model discrimination is largely reliant on experimental data for comparison, and second, the assumptions about possible system structures for constructing competing models were taken directly from biological observations measured under different conditions. By contrast, in our study, no biological information about the trypanothione metabolic system is available to aid the definition of candidate models. It is not surprising that for such a complex and poorly understood system, relying on repetitive *in vivo* experiments to discover unknown regulatory mechanisms or to clarify ambiguous concepts about the system would be impractical.

We made great efforts by means of optimization-based simulation to investigate physical grounds of the underlying biological processes. We proposed two kinetic mechanisms for arginine metabolism from the two contradictory sets of experimental data. Both proposed mechanisms imply a potential regulatory link between the enzymes of polyamine biosynthetic sub-pathway. We also identified the parts of the model for which structure exploration via computational simulation is not allowed due to the absence of sufficient biological observations. Our *in silico* investigations provide advice for biologists on design of *in vivo* experiments and analysis of the experimental results in order to enable the exploration of model structure, to discriminate between rival hypotheses and to gain new insights about the system.

Recently, a Bayesian approach has been applied to model selection of biological networks (Vyshemirsky 2007), which uses the rules of probability theory to select among different models. The probability-based approach for model discrimination was first proposed by Box and Hill (Box and Hill 1967) based on the concept of entropy. The mathematical method developed by Box and Hill can also be applied to guide the design of 'new' experiments so as to attain maximum expected discrimination among rival models that would fit the data equally well. The authors used the method to identify the operating point (e.g. time, temperature, etc.), at which rival models can be discriminated.

As stated by Box and Hill (Box and Hill 1967), experiments in any given situation can only be conducted within the physical constraints of the experimental setup, e.g. the time span or operating temperature. This is the so-called operability region. In the context of biological experiments, the operability region frequently corresponds to the time span, which can be very extensive, and it may be impossible to cover the full range. In terms of an experimental design for model discrimination, our *in silico* investigations can help improve the efficiency of 'new' biological experiments by advising experimentalists to focus on the operability region where the discriminatory power of the experiments can be increased. Time-series concentration profiles estimated via computational simulation, for instance the results in Figure 7.10 and Figure 7.12, indicate that an enhanced discriminatory capability can be obtained by measuring the system behaviour over the first 6 hours after DFMO addition.

It is worth emphasizing that a mathematical model is only an approximate representation of the phenomena being studied; it is useful for achieving a system-level understanding with the assistance of computational simulation.

However, in reality, no biological systems will follow a mathematical model exactly. When the number or the precision of observed data points increases, it is likely that revision of the model will be necessary.

During the course of system identification, it is always important to be reminded of the saying from Box (Box 1979) that "all models are wrong, only some are useful". Models should be designed and evaluated with respect to their intended use. Asking whether a model is correct or wrong is not appropriate. Rather, one should ask if the model is valid in representing the essential features of the system and if uncertainty in the model will result in noticeable effects on model inferences, and consequently, the confidence in model applicability.

Our study is intended to, given certain experimental data, seek for kinetic mechanisms of the reactions that can produce a reasonably good description of the underlying biological properties. The adequacy of model structures in predicting the system dynamics in different experimental conditions can only be determined when relevant biological evidence is available for the system. We argue that model structure is correct if it can reproduce the existing data consistently within the limit of the modelled biology, whereby models can be assumed as a good representation of reality.

Remarks on Model Decomposition

We noticed that our proposal on model decomposition and the approach proposed by Koh et al. (Koh et al. 2006) appears similar to a certain degree, for instance, modules are defined as closed systems. However, fundamentally, they are distinct approaches as summarized below:

1. In Koh et al.'s approach, model decomposition intends to solve the problem of parameter estimation, assuming the structure as correct. In contrast, in our approach, model decomposition intends to solve the problem of structure identification, where ill-defined reactions can be identified via a qualitative comparison between model simulation results and experimental data.

2. In Koh et al.'s approach, a typical evolutionary procedure is applied to the parameter estimation procedure, which is inactive until all the model components (so-called sub-systems in our approach) are derived. Their approach chooses a particular component for parameter estimation that contains the 'richest' information on kinetic parameters and experimental

data, which enables the remaining components to be solved in sequence. In our approach, an optimization-based simulation is performed once a sub-system is derived and the quality of fit and the availability of experimental data determines if further levels of model decomposition are required.

3. In Koh et al.'s approach, model decomposition begins with a single state variable and components are derived by adding reactions and metabolites associated with the state variable according to predefined criteria. In our approach, model decomposition begins with an entire model and at each stage, 'unmeasured metabolites'[1] and associated reactions are removed in a recursive manner until 'known metabolites' are obtained which can be used as module boundaries. It is not clear how Koh et al. initiated the decompositional procedure, particularly, the selection of the first state variable. Our approach takes advantage of the relationship between structural topology and functional modules discovered via structural modelling to initialize the decompositional procedure.

4. In Koh et al.'s approach, model components share inputs or outputs, however in our approach sub-systems may be subsets of other sub-systems.

The modularity of the trypanothione metabolic pathway (shown in Figure 4.1) was also explored using a topology-based decomposition strategy. In order to detect sub-networks, MatlabBGL package[2] was used to determine a betweenness measure based on the shortest path (see Section 2.5 in Chapter 2). An adjacency matrix was formulated as an input to the algorithm, with each path (reaction) given equal weight for the pathway vertices (metabolites). An adjacency matrix (A) of a directed graph (such as the trypanothione pathway) represents which vertices are adjacent to which other vertices; the entry $A(i,j)$ is equal to 1 if there is an edge from vertex i to vertex j, otherwise it is 0. The highest edge betweenness values were obtained for the paths between the following pairs of metabolites: spermidine, glutathione and trypanothione, and trypanothione and TS2. These two paths divide the pathway into two topologically isolated sub-networks, which corresponds to the functionally isolated sub-networks identified in Chapter 3. This observation supports our decomposition approach, where trypanothoine, which is the joint between the two

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[1]in our context, unknown metabolites refer to metabolites for which no experimental data is available.

[2]http://www.stanford.edu/~dgleich/programs/matlab_bgl/

sub-networks, is selected as the initial point. In this way, each sub-network can be explored independently, which is complemented by an investigation of interactions among the modules.

Remarks on Model Structure Exploration

The phase of *Model Structure Exploration* in our proposed framework shares some similarities with the concept of *Model Selection.* Model Selection refers to the process of selecting the 'best' model from a given set of potential models (Anderson et al. 2000). A typical selection strategy is, starting from the smallest model or from the full model, to add or delete one parameter until a predefined critical significance is reached. The present study of structure exploration differs from the framework of model selection in several aspects, including

- First, how a set of candidate models was defined. In the current study, candidate models were designed by revising the kinetic characters of reactions of the intact model, which is guided by available information from the literature. When a candidate model is proposed, a global optimization task is applied to examine the model correctness in terms of whether the simulation results can reproduce the observed experimental data. The number of optimization tasks to be performed is equal to the number of candidate models proposed, the network topology of which remains fixed for individual tasks. This is in contrast to the (automatic) procedure of model selection, which is often realized via a single optimization task that consists of a number of runs. At each run, the network topology examined is distinct in terms of which parameters are contained.

- Second, how the 'best' candidate is selected. In our study of structure exploration, a qualitative comparison method is applied, which indicates that the model with simulation curves most similar to the experimental data is retained as a good candidate. On the contrary, a pre-specified criteria is often set in the model selection procedure for a quantitative comparison of model variants, and the one with the smallest fitness value is chosen as the 'best' model.

Both *Model Selection* and *Structure Exploration* procedures are computationally expensive, particularly for applications when, for Model Selection, no prior knowledge is given, regarding the sequence or combinatory form in which

the parameters should be considered and, for Structure Exploration, regarding the types of kinetic characters to be introduced and on which enzymatic reactions they should be investigated. The automatic procedure of model selection is more likely to lead to models without biological interpretation, thus models without biological support should be excluded from the set of candidate models.

In summary, through the processes of model decomposition, model structure exploration and model reconstruction, a sub-system of the initial mathematical model (polyamine metabolism) was evaluated and refined to a structurally correct variant. The polyamine biosynthetic sub-pathway will now be considered in the phase of model validation.

7.9   Model Validation of Polyamine Metabolism

Model validation is concerned with proving that the model is an adequate representation of reality (Neelamkavil 1987). The substantial value of mathematical models lies in their power to predict system behaviour characterized by new and fresh data. As previously stated, there is no ultimate proof of correctness of a mathematical mechanistic model; knowledge about physical insights of the underlying processes will always increase as more biological evidence becomes available. In this section, we aim at investigating the general applicability of the model of the polyamine biosynthetic sub-pathway. Model validation may challenge the model credibility and encourage further model refinement.

Comparisons of *in silico* predictions with experimental data enable the evaluation of model credibility in interpretation and prediction of biological phenomena. Discrepancy in the comparisons can be used to systematically identify potential improvements to the model and suggest specific experiments to verify hypothesized model modifications.

For example, Garfinkel (Garfinkel 1971) suggested computer simulation of liver gluconeogenesis as a resolution to elucidate the contradictory results (lower malate concentration vs. higher malate concentration) observed by two groups of investigators. A critical computer experiment performed in the work is the simulation of the second data set using the model developed for the first data set, which revealed the parameters that caused the apparent discrepancy between the findings. This *in silico* experiment shows that the same model with different parameter values can produce behaviour qualitatively consistent with individual data sets.

Model applicability of the polyamine biosynthetic sub-pathway is assessed by comparison between the model predictions and an independent data set obtained in a distinct dynamic state of the system. The experimental data from DFMO induction and gene knockdown were collected where the system was initiated with different conditions[3]. We use physiological steady-state concentrations and DFMO-induced data as inputs to the model validation procedure, tackled by the multi-objective optimization algorithm, MoPSwarm, proposed in Chapter 6. Gene-perturbed data (so-called *validation data*) is used to assess the model applicability with the parameter estimates in response to different chemical interventions.

7.9.1 Objective Function

Parameter values are refined by comparing simulation results with experimental measurements. A modified *G-test* was used as the objective function to measure the goodness of fit of the model defined in both conditions – polyamine steady-state model and polyamine perturbed model. The general formula for the test statistic G is

$$G = 2 \cdot \sum_i O_i \cdot \ln(\frac{O_i}{E_i})$$

In our study, the term O_i multiplying the natural logarithm in the formula was removed in order to prevent metabolites with large concentrations dominating the objective function value. This leads to the following expression

$$G(\mathbf{p}, t) = \sum_t \ln(\frac{O(t)}{E(\mathbf{p}, t)}) \tag{7.9}$$

where **ln** denotes the natural logarithm and the sum is taken over all sampling points. In the context of optimization of biological pathways, there are discrete time points. Here, $\mathbf{p}$ is the decision vector to be tuned. $O(t)$ and $E(\mathbf{p}, t)$ are the observed data and model simulation, respectively, given at time point t.

As illustrated in Figure 5.3 in Chapter 5, objective functions based on residual errors are insufficient for parameter estimation problems when the optimization performance is measured by more than one criteria. This is the case for

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[3]Large differences in initial conditions of model simulation and experimental settings cause difficulty in presenting data in the same figure without losing the dynamic trends. A logarithmic axis is used for some metabolites whose initial conditions differ considerably in the simulations and experimental observations.

polyamine steady-state model, where the concentrations of model variables are constrained by two criteria, namely (1) the residual of observed data and simulation results and (2) the trend of change of concentrations. A feasible set of solutions should be tuned for the acquisition of both criteria. We propose the use of a dynamic penalty function to tackle the second criterion – the trend of change of concentrations.

A general form of distance based penalty method incorporating a dynamic aspect based on length of search, l, is as follows, according to the description given by Smith and Coit (Smith and Coit 1997)

$$f_p(\mathbf{x}, l) = f(\mathbf{x}) + \sum_{i=1} S_i(l) d_i^k$$

where $S_i(l)$ is a function monotonically non-decreasing in value with l. d_i is the distance matric of constraint i (e.g. number of generations or the number of solutions searched) applied to solution $\mathbf{x}$ and k is a user-defined exponent, with values of k of 1 or 2 often used.

The penalty function proposed in this study is defined as follow:

$$P(\mathbf{p}) = \sum_{n=1}^{m} n \cdot \ln \left(1 + \left| \frac{E(\mathbf{p}, n) - E(\mathbf{p}, n + 1)}{E(\mathbf{p}, n)} \right| \right) \tag{7.10}$$

where $\ln(1 + |\cdot|)$ is designed to accurately compute (small) absolute values in $|\cdot|$ that quantify the change in concentration of simulation results calculated at the boundaries of the time interval. n is the index of the time interval for $n = 1$ to m intervals in a time span divided into discrete time points. n is monotonically non-decreasing, acting as $S_i(l)$ in the general expression.

The idea of introducing the penalty function is to incorporate a dynamic aspect, which increases the severity of the penalty as the integration or simulation progresses. The objective function for the polyamine steady-state model with both criteria is then formulated as below (for a minimization problem):

$$G_p(\mathbf{p}, t) = G(\mathbf{p}, t) + P(\mathbf{p}) \tag{7.11}$$

where $G(\mathbf{p}, t)$ is the unpenalized objective function and $P(\mathbf{p})$ is the penalty function.

