Amino acid transporters in *Trypanosoma brucei brucei*

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Abstract

Trypanosomes are protozoan parasites causing sleeping sickness in humans. The parasites live free in the bloodstream and the central nervous system of mammalian hosts and also within the midgut of the vector, the tsetse fly. The parasite plasma membrane represents the interface between both hosts and parasite, and trypanosomes accumulate many essential metabolites via specific transport processes.

Very little is known about amino acid requirements in trypanosomes or about amino acid metabolism. The aims of this thesis are to investigate amino acid transporters with a particular interest in bloodstream form trypanosomes. The long term goal underlying this study is to assess the possibility of using amino acid transporters as gate ways for entry for drugs into the parasite. From this perspective, transporters for amino acids of low abundance in human serum are of particular interest since the quantity of drugs that would be needed to compete with the natural substrate should be lower than for a more abundant substrate.

Two approaches have been used to investigate the nature of amino acid transport in *T. brucei*. The first approach involved studying the uptake of radiolabelled amino acids by the parasite, the second approach involved the identification, cloning and expression in a heterologous system of a gene encoding for a putative amino acid transporter.

The biochemical approach revealed that methionine uptake was of relatively high affinity in procyclic and bloodstream forms and dependent on a proton motive force. The recognition motif of this transporter encompasses the amino acid core as well as the two carbon atoms of the side chain.

The anionic amino acids, aspartate and glutamate, were shown to be taken up poorly or not at all by specific transport mechanisms in the two forms of the parasite. The aromatic amino acids, phenylalanine, tyrosine and tryptophan, are taken up via several different routes indicating the importance of these amino acids for the parasite.

The cloning and expression of *TbAATP1* in Xenopus oocytes revealed that this gene encodes for an amino acid transporter that is able to transport tyrosine, glutamine and glutamate.

TbAATP1 is the first member of what was subsequently shown to be a family of genes, encompassing at least twelve members present on three different chromosomes (IV, VI, VIII). On chromosome IV, six of these genes appeared to form a cluster.

Acknowledgements

First, I would like to thank Dr. M. P. Barrett not only for his supervision and help throughout my PhD but also for being a source of inspiration. I would like to thank Prof. S. Phillips and Prof. P Hagan for making it possible for me to work in the department and to the Sir Halley Stewart Trust for their financial support. I am also very grateful to Dr Sanjeev Krishna and to Dr John Connolly for their help with oocytes expression.

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Declaration

This thesis and the results presented in it are entirely my own work, except where indicated.

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LIST OF ABBREVIATIONS

BBB	blood brain barrier
bp	base pair
°C	degree celcius
CCCP	carbonyl cyanide chlorophenylhydrazone
CNS	central nervous system
DEAE	diethyl-amino-ethyl
DNA	deoxyribonucleic acid
FCCP	carbonylcyanide-p-trifluoromethoxyphenylhydrazone
HDL-R	high density lipoprotein receptor
Kb	kilobase
KTMB	α-ketomethiobutyrate
LDL-R	Low density lipoprotein receptor
M	molar
min	minutes
ml	millilitre
µl	microlitre
mRNA	messenger RNA
mmole	millimole
µmole	micromole
NEM	*N*-ethylmaleimide
ng	nanogram
PARP	procyclic acidic repetitive protein
PBSG	phosphate buffered saline containing 1% glucose
PCR	polymerase chain reaction
Pfu	*Pyrococcus furiosus*
RNA	ribonucleic acid
SDS	sodium dodecylsulfate
SHAM	salicylhydroxamic acid
SSC	saline sodium citrate
Taq	*Thermus aquaticus*
TbAATP	Trypanosoma brucei amino acid transporter

Tf-R	Transferrin receptor
TM	transmembrame
Tm	Melting temperature
TREU	Trypanosomiasis Research Edinburgh University
UV	ultraviolet
VSG	variant surface glycoprotein
X-gal	5-bromo-4-chloro-3-indol-β-D-galactopyranoside

La parole est a moitie a celuy qui parle,
moitie a celuy qui escoute

Montaigne

Chapter I

Introduction

1.1. The parasite

1.1.1. Parasitism

What is a parasite?

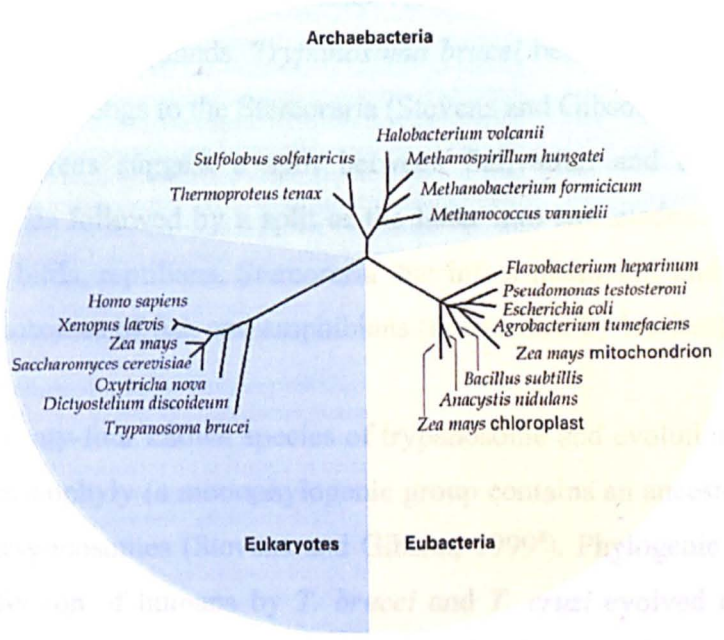
The textbook definition of a parasite is: "Any organism that spends all or part of its life cycle in (endoparasites) or on (ectoparasites) another living organism of a different species (its host), from which it obtains nourishment and/or protection, and to which it is usually detrimental" (Oxford Dictionary of Molecular Biology and Biochemistry, 1997).

Read gave a more refined definition of parasitism describing three main properties: **infectiousness, establishment** and **transmission**. Infectiousness is a short-term process involving a degree of tolerance to an instantaneous exposure to a new environment. Transmission depends on a successful establishment (growth and persistence). One key issue for a successful establishment of the parasite is its ability to tolerate or to evade the host immune system (Zelmer, 1998). As it will appear in this introduction, *Trypanosoma brucei* is a parasite highly adapted to its mammalian host.

1.1.2. Taxonomy

African trypanosomes belong to the order of Kinetoplastida and to the class of the Mastigophora and of the phylum Protozoa. The order of Kinetoplastida is divided into two suborders: the Bodonina, which contain two flagellar and the Trypanosomatina, containing one flagellum (Vickerman, 1994).

The family of the *Trypanosomatidae* contains eight different genera among which are found numerous pathogens. *Trypanosoma brucei* (the agent of sleeping sickness) and *Trypanosoma cruzi* (causing Chagas' disease) are both members of the same genus *Trypanosoma* while *Leishmania spp* (the agent of leishmaniasis) are incorporated the *Leishmania* genus. The genus *Trypanosoma* is subdivided into seven subgenera which are subdivided into two groups: the Stercoraria and the Salivaria.



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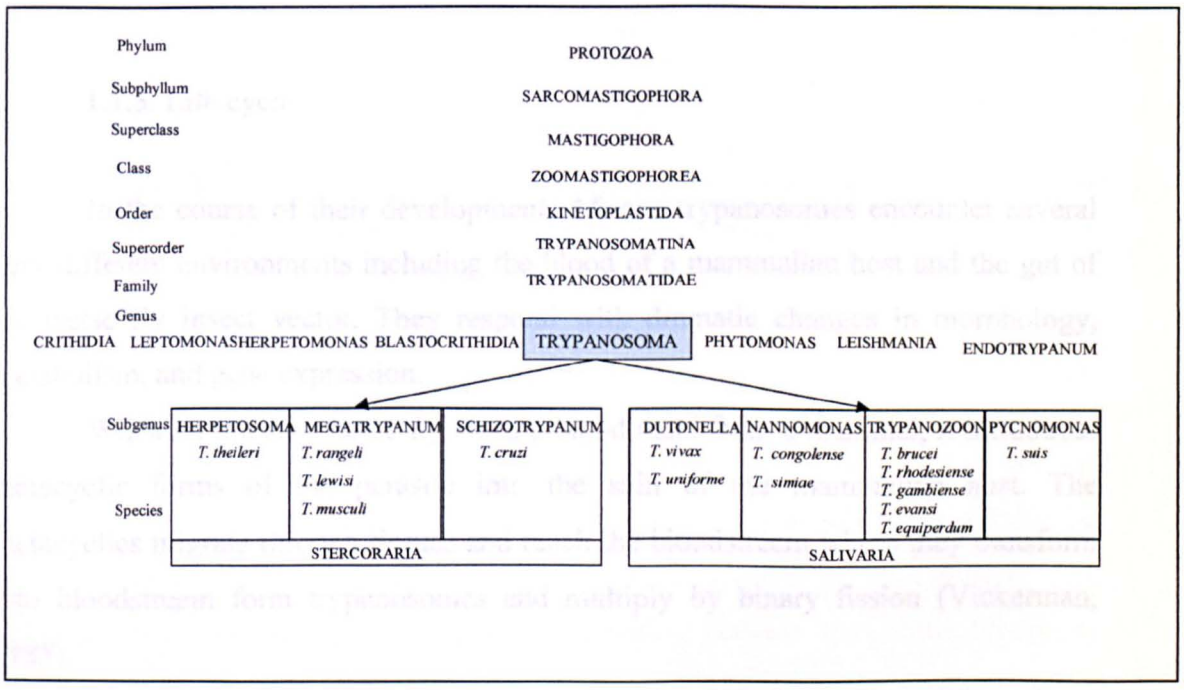


Figure 1.1: A) **An evolutionary tree of life** (copied from Pace *et al.*, 1986) B) Phylogenetic tree representing members of the Kinetoplastidae

This subdivision is based on where the parasite proliferates in the insect. The Stercoraria develop in the hindgut of their insect vector and the Salivaria develop in the insect foregut and salivary glands. *Trypanosoma brucei* belongs to the Salivaria and *Trypanosoma cruzi* belongs to the Stercoraria (Stevens and Gibson, 1999[a]).

Evolutionary trees suggest a split between Salivarian and all the non-Salivarian trypanosomes followed by a split of the latter into two clades. One clade contains parasites of birds, reptilians, Stercoraria that infect mammals, and a second clade contains trypanosomes of fish and amphibians (transmitted by leeches) (Haag *et al.*, 1998).

There are twenty-four known species of trypanosome and evolutionary trees support the idea of monophyly (a monophylogenic group contains an ancestor and all its descendents) of trypanosomes (Stevens and Gibson, 1999[a]). Phylogenic evidence suggests that the infection of humans by *T. brucei* and *T. cruzi* evolved over very different time scales. *T. brucei* seems to have co-evolved with primates over 15 millions years and with the genus *Homo* over 3 million years. Human infection by *T. cruzi*, however, could not have originated more than 30, 000-40, 000 years ago, when humans started to migrate to the Americas (Stevens and Gibson, 1999[b]).

1.1.3. Life cycle

In the course of their development, African trypanosomes encounter several very different environments including the blood of a mammalian host and the gut of the tsetse fly insect vector. They respond with dramatic changes in morphology, metabolism, and gene expression.

When an infected tsetse fly takes a blood meal from a mammal, it introduces metacyclic forms of the parasite into the skin of the mammalian host. The metacyclics migrate through tissues and reach the bloodstream where they transform into bloodstream form trypanosomes and multiply by binary fission (Vickerman, 1985).

In the bloodstream, the trypanosomes initially injected as metacyclic forms by the insect transform into two different forms: a proliferative **long-slender form** and a non-proliferative **short-stumpy form**. A characteristic of trypanosomal infections is the fluctuation of the parasitemia due to the ability of the parasites to evade the host's

immune system. This evasion is achieved by modification of the surface coat of the parasite made of variable surface glycoprotein (VSG). This process is named **antigenic variation** (Turner *et. al*, 1999). The slender forms divide and the parasite population increases reaching a peak. Above a critical cell density the population of slender forms initiates the differentiation into stumpy forms which are incapable of division. The humoral immune response produced by the host induces a clearance of the slender forms first and then the stumpy forms. The parasites that have undergone an antigenic switch can proliferate and create a new peack of parasitemia (being ahead of the immune system) (Matthews, 1999).

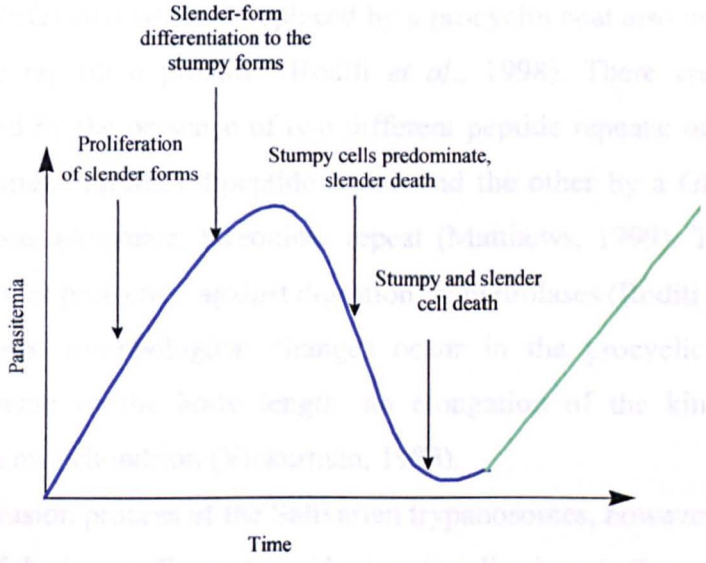


Figure 1.2: Representation of the different phases of the course of a *Trypanosoma brucei* bloodstream parasitemia. A first wave of parasitemia consisting in three phases: 1) proliferation, 2) a maximum parasitemia, 3) a decrease of parasitemia as a result of the antibody-mediated clearance is represented in blue. In green is an outgrowth of slender form parasites that have undergone antigenic switching (modified from Mathews, 1999).

The stumpy forms are unable to divide in the bloodstream but they are the only forms capable of continuing their life cycle in the vector (Matthews, 1999). On the other hand the slender forms are very well adapted to the glucose rich environment of the bloodstream. This differentiation between two forms present in the bloodstream enables the parasite to fulfil two vital requirements: **transmission to the vector** and **proliferation**. The life cycle of *T. brucei* offers a good illustration of the characteristics of parasitism defined earlier: the parasite is able to adapt quickly to

a new environment (infectiousness), it can grow and persist inside its host (establishment) and be transmitted (via the stumpy forms).

The transmission of the parasite from its mammalian host to another tsetse fly takes place when the insect takes a blood meal from an infected mammal. The passage from the blood environment to the midgut of the fly provokes the death of the slender forms and the transformation of the stumpy forms into **procyclic** forms. This transformation occurs in the posterior part of the midgut of the insect and involves dramatic changes for the parasite.

One of the major changes at this stage is the **loss of the variable surface glycoprotein** (VSG) coat which is replaced by a procyclin coat also named PARP for procyclic acidic repetitive proteins (Roditi *et al.*, 1998). There are two types of procyclin defined by the presence of two different peptide repeats: one is composed of an EP (glutamate, proline) dipeptide repeat and the other by a GPEET (glycine, proline, glutamate, glutamate, threonine) repeat (Matthews, 1999). The role of this PARP coat includes protection against digestion by hydrolases (Roditi *et al.*, 1998).

Additional morphological changes occur in the procyclic forms. These include an increase of the body length, an elongation of the kinetoplast and a branching of the mitochondrion (Vickerman, 1985).

The invasion process of the Salivarian trypanosomes, however, does not stop in the midgut of the insect. From the midgut, procyclics invade the oesophagus, then the mouthparts before reaching the salivary duct and then the salivary glands. The glands contain a population of four different stages of trypanosomes. The **epimastigote** stage is a highly proliferative form of trypanosomes which attaches by their flagellum to the epithelial cells of the gland lumen. The **trypomastigote**, also called the pre-metacyclic stage, are dividing forms with reduced flagellipodia (dendritic outgrowths of the flagellum) (Vickerman, 1985). The **metacyclic** stage in which the mitochondrion is unbranched and the glycosomes are more round. **Mature metacyclics** are covered in a coat of variable antigens and are unable to divide. Mature metacyclics reside free in the lumen of the gland, which distinguishes them from nascent metacyclics, and the mature forms are ready to be injected into the host. An infected tsetse fly can harbor 50, 000 metacyclics (Donelson and Rice-Ficht, 1985).

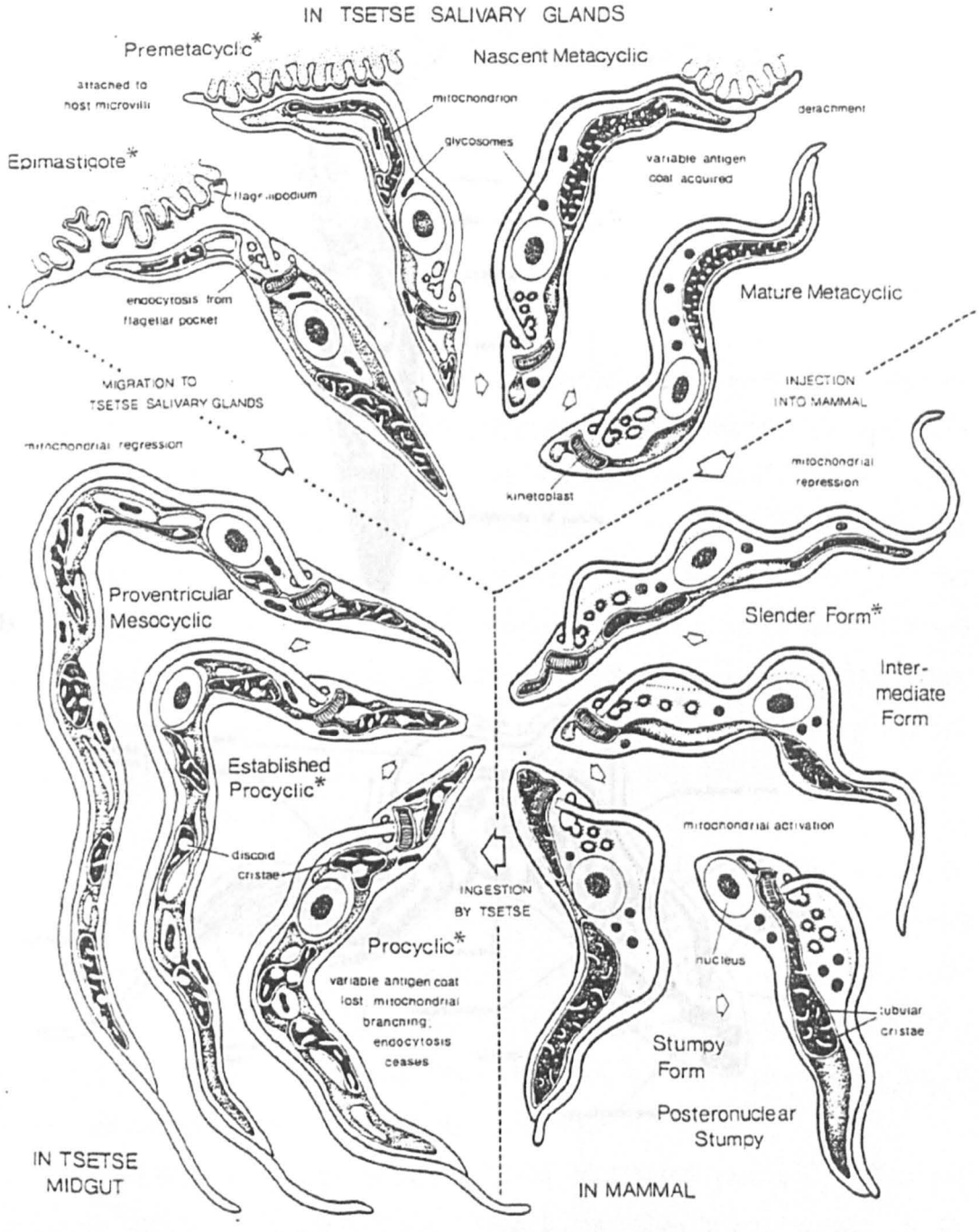
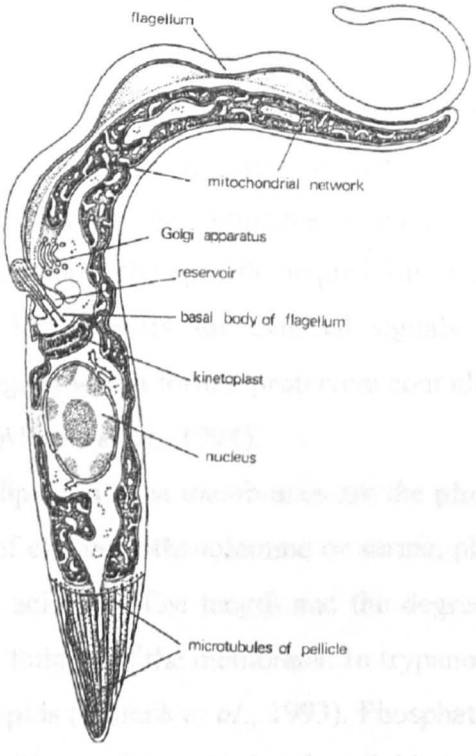


Figure 1.3: Schematic diagram of the *Trypanosoma brucei* developmental cycle in mammals and the tsetse fly (copied from Vickerman, 1985).

1.1.4. Morphology, molecular biology and biochemistry.

A



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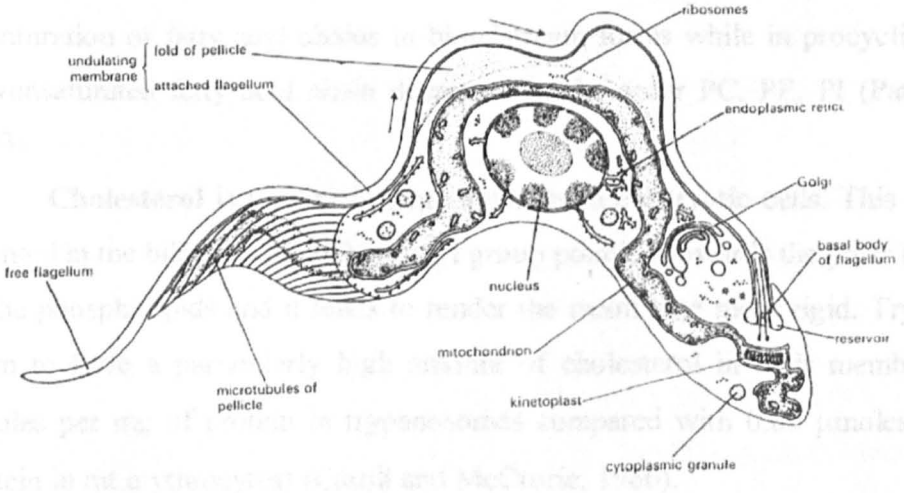


Figure 1.4: Diagram of *Trypanosoma brucei rhodesiense* as seen at the level of the electron microscope A) epimastigote stage B) bloodstream trypomastigote (copied from Mulligan, 1970).

1.1.4.1. Plasma membrane:

In any cell type, the plasma membrane encloses the cell and maintains the essential difference between the cytosol and the extracellular environment. It is a dynamic structure composed of lipids arranged as a bilayer which provides the basic structure of the membrane and creates a barrier to the entry of most hydrophilic molecules. The plasma membrane also contains a series of different proteins, including transporters, which allow the specific acquisition of some nutrients by the cell, receptors which act like sensors for external signals and, in the case of trypanosomes, variable antigens which form a protective coat allowing the parasite to avoid the immune system (Alberts *et al.*, 1994).

The most abundant lipids in most membranes are the **phospholipids**. They are composed of a polar head of choline, ethanolamine or serine, phosphate and glycerol and of a hydrophobic fatty acid tail. The length and the degree of saturation of the fatty acid tail determine the fluidity of the membrane. In trypanosomes, phospholipids represent 80% of the total lipids (Patnaik *et al.*, 1993). Phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE) are the most abundant lipids in both bloodstream and procyclic forms. There is no significant difference in the chain length or in the degree of saturation of fatty acyl chains in bloodstream forms while in procyclic forms the polyunsaturated fatty acid chain decreases in the order PC, PE, PI (Patnaik *et al.*, 1993).

Cholesterol is present in large amounts in eukaryotic cells. This molecule is oriented in the bilayer with its hydroxyl group pointing towards the polar head groups of the phospholipids and it tends to render the membrane more rigid. Trypanosomes seem to have a particularly high amount of cholesterol in their membranes (13.8 μmoles per mg of protein in trypanosomes compared with 0.89 μmoles per mg of protein in rat erythrocytes) (Caroll and McCrorie, 1986).

Glycolipids are sugar-containing lipid molecules present in the non cytoplasmic phase of the lipid bilayer. Gangliosides, the most complex of the glycolipids, have been found at an exceptionally high level in trypanosomes comparable to the level of phospholipids (344 and 359 μg per mg of protein respectively for the gangliosides and the phospholipids) (Caroll and McCrorie, 1986).

Trypanosoma brucei has a very limited capacity to synthesized fatty acids and depends mainly on exogenous sources.

The membrane bilayer also contains numerous proteins associated in different ways to the plasma membrane. Some proteins are embedded in the bilayer as α-helices non-covalently attached to the lipids. An example, in trypanosomes of proteins forming a **single helix** in the bilayer are those belonging to the family of adenylate/guanylate cyclases which is probably a receptor family, transducing host derived signals (Borst and Fairlamb, 1998). **Multipass proteins** are represented mainly by channels which allow ions to cross the membrane and by transporters for the uptake of specific nutrients.

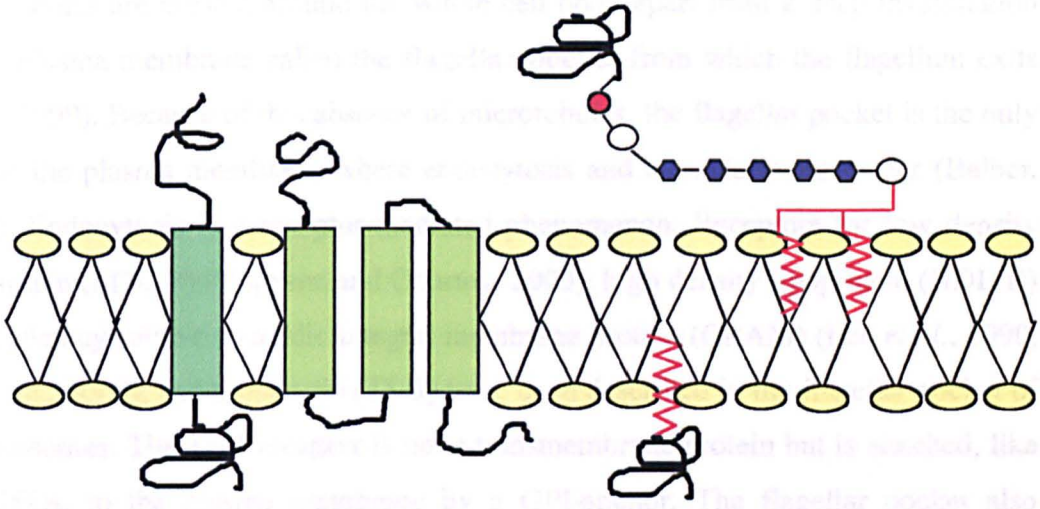


Figure 1.5 : Ways of association of membrane proteins with the lipid bilayer. 1) single pass 2) multipass 3) covalently attached lipid or 4) via a oligosaccharide attached to a phospholipid. (Modified from Alberts *et al.*, 1994).

Other membrane proteins are simply attached to the membrane by a **fatty acid** molecule (myristyl anchor) or a prenyl group (farnesyl anchor) present in their structure.

Most proteins are anchored to the membrane via an oligosaccharide attached to a phospholipid, like **glycosylphosphatidylinositol** (GPI) (Ferguson, 1999). This mode of attachment to the membrane is, in trypanosomes, of particular interest because it is the mode of attachment of the variant surface glycoprotein (VSG). Bloodstream form trypanosomes are entirely covered by a densely packed single

species of VSG. These VSGs form a monolayer acting as a physical barrier protecting the plasma membrane against the components of the humoral immune response (Turner, 1999). The protection against the specific immune response is achieved through the switching of expression between VSG genes. The switching rate is very high: 1 in 100 trypanosomes switch their VAT per generation. Each VSG is about 500 amino acids long and is composed of an N-terminal signal peptide of about 20 amino acids, a variable region (360 amino acids) and a C-terminal region of about 120 amino acids. The C-terminal portion is in contact with the membrane and the N-terminal part is the antigenic determinant (Donelson and Rice-Fitch, 1985).