In practice, penalty functions typically require problem specific tuning to perform well. A major difficulty of combining all objectives into a single objective

lies in determining a multi-criteria fitness value to summarize the degree of satisfaction of all the individual objective. We investigated the appropriateness of several definitions of the penalty function $P(\mathbf{p})$ differing in the explicit form of $S_i(l)$. The penalty function defined in Equation (7.10) was found to be the most suitable expression for the problem of interest.

7.9.2 Hill Equation Approximation to Spermidine

In order to evaluate the model predicability, and thus assess its applicability, interaction of the polyamine biosynthetic module with the rest of the trypanothione metabolic system has to be modelled explicitly (polyamine biosynthetic module is represented as a closed system in the previous phases), which includes spermidine consumption in GspdSH production and its regeneration from GspdSH hydrolysis. However, as indicated in the study carried out in the phase of model reconstruction, the correctness of the current mathematical equations used to model the bi-functional enzyme trypanothione synthetase is uncertain. To render predictive power to this module, a black-box structure has to be applied to the module output – spermidine, to approximate its participation in other biochemical reactions.

Selecting an appropriate black-box structure to describe non-linear dynamics is a difficult problem. As introduced in Chapter 2, model structures can take the form of power series polynomial, fuzzy logic or neural network. We employ the *Hill equation* in the form of a regressive function to approximate spermidine participation in other metabolic functions of the system, as shown below

$$V_{spdout} = V_{max}^{spdout} \cdot \frac{\left(\frac{[Spd]}{K_m^{spdout}}\right)^n}{1 + \left(\frac{[Spd]}{K_m^{spdout}}\right)^n} \tag{7.12}$$

Thereby, spermidine dynamics is modelled by the following differential equation

$$\frac{d[Spd]}{dt} = V_{SpdS} - V_{spdout}$$

where the rate equation of enzyme V_{SpdS} responsible for spermidine production is given in Equation (4.16) in Chapter 4.

An advantage of using the Hill equation (Equation (7.12)) to approximate the non-linear dynamics of spermidine participation is that the parameters involved

Time-dependent Variables	Differential Equations
$[Met]$	$\frac{d[Met]}{dt} = V_{MetPt} - V_{MAT} + V_{MetRcy}$
$[AdoMet]$	$\frac{d[AdoMet]}{dt} = V_{MAT} - V_{AdoMetDC^T} - V_{AdoHcy}$
$[dAdoMet]$	$\frac{d[dAdoMet]}{dt} = V_{AdoMetDC^T} - V_{SpdS}$
$[Arg]$	$\frac{d[Arg]}{dt} = V_{ArgPt} - V_{ARG}$
$[Orn]$	$\frac{d[Orn]}{dt} = V_{ARG} - V_{ODC}$
$[Put]$	$\frac{d[Put]}{dt} = V_{ODC} - V_{SpdS}$
$[MTA]$	$\frac{d[MTA]}{dt} = V_{SpdS} - V_{MetRcy}$
$[Spd]$	$\frac{d[Spd]}{dt} = V_{SpdS} - V_{spdout}$

Table 7.1: Differential equations for the different metabolites included in the polyamine biosynthetic sub-pathway.

in the structure have direct biological meaning: V_{max}^{spdout} – maximum velocity of spermidine participation, K_m^{spdout} – half-saturation constant, and n – hill coefficient. This avoids the problem of a lack of physical insight in most cases when black-box structures are used.

The structure of the reaction diagram of polyamine metabolism in *T. brucei* is shown in Figure 7.13. The difference between this diagram and Figure 7.3(C) lies in the aspect that intracellular spermidine is modelled as a time-dependent variable rather than a given output approximated with measured dynamics.

Table 7.1 gives the differential equations for the different metabolites in the polyamine biosynthetic sub-pathway.

We first carry out an optimization-based simulation of the polyamine biosynthetic sub-pathway to examine if the black-box structure in the form of a Hill equation is sufficient to reproduce observed behaviour of intracellular spermidine. The simulation results of spermidine dynamics (see Figure 7.14) show a good match with the experimental data, supporting the proposed model structure.
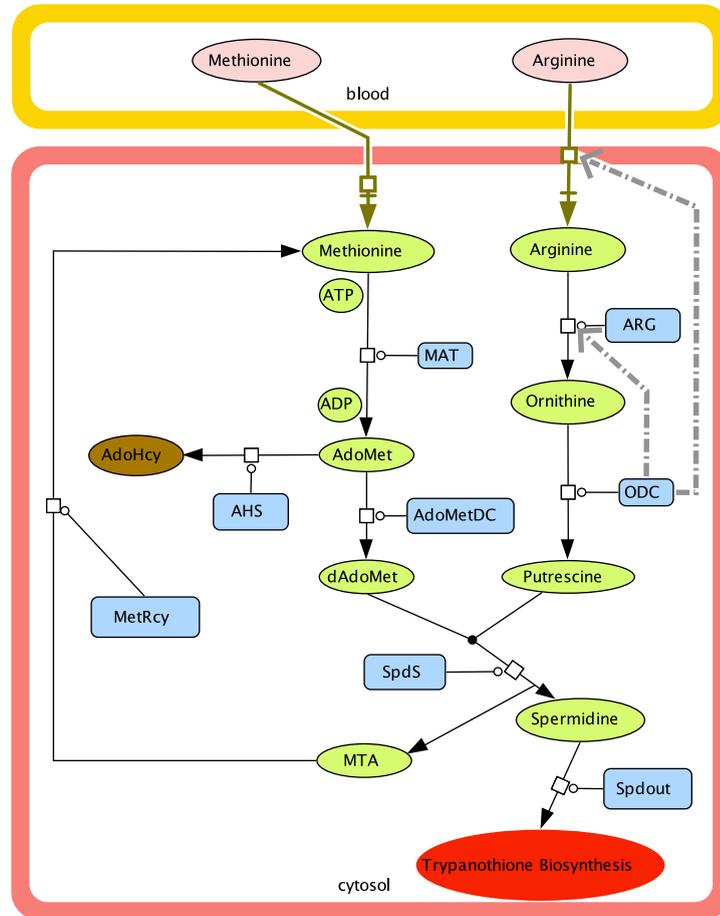
Figure 7.13: The schematic representation of polyamine biosynthetic sub-pathway. Metabolites in pink, brown and green ovals represent model components that are exogenous, constant and variable, respectively. Edges represent chemical conversions between model components with arrows indicating reaction directionality. Enzymes included in this sub-pathway are denoted with blue boxes. Intracellular spermidine is considered as a time-dependent variable. The red oval – trypanothione biosynthesis stands for the rest of the trypanothione pathway. The dot-dashed edges represent the regulatory links predicted between enzymes of ODC, ARG and the arginine transport enzyme in Section 7.6.

7.9.3 Experimental Results

As introduced in Chapter 6, a common approach to model validation involves a two-step procedure to determine the vector of estimated parameters that can correctly describe a system's response to perturbations. This involves fitting model parameters to experimental data generated by a reference cell type (wild type) and then testing this model on data generated by a variation (mutant).

The polyamine steady-state model contains, in total, 28 unknown parameters
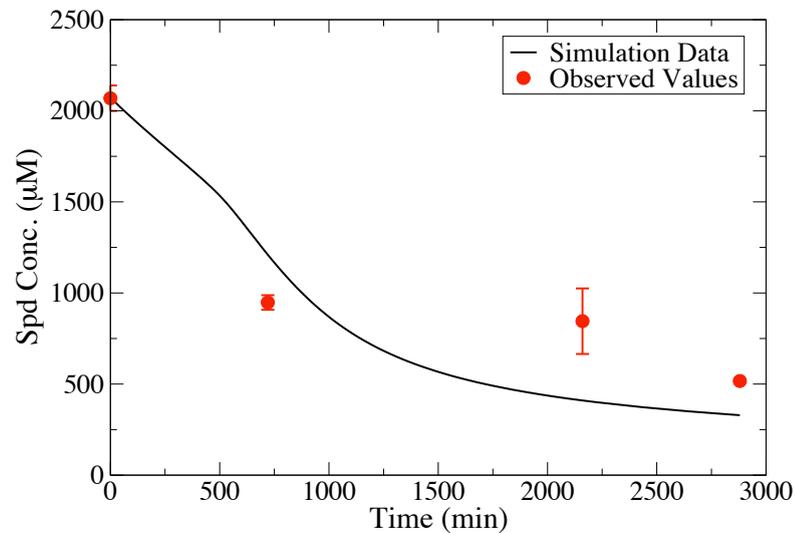
Figure 7.14: Simulation profiles for spermidine dynamics of polyamine biosynthetic sub-pathway compared to experimental data. Error bars are presented where appropriate data was available in the original papers.

including 20 kinetic parameters and a set of 8 parameters for initial concentrations of the model components. Our initial choices of the protein concentrations for model simulation vary within $\pm 20\%$ of the physiological levels of polyamines reported by Fairlamb et al. (Fairlamb et al. 1987) and experimentally measured by the group of Mike Barrett (University of Glasgow). Following the conventional approach, a set of parameters that can satisfy the steady-state constraint of the sub-pathway was solved using the global optimizer PSwarm for the simulation study.

Two hundred runs of the steady-state optimization were executed in Matlab. Each run starts with a different initial population of particles and is computed over a simulated time span of 96 hrs (5760 minutes). The set of parameters that gives the minimum of the objective function, G_p in Equation (7.11), is retained for testing on DFMO inhibition data in the second step. To perform computational simulations of DFMO treatment, the time-independent parameter of ODC concentration in the steady-state model is replaced with the exponential decay function defined in Equation (7.4). Computational simulation of the perturbed model failed to produce results consistent with the true perturbation data, even with the 'best' set of parameters estimated from the steady state.

The failure in model validation of the polyamine sub-pathway demonstrates that the conventional approach is incapable of tackling the problem of interest, which is characterized by complex non-linearity of kinetics and high-dimentional parameter space. The critical challenge of relying on the parameter estimates solved from the steady state, serving as the first step of the conventional model validation procedure, carries with it an important message that fitting a steady state model is a weaker criterion than that of a model of time dependent processes describing the temporal behaviour of the model components.

We chose to apply the proposed approach – MoPSwarm – to model validation of the polyamine biosynthetic sub-pathway. Of 28 unknown parameters, 20 kinetic parameters (k_1 to k_{20}) are common to both steady-state and perturbed model. The set of 8 parameters of initial concentrations (k_{21} to k_{28}) is specific to the steady-state model, assumed to vary within $\pm 20\%$ of the reported physiological levels. In terms of the mathematical representation, ODC concentration is a time-invariant parameter for the steady-state model and expressed as a time-dependent exponential decay for the perturbed model. This aims to mimic the time-dependent behaviour of DFMO transport across the cell membrane and into the cell to react with ODC.

Trade-off solutions between the two objectives, defined by Equation (7.11) for the steady state and Equation (7.9) for the perturbed state, are solved with MoPSwarm and presented in Figure 7.15. The maximum number of iterations was set to 1000 and 20 particles were used in the initial population. The search process of the algorithm terminated at 807 iterations due to there being only a single particle remaining. Ten optimization runs with different initial random populations seeds were performed with MoPSwarm.

The selection of good parameter sets is conducted according to a quantitative comparison method. This type of comparison method is based on a goodness-of-fit criterion, which is generally applied in the optimization problems where the emphasis is on the accuracy of the results. We employed the Root Mean Square (also known as the quadratic mean) to rank the solutions from all ten simulations. This measure is a common method of defining the importance of solutions with respect to satisfying both objectives, and is given as

$$Rms = \sqrt{\left(\frac{\text{Obj}_{ss}^i}{\max(\text{Obj}_{ss}^i)}\right)^2 + \left(\frac{\text{Obj}_{ptb}^i}{\max(\text{Obj}_{ptb}^i)}\right)^2} \qquad (7.13)$$
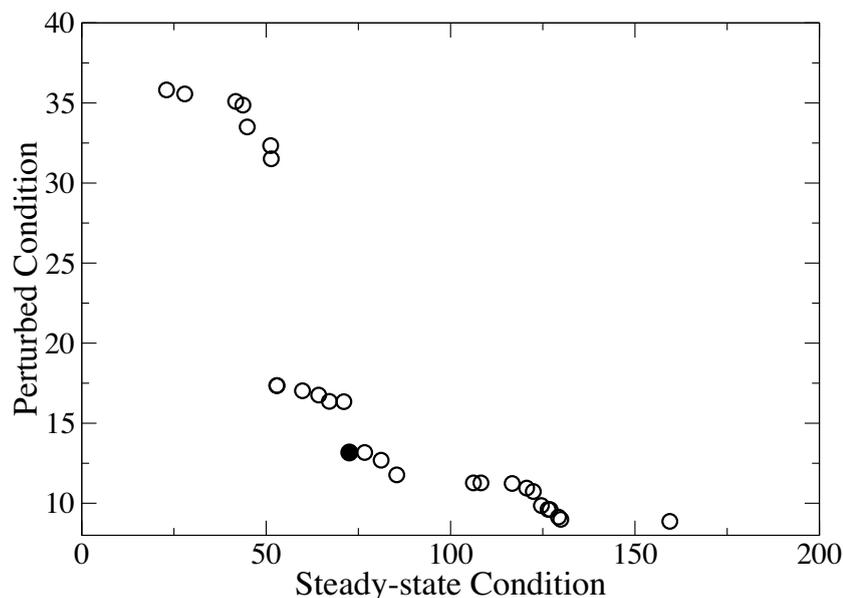
Figure 7.15: Trade-off solutions of the polyamine model solved by MoPSwarm. The solutions show a good diversity covering a wide range of the fitness landscape, and the corresponding Pareto front is discontinuous. The point denoted with a *filled circle* is the 'best' solution of all ten runs as defined by Equation (7.13).

where Obj_{ss} and Obj_{ptb} are the values of objective functions responsible for the steady state and perturbed state given by individual solution i. Note that the objective function values have been normalized so that they fall in the range of 0–1, which prevents large absolute values from skewing the metric and gives equal weighting to both objectives.

The 'best' solution from all ten runs, as defined by the above measure, is selected for investigation and the Pareto front shown in Figure 7.15 corresponds to the simulation that yielded this solution. Table 7.2 provides the predicted values for the unknown kinetic parameters common to both objectives, obtained from this solution (denoted with a *filled circle* in Figure 7.15). Rate equations of the corresponding pathway enzymes are indicated explicitly in the table.

It is noteworthy that our model predications of the unknown parameters are consistent with the qualitative biological information about the system, which was detailed in Chapter 4 (Section 4.3.1). The predicated value of 778 μM for $K_{idAdoMet}^{AdoMetDC^{O}}$ confirms the postulation by Pegg and Jacobs (Pegg and

Jacobs 1983) that *T. brucei* AdoMetDC is insensitive to the reaction product dAdoMet. A value of 2.9 $\mu mol/min/mg$ for V_{max}^{SpdS} indicates that the maximum velocity of *T. brucei* SpdS is comparable to the enzyme activity in mammals as argued by Bitonti et al. (Bitonti et al. 1984) (between 0.43 and 10.95 $\mu mol/min/mg$) and disagrees with the value reported by Taylor et al. (Taylor et al. 2008). Additionally, a large value of 40 $\mu mol/min/mg$ for V_{max}^{MetRcy} reflects the observation that the recycling path from MTA to methionine is an important source of this amino acid.

Predictions on the unknown kinetic parameters		
Enzymes − Rate Equations	**Parameters**	**Estimated Values**
ODC – Equation (7.5)	[ODC]	0.012 μM
AdoMetDC – Equation (7.1) & (7.2)	$[AdoMetDC^L]$ $K_{idAdoMet}^{AdoMetDC^O}$ β	1 μM 778 μM 0.94
MAT – Equation (4.14)	V_{max}^{MAT}	1.46 $\mu mol/min/mg$
SpdS – Equation (4.16)	V_{max}^{SpdS} K_{iMTA}^{SpdS}	2.9 $\mu mol/min/mg$ 10 μM
MetRcy – Equation (4.17)	V_{max}^{MetRcy} K_{mMTA}^{MetRcy}	40 $\mu mol/min/mg$ 959 μM
AHS – Equation (4.18)	V_{max}^{AHS} $K_{mAdoMet}^{AHS}$	38.9 $\mu mol/min/mg$ 1000 μM
ArgPt – Equation (7.7)	V_{max}^{ArgPt} K_{m}^{ArgPt} K_{aODC}^{ArgPt} γ_2	0.75 $\mu mol/min/mg$ 1 μM 1e-4 μM 0.21
ARG – Equation (7.6)	K_{aODC}^{ARG} γ_1	1e-4 μM 9.94
Spdout – Equation (7.12)	V_{max}^{spdout} K_{m}^{spdout} n	3.73 $\mu mol/min/mg$ 1000 μM 3

Table 7.2: Estimated values of unknown parameters of the polyamine biosynthetic sub-pathway.

Computational simulation of the model described in Table 7.1 using the parameter estimates in Table 7.2 thus yielded the steady state shown in Figure 7.16. The model achieved steady state in less than two days and maintained it until day 6 (8760 minutes). The initial condition of the steady-state model predicted
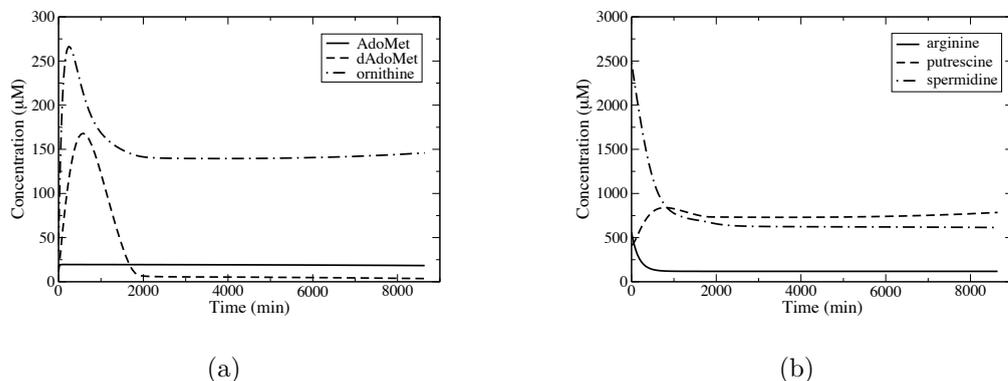
Figure 7.16: Predicted concentration profiles of the steady-state model of the polyamine biosynthetic sub-pathway. All the metabolites reach a steady state within 2 days and maintain this over the next 4 days. Figure 7.16(a): AdoMet (*continuous line*), dAdoMet (*dashed line*) and ornithine (*dashed-dotted line*) and Figure 7.16(b): arginine (*continuous line*), putrescine (*dashed line*) and spermidine (*dashed-dotted line*).

with MoPSwarm is given below:

$$[Met]_{t=0} = 4557.6 \ \mu M, \ [AdoMet]_{t=0} = 16.1 \ \mu M, \ [dAdoMet]_{t=0} = 10.8 \ \mu M,$$

$$[Arg]_{t=0} = 560 \ \mu M, \ [Orn]_{t=0} = 34.4 \ \mu M, \ [Put]_{t=0} = 413.6 \ \mu M,$$

$$[MTA]_{t=0} = 16 \ \mu M, \ [Spd]_{t=0} = 2482.8 \ \mu M.$$

We call the resulting steady state acquired by the model a *basal condition*. Table 7.3 shows that concentrations of polyamine metabolites in this basal condition fit well with actual values observed from experimentally available data. Deviations of the basal condition from the observed values can be ascribed to the fact that polyamines are distributed among free and non-covalently bound polyamine pools *in vivo* (Shim and Fairlamb 1988) and experimentally measured data normally corresponds to total intracellular concentrations. In this study, the model was tuned to respond to free polyamines, and total polyamine concentrations can be derived when the ratio of free polyamine concentration to total polyamine concentration is known.

Computational simulations of the perturbed model shows an agreement with experimental data on ODC suppression, in which case, the basal condition acts as the initial model condition for simulation. The predicted concentration profile of intracellular metabolites of the sub-pathway in response to DFMO treatment

	Polyamine Concentrations (μM)							
	Met	AdoMet	dAdoMet	Arg	Orn	Put	MTA	Spd
from Model	1957	18.3	3.6	118	146	785	17	614
from refs.	3978	19	9	700	43	517	20	2069

Table 7.3: Polyamine concentrations considered as the *basal conditions* of the polyamine model compared with those reported by Fairlamb et al. and experimentally measured by Mike Barrett.

is presented in Figure 7.17. Our simulations using the 'best' solution over an interval of 48 hours predict well the real experimental responses, with a drastic decrease of putrescine that caused a significant increase of dAdoMet level and a decrease of spermidine. As expected, AdoMet is almost unchanged with a slight decrease of the concentration level at the end of the time period. An increase of ornithine and arginine was observed within first 12 hours of DFMO inhibition, followed by realization of a different steady state (the arginine concentration level stabilizes at 825 μM within a simulation interval of 72 hours).

To summarize the results of model validation with MoPSwarm, predictions of the steady-state model and the perturbed model made using the 'best' solution computed from the algorithm describe sufficiently well the physiological steady-state concentrations and DFMO inhibition data. Our *in silico* investigation further supports the biological postulation that dramatic changes in the concentration levels of putrescine and dAdoMet are not accompanied by similar changes in AdoMet within the given time span, and arginine concentration may not be significantly changed under the DFMO treatment. Next, we assess the model applicability of the polyamine biosynthetic sub-pathway by examining responses to independent data sets obtained in distinct dynamic states of the system. To the end, data from available gene perturbation experiments on prozyme, ODC and AdoMetDC are used as *validation data*. *In silico* analysis on some parameters of interest is also presented.

Model predictions on the effects of *in vivo* ODC knockdown

The similar tendencies in polyamine levels induced by DFMO inhibition are also observed by a 90% ODC activity down-regulation. The simulation results, shown in Figure 7.18, accurately capture the transient changes of the metabolite concentrations. In all figures, the experimental data measured by Xiao et al. (Xiao et al. 2009) have been normalized to the initial concentration of the simulation

(a) AdoMet

(b) dAdoMet

(c) arginine

(d) ornithine

(e) putrescine

(f) spermidine
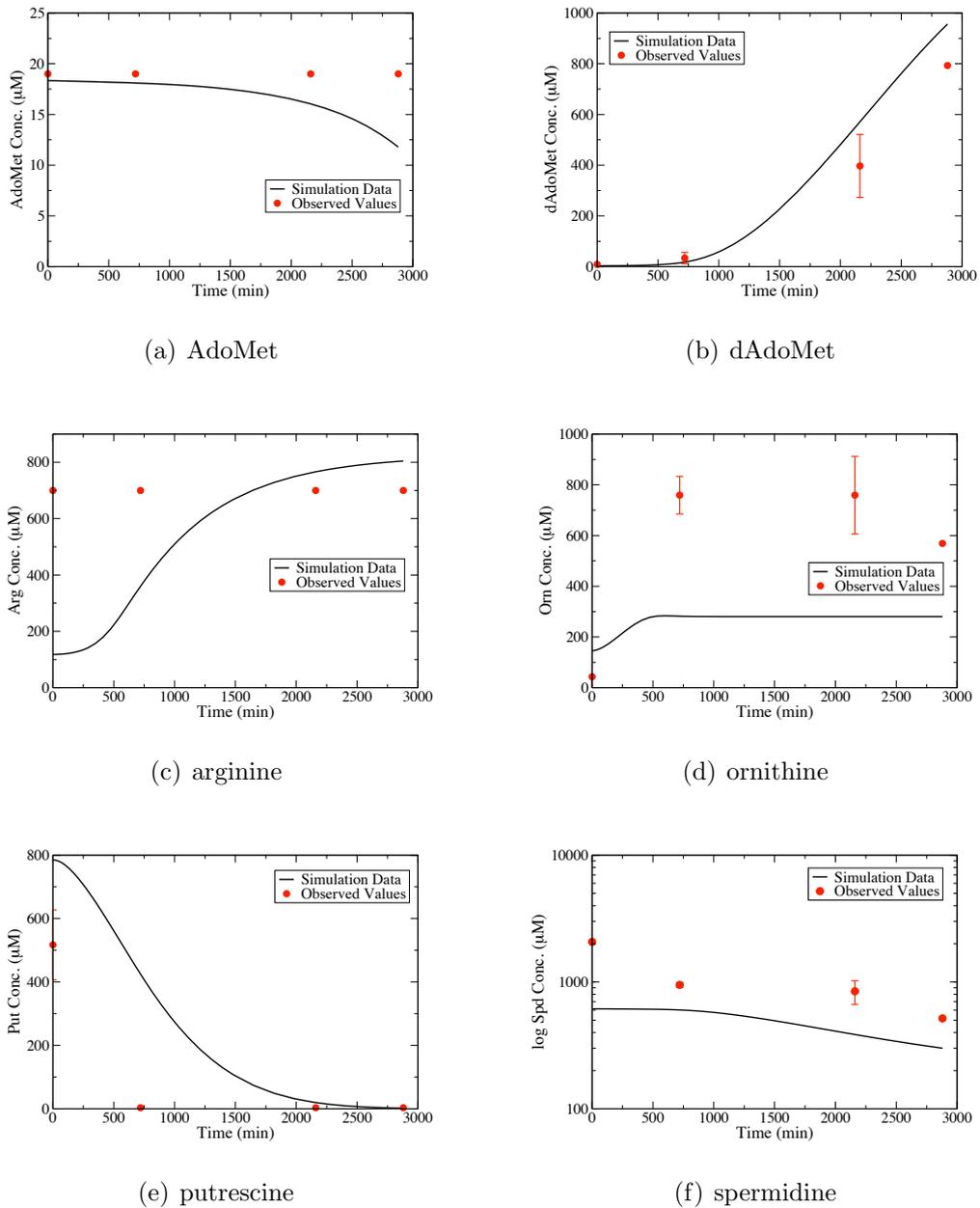
Figure 7.17: Predicted concentration profile of the perturbed model of the polyamine biosynthetic sub-pathway. DFMO effects on polyamine levels in time-response simulations (*continuous lines*) are compared with experimental data (*red dots*). ODC concentration was modelled as a time-dependent variable. In Figure 7.17(a), AdoMet dynamics observed by Xiao et al. were adopted as experimental data. Logarithmic axis is used for spermidine in order to better present the data. Error bars are presented where appropriate data was available in the original papers.

data in order to better show the dynamic trend. Effects on the polyamine levels caused by ODC activity down-regulation are smaller compared to DFMO inhibition. In particular, when simulated with a DFMO treatment versus the ODC activity down-regulation, putrescine is reduced to 0.2% versus 11% of the basal condition, and spermidine is reduced to 49% versus 67% of the basal condition, respectively.