A corset of cross-linked microtubules underlies the plasma membrane. These microtubules are present around the whole cell body apart from a deep invagination in the plasma membrane called the **flagellar pocket** from which the flagellum exits (Gull, 1999). Because of this absence of microtubules, the flagellar pocket is the only part of the plasma membrane where endocytosis and exocytosis can occur (Balber, 1990). Endocytocis is a receptor-mediated phenomenon. Receptors for low density lipoprotein (LDL-R) (Coppens and Courtoy, 2000), high density lipoprotein (HDL-R) also called cysteine-rich acidic integral membrane protein (CRAM) (Lee *et al.*, 1990, Liu *et al.*, 2000), and transferrin (Tf-R) have been described in the flagellar pocket of trypanosomes. The Tf-R receptor is not a transmembrane protein but is attached, like the VSGs, to the plasma membrane by a GPI-anchor. The flagellar pocket also harbours a putative receptor for the trypanosome lytic factor which appears to kill trypanosomes by generating peroxides (Hager *et al.*, 1994, Molina Portela *et al.*, 2000).

Thus the plasma membrane of trypanosomes appears to be much more than a simple structure enclosing the cytosol. It is a very elaborate and dynamic system combining two functions: a barrier protecting the trypanosome against the host immune system and gateway for nutrients.

1.1.4.2. The key cytosolic metabolic pathways

Amino acid and S-Adenosylmethionine metabolism:

Amino acids metabolism has been poorly studied in trypanosomes and there is a paucity of data on this subject.

Chappell *et al.* (1972) measured free amino acids in the cytosol of bloodstream forms of *T. b. gambiense*. **Alanine** was the most abundant amino acid and represents 47 % of the free pool. Incubation of the parasite in the presence of radiolabelled glucose led to the incorporation of radiolabel into 70 % of the alanine pool. Glutamate represents the second most abundant amino acid (12 %), followed by arginine (8.2 %) and taurine (6.2%). The aromatic amino acids (phenylalanine and tyrosine), methionine, glutamine, proline, valine were detected at trace levels only (Chappell *et al.*, 1972).

Cysteine is an essential growth factor for bloodstream form trypanosomes and is involved in protein biosynthesis, and in the production of glutathione and trypanothione (Duszenko *et al.*, 1992).

Methionine, apart from its role in protein synthesis is also adenosylated by S-adenosylmethionine synthase to form S-adenosylmethionine (Bacchi *et al.*, 1995). S-Adenosylmethionine is an important metabolite in trypanosomes as in its decarboxylated form it is the source of aminopropyl groups for polyamine synthesis, and it is also a donor of methyl groups for protein and lipid methylation ([a]Golberg *et al.*, 1997). Methionine is used at least ten times more in methylation reactions than it is incorporated into protein ([b]Goldberg *et al.*, 1997). The transfer of a methyl group occurs when S-adenosylmethionine is transformed to S-adenosylhomocysteine by a methyltransferase. S-adenosylhomocysteine is then hydrolysed to homocysteine and adenosine and methionine is regenerated by transfer of a methyl group from N-methyltetrahydrofolate to homocysteine by homocysteine methyltransferase (Bacchi *et al.*, 1995).

Aromatic amino acids (tyrosine, phenylalanine, tryptophan) are involved in recycling the methionine pool by transamination to α-ketomethiobutyrate (KMTB) (Berger *et al.*, 1996).

Proline is the preferred oxidizable substrate for the procyclic forms (Evans and Brown, 1972). The proline ring is opened and oxidized to α-ketoglutarate and subsequently to succinate which enters the Krebs' cycle.

Polyamine biosynthesis:

Putrescine and spermidine are present in procyclic and bloodstream forms of *T. brucei*. Unlike *Trypanosoma cruzi*, *T. brucei* has very little or no spermine. The polyamine biosynthetic pathway begins with the decarboxylation of ornithine by onithine decarboxylase (ODC) to form putrescine. Spermidine is then formed by the transfer of an aminopropyl group coming from the decarboxylated form of S-adenosylmethionine (dAdoMet) (Fairlamb and Le Quesne, 1997).

The role of polyamines in trypanosomes is not known but inhibitors of ODC like D,L-α difluoromethylornithine lead to an arrest of trypanosomal growth. Moreover, polyamine biosynthetic pathway is critical in the synthesis of a unique thiol-spermidine conjugate called **trypanothione** (Fairlamb and Le Quesne, 1997) in these organisms.

Thiol metabolism:

As a defense against reactive oxygen molecules (which arise internally through respiration or from the host), African trypanosomes possess a low molecular weight thiol conjugate that plays a similar role to that of glutathione in mammals. This molecule, called trypanothione, consists of two glutathione molecules linked by a spermidine moiety. The biosynthetic pathway of trypanothione comprises two steps: 1) the formation of glutathionylspermidine from spermidine and glutathione by glutathionylspermidine synthetase, 2) the formation of trypanothione from glutathionylspermidine and glutathione by trypanothione synthetase (Fairlamb, 1990).

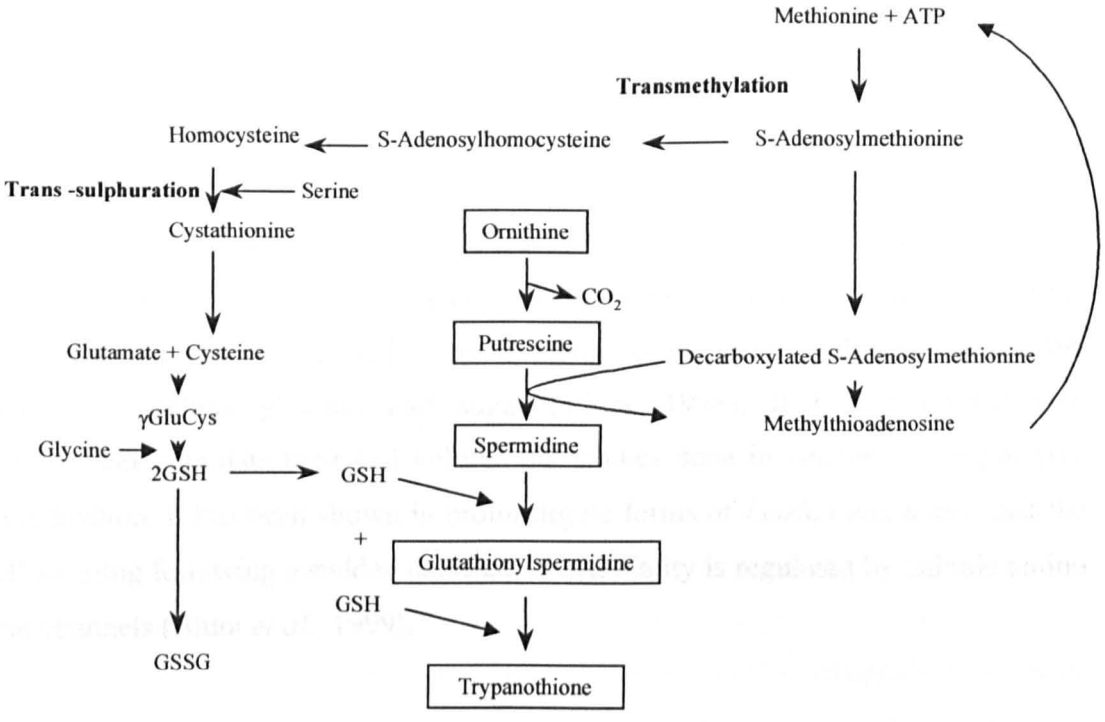


Figure 1.6: Polyamine biosynthetic pathway in African trypanosomes. GSH: glutathione.

The pentose-phosphate pathway:

The pentose phosphate pathway is a metabolic pathway in which glucose 6-phosphate is converted to ribose 5-phosphate and provides NADPH. This later serves as a hydrogen donor important in biosynthetic processes but also plays an important role in the defence against oxidative stress. The first three enzymes of this pathway, glucose-6-phosphate dehydrogenase, 6-phosphogluconolactonase and 6-phosphogluconate dehydrogenase have been cloned and characterised. Most of the enzymes of the pentose phosphate pathway are present in the cytosol, however the three first enzymes of the pathway seem to be mainly located in the glycosome (Barrett, 1997, Duffieux *et al.* , 2000).

Most of the enzymes involved in these pathways are pH sensitive and the parasite needs to regulate its cytoplasmic pH. Unlike in *Leishmania* where the process is regulated by the combined action of H^+ pumps and Cl^- channels, trypanosomes appear to regulate their pH mainly by the H^+ pump (Viera, 1998).

Like any other cell type, trypanosomes need to have the ability to regulate their cell volume in response to hypo or hyper-tonicity. In plants, fungi and lower eukayotes this process is achieved utilizing amino acids (alanine, glutamate, glutamine, proline, glycine) and sugar (Viera, 1998). It is not known how trypanosomes regulate their cell volume but studies done in *Leishmania* might give an indication. It has been shown in promastigote forms of *Leishmania major* that the cell swelling following a sudden decrease in osmolality is regulated by anionic amino acid channels (Blum *et al.*, 1999).

1.1.4.3. The glycosomes

Glycosomes are organelles found in all members of the order Kinetoplastida. In trypanosomes they are present in procyclic and bloodstream forms and represent approximately 4 % of the total volume of the cell (Vickerman, 1985). It appears that these organelles, surrounded by a single membrane, are related to peroxisomes of other eukaryotes. The term glycosome was coined to reflect the fact that these organelles contain the first seven enzymes of glycolysis (Michels *et al.*, 2000). Bloodstream forms, because of the lack of a functional Kreb's cycle and respiratory chain, are entirely dependent on glycolysis for ATP production and most of the glycolytic enzymes are localized in the glycosomes.

Glucose is the main energy source but fructose, mannose or glycerol can be used as alternatives. All of the enzymes involved in the glycolytic pathway from glucose to 3-phosphoglycerate are located in the glycosome while the last three enzymes leading to the production of pyruvate are cytosolic. Thus, net ATP synthesis occurs only in the cytosol.

The reason why most of the glycolytic enzymes are compartmentalized is still a subject of interrogations (Michels *et al.*, 2000).

Pathways other than glycolysis are also present in glycosomes for example some enzymes involved in β-oxidation of fatty acids have been found in glycosomes as well as enzymes for purine salvage, pyrimidine biosynthetic, ether-lipid and sterol pathways (Michels *et al.*, 2000, Clayton and Michels, 1996).

Thus, it seems reasonable to envisage glycosomes not as an organelle devoted only to glycolysis but also involved in a range of other metabolic pathways.

1.1.4.4. The mitochondrion

Trypanosomes are unusual in that they possess only a single mitochondrion. This mitochondrion undergoes major changes during the life cycle of the parasite. In procyclic forms the mitochondrion is fully developed into a branched network of inner membrane foldings (cristae), while in bloodstream forms the mitochondrion is reduced to a linear form lacking cristae. The relative mitochondrial volume decreases from 25 % to 5 % of the total volume of the cell during this transformation (Vickerman, 1985).

In procyclic forms the respiratory chain (cyanide sensitive) is present and coupled to oxidative phosphorylation as well as a fully functional Kreb's cycle leading to the production of CO_2 as an end product (Clayton and Michels, 1996). In procyclic forms, the end product of glycolysis is pyruvate which is subsequently metabolized inside the mitochondrion and transformed to acetyl-CoA. Some acetyl-CoA then enters the Krebs' cycle and some is metabolized into acetate by an acetate/succinate CoA transferase generating ATP (Tielens and Van Hellemond, 1998).

In bloodstream forms a functional Krebs' cycle is absent as well as the classical respiratory chain (Chaudhuri *et al.*, 1998). However a plant like alternative oxidase is present. This enzyme is insensitive to cyanide but sensitive to salicyhydroxamic acid (SHAM). The FAD linked glycerol-3-phosphate dehydrogenase (which converts glycerol 3-phosphate, derived from glycolysis, to dihydroxyacetone phosphate) and the complex I of NADH-dehydrogenase are both electron donors for the ubiquinone - ubiquinol pool. This pool then acts as the electron donor for the alternative oxidase (which converts O_2 into H_2O) without ATP

production. However NADH-dehydrogenase does pump a proton out of the inner membrane of the mitochondrion thus generating an electromotive force across the membrane (Tielens and Van Hellemond, 1998).

The trypanosome alternative oxidase is encoded by a single copy gene and the transcription of this gene is down regulated during the differentiation of bloodstream to procyclics (Chaudhuri *et al.*, 1998).

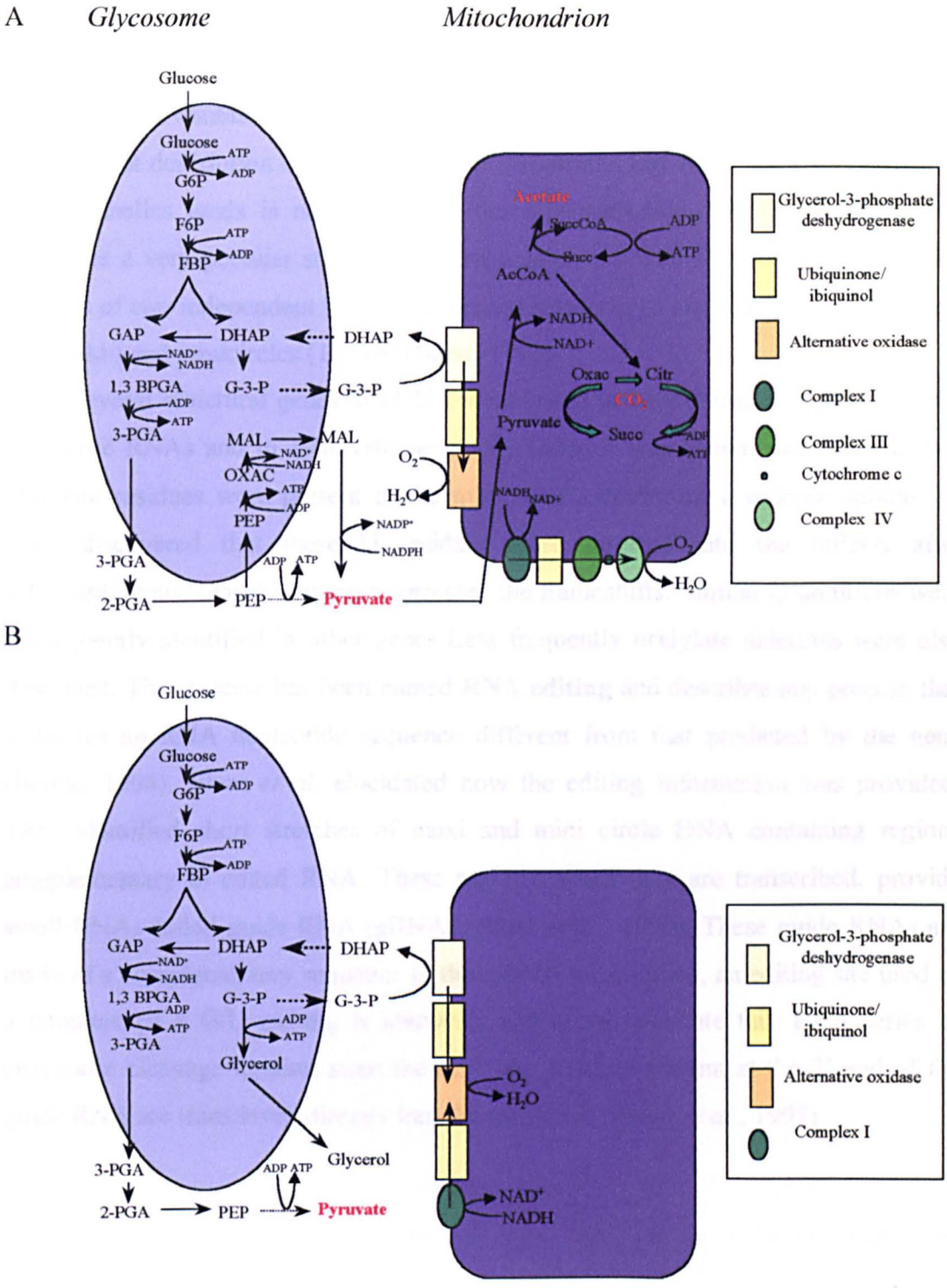


Figure 1.7: Major pathways of carbohydrate degradation in *T. brucei* procyclic (A) and bloodstream foms (B). End products are shown in red. Abbreviations: AcCoA, acetyl-CoA; Citr, citrate; DHAP, dihydroxyacetone phosphate; FBP, fructose 2,6-bisphosphate; F6P, fructose-6-phosphate; GAP, glyceraldehyde 3-phosphate; G-3-P, glycerol 3-phosphate, G6P, glucose 6-phosphate, MAL, malate; Oxac, oxaloacetate, PEP, phosphoenolpyruvate; Pyr, pyruvate; Succ, succinate; SuccCoA, succinyl-CoA, 1,3 BPGA, 1,3-biphosphoglycerate; 2-PGA, 2-phosphoglycerate; 3-PGA, 3-phosphoglycerate (Modified from Tielens and Van Hellemond, 1998).

Most mitochondrial proteins are encoded by nuclear genes, however the mitochondrion contains an additional genome called the **kinetoplast**. The kinetoplast was the first description of the existence of an extranuclear DNA and the presence of this organelles lends is name to the order Kinetoplastida. The kinetoplast DNA possesses a very peculiar structure and replication process. The structure of kDNA consists of two independent but intercatenated networks of circular DNA called maxi (20-40 kb) and minicircles (1-3 kb) (Borst, 1991).

Several structural genes in kDNA were noted to have frameshifts with respect to mature RNAs and in 1986 (Benne *et al.*, 1986) it was shown that non-encoded uridylate residues were present in the mRNA of cytochrome c oxidase subunit II. They discovered that these U residues were inserted into the mRNA after transcription and that this process corrected the frameshifts. Similar U-additions were subsequently identified in other genes Less frequently uridylate deletions were also described. This process has been named **RNA editing** and describes any process that generates an RNA nucleotide sequence different from that predicted by the gene (Benne, 1994). Blum *et al.* elucidated how the editing information was provided. They identified short stretches of maxi and mini circle DNA containing regions complementary to edited RNA. These regions, when they are transcribed, provide small RNAs called guide RNA (gRNA) (Blum *et al.*, 1990). These guide RNAs are made of a complementary sequence to the mRNA to be edited, an editing site used as a template (if a G/U pairing is allowed), and a polyuridylate tail. By a series of enzymatic cleavage-ligation steps the uridylate residues present at the 3' end of the guide RNA are transferred directly into the transcript (Stuart *et al.*, 1997).

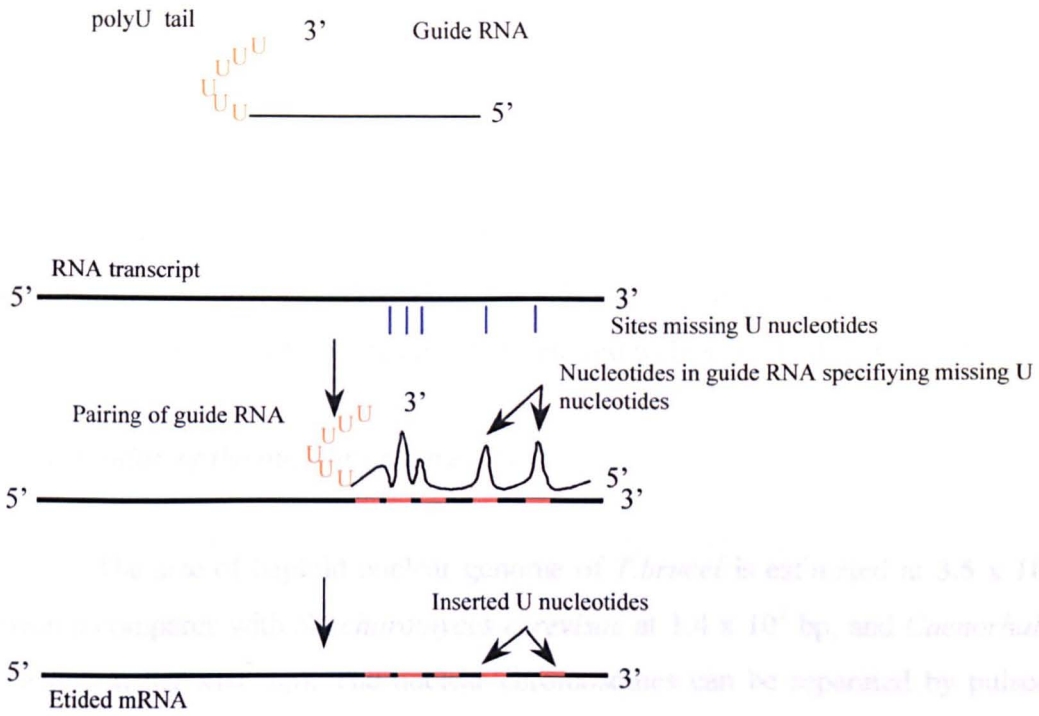


Figure 1.8: RNA editing in the mitochondria of trypanosomes. Guide RNAs contain a stretch of poly U at their 3' end. U nucleotides are donated to sites on the RNA transcript that mispair with the guide RNA. Editing generally starts near the 3' end and progresses towards the 5' end of the RNA transcript (modified from Alberts *et al.*, 1994).

1.1.4.5. Acidocalcisomes.

Bloodstream and procyclic trypanosomes possess an acidic compartment involved in the storage of Ca^{2+} named the **acidocalcisome**. The acidic component of these vacuoles is generated by the presence of a H^+-ATPase and the uptake of Ca^{2+} occurs in exchange for H^+ and is coupled to a Ca^{2+} ATPase. Ca^{2+} release occurs in exchange for H^+ by a Ca^{2+}/H^+ antiporter and is favoured by a Na^+/H^+ antiporter (Vercesi and Docampo, 1996).

1.1.4.6. The nucleus.

The nucleus of *T. brucei* is made of an envelope containing numerous nuclear pore complexes and a nucleoplasm containing a large nucleolus of 0.5-1 μm. Chromatin and histones (H2A, H2B and H4) are present in both forms. Bloodstream forms seem to be more chromatin dense than procyclics (Ersfeld *et al.*, 1999).

Organization of the nuclear genome:

The size of haploid nuclear genome of *T.brucei* is estimated at **3.5 x 10^7 bp** (which compares with *Saccharomyces cerevisae* at 1.4 x 10^7 bp, and *Caenorhabditis elegans* at 9.7 x10^7 bp). The nuclear chromosomes can be separated by pulsed gel electrophoresis (PFGE) and they have been grouped into three general classes according to their sizes. The **megabase chromosomes** (1 to 6 Mb), the **intermediate chromosomes** (from 200 to 900 kb) and the **minichromosomes** (50-150 kb) (El-Sayed *et al.*, 2000).

There are 11 pairs of megabase chromosomes carrying most active genes with the exception of *VSGs* and *ESAGs* (expression site-associated genes).

Intermediate chromosomes have an uncertain ploidy and a variation in number and sizes between stocks. There are between 1 and 5 intermediate chromosomes of 200 to 900 kb and they are known to contain VSG expression sites (*VSG-ES,* B-ESs for bloodstream forms and M-ESs for metacyclic forms) (El-Sayed *et al.*, 2000).

Ninety percent of the sequence of the minichromosomes is made of a 177 bp repeat of an unknown function. Between one of these repeats and one telomere, two GC (74 bp) rich domains are found separated by an AT rich domain of 155 bp. These telomeric regions contain conserved repeat elements that are highly susceptible to recombination and may enable VSG gene copies (also present on minichromosomes) to translocate to potential expression sites on large chromosomes (Ersfeld *et al.*, 1999).

There are approximately a thousand VSG genes (*VSGs*) in the genome of *Trypanosoma brucei* and only one gene is expressed at the time. The silent genes are mainly located at telomeric regions of the minichromosomes.

A typical expression site comprises a promoter, followed by at least eight expression site associated genes (ESAGs), repeats of a 76 bp unit and finally the *VSG*. The VSG genes move into and out of the expression site by different molecular mechanisms (see for review Borst *et al.*, 1998; Cross *et al.*, 1998).

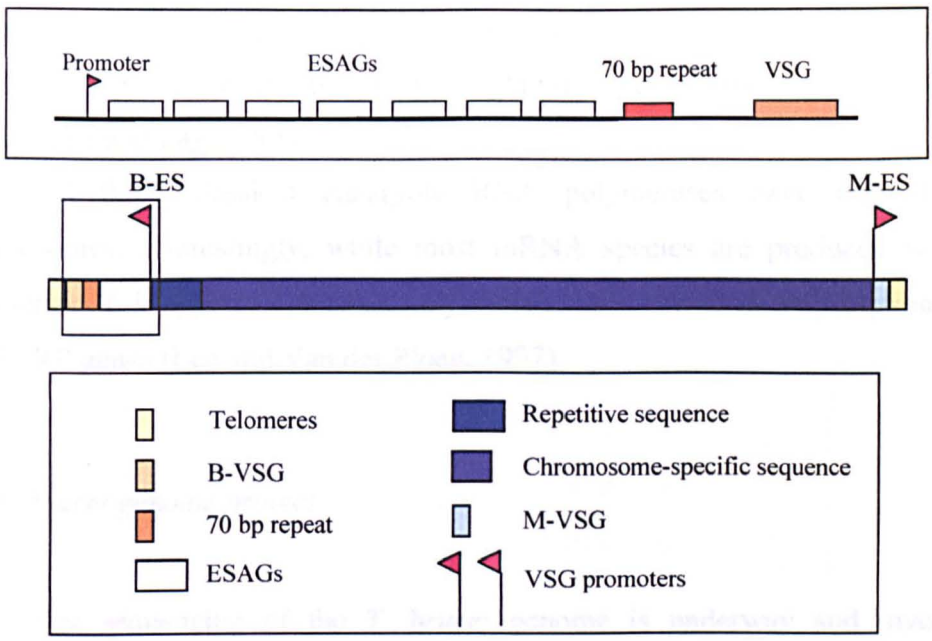


Figure 1.9: Schematic organisation of a *T. brucei* megabase chromosome. Each telomeric end harbours a variant glycoprotein (VSG) expression site (ES). The diagram shows a bloodstream form ES (B-ES) at the left end, containing a repetitive sequence adjacent to the VSG gene, co-transcribed genes (expression site-associated genes, ESAGs) and a distant promoter; a metacyclic ES (M-ES) is shown at the right end, with fewer ESAGs and a *VSG*- specific promoter. The B-ES is separated from the remainder of the chromosome by large tracts of repeated sequences. The housekeeping genes are found in the mainly chromosome specific core of the chromosome.

Gene expression

In trypanosomes, genes are often organized in large clusters of repeats of particular open reading frames (glucose transporter genes are an example) (Vanhamme and Pays, 1995). Trypanosome genes lack the presence of introns. Transcription of arrays leads to the production of long polycistronic units of pre-mature RNA. This polycistronic organization might relate to the fact that few promoters have been found in trypanosomes (ribosomal, procyclin, and VSG genes) (Vanhamme and Pays, 1995).

The transformation of primary transcript into mature mRNA requires the addition of a cap at the 5' end and a poly(A) tail at the 3'end. The capping process is known as ***trans* splicing**. Small nuclear RNA ensures the addition at the 5'end of the cleavage site of a pre-capped spliced leader. There is no known consensus signal for polyadenylation but the two events are coupled. The choice of the splicing site (a polypyrimidine stretch in the intergenic region) of the downstream transcript seems to determine the choice of the polyadenylation site of the upstream transcript (Vanhamme and Pays, 1995).

All three classical eucaryote RNA polymerases have been found in trypanosomes. Interestingly, while most mRNA species are produced with RNA polymerase II, it appears that RNA polymerase I is involved in transcription of VSG and PARP genes (Lee and Van der Ploeg, 1997).

The T. brucei genome project

The sequencing of the *T. brucei* genome is underway and involves the collaboration of at least ten different laboratories. The coordination is under the responsibility of the WHO /Special Programme for Research and Training in Tropical diseases (TDR). This network involves different approaches leading to different types of databases.

- Sequences of cDNA from bloodstream and procyclic forms of trypanosomes can be found in the **expressed sequence tags** databases (dbEST).
- Random **genomic sequences** can be found in EMBL or GENBANK (75% of the genome has been sequenced to date).
- A **chromosome sequencing** project has started at the Sanger Center (http://www.sanger.ac.uk/) and at TIGR (http://www.tigr.org).