(a) AdoMet

(b) dAdoMet

(c) putrescine

(d) spermidine
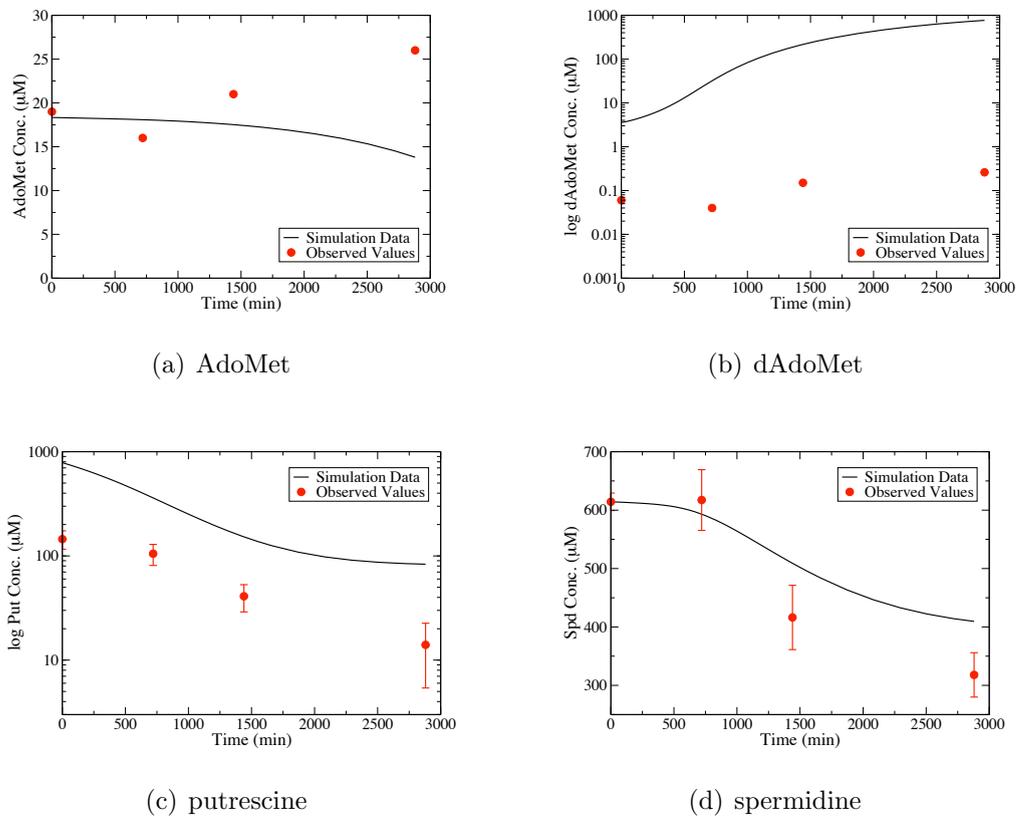
Figure 7.18: Effects of ODC knockdown on polyamine levels in time-dependent model simulations. In this case, ODC activity is considered a time-independent parameter. Logarithmic axis is used for dAdoMet and putrescine in order to better present the data. Error bars are presented where appropriate data was available in the original papers.

Model predictions on the effects of *in vivo* AdoMetDC knockdown and prozyme knockout

Willert and Phillips (Willert and Phillips 2008) studied the potential for prozyme and AdoMetDC to function as regulators in polyamine biosynthesis. The authors observed that loss of AdoMetDC or prozyme leads to decreases in spermidine and

(a) putrescine   (b) spermidine
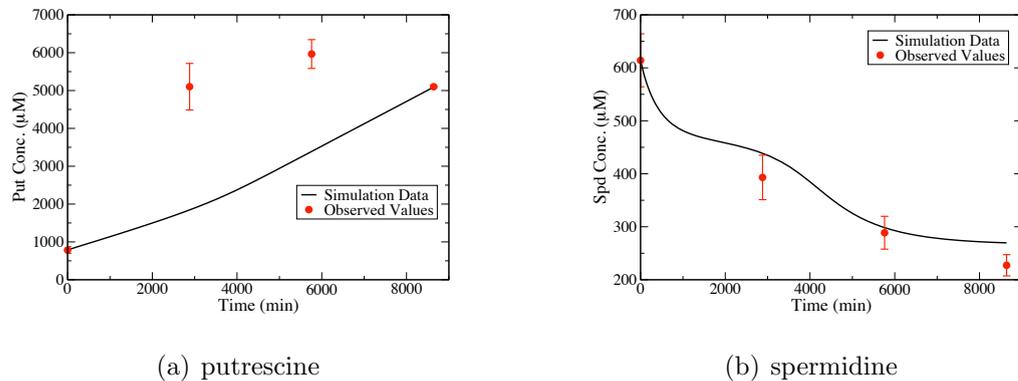
Figure 7.19: Effects on polyamine levels in time-dependent simulations induced by AdoMetDC knockdown. In this case, AdoMetDC activity is considered a time-independent parameter. Error bars are presented where appropriate data was available in the original papers.

trypanothione and to cell death. When a 70% AdoMet activity down-regulation and prozyme knockout were applied, simulations of the polyamine model show a dramatic increase of putrescine and a decrease of spermidine, which is in good agreement with the tendencies described in the real experimental observations reported by Willert and Phillips.

Simulation of the time-dependent effects on polyamine levels, putrescine and spermidine, induced by AdoMetDC knockdown and prozyme knockout are shown in Figure 7.19 and Figure 7.20. In both figures, the experimental data has been normalized to the initial concentration of the simulation data in order to better show the dynamic trend.

In our model, simulations of prozyme knockout and AdoMetDC knockdown both resulted in a large increase in putrescine levels (approximately 7-fold increase versus the 10-fold increase observed *in vivo*), whereas prozyme knockout leads to a more substantial reduction in spermidine than AdoMetDC knockdown – a 75% reduction due to prozyme knockout versus 55% reduction from AdoMetDC knockdown. This matches well with the biological observations by Willert and Phillips regarding the essentiality of prozyme for growth in *T. brucei*. *In silico* simulation also indicates that an increase of putrescine as a consequence of a 70% AdoMetDC knockdown or prozyme knockout may lead to a concomitant decrease of dAdoMet and methionine; however the concentration levels of AdoMet and arginine are largely unchanged. These model predictions can be verified when the relevant experimental data is available.

(a) putrescine

(b) spermidine
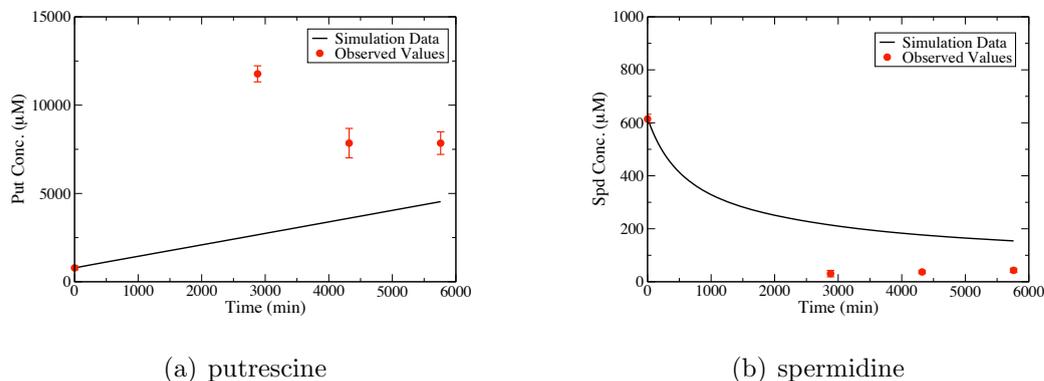
Figure 7.20: Effects on polyamine levels in time-dependent simulations induced by prozyme knockout. In this case, the factor $1 - \beta$ representing the percent of the complex AdoMetDC|prozyme taking up the total enzyme AdoMetDC is given zero. Error bars are presented where appropriate data was available in the original papers.

To further investigate the role that prozyme plays in the regulation of polyamine metabolism, we performed simulations of polyamine levels with variations in the value of β (used in Equation (7.1) and Equation (7.2)). In the model, $1 - \beta$ accounts for the percentage of the complex compound AdoMetDC|prozyme taking up the total enzyme concentration. The value of β was assumed to vary between 0.5 and 1 in order to reflect experimental observation and still allow the compound to change within a physiologically feasible range. When β is close to 1, the AdoMetDC|prozyme compound taking up the total AdoMetDC concentration is minimal.

Figure 7.21 plots the polyamine levels obtained at the end of the simulated time span (2880 minutes) using different values of β taken from the range [0.5, 1]. The results show that there is little effect on the polyamine levels for values of β between 0.5 and 0.9. Significant changes in the polyamine concentrations were observed as β approaches 1, however. The simulations indicate that the compound AdoMetDC|prozyme accounts for a limited quantity of the total enzyme AdoMetDC, as the percentage of the compound taking up AdoMetDC concentration is approximated by $1 - \beta$, where the estimated value of β is 0.94. Prozyme reacting with AdoMetDC is a limiting factor on the AdoMetDC activity and polyamine biosynthesis, since significant changes in polyamine levels were only observed when the value of β falls into a small sub-range.
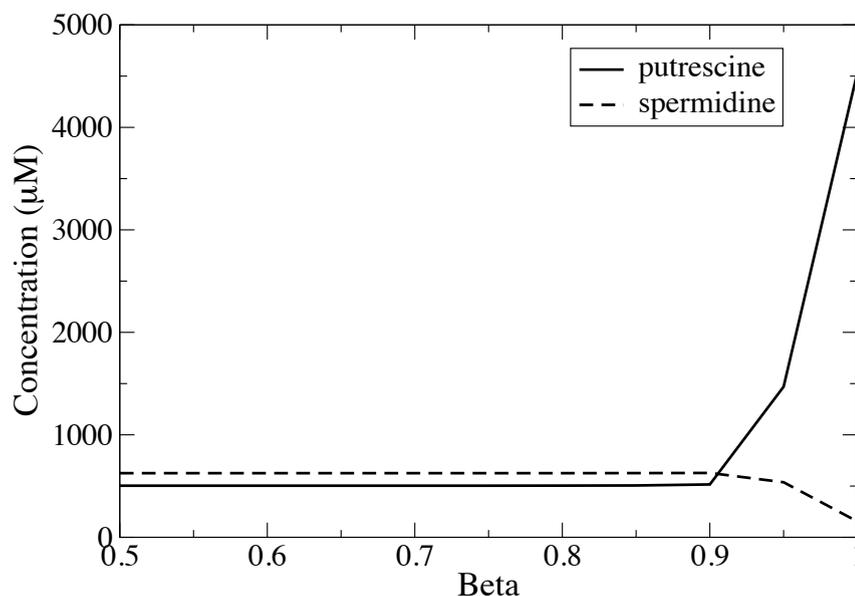
Figure 7.21: Effects of changes in parameter β on polyamine levels computed at the end of simulation (2880 minutes): putrescine (*continuous line*) and spermidine (*dashed line*).

Model predictions on the effects of 'Methylation index'

The ratio of AdoMet to AdoHcy, termed the 'Methylation index' indicates the transmethylation potential of a cell (refer to Section 4.3.1 in Chapter 4). Normal methylation was shown to range from 2:1 to 9:1 (Bacchi et al. 1995). A substantial flow from AdoMet to AdoHcy was observed in our model, with a value of 38.9 $\mu mol/min/mg$ estimated for the maximum velocity of the enzyme AHS, which catalyzes the chemical conversion between the metabolites. Over the simulated 48 hours of DFMO inhibition, AdoMet concentration level does not change significantly, resulting in a constant 'Methylation index' (AdoHcy is a constant model component). Our model predictions differ from the experimental observations given by Yarlett et al. (Yarlett and Bacchi 1988), which showed that the ratio increased from 6.5 to 114 within 24 hours of DFMO treatment, due to a large increase in AdoMet. This disagreement results from the differing observations on the concentration of AdoMet, as observed by Xiao et al. (Xiao et al. 2009).

Under normal conditions, a decline in the Methylation index can induce the

so-called hypermethylation status leading to cell death (Reguera et al. 2007). In our model, the use of AdoMet for AdoHcy production is controlled by the maximum velocity of the enzyme AHS (V_{max}^{AHS}). Simulations of a three-fold AHS activity up-regulation, as shown in Figure 7.22, result in a significant depletion of AdoMet, dAdoMet and spermidine, accompanied by a large increase of putrescine. The model predictions are in a good agreement with biological observations (Reguera et al. 2007) that a decline of the index reduces intracellular spermidine level, thus leading to cell death.