Table 1: Status of the *T. brucei* chromosome sequencing project.

Chromosome number	Sequencing status	Sequencing center
I	Complete	Sanger center
II	Almost complete	TIGR
III	Not started	TIGR
IV	In progress	TIGR
V	Not started	TIGR
VI	In progress	TIGR
VII	Not started	TIGR
VIII	Not started	TIGR
IX	Not started	Sanger center
X	Not started	Sanger center
XI	Not started	Sanger center

- Four genomic libraries containing DNA from *T. brucei* TREU927 are in use and high-density filters and clones from a bacteriophage P1 library and a cosmid library are now available.

- A **genetic map** is also under construction.

1.1.5. Sleeping sickness:

1.5.1. An overview of the history of sleeping sickness

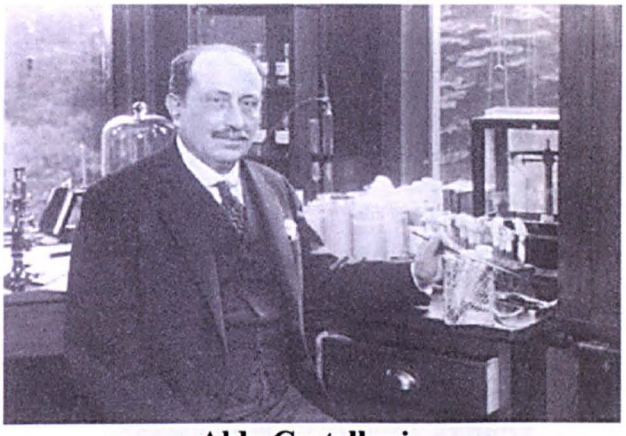
Before the etiology of sleeping sickness was understood it was already known that the bite of the tsetse fly was "poisonous" and long term lethargic effects occurring after the bite were described by Livingstone in 1857. At this time trypanosomes had already been described by Gruby in 1843 in the blood of a salmon. The name "trypanosome" was given to this organism whose motion suggested the movement of a corkscrew. The first observations of the parasite in mammalian blood were made by Lewis (1879) and Evans (1881) who observed trypanosomes respectively in the blood of rats (*Trypanosoma lewisi*) and horses and camels (*Trypanosoma evansi*).

It was David Bruce in 1895 who correlated the presence of trypanosomes in the blood of animals and the disease called nagana. Bruce also elucidated the role of the tsetse fly as the vector from which transmission occurs. David Bruce had been called by the Governor of Zululand to investigate the nature of *nagana* decimating the livestock of the country. The mission was a real success (Cox, 1996).

However the correlation between nagana and sleeping sickness in humans was not immediately clear. Aldo Castellani was sent to Entebbe to investigate the bacterial cause of sleeping sickness. Castellani's hypothesis was that streptococci were responsible for the disease. However in 1903, he observed the presence of trypanosomes in the bloodstream and the cerebrospinal fluid of patients with sleeping sickness. At that time David Bruce was sent to take control of the investigation and a controversy arose as to whether Bruce or Castellani was first to identify trypanosomes as the etiologic agent of sleeping sickness (Vickerman, 1997).

David Gruby
(1810-1898)

Griffiths Evans
(1855-1935)

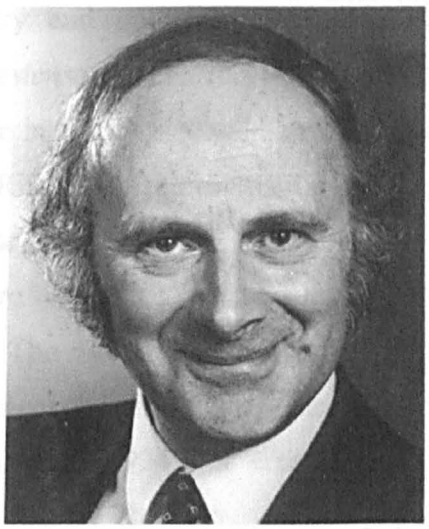


Aldo Castellani
(1878-1971)

David Bruce
(1855-1931)

Figure 1.10: Photographs of the key figures involved in the discovery of trypanosomiasis and the description of the parasite (These photos belong to the Wellcome Institute Library).

At the beginning of the twentieth century the cause and the mode of transmission of the disease had been elucidated. In the second half of the century that interest in sleeping sickness regained interest and investigations on the biochemistry, immunology and molecular biology of the parasite started. Professor Keith Vickerman pioneered these studies has to be cited. His detailed work on electron microscopy revealed the structure of trypanosomes throughout their life cycle and led to the discovery of a coat of antigens at the surface of the parasite responsible for antigenic variation (Vickerman, 1969). These antigens were later characterised biochemically by George Cross (Cross, 1975).



Keith Vickerman

Figure 1.10. *(continued) (Personal source)*

1.1.5.2. Geographical distribution and prevalence

The disease is distributed over 36 African countries situated in the sub-equatorial part of Africa between 14° North and 29° South of the equator (Kuzoe, 1993). The distribution of the disease follows the distribution of the vector, *Glossina*, more commonly called the tsetse fly. In West and Central Africa, the endemic species of tsetse belong to the *Palpalis* group with three species *G. palpalis*, *G. tachinoides* and *G. fucipes*. These species of tsetse fly require a sustained level of humidity and have a preference for the riverine habitat. They transmit *Trypanosoma brucei gambiense*. The reservoir of the infection is mainly humans (Smith *et al.*, 1998). In East Africa, the transmission is due to the presence of tsetse fly from the *Morsitans* group: *G. morsitans*, *G. pallipides* and *G. swynnertoni*. These species of tsetse fly have a preference for dry and open areas of savannah. They are vectors of *Trypanosoma brucei rhodesiense*. The reservoir of the infection in West and Central Africa is usually man while in East Africa wild and domestic animals also play a role (Smith *et al.*, 1998). The Rift valley represents the geographical separation between the two forms of the disease (Welburn *et al.*, 2001).

Currently there are estimated to be three hundred thousand cases of trypanosomiasis annually. The most severely affected countries are the Democratic Republic of Congo and Angola. In Zaire (now the Democratic Republic of Congo) the prevalence of the disease in 1994 was estimated to be the same as in 1930. Epidemic outbreaks occur due to the movement of populations or interruption of control programmes caused by civil wars or social upheaval (WHO Report, 1998, Barrett, 1999).

Figure 1.11: The geographic distribution of *Trypanosoma brucei gambiense* (in yellow) and *Trypanosoma brucei rhodesiense* (in red). Foci follows closely the western edge of the African Rift Valley, shown here on a digital elevation model of the continent (USCS digital elevation model) (Copied from Welburn *et al.*, 2001)

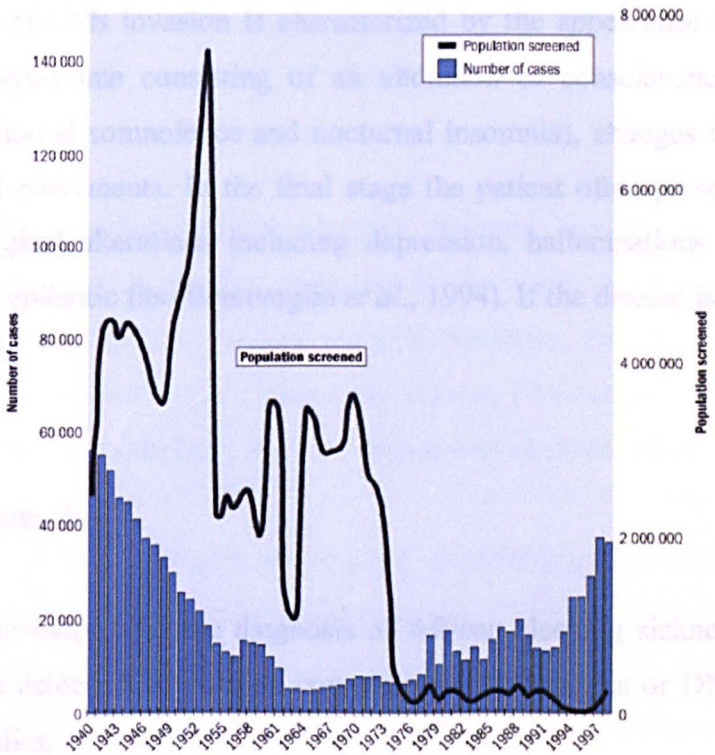


Figure 1.12: Number of reported cases of African trypanosomiasis and population screened between 1940 and 1998 (copied from Chapter 8 of WHO Report on Global Surveillance of Epidemic-prone Infectious Diseases).

1.1.5.3. The disease:

In the days following the bite of an infected tsetse fly, parasites proliferate at the site of infection. The site of injection becomes swollen and presents a self-healing chancre for three to four weeks. The parasite progressively invades the lymph nodes and the bloodstream. In the early stages of the infection the principal clinical symptoms are intermittent fever and a splenomegaly and gangliopathy. Swollen lymph nodes may appear in the neck and are known as Winterbottom's sign.

In the second stage of the disease the parasite invades other organs and especially the central nervous system (CNS). The progression of the disease can take several years after an infection due to *T. b. gambiense*. However, in the case of an infection by *T. b. rhodesiense*, the form of the disease is acute and develops in a matter of weeks.

Trypanosomes are found in several areas of the brain including the thalamus and the hypothalamus and the blood brain barrier (BBB) is usually extensively damaged (Philip *et al.*, 1994).

This phase of CNS invasion is characterized by the appearance of complex neuro-psychiatric syndrome consisting of an alteration of consciousness and the rhythm of sleep (diurnal somnolence and nocturnal insomnia), changes in muscular tone and abnormal movements. In the final stage the patient often presents a large range of psychological alterations including depression, hallucinations and manic episodes as well as epileptic fits (Bentivoglio *et al.*, 1994). If the disease is not treated it is invariably fatal.

1.1.5.4. Diagnosis

The tools developed for the diagnosis of African sleeping sickness fall into two categories: the detection of parasite components like antigens or DNA and the detection of antibodies.

A card indirect agglutination test for trypanosomiasis (CIATT) due to *T. b. gambiense* and *rhodesiense* has been developed and is based on antigen detection

using an indirect latex agglutination assay using whole blood obtained by finger prick. The specificity of this test is evaluated at 99 % (WHO report).

A card agglutination test for trypanosomiasis (CATT) is also available and is based on antibody detection. This test does not identify infections due to *T. b. rhodesiense.*

Positive diagnosis using one of these techniques must be followed by positive identification of the parasite. Positive diagnostic depends upon microscopic identification of the parasite. Different types of samples can be used: tissue fluids, thick blood smears, aspiration of chancres or cervical nodes (WHO Expert Committee, 1998).

The symptoms developed during the first phase of the infection are very general and non-specific to sleeping sickness and a patient suspected to have the disease will probably be in the second phase of infection and have trypanosomes present in the CNS.

1.1.5.5. The control of the disease

Treatment

Drugs in use

There are no vaccines against sleeping sickness and the prospects for prophylactic immunization are severely hampered by the ability of the trypanosomes to escape from the host immune system by antigenic variation. Hence, chemotherapy is the principal means of intervention against the disease. Four drugs are currently in use: **suramin, pentamidine, melarsoprol** and **difluoromethylornithine** (DFMO).

Suramin is a highly negatively charged naphthalene derivative with six sulphonic acid groups which are deprotonated under physiological conditions (Voogd *et al.*, 1993). Such a highly charged molecule would not cross the plasma membrane of the parasite by simple diffusion in its anionic form. Suramin has been shown to bind to numerous serum proteins including albumin and low density lipoprotein (LDL). Two types of LDL receptor have been identified in the flagellar pocket of

bloodstream forms. Thus, LDL receptors have been proposed as the gate of entry of suramin into trypanosomes (Coppens and Courtois, 2000).

The trypanocidal action of suramin has not been identified. Inhibition of some essential enzymes like glycerol-3-phosphate oxidase, protein kinases and phospholipases have been suggested. On the other hand suramin decreases the binding of LDL to the LDL receptor of trypanosomes and thus may interfere with cholesterol and fatty acid import (Coppens and Courtois, 2000).

The side effects of the drug are mainly a febrile reaction to the injection, rash, malaise and more severely renal damage with variable recovery (Voogd *et al*, 1993).

Pentamidine is an aromatic diamidine. The drug probably enters trypanosomes principally via an amino-purine transporter named P2. Loss of this transporter is associated with a cross-resistance to diamidines and melaminophenyl arsenicals. However, two other transporters associated with pentamidine uptake have also been identified (De Koning, 2001).

The parasites accumulate high concentrations of the drug (millimolar concentration) (Carter *et al.*, 1995). The mode of action is not fully understood but pentamidine binds to trypanosomal kinetoplast DNA via specific interactions with sites rich in adenine-thymine bases. The molecule does not cross the blood brain barrier and is used only in the hemolymphatic phase of the disease (Sands *et al.*, 1985).

The side effects of pentamidine after injection are mainly nausea, hypotension, hypo or hyper glycemia. Pain and necrosis can occur at the injection site (Sands *et al.*, 1985).

Melarsoprol is a melaminophenyl based organic arsenical introduced as a drug by Freidheim in 1949. Melarsoprol may enter the trypanosomes by diffusion but also via the P2 nucleoside transporter. Melaminophenylarsenicals have been shown to inhibit the uptake of adenine via the P2 transporter and trypanosomes resistant to sodium melarsen show a loss of the P2 transporter (Carter *et al.*, 1995). The mode of action of the drug has not been elucidated yet. Trypanothione, a trypanosomal equivalent of the mammalian glutathione, has been suggested as a target. Arsenic is

known to form stable interactions with thiols, however only a small fraction of the arsenicals have shown their ability to bind to trypanothione.

Melarsoprol is a lipophilic molecule capable of crossing the blood brain barrier and is one of the few drugs that can be used in the late stage of the disease (Onyango *et al.*, 2000).

Melarsoprol has been used for a long time, and it is highly toxic which illustrates problems imposed by the lack of trypanocidal drugs available on the market. It is administrated by intravenous injection as a 3.6% solution in propylene glycol (Keiser and Burri, 2000). The adverse effects are generally fever, headache, diarrhea, polyneuropathies, exfoliative dermatitis, pruritus and abdominal and chest pains (Burri *et al.*, 2000) but the worst adverse effects are certainly the development of encephalopathy in 10% of the treated patients. These encephalopathies are lethal in 10-50% of cases (WHO, 1998). Until recently the administration of melarsoprol consisted of series of three to four injections of an increasing dose of melarsoprol from 1.2 to 3.6 mg/kg with an interval of seven to ten days between the injections.

This treatment schedule imposed a hospitalization of the patient for twenty-five to thirty-six days, which is a serious drawback for the patients and their families. An alternative schedule has recently been proposed by Burri *et al.* consisting in daily administration of 2.2 mg/kg of melarsoprol over a period of ten days. The treatment showed the same level of efficacy and the same level of adverse effects but a better compliance of the patient probably due to the advantage of a shorter period of treatment (Burri *et al.*, 2000).

Eflornithine, also named difluoromethylornithine (DFMO), is an analogue of ornithine that inhibits ornithine decarboxylase (ODC). Eflornithine inhibits mammalian and trypanosomal ODC but the specificity of the drug against trypanosomes relies on the fact that the trypanosomal enzyme is degraded within the cell and replenished at a much slower rate than in mammals (Bacchi *et al.*, 1983). Thus, the parasite is rapidly depleted in polyamines and this leads to a cessation of parasite growth. The immune system is then capable of killing the growth arrested trypanosomes.

There are several limitations to the use of DFMO: It lacks activity against *T. b. rhodesiense* and its availability is limited (ten times more expensive than

melarsoprol treatment). The WHO has recently reached an agreement with Aventis to produce the drug for use in sleeping sickness. Recently the drug has been included in a formulation that removes facial hair and there is renewed optimism that the drug will become available to treat sleeping sickness.

The side effects of DFMO are limited to an anemia and to a reduction in other blood cell number. DFMO presents another advantage in that it can be administrated orally.

New drugs in development:

The lack of new drugs aginst sleeping sickness has stimulated WHO-TDR to establish three centres to screen new drugs.screening of drugs is an approach that the TDR supports through a financial support at the London School of Hygiene and Tropical Medicine, the Swiss Tropical Institute and the Janssen Research Foundation (Belgium) (TDR report)

Two classes of compound are in pre-clinical development: the triazine derivative SIPI 1029 and a nitroimidazole, megazol.

The putative mode of action of SIPI 1029 is an inhibition of the S-adenosylmethionine decarboxylase. The molecule does not cross the BBB but present a low toxicity as well as an activity against both *T. b. gambiense* and *T. b. rhodesiense* (Keiser *et al.*, 2001).

Megazol is a molecule originally synthesized in 1968 and abandoned because of putative risks of mutagenicity. The product has recently received renewed interest because of its capacity of to cross the BBB (Enanga *et al.*, 2000). Its toxicity is currently under evaluation.

A compound named CG40215, an inhibitor of S-Adenosylmethionine pathway has also been investigated against trypanosomes. Its action is limited to infections by *T. b. gambiense* and to the early phase of the disease. Investigation on this molecule has therefore been stopped (Keiser *et al.*, 2001).

Putative new targets:

Extensive studies on the molecular biology and biochemistry of the parasite have identified a number of potentially attractive targets.

Topoisomerases: Trypanosomatids contain an unusual network of mitochondrial DNA, enclosed in a structure called the kinetoplast. Topoisomerases are enzymes involved in the control of the structure of the network. Several compounds, known for their capacity to inhibit these enzymes, have been tested *in vitro* against trypanosomes. The methylendioxy derivatives of Camptothecin (specific to topoisomerase I) gave promising results (Shapiro, 1993, Keiser *et al.*, 2001).

Polyamine pathways: Bloodstream forms need to synthesize polyamines to proliferate. DFMO, an inhibitor of one of the enzyme of the polyamine pathway, ornithine decarboxylase, is one successful compound used therapeutically. Other enzymes of the pathway have also been investigated. Inhibitors of S-adenosylmethionine decarboxylase such as methyl glyoxalbis(guanylhydrazone) and CGP40215 either failed to clear the infection *in vivo* or were not active against the late stage of the disease (Keiser *et al.*, 2001).

Glycolysis: Bloodstream forms rely entirely on glycolysis to produce ATP and numerous enzymes of the glycolytic pathway have been the subject of intense study (Glyceraldehyde-3-phosphate dehydrogenase, phosphofructokinase, pyruvate kinase) but none of the inhibitors have yet been tried *in vivo*.

Trypanothione: In place of glutathione as the major low molecular weight thiol, trypanosomatids possess a glutathione-spermidine conjugate called trypanothione. The enzyme trypanothione reductase is pivotal in keeping trypanothione reduced. Different inhibitors, targeting this enzyme have been tested (peptide inhibitors, phenothiazines, and diphenylsulfides). The activity of these compounds has been demonstrated *in vitro* but the activity *in vivo* needs to be improved.

GPI: Glycosylphosphatidyl inositol (GPI) molecules serve as anchors at the plasma membrane for different proteins and especially for Variant Surface Glycoproteins (VSGs). These molecules contain a myristate unit and the GPI myristoylation has been explored as a putative chemotherapeutic target.

Protein farnesyltransferase: A number of cellular process including signal transduction require the attachment of farnesyl or geranylgeranyl groups to specific proteins. Inhibitors mimicking the substrate farnesyl pyrophosphate have shown to inhibit the growth of procyclic and bloodstream forms at concentrations that do not affect the proliferation of fibroblasts (Yokoyama, 1998).

The treatment of African trypanosomiasis is still unsatisfactory. Most of the drugs in use to date where developed in the first half of the twentieth century. Only one drug has been developed recently but its use is limited by its cost. Unfortunately the lack of new drugs is not the only factor contributing to the problems of chemotherapy. Some health centers are still not sufficiently well equipped to permit a correct diagnosis or to differentiate the stages of the disease. Frequently patients with sleeping sickness present co-infections with other parasites (*Plasmodium*, intestinal helminths, filariasis) and a background of malnutrition reduces their ability to survive. The follow-up of the treatment is long (24 months) and requires at least four lumbar punctures and which results in a poor compliance (Atougia and Costa, 1999).

Sleeping sickness in rural communities dependent on agriculture leads to serious economic problems when working people are infected. The disease affecting the livestock (nagana) can also endanger farming activity. The impact of the disease is not only economical but also social. Women infected by sleeping sickness are under sociocultural constraints (shame, ostracism, and financial dependency) and seek treatments less readily than men (WHO). Sleeping sickness also has an impact on children. Physiologically a lower weight and a delay of puberty has been observed compared to non infected children and psychologically infected children are academically less fit and drop out of school more often.

Vector control

While drugs represent the main tools of trypanosomiasis today, the other method of controlling the disease that has had a success historically is the control of the tsetse fly vectors of the disease

Since the discovery of DDT in 1945, the use of insecticides has been the main line of defense against tsetse flies. In the middle of the century very large areas were

sprayed and in some countries including Cameroon, and Nigeria trypanosomiasis was kept under control for several years.

Towards the end of the 20th century the most commonly used technique were bait techniques. These approaches involve the attraction of the tsetse using visual or olfactory bait to traps where the insect can be killed. The technique was then developed using life bait that have been dipped or sprayed with DTT or pyrethroides (more effective against the fly and less toxic for mammals) (Atouguia and Costa, 1999).

Tsetse flies are very susceptible to insecticides but a limitation of the vector control approach is its sustainability. Inadequate follow-up, leading to the re-infestation of regions by the tsetse and the failure of the control programme, is often due to political instability (civil wars) and the lack of finance.

1.2. Transport phenomena.

1.2.1. Principles:

The process by which a molecule is able to cross the plasma membrane depends on the nature of the molecule. Water and nonpolar molecules permeate by simple diffusion across the membrane whereas various polar molecules (sugars, ions, amino acids etc) are transported using special membrane proteins named **membrane transport proteins**. There are two main classes of membrane transport protein: the **carrier proteins** (also called transporters or permeases) and the **channel proteins.**

The transport of molecules through channels and some carriers is a **passive phenomenon** (also called **facilitated diffusion**). If the molecule is uncharged the driving force is the concentration gradient of the molecule only. If the molecule is charged the driving force is a combination of the concentration gradient and the membrane potential (the electrical potential difference across the membrane). The combination of the concentration gradient and the membrane potential is named the **electrochemical gradient.**

Cells are also required to transport some solutes against their electrochemical gradient. This process is called **active transport** and is always mediated by a protein carrier tightly coupled to a source of metabolic energy (ATP hydrolysis or an ion gradient). If the carrier transports only one solute it is called an uniporter. For coupled transport, the transport of one solute depends on the transfer of a second one either in the same direction (symport) or in the opposite direction (antiport) (Alberts *et al.*, 1994).

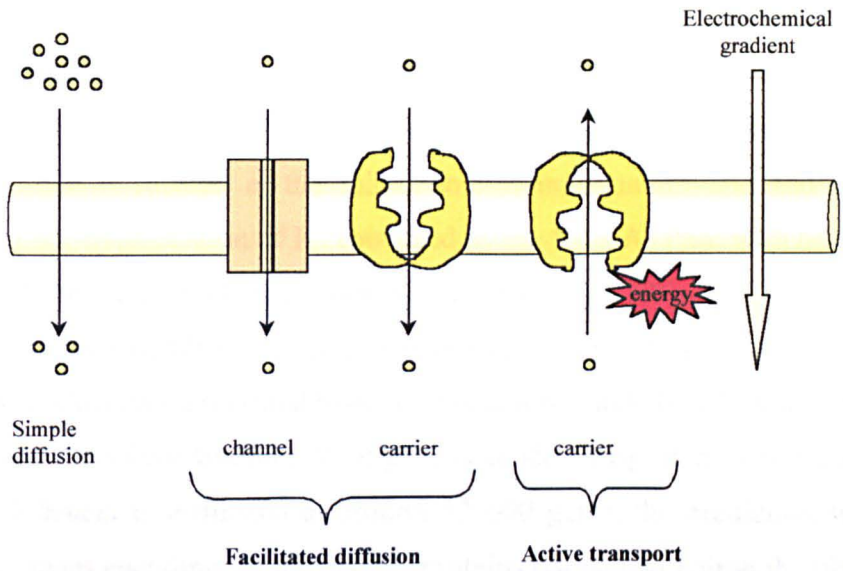


Figure 1.13: Comparison between passive diffusion, facilitated diffusion and active transport. Passive and facilitated diffusion occur spontaneously while active transport requires an input of metabolic energy. Facilitated diffusion can be carried out by channels or carrier proteins while active transport is mediated by carriers only.

When the energy of translocation of one solute is supplied by (electro)chemical gradients of other solutes the transport is named **secondary active transport**. When light or chemical energy is directly converted into an electrochemical energy (an ion concentration gradient for example), the type of transport is called **primary active transport**. The different types of ATPases (P-type at the plasma membrane, V-type in vacuoles and F-type in the mitochondrial inner membrane) fall into this category. These ATPases hydrolyse ATP and create an electrochemical gradient of protons across the membrane. Secondary and primary transport can be coupled. In trypanosomes most of the nutrient transporters studied so far have been shown to be dependent on a proton motive force. The nutrient is symported with a proton which enters the cell because of the electrochemical gradient which is generated by a proton ATPase. The **proton motive force (PMF)** is a combination of two components: the membrane potential and the pH gradient across the membrane and can be calculated using equation 1 (Appendix-Chapter I). In procyclic forms the PMF has been estimated at –80 mV (de Koning and Jarvis, 1997) and for the bloodstream forms the value varies according to the authors between –52 mV (Nolan and Voorheis, 2000) and –95.4 mV (De Koning and Jarvis, 1997).

In the bloodstream forms of the parasite the membrane potential is composed of two distinct electrical gradients. One is located at the plasma membrane and the largest factor contributing to its generation is the potential due to the diffusion of K^+. The other one is located at the mitochondrial inner membrane and is generated entirely by the translocation of H^+ catalysed by the F_1F_0-ATPase with no contribution from the electron transport chain (Nolan and Voorheis, 2000).

The total number of plasma-membrane associated proteins in trypanosomes is unknown. However a reasonable prediction can be made based on the analogy with other organisms where around 5 % of genes encode transporters. The total number of genes in *T.brucei* is estimated at around 12 000 genes the prediction would be of about 600 genes encoding for transporter proteins (Borst and Fairlamb, 1998).

1.2.2. Mechanism of transport:

Many descriptions for the kinetics of transport rely on an analogy to typical reactions catalyzed by soluble enzymes. In this model, when the carrier is saturated, the rate of transport is maximal determining a $\mathbf{V_{max}}$ value characteristic of the transporter. Each carrier is also characterized by a $\mathbf{K_m}$ value equal to the concentration of substrate when the rate of uptake is half of its maximum value and is inversely proportional to the apparent affinity of the substrate for the transporter.

This view has been, and is still, extensively used although it is an over simplistic way of looking at transport mechanisms. Unlike soluble enzymes, transporters are vectorial catalysts that operate between two compartments and need to be approached differently.

Another model, specifically concerned with the transporter case was published by Deves in 1991. The mechanism of transport relies on four consecutive events: 1) the **binding step** of the substrate to the recognition site of the transporter, 2) the **translocation step** of the complex carrier-substrate, 3) the **dissociation** of the substrate from the binding site and 4) the **return of the free carrier** to its original position (Deves, 1991).

1.2.2.1. The binding step:

The **binding step** is the interaction of the substrate with its recognition site on the carrier. In the case of coupled transport the question of an order in binding steps of the two solutes can be raised. Separate studies on Na^+/glutamate (Yamato, 1992) and Na^+/glucose transport (Krupka, 1989) and the lactose permease (Sahin-Toth *et al*, 2000) have shown that the cation binds first to the carrier and induces a conformational change that exposes the substrate binding site.

1.2.2.2. The translocation of the complex .

Different models have been proposed to illustrate how a protein carrier can conduct its substrate from one side of the membrane to the other. In one model, mainly used for non-coupled transport, the substrate site only shifts and the carrier is seen as a fixed membrane protein in equilibrium between two conformations. In coupled transport the hypothesis is that the complex substrate-carrier undergoes a reorientation involving a fall in the energy barrier between the inward and the outward-facing conformations. The energy source necessary for this fall of the energy barrier most probably comes from the binding forces of the complex (Krupka, 1989). The change in protein conformation improves the fit between the substrate and the transporter site. This gain in energy is the driving force for the carrier from the immobile form in the membrane to the mobile state (Krupka, 1989) and determines the rate of translocation. Considering transport in this way has several consequences. If the exchange rate depends on the increase of the binding forces that arise in the transition state, a good substrate is a molecule that is initially bound weakly in the complex and tightly bound in the transition state. This implies that the design of a good inhibitor should complement the transporter site in its original conformation and that a good analogue should be complementary to the site in the transition state.