(a) AdoMet

(b) dAdoMet

(c) putrescine

(d) spermidine
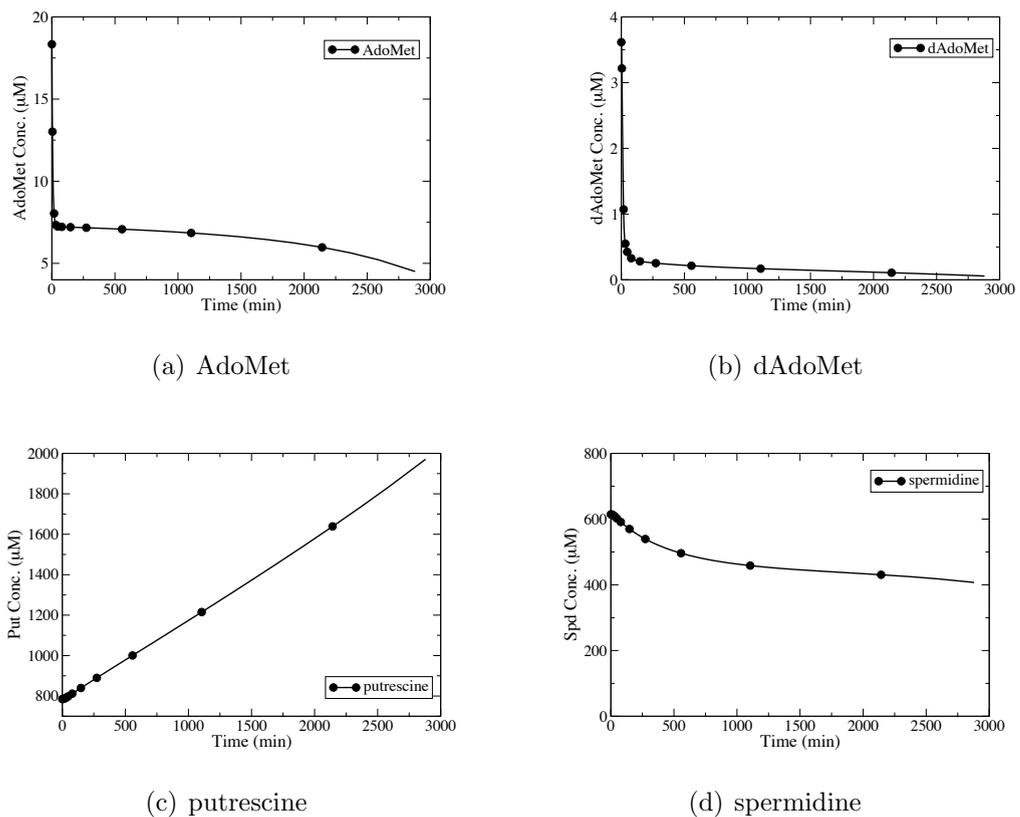
Figure 7.22: Effects of a three-fold AHS activity up-regulation on polyamine levels in time-dependent model simulations.

Yarlett et al. (Yarlett et al. 1991) found that in *T. brucei*, transmethylation reactions were very sensitive to AdoHcy inhibition. Figure 7.23 shows the effect of apparent inhibition value $K_{iAdoHcy}^{AHS}$ (representing the AdoHcy inhibition on the enzyme AHS) on AdoMet and dAdoMet intracellular concentrations. AdoMet concentrations simulated at the final time point of the time span 0 to 48 hours were plotted. These are calculated using values of $K_{iAdoHcy}^{AHS}$ taken from the range 0.01–2 fold the observed value of 12.9 μM. Our model predicts a gradual reduc-

(a) AdoMet (b) dAdoMet
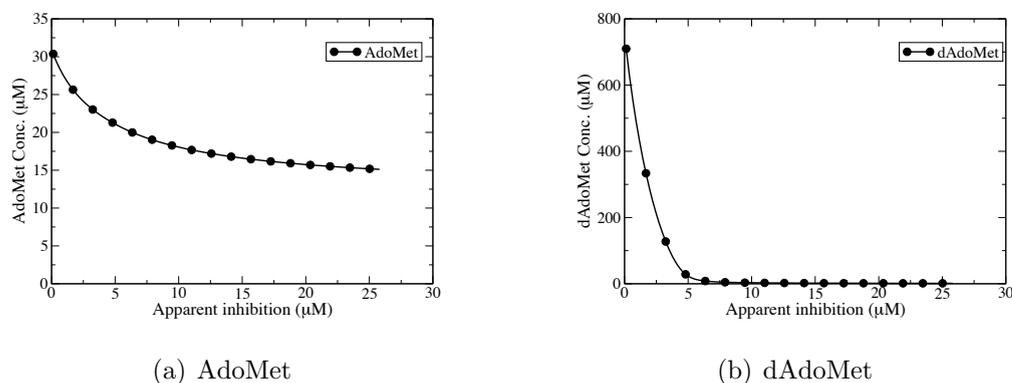
Figure 7.23: Effects of changes in AHS enzyme related apparent coefficient on the concentration levels of AdoMet and dAdoMet computed at the end of simulation (2880 minutes). The coefficient varies in the range of 0.01–2 fold of the observed value.

tion in the final AdoMet level as the apparent inhibition coefficient increases, followed by a rapid depletion of dAdoMet. There is, however, no experimental data available to verify the predictions.

7.9.4 Remarks on the Mathematical Model

We have demonstrated in this section that the proposed mathematical model is an adequate representation of the system of polyamine metabolism. This allows various types of analysis methods to be applied to the model. For example, *Metabolic Control Analysis* (MCA), as defined by Cascante et al. (Cascante et al. 2002), provides a quantitative description of system variables (i.e. fluxes and metabolite concentrations) in response to changes in system parameters. With this method, the relative control exerted by each step (enzyme) in the polyamine pathway on the system variables can be assessed and the enzymatic reactions that have the greatest influence on the regulation of polyamine function and metabolism can be identified. Our mathematical model also offers opportunities to investigate different strategies for targeting this pathway in anti-trypanosomal drug design. For instance, an effective treatment of human African trypanosomiasis could be achieved by enhancing polyamine depletion via combined blockage of polyamine synthesis. With such information, promising targets for anti-protozoal agents can be proposed or less promising possibilities can be eliminated. The details of such analyses are beyond the scope of the thesis.

7.9.5 Structural Modelling vs. Kinetic Modelling

One of the aims of structural modelling is to predict the relative fluxes of all internal reactions in the system via optimization of a specific objective function (see Section 3.5.1 in Chapter 3). The steady-state reaction flux distribution in a metabolic pathway predicted by the mass-balance model can be compared quantitatively to the fluxes predicted by the kinetic model.

Table 7.4 reports the steady-state flux distribution in the polyamine biosynthetic sub-pathway computed by both approaches. The fluxes going though the reactions determined by the kinetic model (the corresponding rate equations are given in Table 7.2) are approximated with the 'best' set of parameters. Note that the flux through the reaction step of exogenous methionine assimilation has a known value of 0.122 μmol per min per mg of cell protein, calculated by Equation (4.19) in Chapter 4.

The structural model of the polyamine biosynthetic sub-pathway can be extracted from matrix (3.12) (defined in Chapter 3). Irrelevant metabolites and reactions to this sub-pathway are removed, which causes the accidental removal of an essential reaction step concerned with AdoMet-dependent transmethylation. To make the model consistent with the schematic representation of the sub-pathway (see Figure 7.13), the reaction step of AdoHcy production from AdoMet is introduced (AdoHcy is set as an additional external metabolite) and a new output reaction describing the conversion of spermidine into trypanothione biosynthesis (catalyzed by *Spdout*) is also added.

The reaction flux going through the step of spermidine consumption into trypanothione biosynthesis is defined as the objective function, in order to allow the two modelling approaches to be compared. The steady-state flux distribution of the sub-pathway via structural modelling is obtained by maximizing this objective function. The flux of the input reaction – exogenous arginine assimilation – is not available from the literature, and the value approximated by the kinetic model is assigned to this reaction flux. The flux of the other input reaction – exogenous methionine assimilation – is fixed as given above. All the internal reaction fluxes are assumed to vary between 0 and 2, with units of μmol per min per mg of cell protein. The maximum flux through the objective function (reaction index 10 in Table 7.4) is constrained with the corresponding flux value predicted by the kinetic model.

Table 7.4 shows a good match between the predictions from structural modelling and kinetic modelling. The internal fluxes predicted by optimizing the

Index	Reaction Name	Stoichiometry	SM	KM
1	Arginase	arginine $\rightarrow$ ornithine	0.7275	0.7275
2	Ornithine decarboxylase	ornithine $\rightarrow$ putrescine	0.7275	0.7280
3	AdoMet synthase	methionine $\rightarrow$ AdoMet	0.8500	1.1584
4	AdoMet decarboxylase	AdoMet $\rightarrow$ dAdoMet	0.7275	0.7280
5	AdoMet-dependent transmethylation	AdoMet $\rightarrow$ AdoHcy	0.1220	0.4287
6	Spermidine synthase	putrescine+dAdoMet $\rightarrow$ spermidine+MTA	0.7275	0.7301
7	MTA recycling	MTA $\rightarrow$ methionine	0.7275	0.7301
8	Arginine assimilation	$Ext_{arg} \rightarrow$ arginine	0.7275	0.7275
9	Methionine assimilation	$Ext_{met} \rightarrow$ methionine	0.1220	0.1220
10	Spermidine consumption	spermidine $\rightarrow$ trypanothione biosynthesis	0.7275	0.7386

Table 7.4: Comparison of the steady-state flux distributions in the polyamine biosynthetic sub-pathway predicted via structural modelling and kinetic modelling. All the values are expressed in the units of μmol per min per mg of cell protein. **SM** and **KM** stand for the structural model and kinetic model, respectively.

structural model are almost identical to the fluxes computed with the kinetic model and the objective function is optimized sufficiently close to the maximum value (0.7275 versus 0.7386).

Fluxes going through the reaction steps – AdoMet synthase and AdoMet-dependent transmethylation – were solved with different values. This can be explained from numerical aspects. AdoMet is a branching point in the polyamine biosynthetic sub-pathway, where one incoming reaction (reaction 3) is split into two outgoing reactions (reaction 4 and 5). In this case, when only one flux is known, in theory, there will be many combinations of flux values to be computed for the other two reactions, and therefore values of these fluxes can only be determined based on constraints imposed by other fluxes.

When solving both models, the reaction step describing the conversion of methionine into AdoMet (reaction index 3 in Table 7.4) is predicted to carry the maximum flux value of all the internal reactions, indicating the significant role that this reaction step plays in satisfying the objective function. This observation is consistent with the analysis results of control-coefficient fluxes representing the importance of each reaction for efficient and flexible operation of the entire pathway. As shown in Figure 3.10 in Chapter 3, of all the polyamine reactions,

the reaction of interest (reaction 3) is calculated with the highest control-effective flux (Note that in the figure reactions 1–7 and 15–16 correspond to reactions 1–9 in Table 7.4).

In summary, with appropriate constraints, a good interpretation of the metabolic capacities of the polyamine biosynthetic sub-pathway can be obtained by optimizing the structural model with respect to a specific objective function. The consistency of predictions of internal reaction fluxes in both types of models gives confidence that the models are an adequate reflection of the real system, given the absence of experimental data. With the predicted metabolic fluxes, an adequate measure of the degree of participation of the polyamine biosynthetic reactions in the process of spermidine participation in trypanothione biosynthesis is obtained.

7.9.6 Computational Issues

MoPSwarm is a reliable and efficient multi-objective optimization algorithm. From the modelling point of view, model validation with MoPSwarm is a promising strategy. The present model structure combined with estimated parameter values is able to consistently interpret the underlying biological processes. This is ascribed to the fact that MoPSwarm allows an integrated study of different states of the polyamine biosynthetic sub-pathway, which is an essential condition for the elucidation of regulatory principles. In the context of parameter estimation, MoPSwarm enables non-dominated solutions covering a well-distributed Pareto front to be found with a small population of particles (20 initial particles) and with a small number of generations (less than 1000 generations). The proposed algorithm is not only able to optimize problems exhibiting straightforward convex tradeoffs but also addresses problems resulting discontinuous Pareto fronts.

Due to the sparse nature of the problem, it is frequently difficult for the ODE solver to integrate the differential equations. Some regions of the parameter space are numerically unstable and the solver may stall or produce invalid results, such as NaNs (Not a Number) and integration failures. It is necessary to implement error handlers in MoPSwarm to deal with these problems; the Matlab solver `ode15s` was modified to include an extra error handler to prevent stalling due to invalid results. For a difficult optimization problem, such as this, we replaced '$2 \times f_i(\mathbf{x})$' with '$10 \times f_i(\mathbf{x})$' in the mathematical definition of 'Partially

Dominated Solutions' (see Definition 6.5 in Chapter 6); that is, a particle may be up to 10x as bad in any one objective if there is any improvement in the other. This was designed to prompt a good diversity in the Pareto-optimal solutions by easing the strict constraint imposed on the selection of 'good' particles.

During the search process, MoPSwarm was frequently confronted with difficult search regions with many invalid positions found for both the steady-state and perturbed model. For example, in the course of identifying the Pareto front shown in Figure 7.15, MoPSwarm retrieved only 28 trade-off solutions in the parameter search space with variations in the parameter values covering 5 orders of magnitude, with 3145 and 2441 invalid results found for the steady-state model and the perturbed model, respectively. This may explain why the resulting approximate Pareto-optimal front is concave and discontinuous, being comprised of four disconnected Pareto-optimal sets and a single point.

Despite the good results achieved by MoPSwarm, the number of fitness function evaluations may be very large, and the computational cost of using this approach can vary significantly when solving other systems. The most likely cause of this is from variations in the time taken to evaluate the model in order to determine the objective function value. In our case, the average computation time over ten simulation runs of model validation is approximately 19 hours on AMD Opteron 2000-based cluster nodes.

7.10 Conclusions

Mathematical modelling of biological systems involves the identification of chemical relations between system components and the formulation of kinetic mechanisms representing the enzymatic catalysis. Constructing a detailed mechanistic model for the trypanothione metabolic pathway is a challenging task for several reasons:

- First, the system is ambiguously determined in terms of both network topology (chemical relations between system components) and kinetic characters (rate laws governing the reaction velocities). A systematic identification procedure for model structure correctness is necessary.

- Second, the search space is very complex in terms of both scale and dimensionality. Feasible region(s) – where optimal solutions are located in the search space can be very small and the search can be complicated by

the existence of infeasible region(s) where no solutions can be achieved and the system may be numerically unstable.

- Third, the very limited number of experimental observations imposes a significant challenge to optimization methods. Parameter estimation is particularly difficult when the dimensionality of parameters to be resolved is much larger than the number of measurement samples.

In this chapter, we proposed a methodological framework (Figure 7.1) in order to tackle the challenge imposed by data sparsity and incompleteness of prior knowledge of the system. The purpose of the proposed framework is to reduce the whole system into sub-systems through an iterative process and then study the sub-systems in a modular manner, which supports the model refinement in a functionally independent manner. Module-based representation allows the understanding of the entire system through parsing the sub-systems that are smaller in terms of both the number of state variables and kinetic parameters, by means of which complexity of the original system can be largely reduced. Module-based representation is thereby a very useful strategy in the development of mathematical models for large-scale non-linear dynamic systems.

One major difficulty of system identification lies in the design of correct model structures to consistently interpret the underlying processes, which involves seeking missing reactions or refining ill-characterized kinetic mechanisms. When no or limited biological evidence is given, it is almost impossible to make biologically meaningful assumptions about suitable model structures. The design principle of model decomposition in the proposed methodological framework is to direct the search for ill-characterized kinetic characters of the pathway in an efficient manner. This methodological framework has the potential to solve a wide range of cellular processes, including gene regulatory pathways, signalling pathways and metabolic pathways.

The procedure for model structure exploration does not lead to verification of models; rather to their falsification. Given conflicting experimental data, different mathematical models are expected, which may also show major variations due to imprecise measurements. It of course must also be remembered that proposed model structures are consistent in describing the biological data only within limits of experimental noise and the biological phenomena modelled.

By applying the proposed methodological framework, a novel kinetic model of the polyamine biosynthetic sub-pathway is successfully constructed. The module

predicability is preserved by using the Hill function to approximate the module interaction with the rest of the pathway. The regulatory principles of the polyamine biosynthetic sub-pathway were elucidated by studying the system in different physiological states. The agreement between model simulation with the estimated parameter values and measured data reported in various experimental conditions shows the good applicability of the kinetic model of the sub-pathway.

The system behaviour that has not yet been observed was also predicted by *in silico* model simulations. We observed an abrupt change on the polyamine levels in response to a gradual decrease of the complex compound – AdoMetDC|prozyme within a physiological range of the AdoMetDC concentration. A regulatory link between the polyamine enzymes was predicted to be an essential feature for arginine metabolism, as inferred from model simulation. An enzyme assay could be designed to verify the prediction by determining the arginine transport rate and arginase activity in *T. brucei* mutants that lack or are deficient in ODC. The regulatory link can be verified if suppression of arginine transport rate and arginine activity is observed.

Chapter 8

Conclusions and Future Work

In this thesis, the challenges associated with the computational modelling of complex biological systems when prior knowledge was restricted by sparse quantitative information and incomplete physical descriptions of the underlying processes have been investigated. In this chapter we summarize the main points of the work and reiterate the contributions drawn. We also discuss interesting future work directions, including possible improvements and extensions on the proposed approaches.

8.1   Conclusions

A systematic approach is necessary to elucidate the complex relationships involving multiple system components governed by non-linear kinetics. The emerging field of Systems Biology provides a powerful foundation and established scientific methods to enable the study of biological pathways at the system level.

In biology, the identification and understanding of system behaviour is not an easy task. The difficulty in achieving a system-level understanding stems from the intrinsic complexity of biological systems where large numbers of functionally diverse components interact non-linearly. It has been widely accepted that the best way to understand complex networks of biological pathways is via the use of computational modelling. However, this is often complicated by incomplete prior knowledge of the system. Inconsistent and sparse experimental data of the network also creates a multitude of problems for the modelling process.

Constructing a sound mathematical model to understand biological systems is a challenging endeavor. Mathematical models with a high degree of precision and quality are necessary for drug discovery and treatment optimization, where

multiple aspects of the biological processes have to be integrated and the model predictions must be validated with the experimental observations. Mathematical modelling is also a useful tool for the development of new processes and in this case has allowed disparate information about the system to be organized and represented in a coherent manner.

Chapter 4 contributes to knowledge of the first detailed kinetic model of an important biological system – the trypanothione metabolic pathway in the protozoan parasite *Trypanosoma brucei*. We focused on continuous and deterministic descriptions of the trypanothione metabolism by using non-linear ordinary differential equations. We took into account the essential information in order to build a useful mathematical model. An extensive literature search about the metabolic pathway was conducted, where scientific papers relevant to individual enzyme kinetic properties were critically reviewed.

We have proposed a new methodological framework to address the modelling challenges and illustrated the feasibility of the framework by studying the trypanothione metabolic pathway in *Trypanosoma brucei*. Metabolism in this parasitic organism is the focus of several investigations for anti-trypanosomal therapeutics, as *Trypanosoma brucei* remains a major problem in developing countries despite the availability of several drugs. To achieve the goals, some existing scientific methods have been reviewed and then adapted and extended in an appropriate way for use in solving the problem of interest.

8.1.1 Metabolic Modelling

As stated in Chapter 1, the type of model should depend on the biological system in question, the type/amount of information available and the intended use of the model. A system-level understanding of the trypanothoine metabolism has been attempted through structural and kinetic modelling approaches. Both approaches are essential to understand the metabolic properties of the pathway and focus on topological and regulatory properties, respectively. Structural modelling and kinetic modelling are complementary approaches, and one enables a determination of a variety of model properties that could not be allowed by the other.

Knowledge about the trypanothione metabolic pathway is currently restricted to a qualitative view and the network topology is better understood than its numerical aspects. Most of the cellular enzymes, such as regulatory mechanisms

are, at best, qualitatively known; detailed enzyme-kinetic models are lacking. This creates severe difficulties for kinetic modelling of the pathway in order to comprehend the trypanothione metabolism in its entirety, which was demonstrated in Chapter 7. In the absence of enough kinetic information, structural modelling is one promising approach for the understanding the trypanothione metabolism as a whole.

Chapter 3 reported the first structural model of the trypanothione metabolic pathway. In this chapter, we applied a theoretical structural modelling approach to study the network metabolism even in the absence of sufficient quantitative information of target enzymes. This structural modelling approach is based on the stoichiometry and the reversibility of the metabolic reactions, which is a structural invariant of the pathway, and uses theoretical tools to investigate the model. Topological properties and metabolic capabilities of the trypanothione pathway to support cell growth were investigated with this approach. Essential genes were predicted by optimizing the mass-balance model of the pathway with respect to given objective functions (biomass yield and H_2O_2 residual).

By structural modelling, we computationally tested the completeness of available topological information based on the elementary mode analysis. When the metabolic flux measurements become available and adequate, the postulated additional reactions of the pathway can be verified. We examined the consistency of given objective functions for linear programming with experimentally observed genotypes. Wrong predictions revealed an incorrect definition of the objective functions, improving our understanding of the growth control of trypanosomes. Two constituting factors of the trypanosomal growth are characterized with two topological modules carrying out specific metabolic functions – the trypanothione biosynthetic system and the trypanothione redox system.

Although the structural model was not used for simulation, it still helped us focus on the essential features of the system and facilitated the construction and simplification of the kinetic model of the pathway. This type of modelling enabled an establishment of the correlation between topological modules (spatially isolated) and functional modules (carrying out a monotonous metabolic function), which is otherwise not easy to achieve. This information was used to aid the initialization of model decomposition in the methodological framework proposed in Chapter 7.

Application of structural modelling to the trypanothione metabolic pathway provided us a relatively simple starting point to understanding the approach.

The work performed demonstrates the feasibility of structural modelling for understanding the system when it extends to the genome level. The predictions of the steady-state flux distribution in the polyamine biosynthetic sub-pathway from structural modelling and kinetic modelling were compared with appropriate constraints. Since the measurement of fluxes is a tedious task, the good agreement of both models indicates that the models are adequate representations of the real system.

However, flux distributions under steady-state conditions do not explain anything about the regulatory principles of the system and the disadvantage of structural modelling is that dynamical aspects of the systems are left implicit. We have demonstrated in Chapter 4 and further discussed in Chapter 7 that a realistic metabolic model, using coupled enzymatic reactions, can only be described by a system of non-linear equations for which no analytical solutions can be easily obtained.

8.1.2 Optimization of Computational Models

Chapter 5 laid the foundation for the optimization-based study, which played an essential role in the methodological framework proposed in Chapter 7. This chapter reviewed some of the basic theory associated with optimization and discussed Particle Swarm global optimization and the derived PSwarm algorithm. PSwarm is a recently developed population-based optimization method and has not yet been widely applied to biological problems. This chapter investigated the performance of the algorithm, which demonstrated that PSwarm is capable and efficient in tackling the inverse problem of complex biological systems. PSwarm was employed as the search engine during the examination of model structural correctness. The problem of parameter estimation suffering from missing experimental measurements and lack of information content was illustrated using the ERK signalling pathway.

Chapter 6 proposed a novel approach MoPSwarm for applying a multi-objective optimization to the validation of perturbation-based models of biological systems. MoPSwarm is a generalized pattern search algorithm, defined by a Search-and-Poll framework, where the standard particle swarm optimization is employed in the search step and the poll step is only activated when the search step failed. Satisfactory simulation results of the ERK signalling pathway using the proposed validation approach demonstrated the reliability and

utility of the proposed algorithm for model validation in comparison with the conventional approach. This chapter, combined with the work on the algorithm presented in Chapter 7, indicated that model validation is the most difficult problem in the modelling process, particularly when the model predicability is reliant on accurate and reliable quantification of parameters.

Despite the great challenge in solving the real-world applications (including the ERK signalling pathway and the polyamine biosynthetic sub-pathway) due to their non-linear nature, MoPSwarm has shown good performance in tackling multi-objective optimization problems characterized with various properties. Experimental results from model simulation of both biological pathways demonstrated that MoPSwarm enables us to find non-dominated solutions covering a wide-spread Pareto front with a small number of population (20 initial particles) and with a small number of generations (less than 1000 generations).

Multi-objective optimization simultaneously takes several, possibly conflicting aspects into consideration. We have demonstrated that with the multi-objective optimization scheme, a population of solutions results from a single run of the algorithm owing to the contradictory objectives and several parts of the objective space can be explored simultaneously. A practical interpretation of the Pareto-optimal front is that different solutions can be selected to describe the system according to the degree of confidence ascribed to individual objective.

We have performed a comparative study investigating some of the factors important to the performance of MoPSwarm in solving multi-objective optimization problems. We have found that the choice of selection scheme for global best guide and the mechanism for updating the archive is critical for the Pareto-based approach, from the perspective of promoting an effective search towards the Pareto front and maintaining diversity in the population. Experimental investigation indicated that MoPSwarm is capable of tackling the multi-modal objective function space. The proposed algorithm is not only able to detect solution spaces exhibiting straightforward convex tradeoffs but also address concave and discontinuous Pareto fronts. By simultaneously optimizing the steady-state and perturbed model, the difficulty of potentially large steady-state degrees of freedom can be relieved by incorporating time-depedent system dynamics. Computational investigations of MoPSwarm underlined the necessity of considering more than one state of the system for reliable parameter estimation.

8.1.3   Computational Modelling

The proposed methodological framework is envisioned as an important contribution to the mechanism-based mathematical modelling of complex biological systems. The novelty lies in the integration of mathematical modelling (analytical approach) and system identification (experimental approach) to enable physical insights about the system to be accounted for in the modelling procedure, particularly when our prior knowledge is restricted. By means of the methodological framework, the mathematical model of the trypanothione metabolic pathway initially described in Chapter 4 was explored strategically.

We have carried out in this thesis one of the few works on studying model inappropriateness. Mismatch between the model responses given a certain structure and experimental observations via the optimization-based study with PSwarm has uncovered the inappropriateness in the model representation.

A major biological use of this work would be to generate predictions about enzyme kinetics and regulatory mechanisms to be tested by *in vivo* approaches. Since mathematical models are manipulable, the basic principles of metabolic regulations via different kinetic characters can be evaluated. Data produced *in silico* combined with collections of *in vivo* experiments motivate the formulation of new hypotheses to be placed in computational experiments for testing and validation. The regulatory link between the polyamine enzymes, including exogenous arginine transporter, arginase and ODC has been hypothesized and validated via *in silico* model simulation that is amenable to further biological studies. After biological experiments have been performed for hypothesis verification, models can be iteratively improved and new insights can be gained.