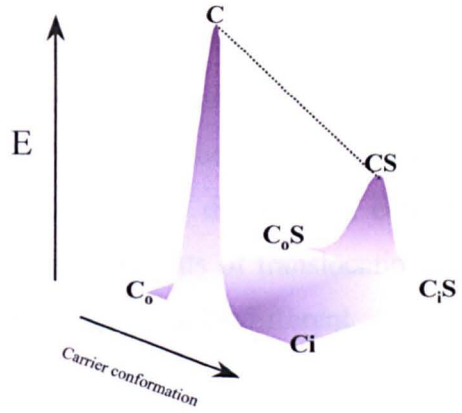


Figure 1.14 : Energy profile for catalysis of carrier movement by the substrate. C_o and C_i are respectively the outward-facing and inward-facing of the carrier. C_oS and C_iS are respectively the outward-facing and inward-facing of the complex carrier-substrate.

1.2.2.3. The dissociation of the complex.

The dissociation occurs when the carrier-substrate complex has translocated to the inner face of the membrane. The conformational changes due to the translocation lead to the liberation of the coupling ion. This liberation then causes a reorientation of the binding site which allows release of the substrate.

1.2.2.4. The translocation of the empty carrier.

Loss of substrate means that the binding forces are no longer present, and the empty carrier in its inward conformation is thermodynamically unstable and returns to its outward conformation.

In the light of these considerations two questions may be asked: What is the real meaning of the maximum rate of transport and what is the affinity for the transport site. The maximum transport rate of a carrier does not only depend on the rate of translocation of the complex substrate-carrier but also on the rate at which the free carrier returns to its original conformation. Three situations can be envisaged: 1) the rates of translocation of the complex and the free carrier are the same 2) the complex translocates faster than the free carrier 3) the free carrier translocates faster

than the complex. This concept can be particularly important in the interpretation of inhibition studies and is known to vary for different transporters (Deves, 1991).

The apparent affinity of the substrate for the transporter can not always be presumed to reflect the half saturation constant (K_m value). The apparent affinity is in fact dependent not only on the true dissociation constant of the substrate carrier complex but also on the rate constants of translocation of the complex and the free carrier, which, as described above, can be different.

1.2.3 Transporters in trypanosomes

1.2.3.1. Sugar transporters:

Bloodstream form trypanosomes rely exclusively on glycolysis as a source metabolic energy (Michels *et al.*, 2000) and glucose transporters are present in *T. brucei* to sustain this requirement. Procyclic forms can proliferate in the absence of glucose preferring amino acids and especially proline as an energy source.

Two genes, THT1 and THT2 have been cloned and found to be part of a tandemly arranged multigene family with 80 % homology (Bringaud and Baltz, 1993). The expression of these two genes throughout the life cycle is different. Bloodstream forms express forty fold more THT1 than THT2, whereas procyclics express only THT2 (Bringaud and Baltz, 1993). The type of transport for THT1 has been shown to be facilitated but it is still debated whether THT2 is a facilitated transporter or an active one with a proton dependency. The discrepancy in the results may be due to differences in the experimental approach.

The recognition motif for glucose is different for THT1 and THT2. In bloodstream forms hydrogen bonds are formed between C-1, C-3, and C-4 positions of glucose and THT1 (Barrett *et al.*, 1995), while in procyclics THT2 forms an additional hydrogen bond in C6 (Tetaud *et al.*, 1997). The apparent affinity of both transporters also differs with a K_m value of 1 mM for THT1 and 40 μM for THT2. These apparent affinities may conceivably be attributed to the number of hydrogen bonds between the substrate and the transporter.

The two transporters encoded by THT1 and THT2 are proteins of 527 and 529 amino acids respectively and possess twelve transmembrane domains as do other hexose transport systems (Tetaud *et al*, 1997).

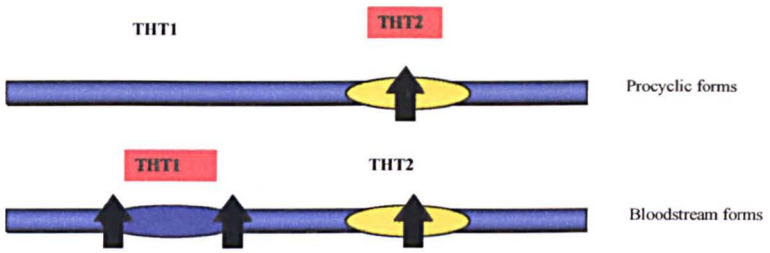


Figure 1.15 : Differential expression of glucose transporter genes *THT1* and *THT2* in procyclic and bloodstream *Trypanosoma brucei*. Bloodstream forms express mainly THT1 whereas procyclics express only THT2.

1.2.3.2 Nucleoside and nucleobase transporters:

Trypanosomes are not capable of *de novo* synthesis of purines and need to scavange them from the host (Hammond and Gutteridge, 1984). In the bloodstream forms two nucleobase transporters have been identified and named H2 and H3. They display a very high affinity for hypoxanthine (K_m 123 nM and 4.7 μM respectively). H2 displays a broader substrate specificity including guanosine, uracil, thymine and the purine bases (hypoxanthine, adenine, guanine and xanthine) with H1 being more specific for purine bases (De Koning and Jarvis, 1997[b]). The procyclic forms have a single purine nucleobase transporter named H1 which is similar to H3 in terms of substrate specificity and in terms of affinity with a K_m value of 9.3 μM. H2 and H1 appear to be H^+ symporters (De Koning and Jarvis, 1997[a]).

A proton dependent uracil transporter (U1) of high affinity (K_m of 0.46 μM) and high selectivity in the procyclic forms has also been characterized (De Koning and Jarvis, 1998). None of the nucleobase transporter genes have been cloned so far.

Two adenosine transporters have been described in *Trypanosoma brucei* (Carter and Fairlamb, 1993). One of these is named P2 and transports adenosine and its nucleobase adenine, while the other, called P1 carries adenosine, guanosine and inosine. The bloodstream forms possess P1 and P2 while the procyclic forms apparently possess only P1.

The P2 transporter stimulated significant interest when Carter and Fairlamb showed that melaminophenyl arsenicals and diamidines could inhibit adenosine uptake and that trypanosomes resistant to sodium melarsen had lost the P2 transporter (Carter and Fairlamb, 1993, Carter *et al.*, 1999). The combination of these data

indicated that the P2 transporter was the gate of entry for melarsen oxide and pentamidine. As an additional argument a recognition motif shared between these drugs and the amino-purines has been found (Barrett and Fairlamb, 1999, De Koning and Jarvis, 1999). The two genes encoding for P1 (*TbNT2*) (Sanchez *et al.*, 1999) and P2 (*TbNT1*) (Maser *et al.*, 1999) have been cloned. The P2 gene was identified by yeast complementation using a *Saccharomyces cerevisae* strain incapable of adenosine uptake or synthesis to screen a *T. brucei* cDNA library. The *TbAT1* gene from an arsenical resistant strain has also been isolated and 6 point mutations, leading to amino acid substitutions, were identified. However TbAT1 expressed in yeast was not inhibited by pentamidine (Maser *et al*, 1999) while the P2 transporter previously described in trypanosomes appears to recognise this substrate.

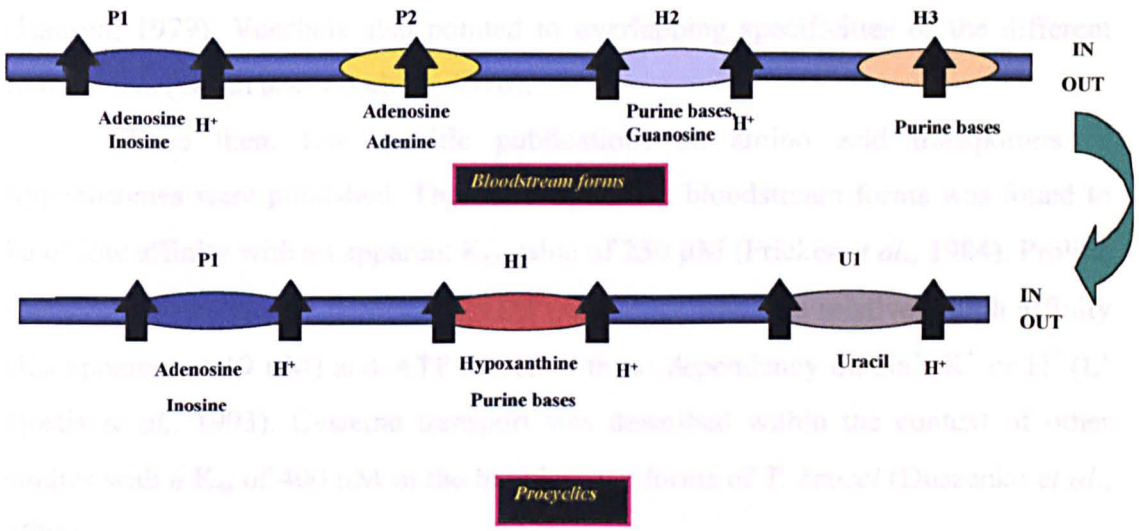


Figure 1.16: Nucleoside transporters in *T. brucei* procyclic and bloodstream forms.

1.2.3.3. Amino acid transporters

The interest in amino acid transporters in trypanosomes started some thirty years ago. Unfortunately many of these studies were performed on different species of trypanosomes (*T. gambiense* (Southworth and Read, 1971), *T. equiperdum* (Ruff and Read, 1974), *T. lewisi* (Manjra and Dusanic, 1972), *T. cruzi* (Hampton, 1970)) which makes difficult to produce a broad picture on amino transporters in

trypanosomes. Secondly, most authors carried out their transport studies using a range of amino acid concentrations from 1 to 10 millimolar. Knowing that the most abundant amino acid in human plasma is glutamine, with a concentration around 700 μM (Brody, 1994) and that half of the amino acids have a concentration below 100 μM makes the physiological relevance of these studies questionable. These early studies were unlikely to identify any transporters of high affinity (Southworth and Read, 1972, Manjra and Dusanic, 1972, Ruff and Read, 1974). However, one key concept did emerge from these studies. The uptake of radiolabeled amino acids was measured in the presence of unlabeled amino acids, and it was shown that one transporter was able to take up different amino acids. Hansen suggested the presence of five different loci: Locus A binding glutamate, arginine, and lysine, Locus B transporting threonine, glycine and alanine, Locus C transporting glutamate, locus D transporting phenylalanine and methionine and locus E taking up lysine and arginine (Hansen, 1979). Voorheis also pointed to overlapping specificities of the different transporters (Owen and Voorheis, 1976).

Since then, few specific publications on amino acid transporters in trypanosomes were published. Threonine uptake in bloodstream forms was found to be of low affinity with an apparent K_m value of 250 μM (Fricker *et al.*, 1984). Proline transport was studied in procyclics. This transporter was of a relatively high affinity (K_m apparent at 19 μM) and ATP driven with no dependency on Na^+, K^+ or H^+ (L' Hostis *et al.*, 1993). Cysteine transport was described within the context of other studies with a K_m of 400 μM in the bloodstream forms of *T. brucei* (Duszenko *et al.*, 1985).

1.2.4. Amino acid transporters in other systems

1.2.4.1 Mammalian amino acid transporters :

The transport of amino acids is comprised of a rather complex system in which amino acids are transported via a number of different carrier-proteins presenting overlapping specificity.

Different amino acid transporter systems have been grouped into four superfamilies: 1) the amino acid/auxin permease family (AAAP), 2) dicarboxylate/amino acid: cation (Na^+ or H^+) symporter family (DAACS), 3) the neurotransmitter sodium symporter (NSS) and 4) the large amino acid /polyamine/choline family (APC). Each of the families is represented in all of the main kingdoms (plants, animals, yeast, bacteria and archea). The APC family is divided into two clusters: the cationic amino acid transporters (CAT) and the leucine amino acid transporter (LAT) system which is involved in the transport of neutral, cationic and anionic amino acids. Interestingly two members of the LAT family have been shown to require association with a glycoprotein belonging to the 4F2hc family, and named gpaATS, for glycoprotein associated amino acid transporters.

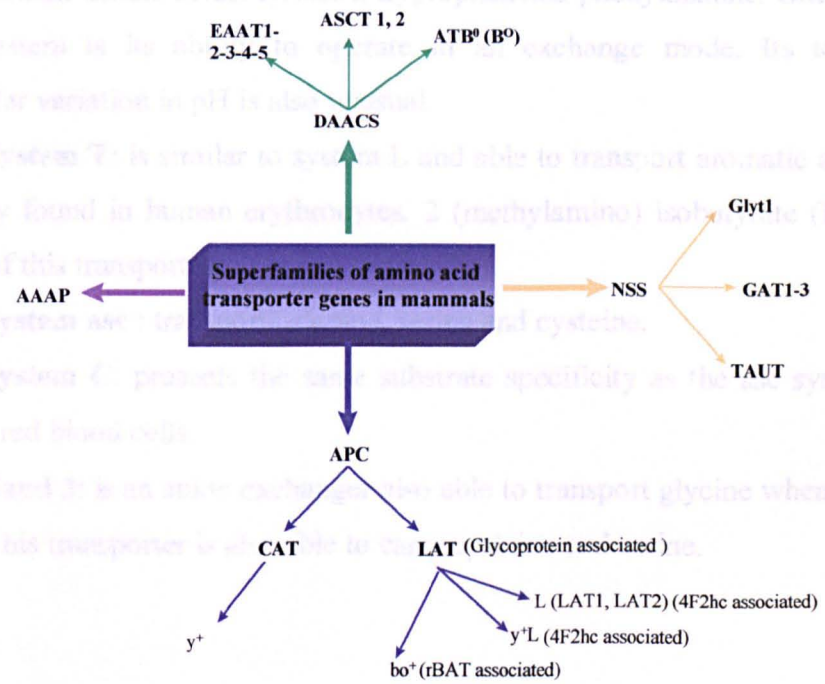


Figure 1.17: Diagram of the superfamilies and families of amino acid transporters.

Mammalian amino acid transporters can be classified in two groups of Na^+ dependent or independent transporters. Combining this with a distribution which can be either very specific to a type of organ / tissue or widespread results in a redundancy of transporters with similar substrate specificity. The main systems found in mammals are the following:

Neutral amino acids :

The following classification is based on the review of Barker and Ellory (1990).

Na^+ independent.

Five systems transporting neutral amino acid in a sodium independent manner have been described. They are named system L, T, asc, C and Band3.

System L: is a widespread transporter which has been named L because of its high affinity for leucine. This transporter is also able to transport large apolar branched chain amino acids: glutamine, methionine, leucine, isoleucine, valine as well as aromatic amino acids: tyrosine, tryptophan and phenylalanine. One peculiarity of this system is its ability to operate in an exchange mode. Its tolerance to extracellular variation in pH is also unusual.

System T: is similar to system L and able to transport aromatic amino acids but is only found in human erythrocytes. 2 (methylamino) isobutyrate (BCH) is an inhibitor of this transporter.

System asc : transports alanine, serine and cysteine.

System C: presents the same substrate specificity as the asc system and is present in red blood cells.

Band 3: is an anion exchanger also able to transport glycine when negatively charged. This transporter is also able to carry cysteine and serine.

Na^+ dependent

System A: alanine, glycine and proline are transported in a sodium dependent manner via this sytem. The activity of this group of transporters is pH dependent and it is expressed in response of to substrate deprivation.

System ASC: this system is equivalent to the asc system and is able to transport alanine, serine, cysteine, threonine within a broad range of pH values.
System A and ASC are widespread.

System N: amino acids with a nitrogen in their side chain: glutamine, asparagine, and histidine are taken up by this system which has a high pH sensitivity. This system is mainly present in liver cells.

System Gly: glycine and sarcosine are substrates for this transporter and transport has a strict dependence on Na^+ and Cl^-. This system is found in erythocytes and hepatocytes.

System β: this system is used to transport taurine (a key molecule in the regulation of cell volume) and β alanine in erythrocytes, cardiac muscle and kidney.

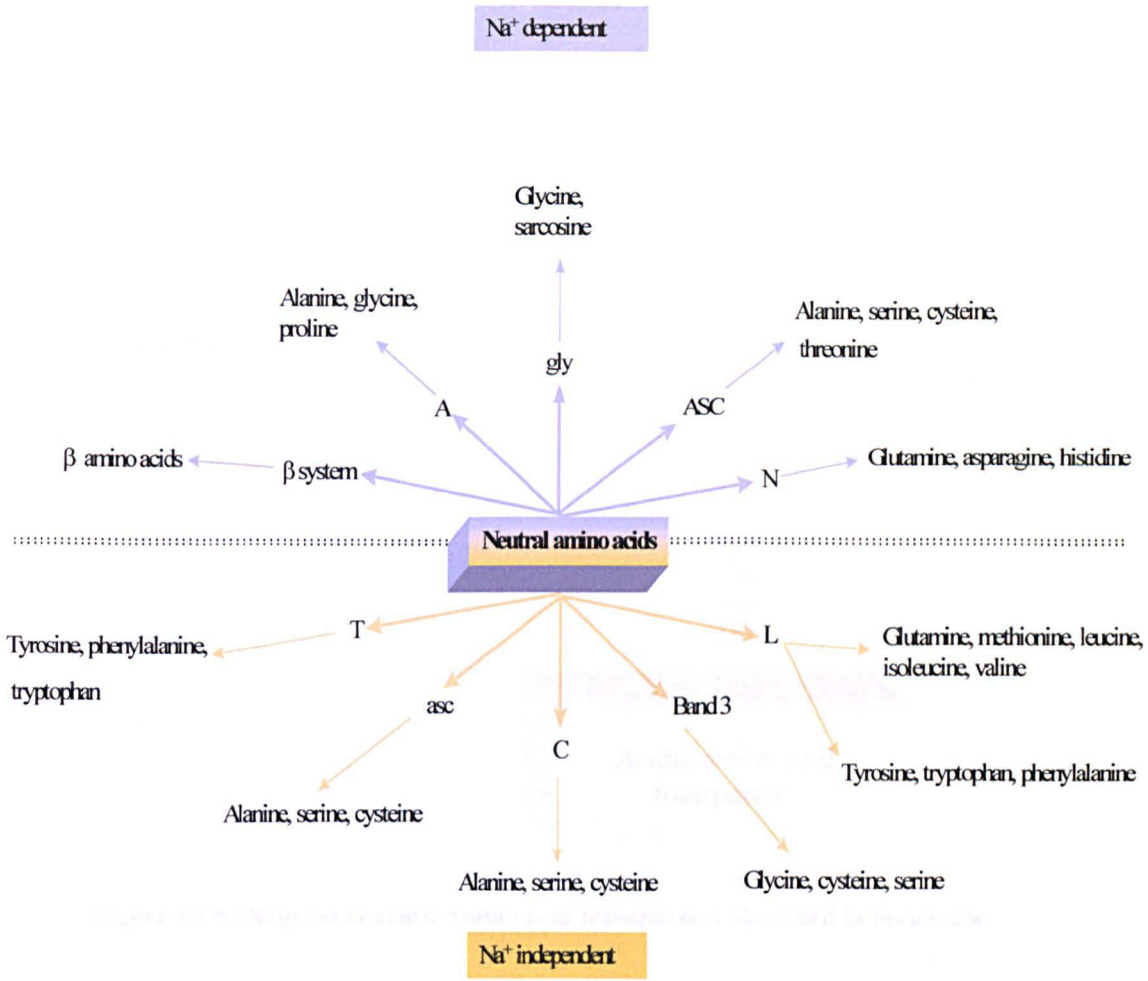


Figure 1.18: Diagram of neutral amino acid transporters identified in mammals.

Anionic amino acids:

The two acidic amino acids, glutamate and aspartate, are transported into mammalian cells via five different transporters: ASC, GLT-1 (Glutamate transporter 1), GLAST (glutamate/aspartate transporter), EAAT4 (excitatory amino acid transporter 4) and EAAC1 (excitatory amino acid carrier 1, also named X^-AG) (Kanai, 1997, Gegelashvili, 2000).

In human brain the glutamate transporter nomenclature is different except for EAAT4. Therefore GLAST is named EAAT1, GLT-1 is EAAT2, EAAC1 is EAAT3 (Kanai, 1997).

Glutamate transporters have channel-like properties allowing the flow of Cl^- when the substrate is present.

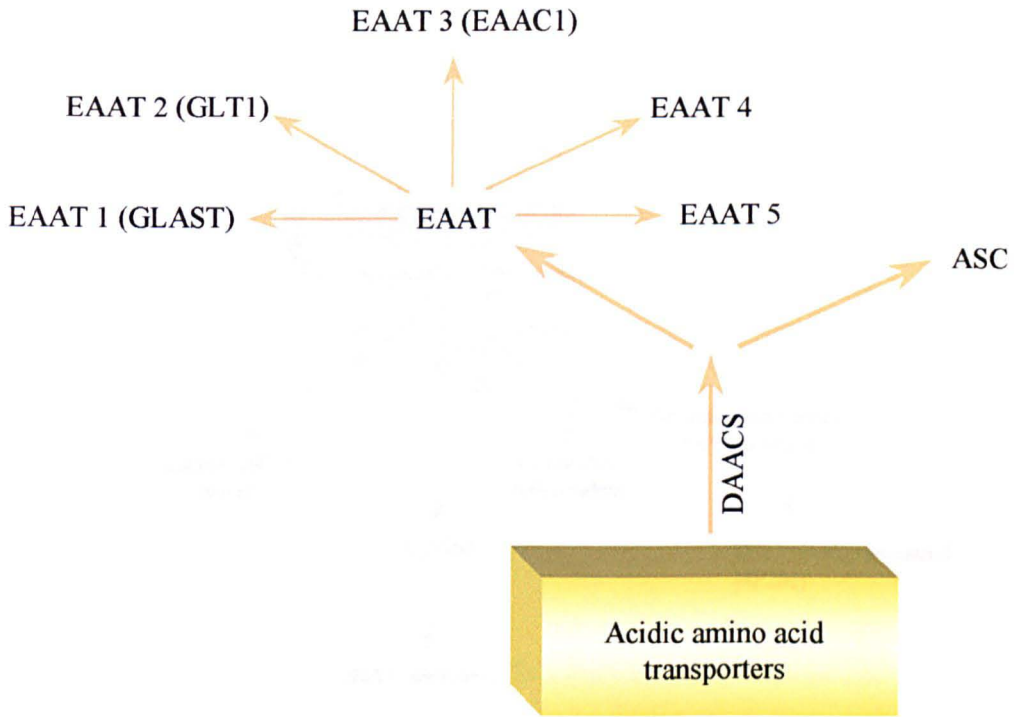


Figure 1.19: Diagram of acidic amino acid transporters identified in mammals

Cationic amino acids:

y+ system: is widespread, sodium independent and transports lysine, histidine and arginine selectively. Two other systems which carry cationic amino acids, have been described in mammals but they also carry neutral amino acids (Rojas and Deves, 1999).

B^{0+} system: transports alanine, valine, lysine and has a distribution limited to blastocyts and oocytes fibroblasts (Deves and Boyd, 1998).

b^{0+} system: is a sodium independent system present in blastocyts, the kidney and the small intestine. The substrate specificity of this system is similar to the B^{0+} system (Deves and Boyd, 1998).

y^+L system has the peculiarity of requiring sodium for the transport of neutral amino acids (leucine, methionine, glutamine) but not for lysine transport (Deves and Boyd, 1998, Kanai, 2000).

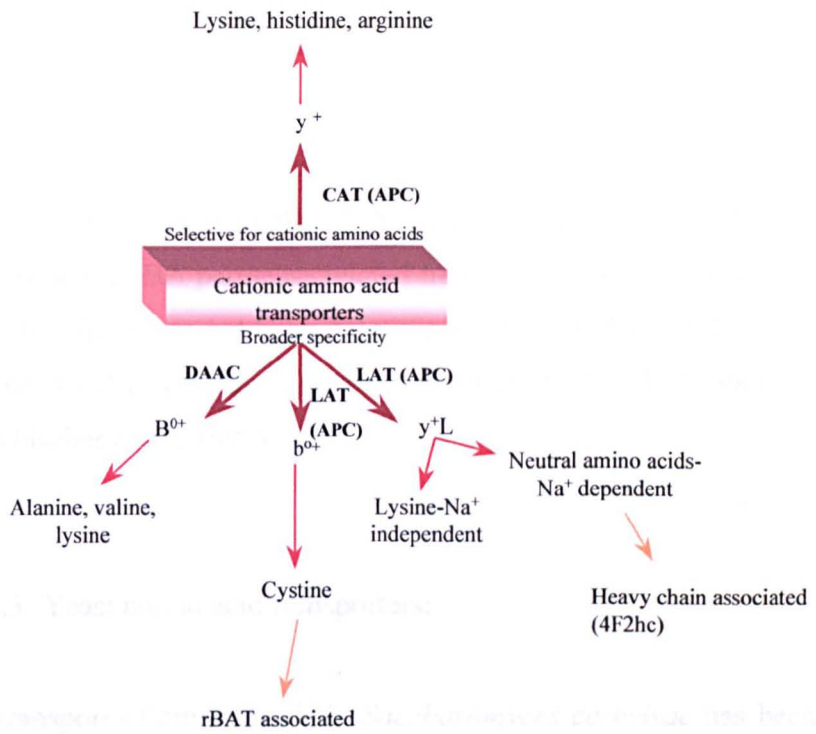


Figure 1.20: Diagram cationic amino acid transporters identified in mammals.

1.2.4.2: Plant amino acid transporters:

Plant amino acid transporters are classified into two large superfamilies named ATF (amino acid transporter family) and APC (amino acid –polyamine-choline facilitator).

The ATF superfamily is the largest and the best characterized. Four families have been distinguished: Prots, AUXs, LHT, AAPs.

The **Prots** family has two members named Prot1 (Schwacke *et al.*, 1999) and Prot2. Both of them show a strong preference for proline.

The **AUXs** family has two family members AUX1 an auxin carrier and ANT1 which is able to transport aromatic amino acids and some other neutral amino acids as well as arginine (Ortiz-Lopez *et al.*, 2000).

The **LHT** family contains three putative family members: LHT1 which carries lysine and histidine (Chen and Bush, 1997), LHT2 and LHT3 have also been identified at the genetic level .

AAPs is a family of six members named AAP1 to AAP6 each with a different tissue distribution. In terms of substrate specificity AAP1, 2, 4, and 6 transport neutral and acidic amino acids whereas AAP3 and AAP5 have an additional high affinity for basic amino acids (Ortiz-Lopez *et al.*, 2000).

The APC superfamily includes the GABA permease related family and the CAT family. In the GABA permease related family only one gene has been found and its substrate specificity has not been determined. In the CAT family two genes have been identified AtCAT1 (basic amino acid transporter) AtCAT2 (putative amino acid transporter) (Fischer *et al.*, 1998).

1.2.4.3: Yeast amino acid transporters:

The transport of amino acids in *Saccharomyces cerevisae* has been shown to be an active process dependent exclusively on protons (Rest *et al*, 1995). Each amino acid is known to be transported by at least two classes of carrier, a general amino acid permease called **Gap1** (Grenson *et al.*, 1970) and **specific permeases** (Isnard *et al.*, 1996). Gap1 can transport all of the natural amino acids in their L form and some in their D form as well as some other compounds like citrulline and ornithine. However the activity of this permease is regulated at the transcriptional and post-transcriptional level by the presence of a nitrogen source like ammonium in the medium. The presence of nitrogen in the medium down-regulates the general Gap1 and amino acids are transported specific permeases (Isnard *et al.*, 1996).

Several genes encoding for specific permeases have now been cloned (Figure 1.21). Like Gap1, some of them (Put4p, Dal5p, Uga4p, Can1) are also down regulated in the presence of nitrogen. Two independent mechanisms of regulation are responsible for this phenomenon. One is named nitrogen catabolite repression (NCR) where repression of transcription of the permease genes, involves two dodecanucleotide sites called the upstream-activating sequence (UAS_{NTR}), situated upstream of the genes. The second is called nitrogen catabolite inactivation (NCI) and inactivates the permeases at a post-translational level (Didion *et al.*, 1998).

Some of the non ammonium sensitive amino acid permeases can also be induced by the presence of particular amino acids in the medium. Didion *et al.* have

shown that the transcription of genes including *BAP2* and *PTR2* is controlled by a family member called *SSY1* which acts as an amino acid sensor with a particularly high affinity to extracellular leucine (Didion *et al*, 1998).