The significance of model decomposition in the early stage of computational modelling has been underlined. The module-based model presentation has allowed us to focus on comparatively well-characterized modules and to study the poorly-defined sub-systems when the relevant biological information is adequate for a mechanism-based model construction. The model decompositional approach assisted in an effective identification of the possible ill-characterized reactions without detailed biological information being available.

In terms of computational modelling, the module-dependent study allowed the selection of appropriate model representations to elaborate the underlying processes of the system described by different amounts of relevant prior knowledge. The usefulness of characterizing different parts of the system with different mathematical model representations has been demonstrated in the computa-

tional modelling of the polyamine biosynthetic sub-pathway in Chapter 7 – the enzymatic reactions for spermidine production were modelled based on detailed physical insights and spermidine participation in the rest of the trypanothione metabolic system was approximated with a non-linear regression function. From the optimization point of view, module-based representation simplified the mathematical description of the system by approximating ambiguously-characterized sub-systems with a parametric function, reducing the degrees of freedom of the parameters to be estimated.

Understanding complex biological systems on the basis of functional modules enables flexible system models to be built from reusable components and allows different calculations to be employed for these components. Module-based models can also assist the analysis of similar modules in related species (e.g. in *T. brucei*, *Leishmania* and *T. cruzi*) to identify mutually-shared or species-specific functions.

The model of polyamine biosynthetic sub-pathway developed in this thesis has enabled a scientific explanation of measured data and a consistent interpretation of the underlying biological processes. We have shown that our model of polyamine interconversion reflects some critical features observed in experimental approaches, such as ODC knockdown, AdoMetDC knockdown, prozyme knockout and variations in the Methlyation index. The modelling activities performed on the trypanothione metabolic pathway have great potential to help experimentalists clarify conceptual ambiguities and to provide a platform for rational identification of potential anti-trypanosomal druge targets.

8.2 Future Work

There are several areas for possible improvements and extensions on the work presented in this thesis.

The first suggestion for future research work is the investigation of the trypanothione metabolism in *Trypanosoma brucei* on the genomic scale. Reconstruction of the metabolic network of the parasite *Leishmaina major* has recently been reported by Chavali et al. (Chavali et al. 2008). The work in Chapter 3 and Chapter 4 provides an opportunity for the genome-scale construction of the metabolic network in the parasite *Trypanosoma brucei*. To achieve the goal, a variety of data sources must be integrated in order to expand the existing structural model, including available genomic, proteomic and

metabolomic data. The computational modelling and systems analysis meth-
ods described in Chapter 3 could be readily applied to the future study of this
parasitic organism.

**The second suggestion for future research work is the integration
of structural model and kinetic model of the trypanothione metabolic
pathway in order to build a whole-cell model.** We have emphasized the
importance of structural modelling and kinetic modelling as individual mod-
elling approach for studying metabolic pathways. A strategy of integrating the
structural and kinetic models appears to be prevalent in practice. Recently, a
framework that combines models of different types of biological processes (e.g.
metabolic and regulatory processes) was developed by Covert et al. (Covert
et al. 2008) for building whole-cell models. Their framework requires the avail-
ability of different models (e.g. structural model and kinetic models) of the same
system in order to create an integrated model. Our work in Chapter 3, Chapter 4
and Chapter 7 developed the structural model and tackled the construction of
kinetic model for *T. brucei*, making the proposed framework by Covert et al.
suitable for application to the development of a whole-cell model in *T. brucei*.

As their approach demands a complete description of the initial conditions
of biomass, enzymes and metabolites of the system, a number of *in vivo* ex-
periments must be performed in order for their approach to be useful. A good
starting point of future research would concentrate on the model of polyamine
biosynthetic sub-pathway, for which some unknown parameters have already
been estimated with MoPSwarm. An interesting question to address could be
concerned with studying the regulation between AdoMetDC and prozyme of
the polyamine sub-pathway. The regulatory mechanism of the two enzymes has
been postulated via the kinetic modelling in Chapter 4. However, the postulated
mechanism cannot be tested due to the lack of measured dynamics essential for
the purpose. We expect that by integrating the kinetic model with the struc-
tural model of the polyamine biosynthetic sub-pathway, more knowledge could
be gained for this regulatory mechanism.

**The third suggestion for future research work is a parallel imple-
mentation of the multi-objective optimization algorithm MoPSwarm
for increased computational speed.** To increase the usefulness of the MoP-
Swarm proposed in this thesis, a potential enhancement to the computational
efficiency of the algorithm is suggested. The Poll step is an important strategy
adopted in the algorithm to guarantee satisfactory convergence to stationary

points and a good diversity of the Pareto-optimal solutions. However, the number of evaluations of fitness functions in the Poll step may be very large and the computational cost of using this approach can vary significantly when solving other systems.

A parallel implementation of the algorithm would be a good solution for improving the computational speed. An attempt has been made to parallelize Particle Swarm for application to computationally demanding single-objective optimization problems (Schutte et al. 2003). Other general strategies could be parallelization onto multiple processors using an interface such as MPI (message passing interface – http://www.open-mpi.org/) or parallelization onto hardware accelerated platform such as GPUs.

The fourth suggestion for future research work is the enhancement of the algorithm MoPSwarm for tackling multi-objective optimization problems. Although MoPSwarm as designed in this thesis in general aims at solving optimization problems constrained by multiple objectives (more than two objectives), the focus has been given to solving problems with two objectives. The requirement to enhance the generalization of MoPSwarm mandates further work on designing appropriate mechanisms in selecting trade-off solutions among objective functions. This may involve assigning different 'weights' to individual objective as importance factors when they are simultaneously solved. MoPSwarm is a stand-alone algorithm and only requires mathematical model (e.g ODEs) and objective functions (e.g. least squares) as inputs, which facilitates future enhancement of the algorithm.

The final suggestion for future research work is the adaptation of the methodological framework for automatic computational modelling. The methodological framework proposed in Chapter 7 intends to tackle the problem of structure identification caused by ill-defined kinetic mechanisms of reaction enzymes, since in most cases the network topology is better understood than regulatory mechanisms. This framework is fundamentally a heuristic approach, where modeller's interactions with the framework in each phase are necessary. Automatic modelling procedure would be appealing in practice, particularly for extreme cases, where no biological information concerning either network topology or kinetic mechanisms is obtainable for *in silico* investigation of system identification problems.

We envision that automation of the modelling methodology could be achieved via a bilevel architecture. The outer level searches through a library of reactions

and/or enzyme kinetics to include so that model simulation can agree with experimental data given a particular model structure. An appropriate (qualitative or quantitative) measure of the level of agreement between model simulation and system responses is crucial for determining 'good' structures. The inner level deals with the problem of model validation for the structure(s) that passed the outer level examination, in which case MoPSwarm can be employed.

However, to make the automated procedure useful, certain issues should be taken into account. One issue is that models without biological interpretation could be derived. Much attention has to be paid in the selection of suitable model structures and models without biological support should not be included in the set of candidate models. Another issue is that computational cost can be enormous with increasing model complexity. This difficulty could be relieved by incorporating expert knowledge about 'good' parts of the model structure, and focusing on only suspected system characteristics during the procedure.

Appendix A

Experimental Data

This section reports the experimental observations that are used for the study in Chapter 7. This data includes DFMO-treated drug responses and single-gene knockdown profiles. Intracellular concentrations of the metabolites are given in μM that are caculated based on a volume of 58 μL/10^9 trypanosomes (Opperdoes et al. 1984).

A.1   DFMO-treated Drug Responses

Table A.1 summarizes the effect of DFMO on bloodstream-form *T. brucei* (Fairlamb et al. 1987) and Table A.2 the effect on procyclic form *T. brucei* (Bellofatto et al. 1987).

Metabolites	Wt	Wt+DFMO 12hrs	Wt+DFMO 36hrs	Wt+DFMO 48hrs
ornithine	43	759	759	569
putrescine	517	3.3	3.3	3.3
AdoMet	19	34	1069	1431
dAdoMet	9	34	397	793
Spermidine	2069	948	845	517
GspdSH	48	34	31	29
glutathione	234	397	414	397
trypanothione	340	257	207	116

Table A.1: Dynamic responses of the intracellular metabolite concentrations in bloodstream-form *T. brucei*. *Wt* (wild-type) and *Wt+DFMO* stand for intracellular concentrations at the steady state and under drug inhibition.

Metabolites	Wt	Wt+DFMO 12hrs	Wt+DFMO 36hrs	Wt+DFMO 48hrs
arginine	603	862	1293	1896
ornithine	43	2069	2069	2069
putrescine	2931	258	0	0
dAdoMet	0	172	431	603
Spermidine	3793	2758	1465	1034
GspdSH	77	34	34	34
glutathione	465	552	862	1000
trypanothione	672	431	379	310

Table A.2: Dynamic responses of the intracellular metabolite concentrations in procyclic-form *T. brucei*. *Wt* (wild-type) and *Wt+DFMO* stand for intracellular concentrations at the steady state and under drug inhibition.

A.2 Single-gene Knockdown Profiles

Table A.3 summarizes the effects of SpdS (spermidine synthase) knockdown on the intracellular metabolite concentrations in *T. brucei* (Xiao et al. 2009).

Metabolites	Wt	Wt+Tet 12hrs	Wt+Tet 36hrs	Wt+Tet 48hrs
putrescine	869	517	445	245
dAdoMet	0.07	2.24	7.07	10.7
Spermidine	1548	881	407	366
GspdSH	11.2	1.2	3	1e-3
glutathione	164	188	198	210
trypanothione	2.6	2.2	2.6	3.4e-2

Table A.3: The effects of SpdS knockdown on the intracellular metabolic concentrations in bloodstream-form *T. brucei*. *Wt* and *Wt+Tet* stand for intracellular concentrations at the steady state and after Tet induction. The addition of Tet (tetracycline) degrades the target enzyme, essentially knocking it down by a given fraction.

Appendix B

Structural Analysis

B.1 The Computational Approach for Detecting Network Gaps

The main idea of the computational investigation is to examine, after adding new branches to one metabolite at a time, whether the elementary modes of the new topology can cover all the enzymatic reactions. Elementary modes are computed with the package *METATOOL*, where the stoichiometric matrix is used as a direct input.

With the assumption that additional reactions occur in isolation, the number of structural topologies to be examined is equal to the number of internal metabolites. Computationally, additional reactions are embodied in the last column of the original stoichiometric matrix, where a non-zero entry in the column represents the involvement of the metabolite in the corresponding reaction.

By way of example, let's assume that the internal metabolite arginine has an additional outgoing branch. Accordingly, the original matrix is expanded with an additional column. There are no changes to the rows as no new metabolites are included. The last column of the expanded matrix is given by the following vector

$$\begin{bmatrix} -1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \end{bmatrix}$$

where the non-zero entry '-1' represents the consumption of arginine in the additional reaction. As we assume that the additional reactions take place in isolation–one reaction at a time, there is only one non-zero entry in the vector.

We also examined the structural topology when the additional reactions of the internal metabolites are assumed to take place in pairs. In this case, two ad-

ditional branches are appended to the end of the original stoichiometric matrix ($\mathbf{S_{tb}}$). The number of scenarios to be examined for network gaps is the number of possible combinations for the selection of 2 metabolites from n pairs, which is $\frac{n!}{k! \cdot (n-k)!}$, where $n!$ denotes the factorial of n, the number of internal metabolites and k is the number of components selected (i.e. 2). When selecting any two from 18 internal metabolites, there are 153 possible pairings to examine. Computational results of this examination are detailed in the section 'Network Gaps Identification' in Chapter 3.