These specific permease genes encode for proteins with a number of predicted transmembrane spanning domains that varies from 10 to 13 (Horak, 1997). It is not known if these discrepancies are real or due to the differences between the predictive algorithms used. However, the high degree of identity at the amino acid level (from 30 to 60%) for these permeases suggests that they belong to the same family (Horak, 1997, Isnard *et al*, 1996). To date most of the yeast amino acid permeases are classified as members of the APC superfamily.

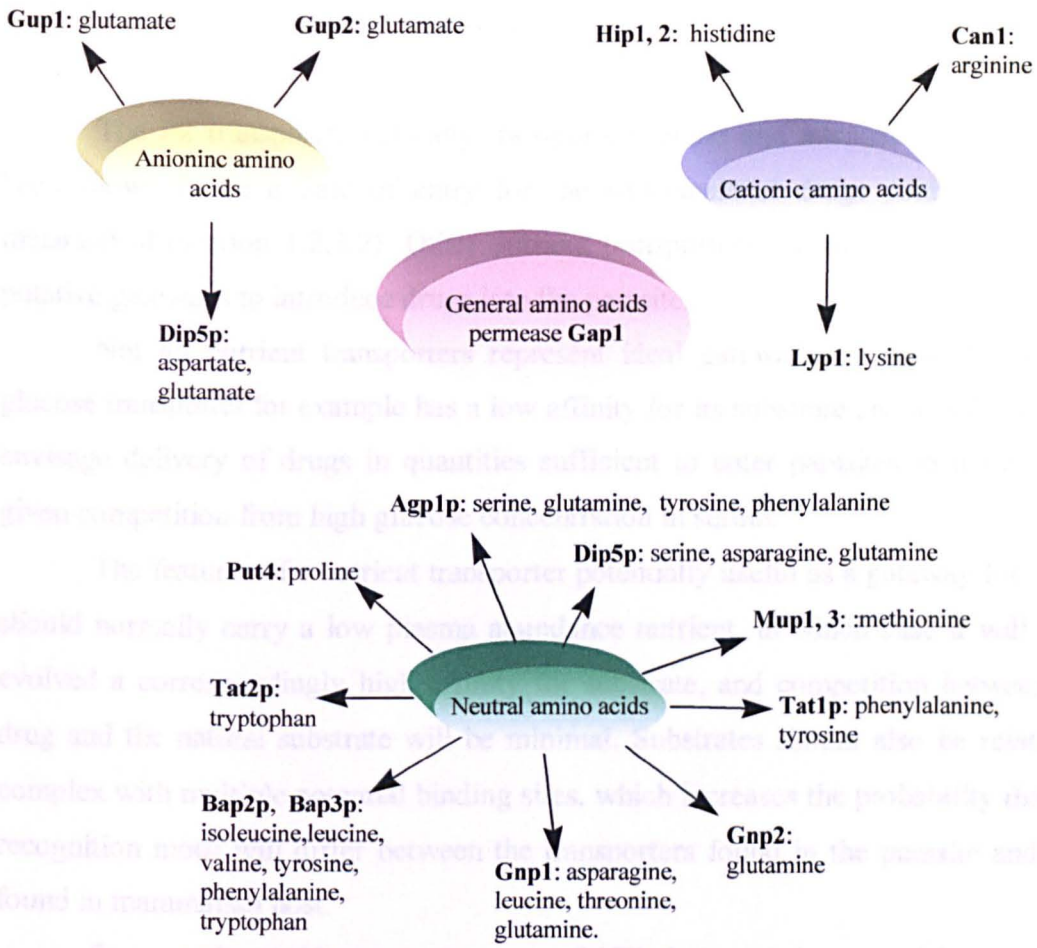


Figure 1.21: Diagram of the amino acid transporters identified in *S. cerevisiae*

1.3- Transporters as gateways for drugs:

The P2 transporter naturally transports adenine and adenosine but has also been shown to be a gate of entry for the trypanosomal drugs pentamidine and melarsoprol (section 1.2.3.2). Other nutrient transporters can also be envisaged as putative gateways to introduce drugs into the parasite.

Not all nutrient transporters represent ideal gateways for new drugs. The glucose transporter for example has a low affinity for its substrate and it is difficult to envisage delivery of drugs in quantities sufficient to enter parasites to toxic levels given competition from high glucose concentration in serum.

The features of a nutrient transporter potentially useful as a gateway for drugs should normally carry a low plasma abundance nutrient, in which case it will have evolved a correspondingly high affinity for substrate, and competition between the drug and the natural substrate will be minimal. Substrates should also be relatively complex with multiple potential binding sites, which increases the probability that the recognition motif will differ between the transporters found in the parasite and that found in mammalian host.

Some amino acid transporters may fulfill these requirements: they play an essential role in the parasite's metabolism and half of the amino acids have a concentration in human plasma below 100 μM (Brody, 1994). Structurally, they also display a certain level of complexity (aromatic amino acids in particular). Because of the paucity of data on amino acid transport in trypanosomes this study represents a first step towards this direction.

Chapter II

Materials and Methods

2.1. Trypanosome Strains

The strain used routinely in this study is *Trypanosoma brucei brucei* strain EATRO 427. This strain was first described as coming from a sheep in Uganda and has been extensively used for experimental purposes. Although the TREU 927/4 strain has been chosen for the *T. brucei* genome sequencing project (http://parsun1.path.cam.ac.uk/), the 427 strain was chosen for this work because a high parasitemia can be achieved in rats leading to the harvesting of a high number of parasites required for uptake studies and because most biochemical analyses in *T. brucei* have used this strain.

2.2. Chemicals.

Radiolabeled L- [^{3}H] glutamine (49 Ci/mmol), L- [^{3}H] glutamate (33Ci/mmol), [3,5-^{3}H] tyrosine (51 Ci/mmol), L- [^{14}C] aspartate (207 mCi/mmol), L-[^{3}H] tryptophan (20Ci/mmol), L-[^{3}H] phenylalanine (118Ci/mmol), were purchased from Amersham Pharmacia Biotech. Radiolabelled [α^{32}P] ATP (3000 Ci/mmol) and L-[^{14}C] methionine (58 mCi/mmol) were purchased from NEN Life Science Products). Oligonucleotides were synthesized by MWG Biotech. Restriction endonucleases and *Pfu* DNA polymerase were obtained from Promega. All other chemicals were purchased from Sigma and at the highest grade available.

2.3. Growth and maintenance of trypanosomes:

2.3.1. Bloodstream form trypanosomes:

Bloodstream form trypanosomes were stored in blood containing 20% glycerol in capillary tubes in liquid nitrogen. The parasites were injected intraperitoneally into Wistar rats. Parasitamia was monitored by examination of a thin tail blood smear using

phase contrast microscopy. All experiments were carried out under license in the animal facility at Glasgow University.

When the parasitaemia reached $1x10^9$ parasites/ml the rat was anaesthetized under CO_2 and blood containing parasites was collected from the rodent by cardiac puncture in a syringe containing 120 U/ml of heparin in separation buffer (Appendix II).

2.3.2. Separation of bloodstream form trypanosomes from blood

Bloodstream form trypanosomes were separated from blood cells by ion exchange chromatography (Lanham and Godfrey, 1970). The separation relies on differences in charges: the red blood cells are negatively charged and adsorb to the DEAE- cellulose (DE 52) while the less negatively charged trypanosomes are eluted using a Phosphate- Buffered-Saline-Glucose (PSG) buffer (Appendix II). The standard conditions of the elution buffer with pH 8, ionic strength of 0.217 and 1% of glucose are crucial for the success of the purification. The blood was collected from rats with high parasitemia as described above and then centrifuged at 1,250 g for 15 minutes at 4°C. The centrifugation led to a preliminary separation of the trypanosomes from the blood with the formation of a white upper layer ("buffy coat") containing the parasites. This layer was carefully removed by pipetting and applied to the DE-52 cellulose column that had been pre-equilibrated using the separation buffer.

2.3.3. Growth and maintenance of procyclic culture form trypanosomes:

Cultures of procyclic trypanosomes were kept in sterile tissue culture flasks at 28°C in SDM-79 medium (Brun *et al.*,1977) containing 10% fetal calf serum that had been decomplemented by incubation at 56°C in a water-bath for 1.5 hours.

The development of the culture was monitored by microscopy and the total number of organisms were determined using a haemocytometer (Counting Chamber, Weber Scientific International Ltd). Cultures reached peak concentrations of around 5 x

10^7 organisms/ml. Late log phase and early log phase contained respectively around $1.5x10^7$ and $1x10^7$ organisms/ml.

Procyclic parasites were preserved at a density of $5x10^6$ cells /ml by diluting in an equal volume of 20% glycerol, cooling slowly in cooling box at –80°C for 24 hours and subsequently transferring vials to liquid nitrogen.

2.4. Transport assays in trypanosomes:

2.4.1. Preparation of cells for uptake assay

Procyclic forms were harvested from mid-exponential phase cultures for transport assays. Bloodstream forms were collected from the infected rat by cardiac puncture using a heparinized syringe and maintained on ice. The cells were washed three times at 4°C in transport buffer (CBSS), then re-suspended in transport buffer at a concentration of 10^8 cells /ml, and stored on ice. Prior to use cells were warmed to room temperature.

2.4.2. Transport assays in trypanosomes

To initiate uptake, a 100 µl sample of parasites was added to 100 µl of transport buffer (CBSS) (Appendix II) containing quantities of radiolabelled amino acid stated in the text. The buffer was layered over a cushion of 90 µl oil [1-bromododecane, 98% (Aldrich); final density 1.038 g/ml] in a 0.5 ml Eppendorf tube. Transport was ended after varying time intervals by centrifuging the tube at 13,000 x g for 60s in a bench top microfuge which separates the parasites from the uptake buffer. Immediately after the centrifugation, tubes were flash frozen using liquid nitrogen. The pellet of parasites was cut from the bottom of the tube into a scintillation vial and lysed with 200 µl of 10 % sodium dodecyl sulphate. 4 ml of scintillation fluid (Ecoscint A, National Diagnostics) were added and incorporated radioactivity was measured using a liquid scintillation counter (LKB Wallac 1219 Rackbeta).

For transport assays in the presence of inhibitors or ionophores, these were dissolved in ethanol (valinomycin, monensin, NEM, CCCP, FCCP, SHAM) or CBSS buffer (KCN, Ouabain). The compounds were added to the final concentration required and pre-incubated with cells for 2 min at room temperature prior to uptake. Control uptakes containing the same percentage of solvent (ethanol) were carried out in parallel.

2.5. Molecular cloning techniques:

2.5.1. Polymerase chain reactions

All PCR reactions were performed using *Pfu* DNA Polymerase (Promega). The oligonucleotides were provided by MWG Biootech. A 50 µl reaction contained 200 µM of dATP, 200 µM of dCTP, 200 µM of dGTP and 200 µM of dTTP, 5 µl of 10X buffer containing $MgSO_4$ (provided by the manufacturer), upstream and downstream primers at the concentration of 1 µM, DNA template in a range of concentrations between 200 ng-300 ng and 1.25 units of *Pfu* DNA Polymerase.

For the amplification of *TbAATP1*, the following primers were designed: 1) forward primer: 5' TAGATCTCAAACAACA**TG**ACCAGCATCAATGCC 3', containing a *Bgl*II site (plain underline), and a Kozak ribosome binding sequence (Kozak, 1986) (dashed underline) placed directly upstream of the initiation codon (bold). 2) the reverse primer, 5' TAGATCTACGCTAATCATCCCACAG 3' containing a *Bgl*II site (plain underlined) upstream of the termination codon (bold).

The amplification was performed using a thermal cycler (Gene AmpPCR System 2400, PerkinElmer). The annealing temperatures used were chosen based on the T_m value of the oligonucleotides. The conditions for amplification were the following: a hot start at 94°C for 1 minute followed by 30 cycles composed of a denaturation step at 94°C for 1 minute, an annealing step at 50°C for 30 seconds and an amplification step at 72°C for 4 minutes, a final extension at 72°C for five minutes.

The product of the reaction was checked by agarose gel electrophoresis and the band corresponding to the size of the expected product was removed and the DNA extracted.

2.5.2. Plasmid vectors:

Two plasmid vectors were used for the cloning of *TbAATP1*: pGEM-T (Promega) was used for subcloning and pSPGT1 was used for *in vitro* transcription for oocyte expression (Krieg and Melton, 1984). The latter was a gift from Dr. G.W. Gould and contained the coding sequence of the rat brain glucose transporter *GLUT1* ligated into a *Bgl* II site and flanked by 89 bp of 5' and 141 bp of 3' untranslated sequence from the *Xenopus* β-globin gene (Gould and Lienhard, 1989).

2.5.3. Preparation of the plasmid

The pGEM-T plasmid was purchased ready to use. pSPGT1 was digested to completion with the restriction endonuclease *Bgl* II in order to remove GLUT1. To prevent recircularization of the vector the DNA was dephosphorylated with Calf Intestinal Phosphatase (CIP, Gibco). The phosphatase was then heat inactivated at 55°C. The product was run on an agarose gel in order to assess the degree of digestion and the integrity of the DNA. The digested product was then extracted using a Qiagen gel extraction kit which also yielded vector without the *GLUT1* insert. The *GLUT1* sequence was replaced by the *TbAATP1* sequence for expression.

2.5.4. Ligation

The principle used in the PGEM-T vector cloning kit is based on the presence of a deoxy-thymidine residue at both ends of the vector providing compatible overhangs for PCR products generated by DNA polymerases adding a single deoxyadenosine at the 3' end of the amplification product in a template-independent manner. However, because we were using *Pfu* DNA polymerase which does not have this characteristic, an additional tailing step was needed. This tailing procedure consisted of a reaction containing the purified PCR fragment generated by the *Pfu* DNA polymerase, 1 μl of *Taq*

DNA Polymerase reaction buffer (10x), 1 µl of 25 mM of $MgCl_2$, 200 µM of dATP, 5 units of *Taq* Polymerase and deionized water to a final volume of 10 µl. The reaction was then incubated for 30 minutes at 72°C. The integrity and the quantity of the DNA were assessed by running 0.5 µl of the reaction on an agarose gel.

Ligation reactions were carried out using a 3 fold-excess in terms of molar ends of the insert DNA and the vector. The reactions were set up in a 10 µl final volume containing 5 µl of ligation buffer (2x), 1 µl of pGEM-T vector (50 ng), and 3 U of T4 DNA ligase. The reactions were incubated at 4°C overnight.

2.5.5. Transformation of *Escherichia coli*

Heat shock sub-cloning efficiency competent cells of the JM109 strain were purchased from Promega. Competent cells were removed from -80°C and placed on ice until they thawed. 100 µl of competent cells was then transferred by pipetting using cold tips, in an ice-cold Eppendorf tube and 5 µl of the ligation mixture (25 ng) was then added. The tubes were put back on ice for 10 minutes and then rapidly transferred to a water bath at 42°C for 50 seconds and immediately put back on ice for two minutes in order to heat-shock the cells. 900 µl of cold LB medium was added to the transformation mix and incubated for one hour at 37°C with shaking (225 rpm). The cells were pelleted by centrifugation at 1, 000g for 10 minutes and resuspended in 100 µl of LB and plated on LB agar plates containing ampicillin (50 µg/ml), X-Gal (50 µg/ml) and IPTG (1 mM).

2.5.6. Plasmid DNA preparation

Single transformed colonies were picked from plates into 5 ml of LB/ampicillin and incubated overnight at 37°C with vigorous shaking. For small scale plasmid preparation 5 ml cultures were taken and spun for 10 minutes at 2, 500 rpm. The pellets were then processed using a Miniprep kit according to the manufacturer's specification (Qiagen).

For the preparation of large scale plasmid DNA a pre-culture of 5 ml was grown overnight and then transferred to 250 mls of LB/amp which was grown overnight at 37°C with shaking. The culture was then spun and the pellet was processed using a Midiprep kit according to Manufacturer's specifications (Qiagen). The quality and the quantity of the plasmid DNA was then assessed by agarose gel electrophoresis.

2.5.7. Restriction digestion of DNA

The digestion of plasmid DNA by restriction endonucleases was carried out in a water bath at 37°C for one or two hours. The reaction was carried out in a volume of 20 µl using 5 units of restriction enzyme, 2 µl of 10x buffer recommended by the manufacturer and 50 ng to 100 ng of plasmid.

The digestion of genomic DNA was carried out in a volume of 200 µl and with 20 units of restriction endonucleases and was incubated at 37°C for at least 6 hours in order to achieve a complete digestion.

2.5.8. Agarose gel electrophoresis of DNA:

Agarose gels were prepared in TAE buffer (Appendix II) according to Sambrook *et al.*(2^{nd} edition) with 1% agarose and ethidium bromide. Gel volumes were either 250 ml and run in 2l of TAE buffer overnight at 25 mV (Southern) or 40 ml run in 200 ml of TAE buffer between 1 and 2 hours at 80 mV.

After running the gels were viewed by trans-illumination over an ultraviolet trans-illuminator (UVP Laboratory Products).

2.5.9. Purification of DNA from Agarose gels

In order to purify particular fragments of DNA from agarose gels, DNA samples were separated by electrophoresis alongside DNA size markers. Fragments were identified by U.V. transillumination and physically excised from the gel using a scalpel. The fragments were collected in an autoclaved Eppendorf tube and processed using a Qiagen gel extraction kit according to the manufacturer's specifications.

2.5.10. Preparation and Purification of DNA and RNA from *Trypanosoma brucei.*

Bloodstream and procyclic forms of trypanosomes were harvested as described above. The RNA was extracted using TRIzol® Reagent (Life Technologies) following the manufacturer's protocol.

The DNA was extracted by adding 450 µl of extraction buffer, 20 µl of 10% SDS, 25 µl of proteinase K (20 mg/ml) to pelleted trypanosomes and incubated for 2 hours at 37 ° C with intermittent gentle shaking. An equal volume of phenol was added and the contents of the tube were mixed for five minutes, then centrifuged for five minutes at 15,000 rpm. The supernatant was collected into a new tube and an equal volume of phenol: chloroform (1:1) was added. The suspension was mixed and centrifuged. To the supernatant an equal volume of chloroform was added and the content of the tube was mixed and centrifuged. The supernatant was collected and the DNA was precipitated using 3M sodium acetate as described in Sambrook *et al.*(2^{nd} edition).

2.5.11. Formaldehyde gel electrophoresis of RNA

A formaldehyde gel was made by adding a solution containing 9.74 ml of formaldehyde, 3 ml of 20x Northern Gel Buffer and 17.3 ml of ddH_2O to solution of 30 ml of 2% agarose melted in a microwave. The mix was poured into a minigel casting-

block form and allowed to solidify. A 1x Northern Gel buffer was prepared by mixing 25 ml 20 x Northern Gel Buffer, 44 ml of formaldehyde and 430 ml of distilled water.

The RNA samples were prepared in a final volume of 20 µl by adding to the RNA sample 1µl of Northern gel buffer, 3.5 µl formaldehyde, 10 µl of formamide and ddH_2O to 20 µl. The mixture was heated at 55°C for 15 minutes, then chilled on ice and 2 µl of RNAse-free loading buffer was added before loading the samples on the gel. The gel run was at 25 V overnight with a magnetic stirrer placed inside the box once the RNA samples had entered the gel matrix. The stirrer keeps the buffer circulated.

2.5.12. Southern Hybridization:

In order to detect restriction fragments in the genomic DNA of *T. brucei* which hybridize to a variety of probes, 3 µg of DNA was digested to completion with restriction endonucleases and electrophoresed through a 1% agarose gel in TAE buffer. After electrophoresis, gels were stained with ethidium bromide and photographed. Following electrophoresis the gel was processed for blotting. The first step involved a depurination of the DNA by placing the gel in a solution of 0.125 M HCl for 20 minutes under a gentle agitation. The gel was then denatured by in a solution containing 1.5 M NaCl and 0.5 M NaOH for 30 minutes and then soaked in neutralization solution containing.

Each step was processed under gentle agitation and at room temperature. Between each step the gel was rinsed in distilled water.

At this stage the DNA was ready to be transferred to the nylon membrane (Hybond N, Amersham) using the blotting technique described by Sambrook *et al.*(2^{nd} edition) using 20x SSC. After transfer the DNA was fixed to the membrane by using an UV cross-linking procedure (Spectrolinker XL-1000 UV linker, Spectronics Corporation).

Once the DNA was fixed to the membrane it was treated for hybridization either at high stringency (to detect sequences with high similarity to the probe of interest) or at low stringency (to detect sequences with a lower homology).

The nylon membrane was pre-hybridized with the hybridization solution containing 50% formamide, 5 x SSC, 10x Denhardt's solution, 0.1% SDS, 20 mM NaH_2PO_4, 5mM EDTA and 0.2 mg/ml of denatured hearing sperm DNA, pH 6.5 for 2 hours at 42°C. The radioactive probe was then added to the solution. Hybridization was then carried out overnight at 42°C. After hybridization, the hybridization solution was removed and the filters were rinsed at room temperature in 3 x SSC and 0.1 % SDS to remove any excess of probe left in the hybridization tube. The washing conditions then varied for high or low stringency Southern blots. For high stringency the membrane was washed 3 times at 60°C for 30 minutes in 0.1 % SDS and 0.1 x SSC. For low stringency washes the membrane was washed 3 times for 30 minutes in 0.1% SDS and 5 x SSC at 42°C.

After washing the membrane was kept wet (using the washing solution), wrapped in plastic film and exposed to X-ray film (Konica Medical Film) using an intensifying screen. The exposure was performed at –70°C for a variable length of time (from 1 to three days). Autoradiographs were then developed using a film processor (X-Ograph imaging system Compact X4) according to the manufacturer's specifications.

2.5.13. Northern hybridization:

RNA was extracted and 4 µg was electrophoresed using denaturing conditions on a formaldehyde gel. The gel was then directly blotted onto a nylon membrane (Hybond N, Amersham) according to the technique described in Sambrook *et al.*(2^{nd} edition), using 20 x SSC.

The nylon membrane was then probed and washed (high stringency conditions) as described for the Southern hybridization.

2.5.14. Labeling of DNA probes:

DNA probes were labelled using the Prime-It II random primer labeling kit (Stratagene).

The procedure relies on the use of random hexanucleotides that anneal to multiple sites along the DNA template. Purified DNA templates (25 ng) were boiled for 5 minutes in the presence of the random oligonucleotides in a final volume of 34 µl. The mix was then allowed to cool and 10 µl of 5 x primer buffer, 5 µl of [α^{32}P] ATP (3000 Ci / mmol, 10 mCi/ml, NEN Life Science Products), and 1µl of Klenow fragment (5U/ml) was added. The reaction was incubated at 37°C for 2 to 10 minutes and stopped by adding 2 µl of stop mix. The probe was then purified to separate the unincorporated dATP from the labeled probe using a spun column procedure (MicroSpin TM S 200HR columns, Amersham Pharmacia Biotechnology, USA). The labeled DNA was then denatured by boiling for 5 minutes.

2.6. Expression of TbAATP1 in the *Xenopus laevis* oocytes :

2.6.1. Preparation of the mRNA:

Capped mRNA was obtained by *in vitro* transcription using the mRNA Message Machine SP6 ™ kit (Ambion) using the *TbAATP1* DNA template in pSPGT1 linearised by *Sal*I. The reaction was carried out according to the manufacturer's indications and incubated for two hours at 37°C. After two hours, 1µl of DNAse 1 was added to the reaction and incubated at 37°C for 15 min in order to degrade the template DNA.

2.6.2. Preparation of the *Xenopus* oocytes:

Mature female *Xenopus laevis* were anaesthetised by immersion in a solution (2g/l) of 3-amino benzoic acid ethyl ester (tricaine) in tap water. Stage V-VI oocytes were harvested and then defolliculated with 1000 U ml^{-1} of Type 1A collagenase (Sigma) dissolved in Barths saline buffer without Ca^{2+} (Colman, 1984). Once defolliculated, the oocytes were washed three times in Barths saline buffer and kept at 16°C in Barth's supplemented with 2.5 mM sodium pyruvate and 2% foetal calf serum.

Twenty-four hours after collagenase treatment, oocytes were micro- injected using glass micro- pipettes with 10 to 20 ng of RNA per oocyte in a volume of 27 nl.

2.6.3. Transport Assays in Xenopus oocytes:

Xenopus oocytes injected with *TbAATP1* or with water as a control, were assayed for transport 3 days after injections. The uptake of [^{3}H]-glutamine, [^{3}H]-glutamate or [^{3}H]-tyrosine was measured on groups of four oocytes at room temperature in transport buffer at pH 7.4 for a length of time stated in the results section. The assays were ended by transferring the oocytes to ice, quickly followed by 3 washes with ice cold CBSS. Each oocyte was transferred individually to a scintillation vial containing 200 µl of 2% SDS and the incorporation of radiolabel was quantified by liquid scintillation spectrometry. Kinetics of tyrosine uptake was obtained by incubation of *TbAATP1* injected oocytes and water injected in a range of tyrosine concentrations from 3.125 µM to 100 µM for 50 min. Assays for inhibition studies were carried out with 0.2 µM [^{3}H]-tyrosine in the presence of 5 mM of each individual amino acid.

2.7. Software

Grafit 4.09 (Erithacus software) was used to handle the biochemical kinetic data. This includes the fitting of the different kinetics of transport of all the amino acids studied and the determination of the kinetic constants and the IC_{50} values.

The Vector NTI suite 6 package was used to handle DNA and protein sequences. This package includes Vector NTI for the handling of DNA and protein sequences (search for open reading frame and restriction sites), AlignX for sequence alignments (based on ClustalW) and ContigExpress which was used to associate overlapping sequences and create contiguous sequences.

ClustalX 1.5 b was used to build the phylogenetic trees.

The different Web pages used in order to search databases or analyze sequences are the following:

Databases

TIGR databases: http://www.tigr.org/tdb/mdb/tbdb/gene.shtml

Parasite-genome (European Bioinformatic Institute): http://www.ebi.ac.uk/blast2/parasites.html.

The Sanger Center: http://www.sanger.ac.uk/Projects/T_brucei/Toolkit/blast_server.shtml

NCBI : http://www.ncbi.nlm.nih.gov/BLAST/

Prediction of transmembrane domains

TMpred: http://www.ch.embnet.org/software/TMPRED_form.html

SOSUI: http://.sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html

Prediction of the presence of signal peptide

SignalP : http://www.cbs.dtu.dk/services/SignalP/#submission

TargetP: http://www.cbs.dtu.dk/services/TargetP/

Chapter III

Transport of Methionine in *Trypanosoma brucei brucei*

3.1. Introduction

Methionine is an amino acid found with relatively low abundance in human plasma (around 30 μM). In trypanosomes, methionine plays a number of crucial roles in the biochemistry of the parasite. In addition to its role as a component of proteins it is adenosylated within the cell by S-adenosylmethionine synthetase, and used as a precursor in various macromolecular methylation reactions ([a,b]Goldberg *et al.*, 1997). S-adenosylmethionine is also decarboxylated by S-adenosylmethionine decarboxylase and the decarboxylated metabolite is used as the source of amino-propyl groups in polyamine synthesis (Bacchi, 1991). The methylthioribose which results from this reaction is reconverted to methionine via a cycle eponymous with this vital amino acid (Berger *et al.*, 1996). Methionine or its precursor in the methionine cycle, α-ketomethiobutyrate (KMTB), can relieve the toxic effect of methionine cycle inhibition (Bacchi *et al.*, 1991), indicating that these metabolites must be taken up by trypanosomes. Considering these characteristics, the methionine transport represents a good candidate to investigate as a potential gateway into trypanosomes. In this chapter are presented the kinetic of methionine uptake in procyclic and bloodstream form trypanosomes as well the substrate specificity's and the ion dependency.

3.2. Results

3.2.1 The uptake of L-methionine

Time course of methionine uptake

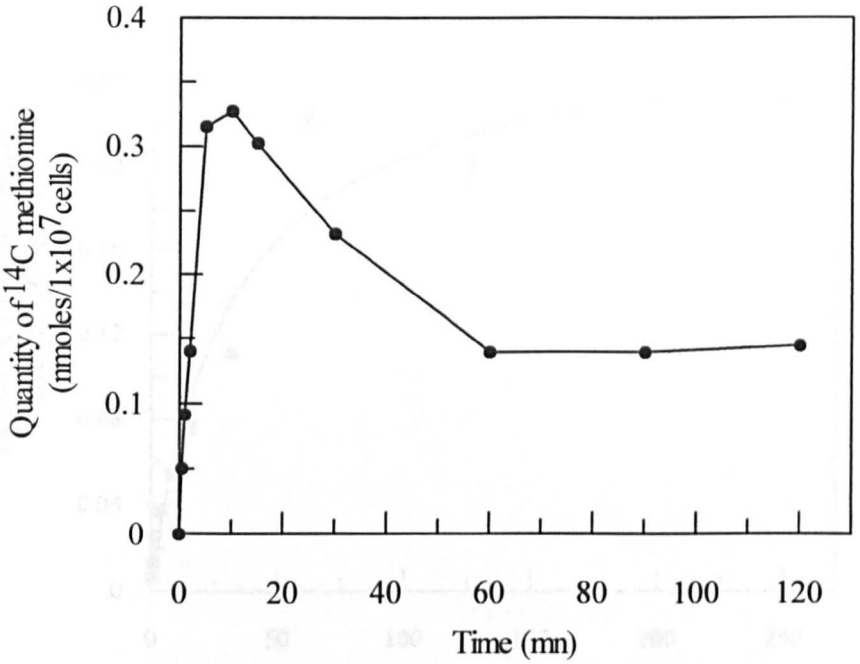
Uptake of 200 μM methionine by procyclic and bloodstream forms was allowed to proceed for 60 minutes, with internalised radiolabel measured at set time points over the uptake period (Figure 3.1). The results showed an accumulation of methionine for 7 minutes followed by a release of methionine to finally reach a plateau. Within the constraints set by the centrifugation through oil technique it appeared that uptake remained linear during this initial phase. To measure the kinetics of initial velocity transport, a 10 second time period was chosen to measure transport as a function of substrate concentration.

Kinetics of methionine uptake

Measuring initial velocity of methionine transport versus substrate concentration for procyclic (figure 3.2.A) and bloodstream forms (figure 3.2.B) revealed the process to fit a typically hyperbolic curve. Taking data points from the curve of the hyperbola, non-linear regression was used to determine a $\mathbf{K_m}$ value of **30.9 ± 7.6 μM** and a $\mathbf{V_{max}}$ value of **16.2 nmol min^{-1} (10^8 cells)$^{-1}$** for procyclic forms and a $\mathbf{K_m}$ value of **32.8 μM ± 3.4** and a $\mathbf{V_{max}}$ of **28.8 ± 0.1 nmol min^{-1} (10^8 cells)$^{-1}$**.

The thermodynamics of the transporter were also studied, measuring the activity of the transporter over a range of temperatures. The accumulation of radiolabelled methionine was dependent upon temperature with temperature quotients $\mathbf{Q_{10}}$ of **2.22** for procyclic forms and **2.43** for bloodstream forms respectively. The activation energy was determined by linear regression from the negative slope of the Arrhenius plot. Apparent $\mathbf{E_a}$ values of **54.2 Kj mol^{-1}** and **63.4 Kj mol^{-1}** were found respectively for procyclic and bloodstream forms (Figure 3.3).

A



B

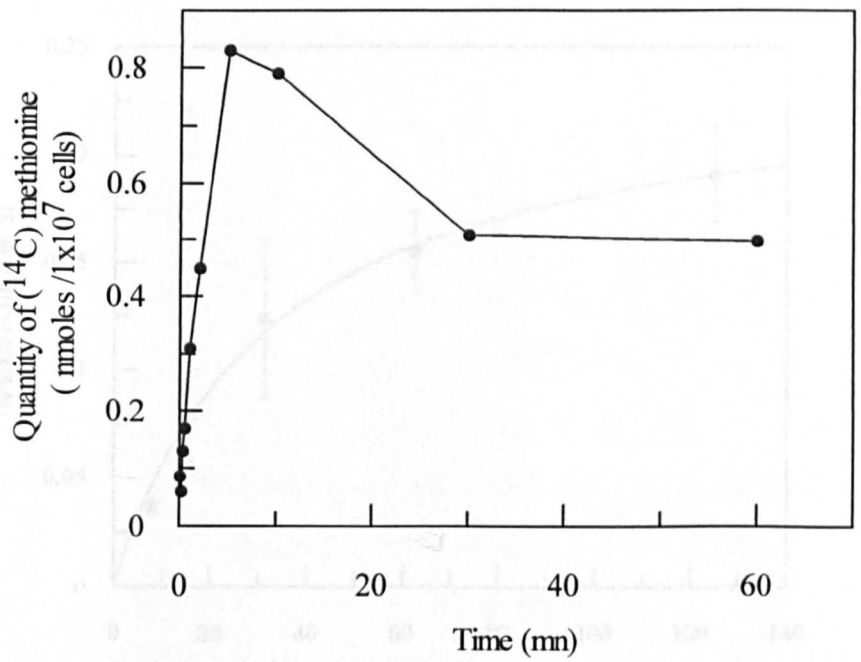


Figure 3.1: Time course of methionine uptake in procyclic and bloodstream forms of *Trypanosomabrucei*. Methionine transport was measured in the presence of 200 µM methionine at 25°C for a range incubation time varying from 5 seconds to 60 minutes. Panel A) Time course for procyclics, Panel B) Time course for bloodstream forms.

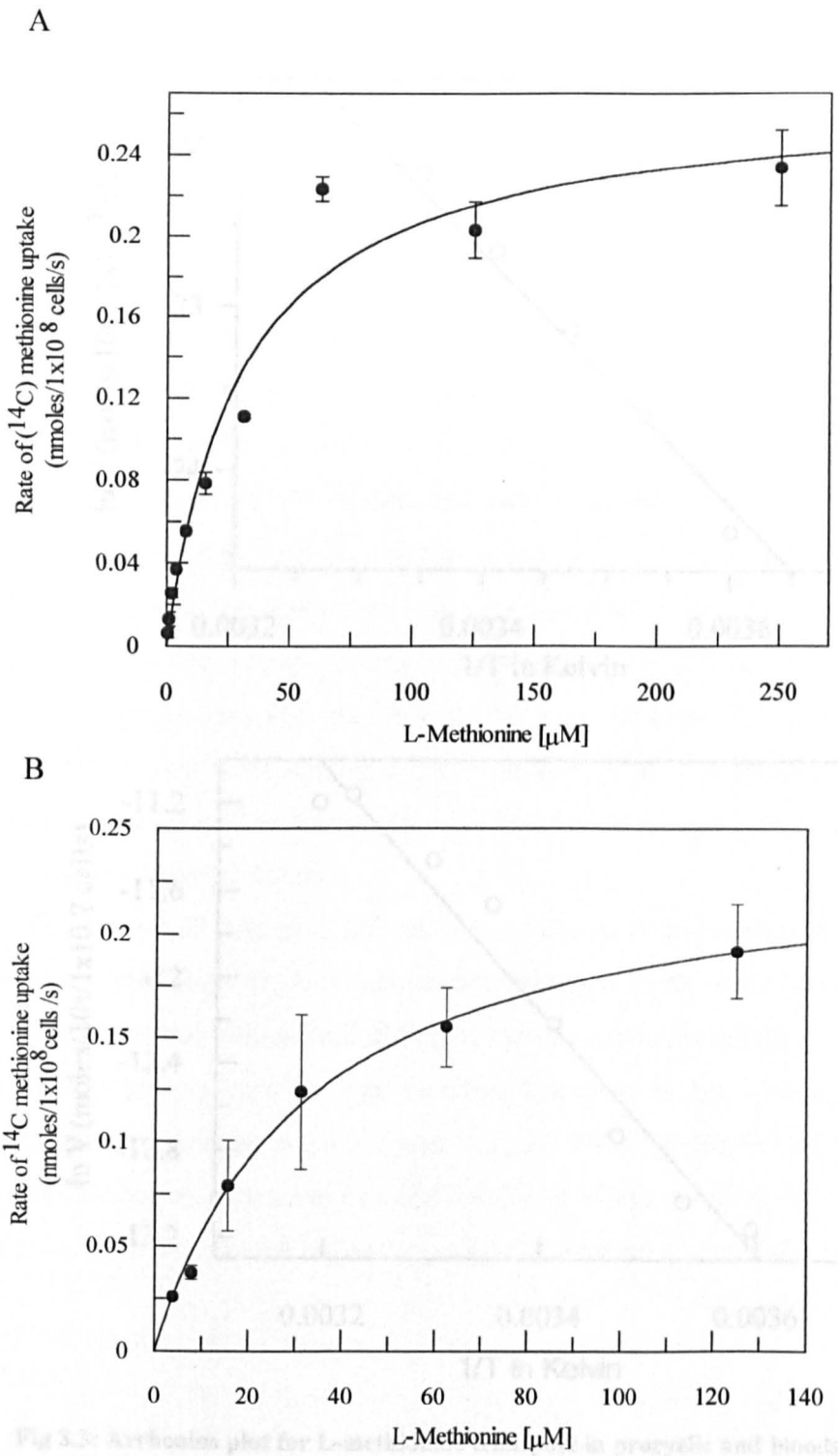
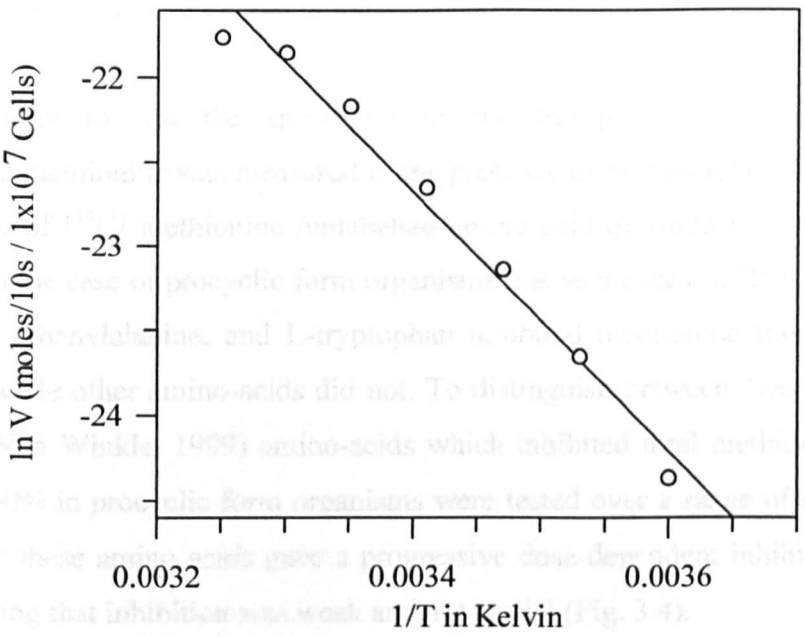


Fig 3.2: **L-Methionine uptake in *T. brucei*.** Methionine transport was measured in the presence of various methionine concentrations at 25° C and at 37°C for the bloodstream forms. Kinetic constants were determined by non linear regression analysis using the Michaelis-Menten equation. Panel A) Kinetic of methionine uptake in procyclic forms, B) Kinetic of methionine uptake in bloodstream forms.

A



B

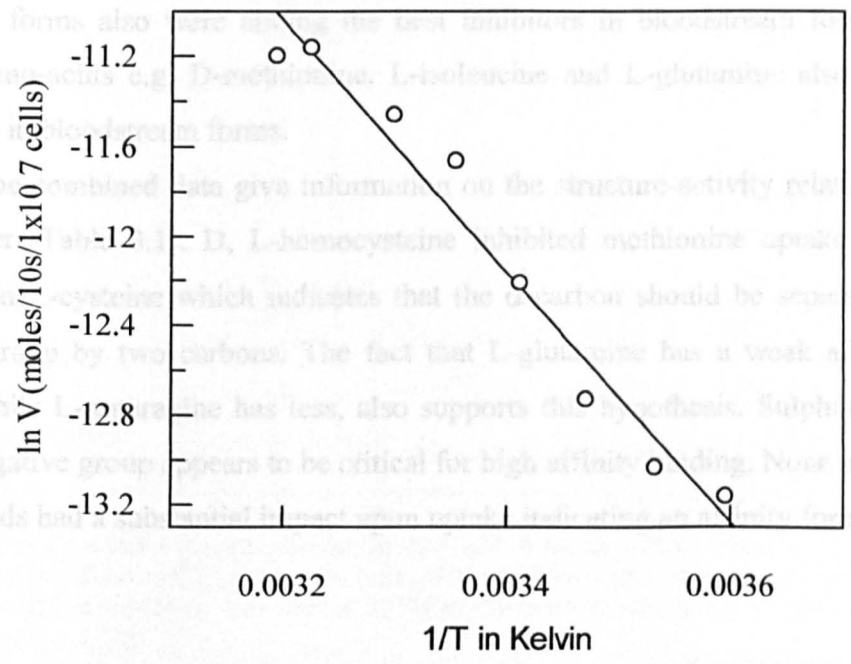


Fig 3.3: Arrhenius plot for L-methionine transport in procyclic and bloodstream forms of *T. brucei*. The rate of L-methionine (200 μM) transport was measured over a range of temperatures from 5°C to 35°C. The Ln of the velocity was plotted versus the inverse of the absolute temperature in Kelvin. The results represent the mean of six points over two experiments. Panel A) Arrhenius plot for procyclic forms, Panel B) Arrhenius plot for bloodstream forms.

3.2.2. Specificity of the methionine transporter

In order to test the specificity of the transporter, zero-*trans* uptake of radiolabelled methionine was measured in the presence of non-radiolabelled amino acids using a ratio of [^{14}C] methionine /unlabelled amino acid of 1/625 to test for inhibition (Table 1). In the case of procyclic form organisms this vast excess of D,L-homocysteine, L-leucine, L-phenylalanine, and L-tryptophan inhibited methionine transport by more than 50%, while other amino-acids did not. To distinguish between "weak" or "partial" inhibition (Van Winkle, 1999) amino-acids which inhibited total methionine uptake by more than 50% in procyclic form organisms were tested over a range of concentrations. In each case these amino acids gave a progressive dose-dependent inhibition to beyond 90% indicating that inhibition was weak and not partial (Fig. 3.4).

The same amino-acids which were the best inhibitors of methionine uptake in procyclic forms also were among the best inhibitors in bloodstream forms, however, other amino-acids e.g. D-methionine, L-isoleucine and L-glutamine also inhibited by over 50% in bloodstream forms.

The combined data give information on the structure-activity relationship of the transporter (Table 3.1). D, L-homocysteine inhibited methionine uptake substantially better than L-cysteine which indicates that the α-carbon should be separated from the sulphur group by two carbons. The fact that L-glutamine has a weak affinity for the carrier while L-asparagine has less, also supports this hypothesis. Sulphur or a similar electronegative group appears to be critical for high affinity binding. None of the charged amino acids had a substantial impact upon uptake indicating an affinity for neutral amino acids.

Table 3.1: Effect the amino acids on the uptake of L-methionine by procyclic and bloodstream forms of *Trypanosoma brucei brucei*.

	Percentage of uptake compared with control	
	Procyclic forms	Bloodstream forms
None	100	100
L-Alanine	84 ± 5	59 ± 5
L-Arginine	91 ± 7	97 ± 7
L-Asparagine	97 ± 8	78 ± 9
L-Aspartic acid	97 ± 8	101 ± 1
L-Cysteine	83 ± 5	65 ± 4
L-Glutamine	62 ± 9	42 ± 2
L-Glutamic acid	104 ± 8	79 ± 3
Glycine	96 ± 3	80 ± 3
L-Histidine	85 ± 11	72 ± 1
L-Isoleucine	75 ± 5	41 ± 5
L-Leucine	40 ± 2	30 ± 5
L-Lysine	90 ± 1	108 ± 3
L-Methionine	8 ± 1	18 ± 4
L-Phenylalanine	45 ± 2	51 ± 2
L-Proline	77 ± 9	78 ± 4
L-Serine	64 ± 8	66 ± 7
L-Threonine	113 ± 3	60 ± 4
L-Tryptophan	48 ± 6	64 ± 4
L-Valine	85 ± 2	82 ± 14
D-L Homocysteine	25 ± 9	16 ± 2
D-Methionine	69 ± 3	48 ± 1
DL-Methylcysteine	65 ± 5	33 ± 5

L-Tyrosine is not soluble at the concentration of 5 mM in the conditions required for this experiment. Consequently inhibition of [^{14}C] methionine uptake was not determined in the presence of tyrosine.
Non radiolabelled amino acids were used at 5 mM and were added simultaneously with [^{14}C] methionine (58 μCi / μmol) at 8 μM.
The results represent the percentage of L-methionine uptake over a time period of 10 seconds against a control containing only 8 μM [^{14}C] methionine.

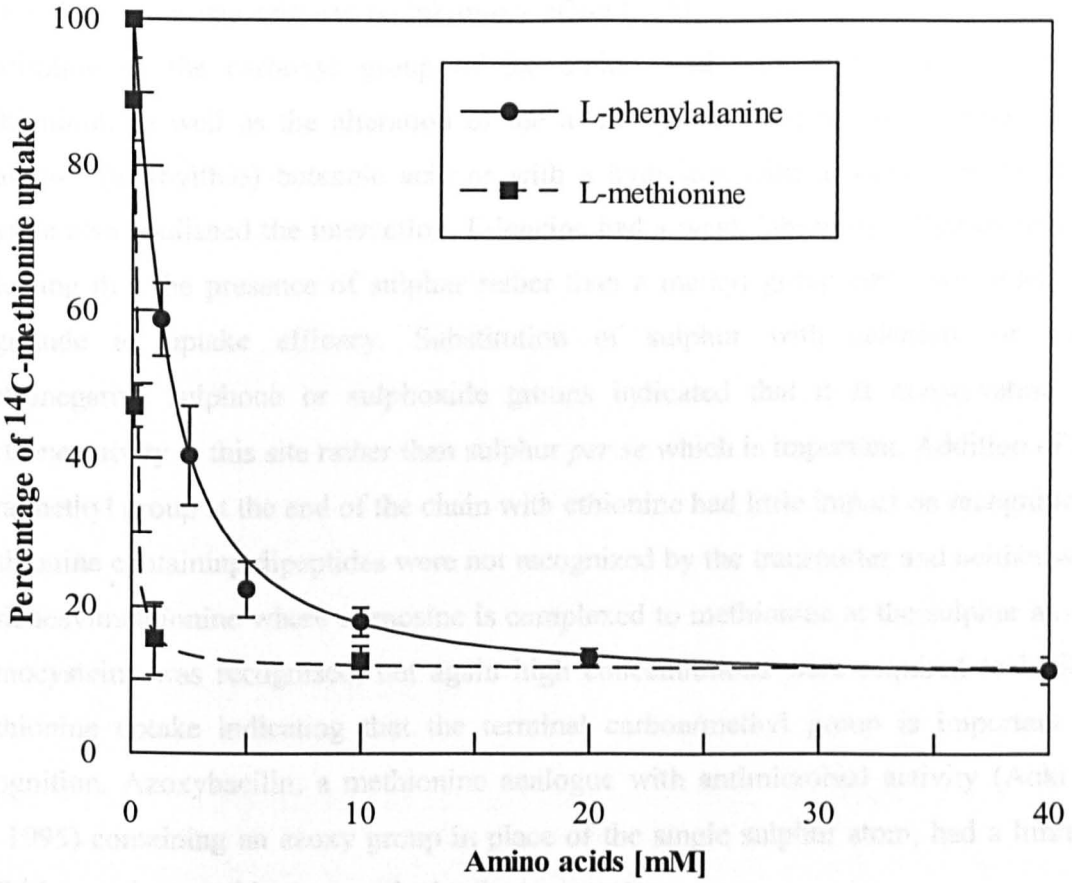


Fig. 3.4 : Inhibition of [^{14} C] L-methionine uptake with phenylalanine. The uptake of ^{14}C L-methionine (8 μM) over 10 seconds was measured in the presence of cold L-methionine (dotted line) or L-phenylalanine (solid line). The concentration range was from 1 μM to 10 mM for L-methionine and 62.5 μM to 40 mM for L-phenylalanine. The percentage of methionine uptake was calculated against a control without unlabelled L-methionine or L-phenylalanine.

The core amino-acid structure appears to be essential since analogues consisting of the side chain alone, including 3-(methylthio)-acetic acid, ethyl methyl sulfide, 3-(methylthio) propionic acid had no inhibitory effect (Table 3.2 and figure 3.5). Moreover, substitution of the carboxyl group of the amino acid with a hydroxyl group in methioninol, as well as the alteration of the amino group by a hydroxyl group in 2-hydroxy-4 (methylthio) butanoic acid or with a hydrogen with methyl-4-(methylthio) butyrate also abolished the interaction. L-leucine had a weak inhibitory effect on uptake indicating that the presence of sulphur rather than a methyl group adds two orders of magnitude to uptake efficacy. Substitution of sulphur with selenium or with electronegative sulphone or sulphoxide groups indicated that it is conservation of electronegativity at this site rather than sulphur *per se* which is important. Addition of an extra methyl group at the end of the chain with ethionine had little impact on recognition. Methionine containing dipeptides were not recognized by the transporter and neither was S-adenosylmethionine where adenosine is complexed to methionine at the sulphur atom. Homocysteine was recognised, but again high concentrations were required to inhibit methionine uptake indicating that the terminal carbon/methyl group is important in recognition. Azoxybacilin, a methionine analogue with antimicrobial activity (Aoki *et al.*, 1995) containing an azoxy group in place of the single sulphur atom, had a limited inhibition against methionine uptake by the transporter.

Table 3.2: Inhibition (IC50) of L-methionine uptake by methionine analogs in procyclic and bloodstream forms of *Trypanosoma brucei brucei*.

Compounds	IC 50 (μM)	
	Procyclic forms	Bloodstream forms
Methionine sulfoxide ($CH_3SOCH_2CH_2CH(NH_2)CO_2H$)	77 ± 10	132 ± 20
Methionine sulfone ($CH_3SO_2CH_2CH_2C(NH_2)HCO_2H$)	78 ± 12	108 ± 10
Selenomethionine ($CH_3SeCH_2CH_2CH(NH_2)CO_2H$)	79± 6	62 ± 12
Ethionine ($C_2H_5SCH_2CH_2CH(NH_2)CO_2H$)	179 ± 24	125 ± 5
Azoxybacilin (CH3NN(O)$CH_2CH_2CH(NH_2)CO_2H$)	ND	775 ± 203

Rates of uptake of 7 μM methionine were plotted against the concentration of inhibitors using a range of concentration from 20 mM to 10 μM except for selenomethionine from 2.5 mM to 10 μM.
Methyl 4 (methylthio) butyrate ($CH_3SCH_2CH_2CH_2CO_2CH_3$), 2 hydroxy 4 (methylthio) butanoic acid ($CH_3SCH_2CH_2CH(OH)CO_2H$), 3(methylthio) acetic acid ($CH_3SCH_2CO_2H$),
Methioninol ($CH_3SCH_2CH_2CH(OH)CH_2OH$), Ethylmethyl sulfide ($CH_3SCH_2CH_3$), 3 (methylthio) propionic acid ($CH_3SCH_2CH_2CO_2H$), S-Adenosyl methionine, Methionine-Glycine, Methionine- Glutamic acid are not inhibiting methionine uptake. ND means not determined

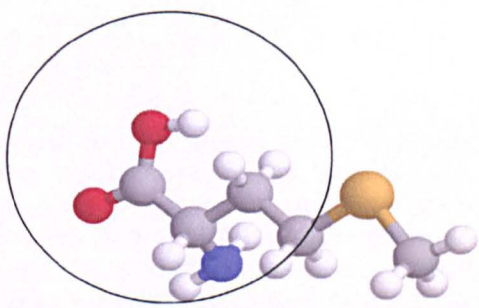


Figure 3.5: Schematic structure of methionine. carbon atoms of are represented in grey, hydrogens in white, oxygens in red, nitrogen in blue and sulfur in yellow. The circle around the molecule represents the part of methionine important for the recognition by the transporter (based on the results shown table 3.2).

3.2.3. Ion dependence for methionine transport

Transport studies were carried out in the presence of a selection of ionophores (Table 3.3) to determine whether co-transport of a counter ion was involved in uptake. Neither the sodium ionophore, monensin, nor ouabain, an inhibitor of the Na^+-K^+ ATPase, had a significant inhibitory effect on methionine transport. Incubation of the cells with valinomycin, (a K^+ ionophore) and different proton ionophores including CCCP and FCCP or NEM (an inhibitor of the plasma membrane proton ATPase), inhibited transport in a dose dependent fashion in both forms of the parasite. The potassium ion electrochemical gradient is the principal component of the membrane potential in both forms of the parasite (Nolan and Voorheis,1991; De Koning and Jarvis, 1997). Protons, migrating with this gradient, appear to be the most common counter ion used by trypanosomes for a number of transport processes (De Koning and Jarvis, 1997). The fact that both protonophores and plasma membrane potential dissipating reagents inhibit uptake indicate that the proton motive force contributes to methionine uptake.

Table 3.3: Effects of ionophores, thiol reagents, KCN and SHAM on the uptake of L-methionine on procyclic and bloodstream forms of *Trypanosoma brucei brucei*.

		Percentage inhibition compared to the control	
		Procyclic forms	Bloodstream forms
KCN	5 mM	53 ± 1	16 ± 6
	2.5 mM	48 ± 3	15 ± 9
SHAM	25 μM	16 ± 3	72 ± 7
	12.5 μM	3 ± 1	68 ± 8
	6.25 μM	13 ± 5	50 ± 17
CCCP	10 μM	75 ± 2	47 ± 9
	5 μM	52 ± 8	50 ± 8
FCCP	10 μM	71 ± 4	53 ± 2
	5 μM	58 ± 1	ND[a]
NEM	2.5 mM	68 ± 5	73 ± 8
	1.25 mM	46 ± 10	52 ± 4
Valinomycin	6 μM	38 ± 1	54 ± 1
	3 μM	ND[a]	44 ± 4
	1.6 μM	29 ± 4	36 ± 1
Monensin	30 μM	21 ± 2	34 ± 4
Ouabain	500 μM	27 ± 8	20 ± 14
	250 μM	19 ± 3	2 ± 1

[a]ND means not determined. A stock of cells was kept on ice and an aliquot was pre-heated at 25 degrees for the procyclic and at 37 degrees for bloodstream forms for 5 min in a heating block and then pre-incubated at the same temperature in the presence of the inhibitor at the concentrations indicated in the table during 2 minutes except for valinomycin and SHAM incubated respectively 6 and 10 minutes before adding L-methionine at the concentration of 6 μM. The results represent the percentage inhibition of L-methionine uptake during 10 seconds compared with a control without any inhibitor.

3.3. Discussion

Methionine is a relatively low concentration amino acid playing a key role in the metabolism of the parasite. Structurally, methionine is characterised by the presence of sulphur within an aliphatic side chain. In both forms of the parasite, methionine is transported using a relatively high affinity transporter (K_m value around 30 μM), consistent with the abundance of this amino acid in plasma.

There have been inconsistencies in the literature about the impact of various inhibitors on the proton motif force in bloodstream for trypanosomes (Viera, 1998). However, our inhibitor studies point to an involvement of both energy and protons in the uptake of methionine. Although this has not been verified using electrophysiological techniques it seems likely that the proton motive force also plays a role in methionine uptake into trypanosomes.

The transporter for methionine in both bloodstream and procyclic form organisms, has similar kinetic constants, a similar substrate recognition profile and similar thermodynamic constants (E_a and Q_{10}).

To determine the specificity of the methionine transporter in procyclic *T. brucei* uptake of methionine was measured in the presence the other amino acids. Of the nineteen amino acids only the aromatic ones (phenylalanine and tryptophan), isoleucine and glutamine demonstrated substantial inhibition of methionine transport. Similar but not identical results were obtained in bloodstream forms. It is not yet clear whether different transporters operate in the different forms or whether the differences in measured function are a result of the experimental protocols used.

Inhibition of overall methionine transport by other amino acids was weak rather than partial. That is to say that total inhibition could be achieved provided sufficiently high concentrations of competitor were used. This indicates that these amino acids are probably poor substrates for a single, measured transporter, rather than potent inhibitors for one of multiple transporters. It is important to stress that inhibition of uptake of a labeled substrate by unlabelled competitors gives information only on the ability of that competitor to inhibit transport of the label. These data are often used to extrapolate

information about the structure-activity relationship between transporter and substrate, however, caution should be exercised in interpreting these results. Hence the role, if any, that the methionine transporter plays in the transport of these other amino-acids remains to be determined.

Analysis of the recognition of the transporter with other amino acids and analogues of methionine revealed that most of the structure is critical for the recognition. The length of the side chain is also crucial to the recognition process.