B.2 Mass-balance Model of the Trypanothione Pathway in ST1

This section presents the mass-balance model and the stoichiometric matrix of the trypanothione metabolic pathway represented by structural topology **ST1**. Refer to texts in Section 3.4 for a detailed description. Structural topology **ST1** introduced a new reaction concerning trypanothione consumption in other metabolic functions to Table 3.1, written as

No.	Label	Reaction Name	Stochiometry
22	v_{tshout}	TSH Consumption	TSH → tsh_end

$$
\begin{aligned}
X_1 &= \tfrac{d[Arg]}{dt} &= 0 =& \; b_{arg} - v_1 \\
X_2 &= \tfrac{d[Orn]}{dt} &= 0 =& \; v_1 - v_2 \\
X_3 &= \tfrac{d[Met]}{dt} &= 0 =& \; b_{met} - v_3 + v_7 \\
X_4 &= \tfrac{d[AdoMet]}{dt} &= 0 =& \; v_3 - v_4 - v_5 \\
X_5 &= \tfrac{d[Put]}{dt} &= 0 =& \; v_2 - v_6 \\
X_6 &= \tfrac{d[dAdoMet]}{dt} &= 0 =& \; v_4 - v_6 \\
X_7 &= \tfrac{d[Spd]}{dt} &= 0 =& \; v_6 - v_{10} + v_{11} \\
X_8 &= \tfrac{d[MTA]}{dt} &= 0 =& \; v_6 - v_7 \\
X_9 &= \tfrac{d[Cys]}{dt} &= 0 =& \; b_{cys} - b_{efcys} + v_5 - v_8 \\
X_{10} &= \tfrac{d[Glu]}{dt} &= 0 =& \; b_{glu} - v_8 \\
X_{11} &= \tfrac{d[Gly]}{dt} &= 0 =& \; b_{gly} - v_9 \\
X_{12} &= \tfrac{d[gGluCys]}{dt} &= 0 =& \; v_8 - v_9 \\
X_{13} &= \tfrac{d[GSH]}{dt} &= 0 =& \; v_9 - 2 \cdot v_{10} + 2 \cdot v_{11} \\
X_{14} &= \tfrac{d[TSH]}{dt} &= 0 =& \; v_{10} - v_{11} - v_{12} + v_{13} - v_{tshout} \\
X_{15} &= \tfrac{d[TS_2]}{dt} &= 0 =& \; v_{12} - v_{13} \\
X_{16} &= \tfrac{d[G6P]}{dt} &= 0 =& \; b_{glc} - v_{14} \\
X_{17} &= \tfrac{d[NADPH]}{dt} &= 0 =& \; -v_{13} + v_{14} \\
X_{18} &= \tfrac{d[NADP]}{dt} &= 0 =& \; v_{13} - v_{14}
\end{aligned}
\tag{B.1}
$$

$$
\begin{aligned}
&\left[\tfrac{d[Arg]}{dt} \quad \tfrac{d[Orn]}{dt} \quad \tfrac{d[Met]}{dt} \quad \tfrac{d[AdoMet]}{dt} \quad \tfrac{d[Put]}{dt} \quad \tfrac{d[dAdoMet]}{dt} \quad \tfrac{d[Spd]}{dt} \quad \tfrac{d[MTA]}{dt} \right. \\
&\quad \tfrac{d[Cys]}{dt} \quad \tfrac{d[Glu]}{dt} \quad \tfrac{d[Gly]}{dt} \quad \tfrac{d[gGluCys]}{dt} \quad \tfrac{d[GSH]}{dt} \quad \tfrac{d[TSH]}{dt} \quad \tfrac{d[TS_2]}{dt} \\
&\quad \left. \tfrac{d[G6P]}{dt} \quad \tfrac{d[NADPH]}{dt} \quad \tfrac{d[NADP]}{dt} \right]^T = \\
&\mathbf{S_{tb}} \cdot \left[v_1 \quad v_2 \quad v_3 \quad v_4 \quad v_5 \quad v_6 \quad v_7 \quad v_8 \quad v_9 \quad v_{10} \quad v_{11} \quad v_{12} \quad v_{13} \quad v_{14} \right. \\
&\qquad \left. b_{arg} \quad b_{met} \quad b_{cys} \quad b_{eff} \quad b_{glu} \quad b_{gly} \quad b_{glc} \quad v_{tshout} \right]^T
\end{aligned}
$$

where

$$\mathbf{S_{tb}} = \begin{bmatrix}
-1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
1 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & -1 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 1 & -1 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 1 & 0 & 0 & 0 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 1 & 0 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & -1 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 1 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 1 & 0 & 0 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & -1 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 0 & 0 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & -2 & 2 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & -1 & -1 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & -1 \\
0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 \\
0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & -1 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0
\end{bmatrix}$$

$$(\text{B.2})$$

B.3 LP Optimization for Maximum Growth Rate

When the mass-balance model in Equation (B.1) is used for the maximization of growth yield, the reaction labelled as v_{tshout} is replaced with the growth rate v_{gr}. The stoichiometric matrix formulated in (B.2) is used for the task of interest. From the biological point of view, the last column in this matrix represents the reaction flux converting the metabolite precursor into biomass components.

B.4 LP Optimization for Minimum Residual Error with Respect to H_2O_2 Production Rate

The following mass-balance model is employed for the prediction of gene essentiality, expressing the residual error between the calculated and measured rate of H_2O_2 production as an objective. The optimal steady-state fluxes of individual reactions are calculated by minimizing the error.

The mass-balance model for LP optimization of the minimum residual error below is based on Equation (B.1) given in Appendix B.2. To enable the H_2O_2 production rate to be computationally simulated, H_2O_2 is considered as a variable metabolite and the corresponding mass-balance equation is formulated, which is specified by X_{16}. This introduces a new reaction flux to the mass-balance model in Equation (B.1), written as below

No.	Label	Reaction Name	Stochiometry
23	$r_{H_2O_2}$	H_2O_2 production	H_2O_2 Source $\rightarrow H_2O_2$

$$
\begin{aligned}
X_1 &= \frac{d[Arg]}{dt} &&= 0 = &&b_{arg} - v_1 \\
X_2 &= \frac{d[Orn]}{dt} &&= 0 = &&v_1 - v_2 \\
X_3 &= \frac{d[Met]}{dt} &&= 0 = &&b_{met} - v_3 + v_7 \\
X_4 &= \frac{d[AdoMet]}{dt} &&= 0 = &&v_3 - v_4 - v_5 \\
X_5 &= \frac{d[Put]}{dt} &&= 0 = &&v_2 - v_6 \\
X_6 &= \frac{d[dAdoMet]}{dt} &&= 0 = &&v_4 - v_6 \\
X_7 &= \frac{d[Spd]}{dt} &&= 0 = &&v_6 - v_{10} + v_{11} \\
X_8 &= \frac{d[MTA]}{dt} &&= 0 = &&v_6 - v_7 \\
X_9 &= \frac{d[Cys]}{dt} &&= 0 = &&b_{cys} - b_{efcys} + v_5 - v_8 \\
X_{10} &= \frac{d[Glu]}{dt} &&= 0 = &&b_{glu} - v_8 \\
X_{11} &= \frac{d[Gly]}{dt} &&= 0 = &&b_{gly} - v_9 \\
X_{12} &= \frac{d[gGluCys]}{dt} &&= 0 = &&v_8 - v_9 \\
X_{13} &= \frac{d[GSH]}{dt} &&= 0 = &&v_9 - 2 \cdot v_{10} + 2 \cdot v_{11} \\
X_{14} &= \frac{d[TSH]}{dt} &&= 0 = &&v_{10} - v_{11} - v_{12} + v_{13} - v_{tshout} \\
X_{15} &= \frac{d[TS_2]}{dt} &&= 0 = &&v_{12} - v_{13} \\
X_{16} &= \frac{d[G6P]}{dt} &&= 0 = &&b_{glc} - v_{14} \\
X_{17} &= \frac{d[NADPH]}{dt} &&= 0 = &&-v_{13} + v_{14} \\
X_{18} &= \frac{d[NADP]}{dt} &&= 0 = &&v_{13} - v_{14} \\
X_{19} &= \frac{d[H_2O_2]}{dt} &&= 0 = &&r_{H_2O_2} - v_{12}
\end{aligned}
$$

(B.3)

Appendix C

System Identifiability

In this appendix we detail the mathematical concepts behind the computational procedures developed for identifiability analysis, including the details for the numerical computation of Jacobian matrix, Fisher information matrix, covariance and correlation matrices, confidence intervals and other related statistical measures.

C.1 *A Priori* Structural Identifiability

Structural identifiability analysis is performed to investigate whether all model parameters can be estimated given the available knowledge of the system. Output sensitivity functions are central to the evaluation of *a priori* local identifiability. Linearly dependent sensitivity functions result in parameter estimates that are correlated. In our study, the numerical method for checking local structural identifiability is based on the description given by Zak et al. (Zak et al. 2003).

Suppose the system is expressed as a set of N_x differential equations with N_x states ($\mathbf{x}$) and N_p parameters ($\mathbf{p}$). Taking $\hat{p}$ as 'true values' of the parameter set, the $N_x \times N_p$ sensitivity matrices of the measured states are calculated at a large enough number of points N where:

$$S_{x(i,j)} = \left(\frac{\partial x_i}{\partial p_j} \right)_{x=x(t,\hat{p}),\ p=\hat{p}}$$

The correlation matrix of the parameters ($\mathbf{M}_c$) was calculated:

$$M_c = \text{correlation}(G)$$

where

$$
G = \begin{bmatrix} S_x(t_1) \\ S_x(t_2) \\ \vdots \\ S_x(t_N) \end{bmatrix}
$$

Parameters that are *locally identifiable* have correlations with all other parameters between -1 and +1. Parameters that are not locally identifiable have correlations of exactly -1 or +1 with at least one other parameter.

Sensitivity matrices S_{xij} have to be calculated for the measured states at a large number of time points to diminish the numerical bias introduced from the lack of points.

Computing the sensitivity matrix involves calculating the gradient of each component of the gradient vector of the system output. One possibility is to evaluate these gradients using finite differences: perturbing each independent parameter **p** in turn, calculating the perturbed gradient of each system state **x**, subtracting the unperturbed gradient, and dividing by the magnitude of the perturbation (Dennis and Schnabel 1983).

The method of finite difference approximation is straightforward in that only the calculation of x_i is required with nominal and perturbed parameters. However, the numerical values obtained may vary significantly with $\Delta\theta_j$, and repeated solutions of the model are required for each parameter. The central finite difference is used in the computation of sensitivity matrix with the form below. This method, compared with the forward and backward finite difference approximation, gives rise to the smallest round-off error.

$$
s_{i,j}(t) = \frac{\partial x_i(t)}{\partial \theta_i} = \frac{x_i(\theta_j + \Delta\theta_j, t) - x_i(\theta_j - \Delta\theta_j, t)}{2 * \Delta\theta_j} \tag{C.1}
$$

C.2 Practical Identifiability with Hessian Matrix

A unified formalism for constructing the Hessian matrix is given in this section.

A Hessian matrix is the second derivative of the objective function **G** with respect to the parameters. Let $\Delta = (\Delta\theta_1, \ldots, \Delta\theta_i)$ be the estimates of $\Delta\theta$, and $\mathbf{G} = (G_1, \ldots, G_j)$ be the objective function values calculated for each of system

states. The Hessian matrix is defined as follows

$$
\begin{pmatrix}
\frac{\partial G_1}{\partial \theta_1} & \frac{\partial G_2}{\partial \theta_1} & \cdots & \frac{\partial G_j}{\partial \theta_1} \\[2ex]
\frac{\partial G_1}{\partial \theta_2} & \frac{\partial G_2}{\partial \theta_2} & \cdots & \frac{\partial G_j}{\partial \theta_2} \\[2ex]
\vdots & \vdots & \ddots & \vdots \\[2ex]
\frac{\partial G_j}{\partial \theta_i} & \frac{\partial G_j}{\partial \theta_i} & \cdots & \frac{\partial G_j}{\partial \theta_i}
\end{pmatrix}
$$

To investigate the practical identifiability, a Hessian matrix $(\mathbf{H}_{ij})$ of partial derivatives of the objective function with respect to the parameter vector has to be formulated.

$$
H_{ij} = (\mathbf{J}^T \mathbf{J})_{ij} + \sum_{i=1}^{n} r_l \cdot \frac{\partial^2 G_{ij}}{\partial \theta_i \partial \theta_j}
$$

where $\mathbf{J}$ is the Jacobian or gradient matrix of the objective function with respect to the parameter vector in the form of

$$
J_{ij} = \frac{\partial G_{ij}(\theta)}{\partial \theta_j} \tag{C.2}
$$

In the presence of small residuals, the Gauss-Newton approximation to the Hessian states that the terms containing the residuals can be ignored, thus giving the following equation:

$$
H_{ij} = (\mathbf{J}^T \mathbf{J})_{ij} \tag{C.3}
$$

The Jacobian matrix $\mathbf{J}$ records the absolute difference between the objective function calculated with the nominal value of θ_j in the parameter vector θ and the perturbed value, which can be computed with the central finite difference in Equation (C.1).

The Hessian matrix is often called the Fisher information matrix. Inverting the Hessian matrix gives the approximate Variance-Covariance matrix as follow

$$
M_{vc} = \begin{pmatrix}
\sigma^2_{i=1,i=1} & \sigma_{i=1,i=2} & \cdots & \sigma_{i=1,i=i} \\
\sigma_{i=2,i=1} & \sigma^2_{i=2,i=2} & \cdots & \sigma_{i=2,i=i} \\
\vdots & \vdots & \ddots & \vdots \\
\sigma_{i=i,i=1} & \sigma_{i=i,i=2} & \cdots & \sigma^2_{i=i,i=i}
\end{pmatrix} \tag{C.4}
$$

The diagonal elements of the Variance-Covariance matrix are the variances,

which can be used to construct confidence intervals for the parameters. The correlation coefficients between parameters p_i and the p_j are approximated by:

$$R_{p_i,p_j} = \frac{C_{p_i,p_j}}{\sqrt{C_{p_i,p_i}C_{p_j,p_j}}}, \quad p_i \neq p_j$$
$$R_{p_i,p_j} = 1, \qquad\qquad p_i = p_j$$

The correlation matrix measures the relationship between the parameters and indicates pairs of parameters where changes in the model output due to a change in one parameter value can be compensated by an appropriate change in an other parameter value.

With the Variance-Covariance matrix, 95% confidence intervals can be calculated for any identifiable parameters, which are given as:

$$CI = \hat{p}_i \pm 1.96\sigma(p_i)$$

where $\hat{p}_i$ denotes the nominal parameter value. Here, symmetry of the confidence region about the nominal values is assumed.

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