None of the cationic or anionic amino acids have any interaction with the transporter. The importance of the sulphur atom was further investigated by replacing it with other electronegative groups (selenomethionine, methionine sulphoxide or methionine sulfone). All of these analogues were recognised by the transporter with affinities in the same order as methionine itself. Substitution of a single sulphur by an azoxy group at this position with azoxybacilin, however, led to a dramatic reduction in affinity.

A methionine transporter previously characterised in *Leishmania tropica* promastigotes (Simon and Mukkada, 1977) showed some similarities to the *T. brucei* transporter measured here. An apparent K_m value of 33 μM was measured and in addition to L-methionine the *L. tropica* transporter also had some affinity for D-methionine, L-leucine, and L-ethionine. However under the conditions used the aromatic amino acids, L-tryptophan and L-tyrosine, did not significantly inhibit the uptake of methionine, unlike in trypanosomes.

Methionine transport has also been characterised in various other systems. The yeast *Saccharomyces cerevisiae* possesses at least three transporters for methionine including a high affinity, high specificity methionine permease (Mup1) (K_m apparent 12 μM) (Isnard *et al.*, 1993). In mammalian cells the situation is complex, with multiple transporters possessing affinity for methionine and other aliphatic amino acids (Mcgivan, 1996). The fact that the *T. brucei* methionine transporter does not appear to have high affinity for other amino-acids, as judged by the inhibition data presented here, indicates that transport of this amino acid differs markedly in trypanosomes and mammalian cells.

The present study suggests that some flexibility in the recognition motif does exist for the *T. brucei* methionine transporter. For example, other electronegative groups

can replace the sulphur atom and additional groups may be added beyond the terminal methyl group.

Chapter IV

Transport of aromatic and anionic amino acids in *Trypanosoma brucei*

4.1. Introduction

Amino acid transporters which have a high affinity for substrate are of particular interest in this study. This chapter presents data on the uptake of the least abundant amino acids in human serum: aspartate, glutamate and the aromatic amino acids: phenylalanine, tyrosine and tryptophan (Brody *et al.* 1994).

Glutamate is a precursor for proline, glutamine and arginine (Alberts *et al.*, 1994). In mammals, serum levels are low (around 45 μM) (Brody *et al.* 1994), while the concentration of glutamate in the cerebrospinal fluid (CSF) is much higher at around one millimolar (Garattini, 2000). Glutamate is one of the main neurotransmitters in the brain and to date five mammalian glutamate transporters have been described (Kanai, 1997). It is also the precusor of another neutransmitter named γ-Aminobutyrate (GABA) (Alberts *et al.*, 1994). Aspartate is the least abundant amino acid in human serum, with a concentration below 10 μM (Brody *et al.*,1994). Structurally glutamate and aspartate are both negatively charged amino acids (at physiological pH) that differ in the presence of an additional carbon in the glutamate side chain.

Phenylalanine, tyrosine and tryptophan are of similar abundance in human serum with a concentration of around 50 μM (Brody *et al.*, 1994). In mammals phenylalanine and tyrosine are key amino acids in the synthesis of L-dopa and dopamine while tryptophan is the precursor of serotonin. Structurally, tyrosine is an analogue of phenylalanine and contains a hydroxyl group on the phenyl ring. Tryptophan is also an aromatic amino acid with the presence of an indole ring that distinguishes this amino acid from phenylalanine and tyrosine.

4.2. Results:

A systematic study of each amino acid, involved the measurement of the rate of uptake and the substrate specificity of each transporter identified.

The rate of uptake of L-phenylalanine, L-tryptophan, L-tyrosine, L- glutamate and L- aspartate was measured in bloodstream forms over a 10s time period as a function of the number of parasites.

Substrate specificity was determined by measuring uptake of radiolabelled amino acids in the presence of an excess of each of the other amino acids. The results were expressed in percentage of uptake compare to a control containing only the radiolabelled amino acid.

4.2.1. Aspartate

Kinetics of transport

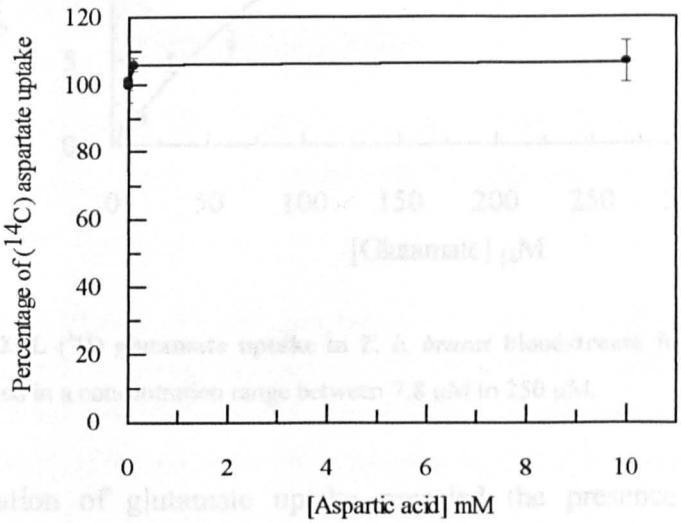


Figure 4.1: Inhibition of L-(^{14}C)aspartate uptake with non-labeled aspartate. The concentration range was from 1 μM to 10 mM. The percentage of aspartate uptake was calculated against a control without unlabelled aspartate.

The uptake of radiolabelled aspartate was not inhibited by an increased concentration of non radiolabelled aspartate, indicative of the absence of a transport phenomenon for this amino acid detectable with the conditions used.

4.2.2. Glutamate

Kinetics of transport

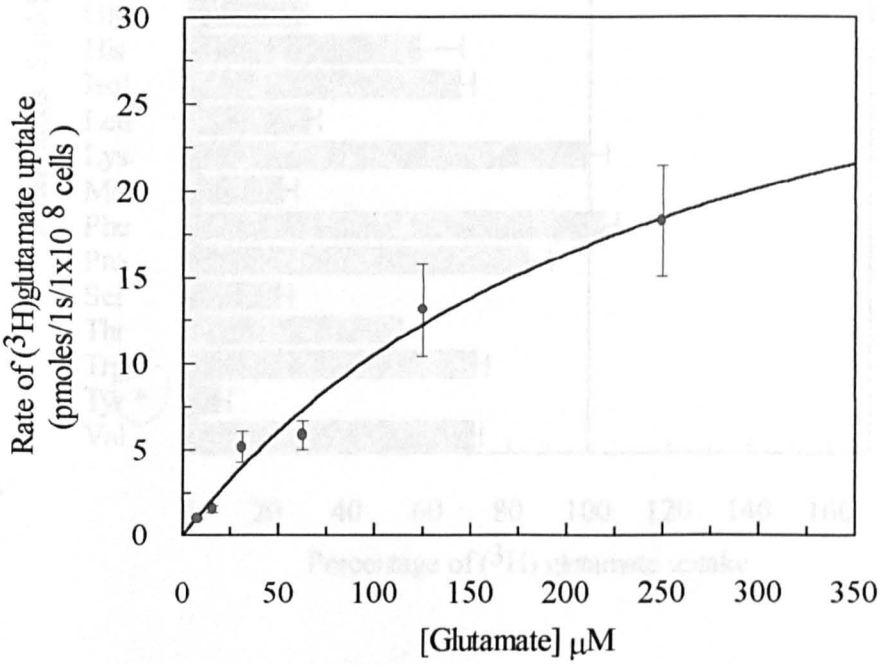


Figure 4.2: L (^{3}H) glutamate uptake in *T. b. brucei* bloodstream forms. Rate of glutamate uptake was measured in a concentration range between 7.8 μM to 250 μM.

Investigation of glutamate uptake revealed the presence of a **low affinity** transporter with an apparent **Km** value calculated at **158 ± 30 μM** and a **Vm** value at **30 ± 5 pmoles/s/1x10^{8}cells**. Kinetics in ranges of concentration from 350 μM to 10 mM and below 10 μM did not reveal the presence of any other transporters.

Specificity of glutamate transport

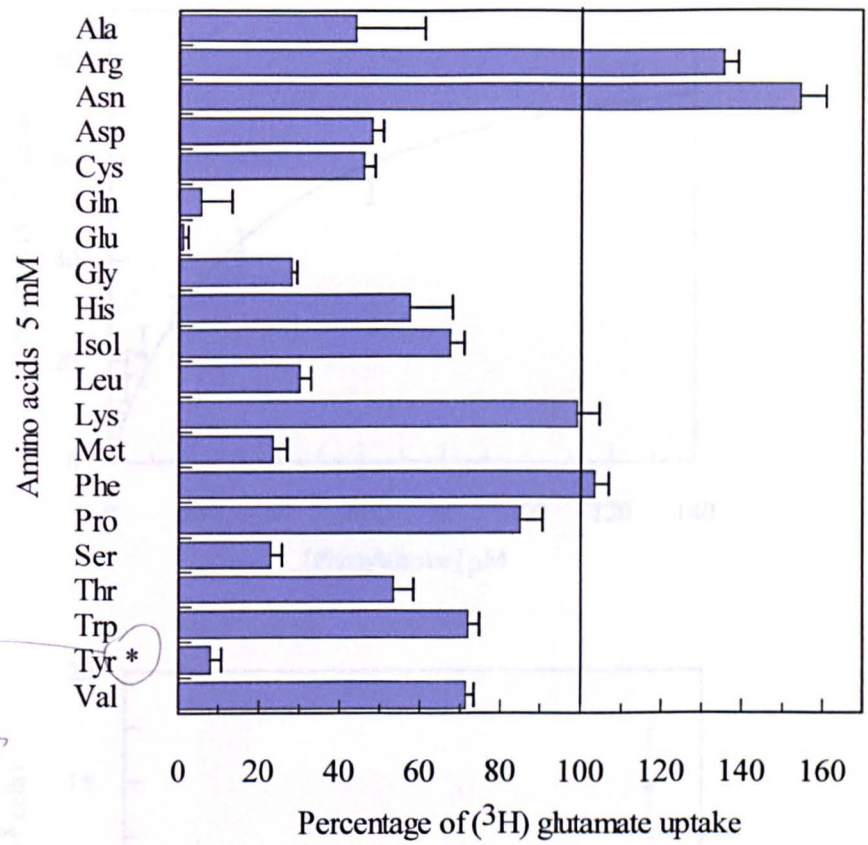


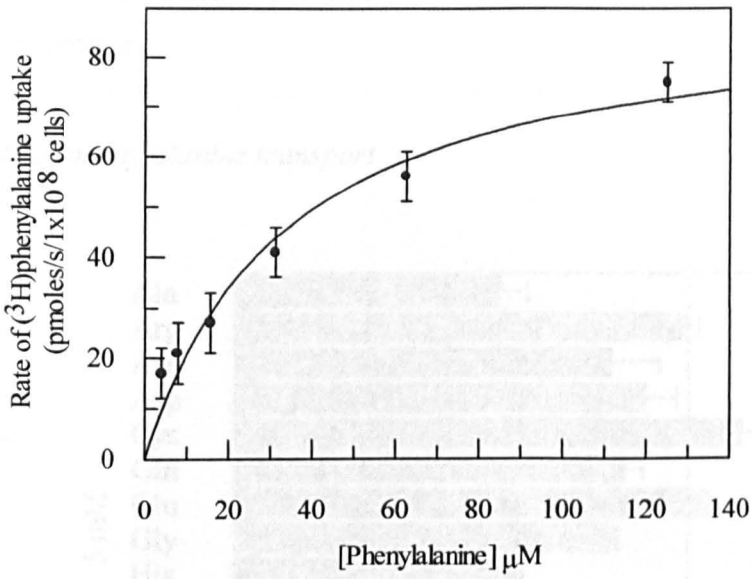
Figure 4.3: Inhibition of L-(^{3}H)glutamate uptake by non radiolabelled amino acids in bloodstream forms of *T. b. brucei*. The uptake of 150 nM of L-(^{3}H) glutamate was measured in the presence of 5 mM of non radiolabelled amino acids. The results represent the percentage of L-glutamate uptake over a time period of 10 s against a control containing only 150 nM of (^{3}H)glutamate.

Glutamate uptake in bloodstream forms was strongly inhibited (90%) by **glutamine** and **tyrosine** and more weakly (70%) by glycine, leucine, methionine and serine. Two amino acids, arginine and asparagine, actually increased glutamate uptake when used in a vast excess.

4.2.3. Phenylalanine

Kinetics of transport

A



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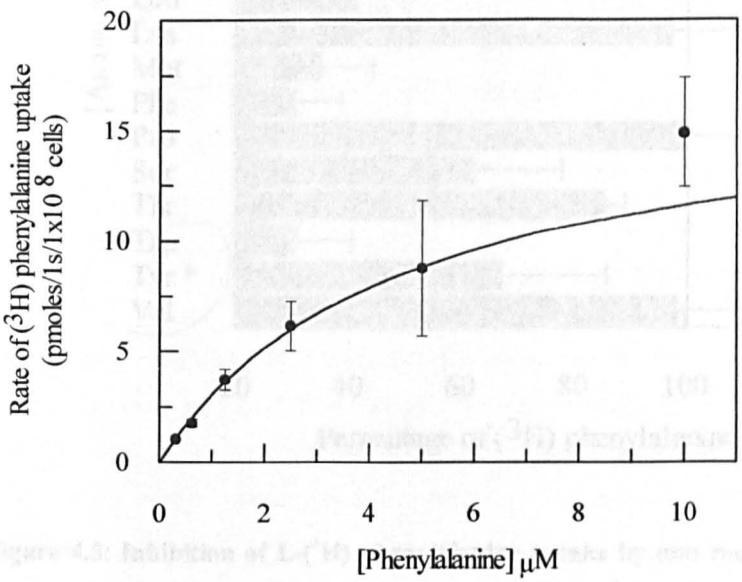


Figure 4.4. L- (^{3}H) phenylalanine uptake in *T. b. brucei* bloodstream forms. Rate of uptake was measured in the presence of a range of concentration from 3.9 μM to 125 μM (panel A) and 0.325 μM to10 μM (panel B).

Measuring initial velocity of phenylalanine as a function of concentration using different ranges of concentration revealed the presence of at least two transporters of **high** and **relatively high affinity**. The apparent **Km** and **Vm** values were calculated at **3.2 ± 1.3 μM** and **16.8 ± 1.3 pmoles /s/ 1x10^8 cells** for the high affinity transporter and at **Km** at **36 ± 9 μM** and **Vmax** at **94 ± 10 pmoles/s/1x10^8 cells** for the relatively high affinity transporter.

Specificity of phenylalanine transport

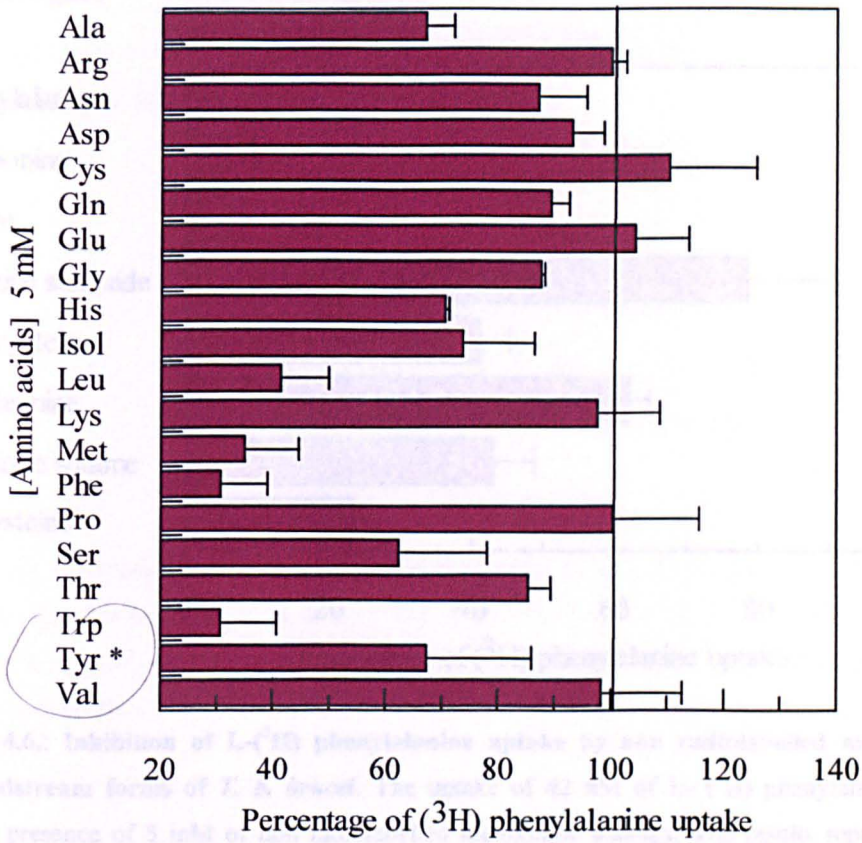


Figure 4.5: Inhibition of L-(^{3}H) phenylalanine uptake by non radiolabelled amino acids in bloodstream forms of *T. b. brucei*. The uptake of 42 nM of L- (^{3}H) phenylalanine was measured in the presence of 5 mM of non radiolabelled amino acids. The results represent the percentage of L-phenylalanine uptake over a time period of 10 s against a control containing only 42 nM of (^{3}H) phenylalanine.

Since more than one transporter is contributing to the overall measured uptake, these results can not be interpreted as indicating substrate specificity of an individual transporter. Among the different amino acids tested for their effect on phenylalanine uptake **leucine**, **methionine**, and **tryptophan** were the most potent inhibitors. A weaker inhibition was observed with serine and tyrosine.

Methionine was shown to be a good inhibitor of phenylalanine uptake, therefore various analogues of L-methionine were also tested for inhibitory activity against phenylalanine uptake.

Methionine analogues

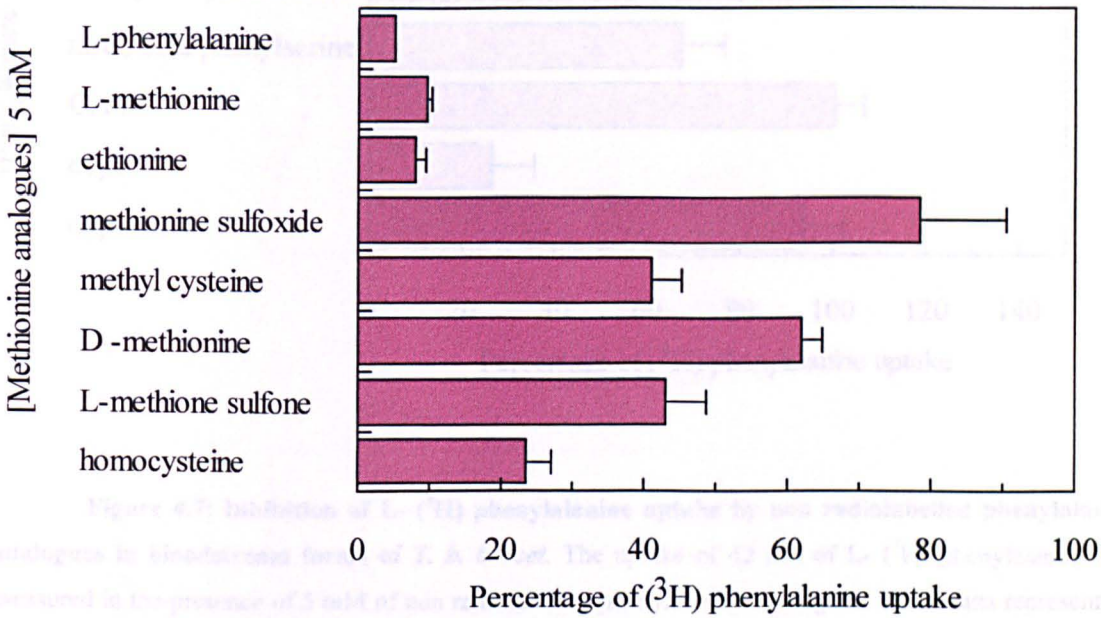


Figure 4.6.: Inhibition of L-(^{3}H) phenylalanine uptake by non radiolabelled methionine analogs in bloodstream forms of *T. b. brucei*. The uptake of 42 nM of L- (^{3}H) phenylalanine was measured in the presence of 5 mM of non radiolabelled methionine analogs. The results represent the percentage of L-phenylalanine uptake over a time period of 10 s against a control containing only 42 nM of (^{3}H) phenylalanine.

Phenylalanine uptake in bloodstream form trypanosomes is strongly inhibited by L- ethionine and homocysteine. The inhibition by methionine was stereospecific for the

L form of the amino acid. Methionine sulfoxide and methionine sulfone gave relatively weak inhibition of L-phenylalanine uptake.

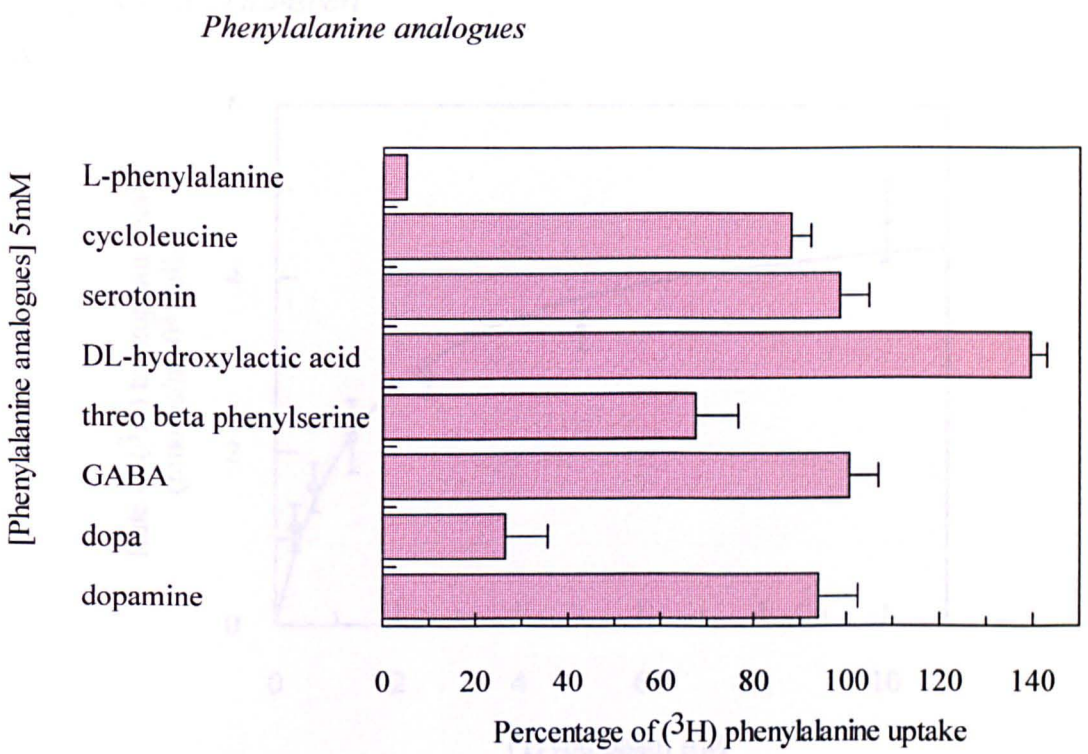


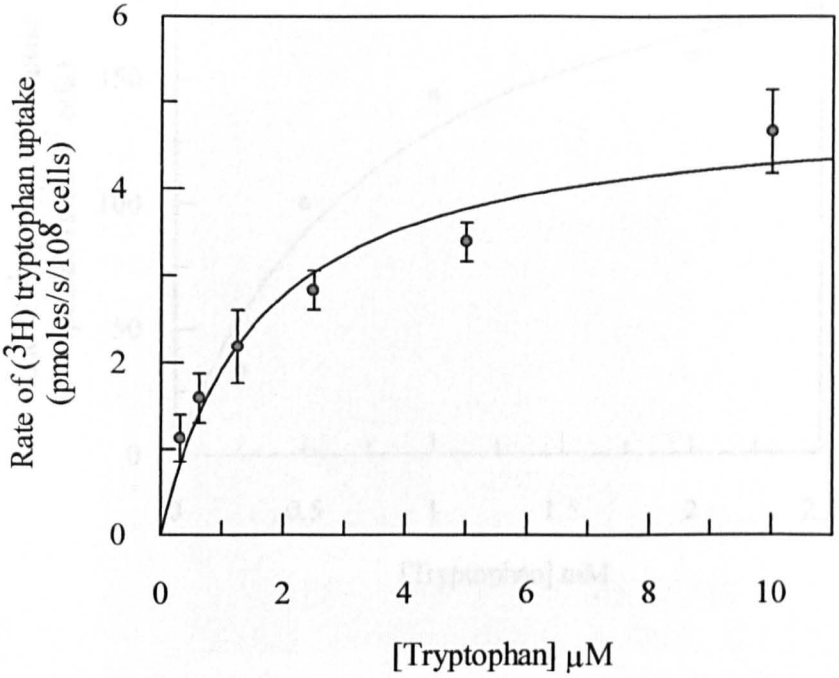
Figure 4.7: Inhibition of L- (^{3}H) phenylalanine uptake by non radiolabelled phenylalanine analogues in bloodstream forms of *T. b. brucei*. The uptake of 42 nM of L- (^{3}H) phenylalanine was measured in the presence of 5 mM of non radiolabelled phenylalanine analogues. The results represent the percentage of L-phenylalanine uptake over a time period of 10 s against a control containing only 42 nM of (^{3}H) phenylalanine.

The uptake of phenylalanine is strongly (76%) inhibited by L-dopa while no significant inhibition was observed in the presence of dopamine. Hydroxylactic acid apparently stimulates phenylalanine uptake.

4.2.4. Tryptophan

Kinetic of transport

A



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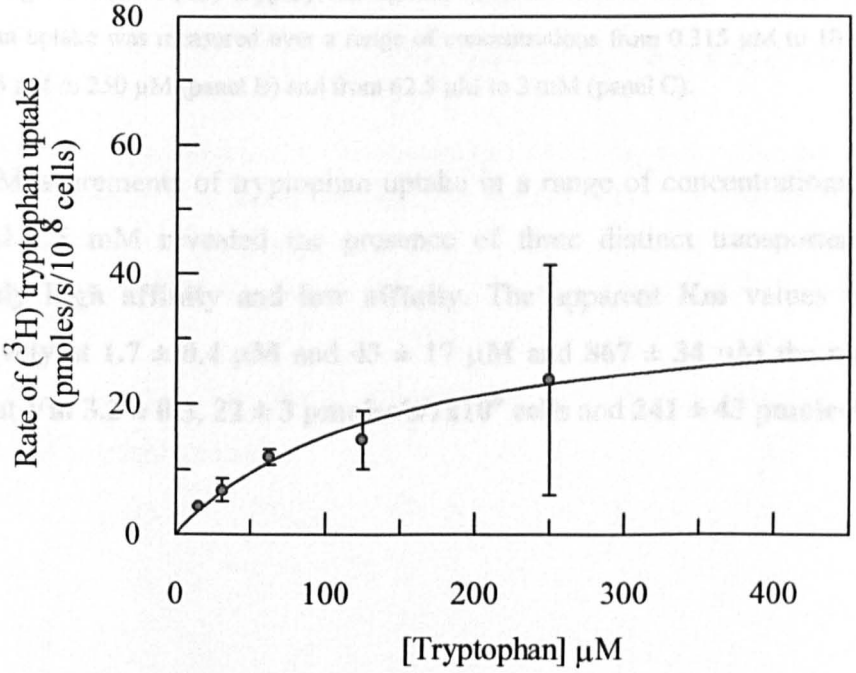


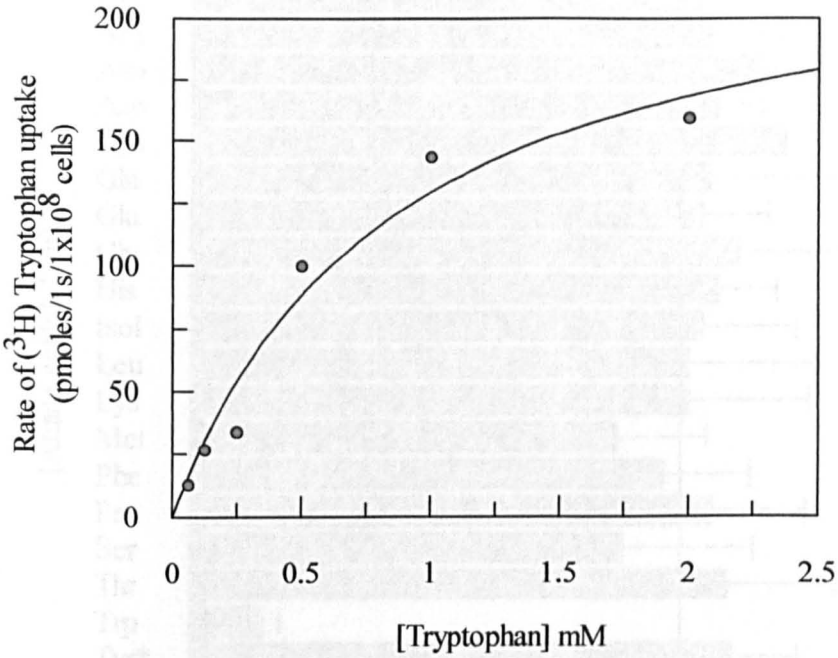


Figure 4.8.: L-(^{3}H) tryptophan uptake in bloodstream forms of *T. b. brucei*. Rate of L-tryptophan uptake was measured over a range of concentrations from 0.315 μM to 10 μM (panel A) and from 15.6 μM to 250 μM (panel B) and from 62.5 μM to 2 mM (panel C).

Measurements of tryptophan uptake in a range of concentrations between 0.315 μM and 2.5 mM revealed the presence of three distinct transporters of **high** and **relatively high affinity** and **low affinity**. The apparent **Km** values were calculated respectively at **1.7 ± 0.4 μM** and **43 ± 17 μM** and **867 ± 34 μM** the maximal rates of uptake at **Vm 3.2 ± 0.3, 22 ± 3 pmoles/s/1x10^8 cells** and **241 ± 43 pmoles/s/1x10^8 cells**.

Specificity of tryptophan transport

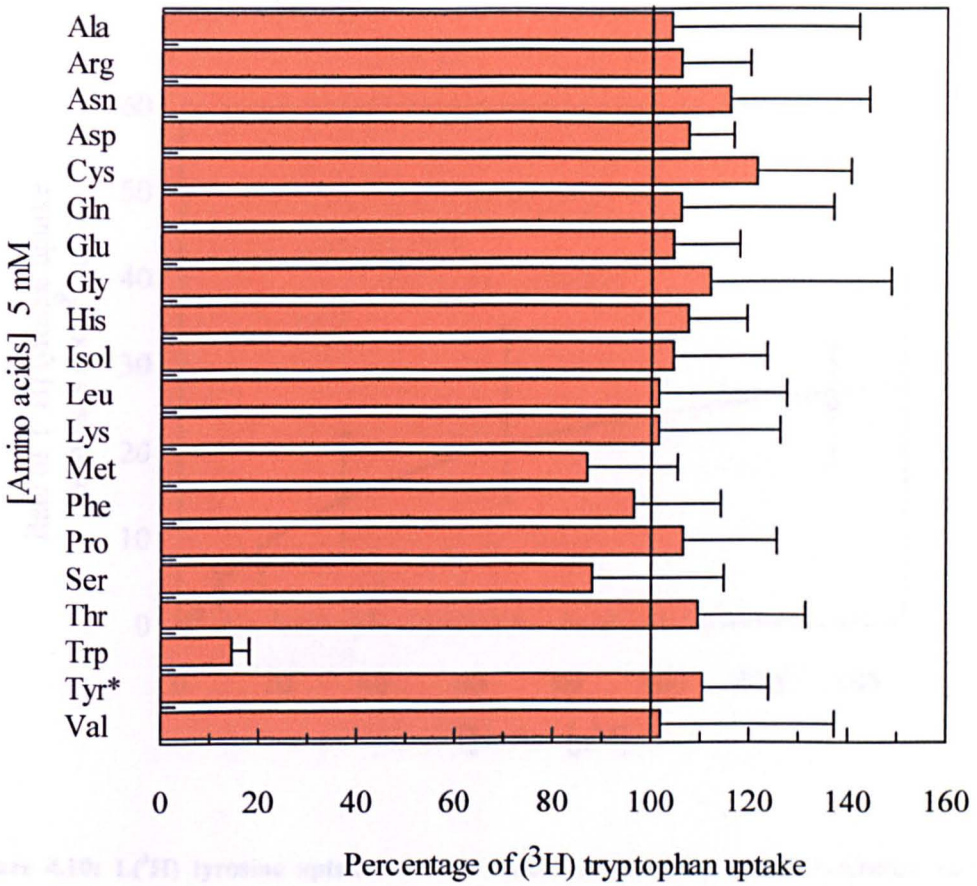


Figure 4.9: Inhibition of L-(^{3}H) tryptophan uptake by non radiolabelled amino acids in bloodstream forms of *T. b. brucei*. The uptake of 250 nM of L- (^{3}H)tryptophan was measured in the presence of 5 mM of non radiolabelled amino acids. The results represent the percentage of L-tryptophan uptake over a time period of 10 s against a control containing only 250 nM of (^{3}H)tryptophan.

None of the amino acids tested significantly inhibited tryptophan uptake with the exception of tryptophan itself.

4.2.5. Tyrosine

Kinetic of transport

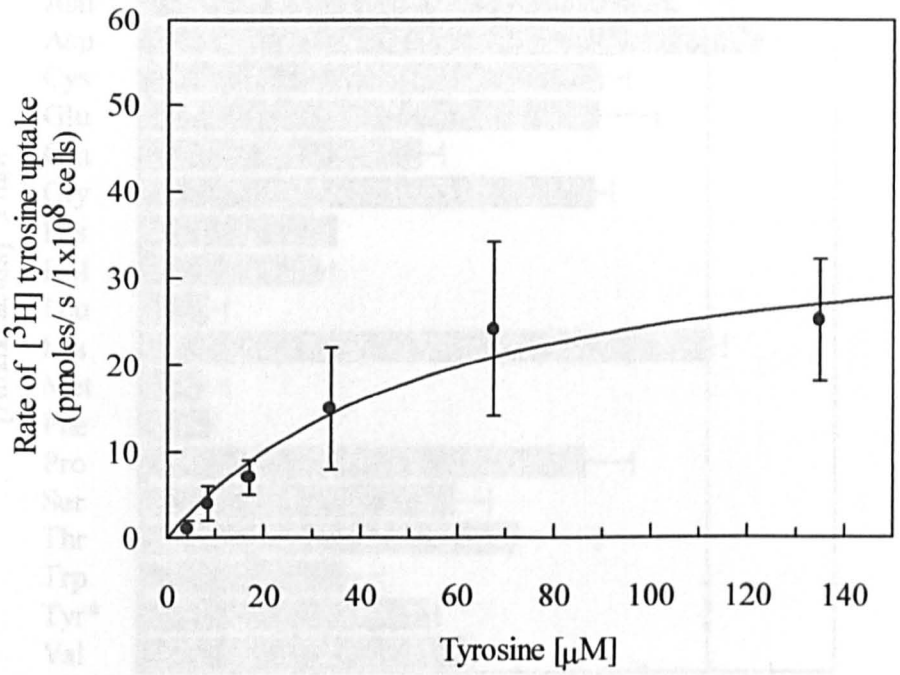


Figure 4.10: L(^{3}H) tyrosine uptake in *T. b. brucei* bloodstream forms. L-tyrosine rate of uptake was measured in a range of tyrosine concentration from 4.2 µM to 135 µM.

Due to the presence of an additional hydroxyl group on the phenyl ring, tyrosine solubility in water is reduced compared to that of phenylalanine, and the highest concentration tested was 135 µM. Over a tyrosine concentration range of 4.2 µM to 135 µM, a single **relatively high affinity** transporter was identified. The apparent **Km** and **Vm** values were respectively determined at **55 ± 20 µM** and **37 ± 6 pmoles/s/1x10^8 cells** respectively.

Specificity of tyrosine transport

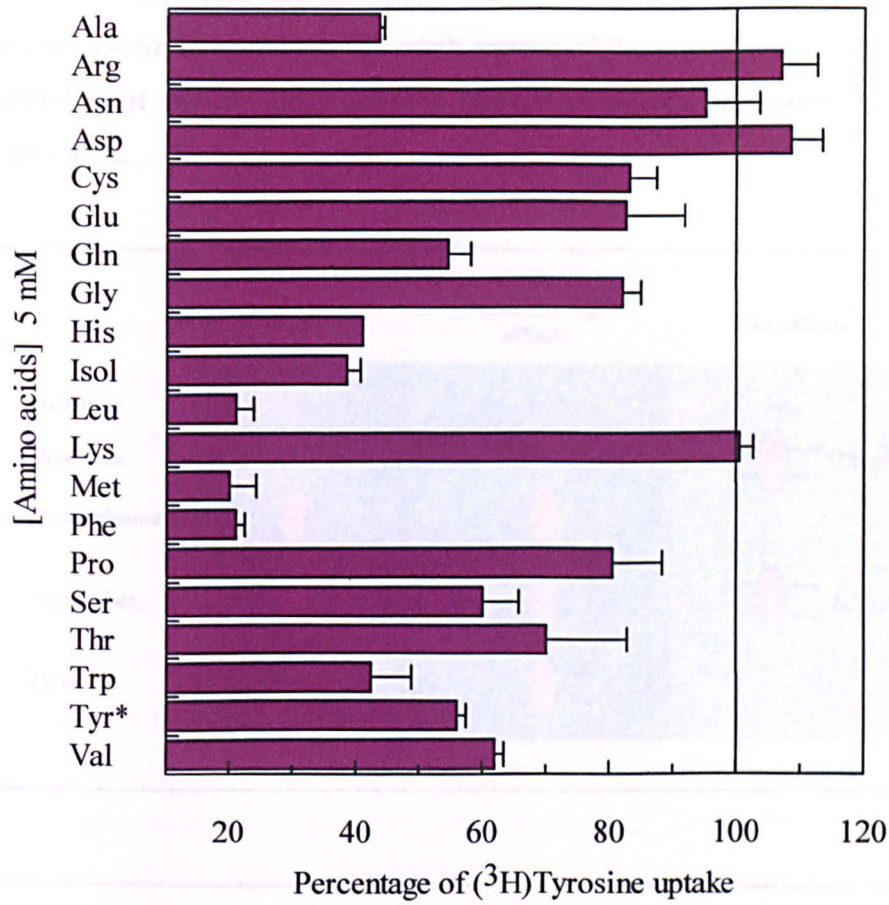


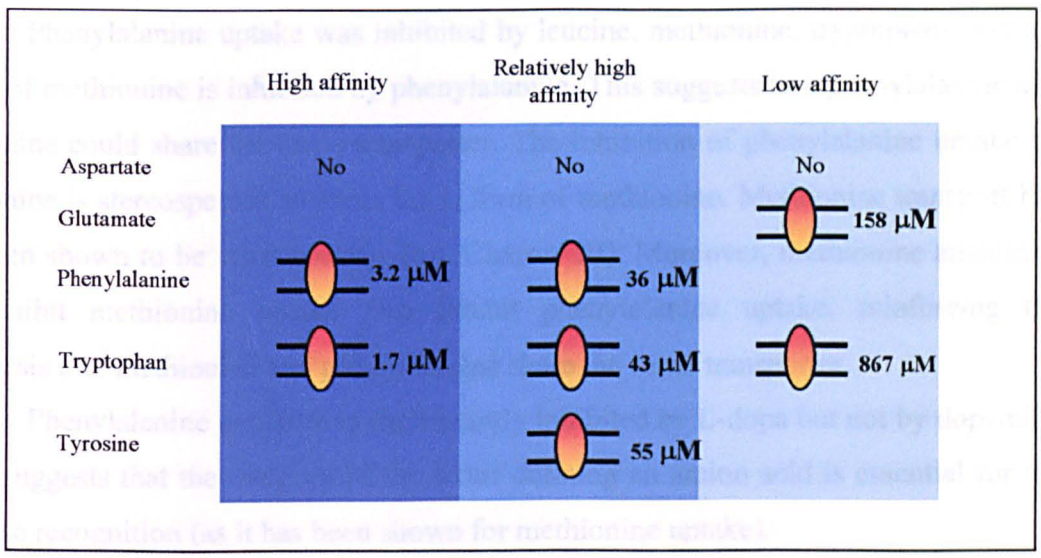
Figure 4.11: Inhibition of L- (^{3}H) tyrosine uptake by non radiolabelled amino acids in bloodstream forms of *T. b. brucei*. The uptake of 98 nM of L- (^{3}H) tyrosine was measured in the presence of 5 mM of non radiolabelled amino acids. The results represent the percentage of L-tyrosine uptake over a time period of 10 s against a control containing only 98 nM of L- (^{3}H)tyrosine.

Tyrosine uptake in bloodstream forms of *T. brucei* was inhibited at around50% by alanine, glutamine, histidine, isoleucine, tryptophan and strongly inhibited (80% inhibition) by **leucine**, **methionine** and **phenylalanine**.

4.3. Discussion.

Bloodstream form trypanosomes have the ability to transport glutamate, phenylalanine, tryptophan and tyrosine while uptake of the anionic amino acid, aspartate, was apparently not carrier mediated. An illustration of the transporters identified is shown figure 4.12.

A



B

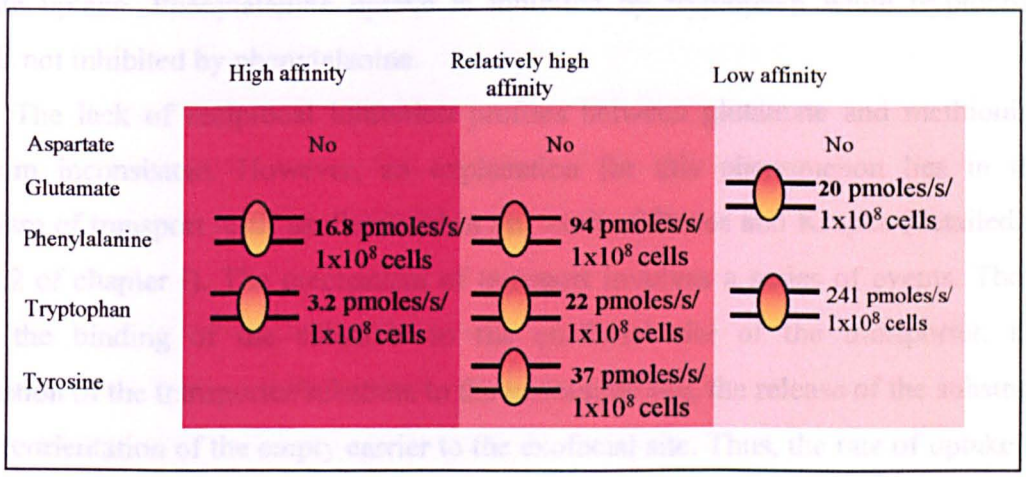


Figure 4.12: Schematic representation of the transporters identified for L-aspartate, L-glutamate, L-phenylalanine, L-tryptophan and L-tyrosine in the bloodstream form of *T. brucei*. Panel A) represents the K_m values of the transporters, Panel B) represents the V_{max} values.

Phenylalanine and tryptophan, have been found to use at least two routes of entry while a single high affinity transporter was identified for tyrosine. In this study, the term "high affinity" is used for K_m values below 30 μM, "relatively high affinity" for K_m values between 30 and 100 μM and "low affinity" for K_m values above 100 μM.

Analysis of the different inhibition profiles of uptake of glutamate, phenylalanine, tryptophan, tyrosine and methionine presented in Chapter III allows a tentative hypothesis to be presented in which some amino acids share the same transporter.

Phenylalanine uptake was inhibited by leucine, methionine, tryptophan, and the uptake of methionine is inhibited by phenylalanine. This suggests that phenylalanine and methionine could share the same transporter. The inhibition of phenylalanine uptake by methionine is stereospecific towards the L form of methionine. Methionine transport has also been shown to be stereospecific (c.f. Chapter III). Moreover, methionine analogues that inhibit methionine uptake also inhibit phenylalanine uptake, reinforcing the hypothesis that methionine and phenylalanine share the same transporter.

Phenylalanine uptake was significantly inhibited by L-dopa but not by dopamine which suggests that the integrity of the motif defining an amino acid is essential for the substrate recognition (as it has been shown for methionine uptake).

Glutamate weakly inhibits methionine uptake but methionine strongly inhibits glutamate uptake. Phenylalanine uptake is inhibited by tryptophan while tryptophan uptake is not inhibited by phenylalanine.

The lack of reciprocal inhibition profiles between glutamate and methionine may seem inconsistent. However, an explanation for this phenomenon lies in the mechanism of transport itself as discussed in the work of Deves and Krupka (detailed in part 2.2.2 of chapter I). The mechanism of transport involves a series of events. These include the binding of the substrate to the exofacial site of the transporter, the translocation of the transporter/substrate to the endofacial site, the release of the substrate and the reorientation of the empty carrier to the exofacial site. Thus, the rate of uptake of a substrate measured by kinetic experimentation is a sum of the rates of these different steps. Considering, as an example, a transporter and two its substrates named A and B. The rate of reorientation of the carrier from the endofacial to the exofacial site after the

release of A or B is the same because the carrier is empty. However the rate of translocation of the carrier when A is substrate can be different from the rate of translocation of B depending on the strength of the binding forces of the substrate with the transporter. The fact that the rate of translocation of these two substrates by the same transporter is different will necessarily lead to a difference in the percentage of inhibition of the uptake of one by the other. In the case of methionine and phenylalanine uptake the rate of methionine uptake is 12 times higher than the "high affinity" phenylalanine transporter and twice as fast as the "relatively high affinity" phenylalanine transporter. This means that measuring the inhibition of methionine uptake by phenylalanine or measuring the inhibition of phenylalanine uptake by methionine does not give the same profile of inhibition because the carrier is faster in the translocation of methionine than in the translocation of phenylalanine.

Although inhibition experiments have been extensively used to characterize the substrate specificity of various transporters, there are serious limitations to the interpretation of these data. A molecule able to significantly inhibit the uptake of a particular substrate is a candidate for being a substrate for this transporter (although this should then be proven directly with radiolabelled substrate). However, a molecule that does not inhibit, or is a weak inhibitor, can not necessarily be excluded from being a potential candidate for this transporter.

The hypothetical model that can be suggested in the context of the inhibition profiles obtained for the different amino acids is that: methionine, phenylalanine, tyrosine and glutamate could share the same transporter. But it is difficult at this stage to draw any definitive conclusion which of the multiple phenylalanine or tryptophan transporters would be involved in the uptake of methione, tyrosine and glutamate.

This model is hypothetical and a definitive answer to the substrate specificity is unlikely to come from a biochemical approach alone. The identification and the expression of the genes encoding these transporters, one by one, will be a key step in this direction. The biochemical approach has, nevertheless, given a broad view on the uptake of these amino acids in the bloodstream forms of the parasite.

Chapter V

Cloning and functional expression of *TbAATP1*, an amino acid transporter gene from *Trypanosoma brucei.*

5.1. Introduction

The functional expression of genes encoding amino acid transporters is a key step in studying amino acid transport in *T. brucei*. Two approaches have been undertaken in attempts to achieve this aim: a yeast complementation approach and the search of the *T. brucei* genome sequence databases.

The yeast complementation technique involved the use of different strains of *Saccharomyces cerevisae* lacking the general amino acid permease gene (gap1) as well as individual transporter genes. These were transformed with a *T. brucei* DNA library in a yeast expression vector.

Transformants were screened on selective media for complementation, which would allow the selection of transformants containing a plasmid bearing a *T. brucei* sequence corresponding to an amino acid transporter gene provided that the gene is expressed. This yeast complementation approach has been used to identify numerous plant amino acid transporter genes and in trypanosomes the cloning of the *TbAT1* nucleoside transporter gene was also identified with such an approach (Maser *et al.*, 1999).

As a second approach the *T. brucei* genome databases were periodically searched at TIGR and the Sanger center using the sequences of amino acid transporter genes from other organisms. The investigation of the databases led to the identification of a putative amino acid transporter gene and the yeast complementation approach was then abandoned. The gene found was named ***TbAATP1*** which stand for *Trypanosoma brucei* Amino Acid Transporter 1.

This chapter presents the identification and molecular characterisation of *TbAATP1* and its functional characterization in *Xenopus laevis* oocytes.

5.2. Results

5.2.1. The identification of *TbAATP1*:

TbAATP1 was identified using the TIGR End Sequences Database and the sequence of an amino acid transporter gene from *Arabidopsis thaliana*. The first sequences encompassing the putative amino acid transporter were 23M11, 16C11, 42I20 rev and 16C11. These sequences were then assembled using the ContigExpress program from the Vector NTI suite. The assembled fragment was 2155 bp in length. An open reading frame (ORF) based on the presence of an ATG start codon and TGA stop codon was identified within the fragment. The ORF of 1404 bp is shown figure 5.1.

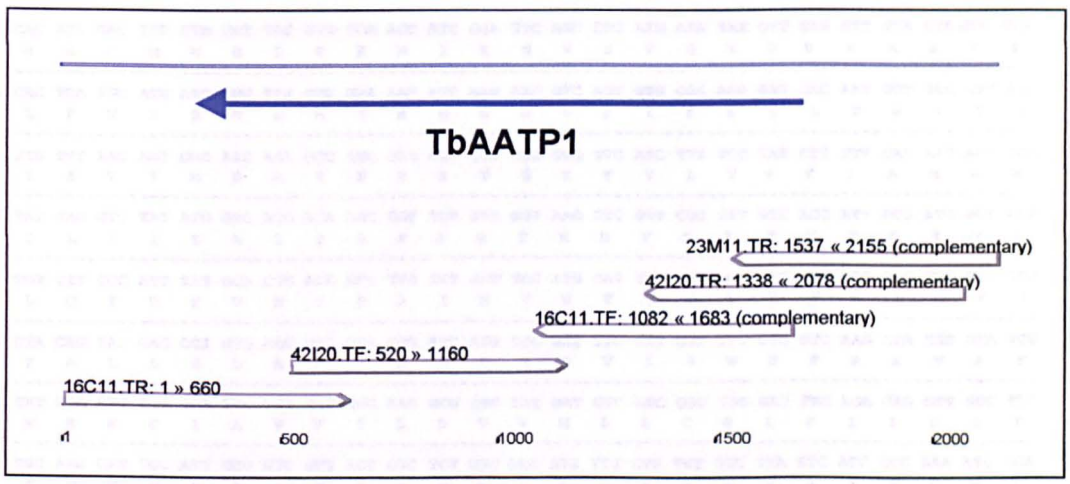


Figure 5.1: Illustration of a contiguous sequence formed by five overlapping sequences 16C11.TR, 16C11.TF, 42I20.TR, 42I20.TF and 23M11.TR from the *T. brucei* genome project. The open reading frame corresponding to TbAATP1 is indicated by a blue arrow.

TbAATP1 was amplified by the polymerase chain reaction using *Pfu* polymerase from *T. brucei* 927 genomic DNA using primers upstream and downstream of the open reading frame. The PCR product was sub-cloned into pGEM-T vector and sequenced. The nucleotide sequence obtained differed only at one nucleotide from the sequence obtained from the database which had no effect on the amino acid translation (Figure 5.2).

```
    M  T  S  I  N  A  Q  P  P  N  S  A  T  Y  P  R  D  D  H  G  S  A  E  A  G
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
  1 ATG ACC AGC ATC AAT GCC CAA CCA CCC AAC TCA GCC ACT TAT CCT CGG GAT GAT CAT GGC AGC GCA GAA GCT GGG
    Q  A  N  A  E  V  E  R  P  Q  T  K  K  Q  K  D  G  G  G  C  F  A  R  V  S
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
 76 CAA GCG AAT GCT GAG GTG GAA CGC CCA CAG ACA AAA AAA CAG AAA GAT GGT GGT GGT TGC TTT GCC AGG GTG AGT
    L  F  M  A  T  I  I  P  P  G  G  I  A  A  S  A  F  N  I  G  S  T  T  I  G
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
151 CTA TTT ATG GCA ACT ATC ATC CCA CCG GGA GGT ATC GCC GCA AGT GCC TTC AAC ATC GGA TCA ACT ACC ATT GGT
    A  G  I  F  G  L  P  A  A  A  N  S  S  G  L  V  M  A  M  I  Y  L  I  I  I
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
226 GCC GGC ATT TTT GGC TTG CCA GCA GCC GCC AAC AGC AGT GGG CTT GTG ATG GCG ATG ATT TAT CTT ATT ATC ATT
    T  A  M  T  I  F  S  I  Y  A  L  G  V  A  A  E  R  T  N  I  R  T  Y  E  G
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
301 ACC GCC ATG ACT ATT TTC TCC ATA TAT GCT CTT GGT GTC GCC GCT GAA AGA ACA AAC ATT CGT ACA TAC GAA GGA
    V  A  R  A  L  L  G  P  W  G  A  F  C  T  A  A  A  R  T  F  F  C  F  S  A
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
376 GTT GCC CGT GCA CTA CTG GGT CCA TGG GGT GCA TTT TGC ACT GCT GCA GCC CGT ACT TTC TTC TGC TTT AGT GCR
    C  V  A  Y  V  I  S  V  G  D  I  L  S  A  T  L  K  G  T  N  A  P  D  F  L
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
451 TGT GTG GCA TAT GTG ATT TCT GTG GGT GAT ATC CTG AGT GCT ACC TTA AAG GGT ACC AAT GCC CCT GAT TTC CTG
    K  Q  K  S  G  N  R  L  L  T  S  L  M  W  L  C  F  M  L  P  L  V  I  P  R
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
526 AAG CAG AAG TCA GGC AAC CGG CTA TTG ACA TCC CTC ATG TGG TTA TGC TTC ATG CTT CCA CTT GTA ATA CCT CGG
    H  I  D  S  L  R  Y  V  S  T  I  A  F  S  L  M  I  Y  V  V  V  V  V  V  V
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
601 CAC ATT GAC TCC CTA CGT TAT GTT TCA ACC ATC GCA TTC AGC CTC ATG ATA TAT GTT GTG GTT GTA GTG GTG GTG
    H  S  C  M  N  G  L  P  E  N  I  K  N  V  S  V  G  K  D  D  N  A  E  I  I
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
676 CAC TCA TGC ATG AAT GGG TTA CCG GAA AAT ATC AAG AAC GTC AGT GTG GGA AAG GAT GAC AAT GCT GAG ATT ATA
    L  F  N  S  G  N  R  A  I  E  G  L  G  V  F  I  F  S  Y  L  F  H  I  T  A
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
751 TTG TTT AAC AGT GGG AAC AGA GCC ATC GAA GGT CTT GGG GTG TTC ATC TTT TCC TAT CTT TTT CAT ATT ACC GCG
    Y  E  V  Y  M  D  M  T  N  R  S  V  G  K  F  V  L  V  V  T  I  A  M  G  M
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
826 TAT GAG GTC TAT ATG GAC ATG ACA AAC CGT TCG GTG GGT AAG TTC GTT CTG GTT GTC ACC ATT GCG ATG GGT ATG
    C  L  P  I  Y  A  L  T  A  F  F  G  Y  M  D  F  G  R  N  V  T  G  S  V  L
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
901 TGT CTT CCC ATT TAT GCA CTG ACA GCC TTC TTT GGT TAC ATG GAT TTT GGT CGC AAC GTT ACC GGC TCT GTG CTC
    L  Q  Y  D  P  V  N  Y  P  A  I  M  V  G  F  V  G  V  L  V  K  L  C  V  S
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
976 CTA CAG TAC GAC CCT GTG AAC TAC CCA GCG ATT ATG GTC GGT TTC GTT GGT GTG CTC GTC AAG CTA TGC GTA TCG
    Y  A  L  L  G  L  A  C  R  N  A  L  Y  D  V  I  G  W  D  F  R  E  V  A  F
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
1051 TAT GCA CTT TTG GGA TTG GCT TGC CGC AAT GCG CTG TAT GAT GTC ATC GGG TGG GAC TTC AGA GAG GTT GCC TTC
    W  K  H  C  I  A  V  V  T  L  S  V  V  M  L  L  C  G  L  F  I  P  K  I  T
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
1126 TGG AAG CAT TGC ATT GCC GTC GTT ACT CTC TCT GTC GTC ATG TTG CTG TGT GGC TTA TTC ATC CCC AAA ATC ACA
    T  V  F  G  F  A  G  S  I  S  G  G  L  L  G  F  I  L  P  A  L  F  F  M  Y
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
1201 ACC GTA TTC GGA TTC GCT GGT TCC ATC TCT GGT GGA TTG TTG GGT TTC ATT CTT CCT GCG CTA TTC TTC ATG TAT
    S  G  G  F  T  W  Q  K  V  G  P  F  Y  Y  I  S  T  Y  V  L  L  I  T  G  V
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
1276 TCT GGT GGC TTT ACG TGG CAA AAG GTT GGT CCC TTT TAT TAC ATT TCC ACT TAT GTT CTA CTG ATC ACA GGT GTT
    I  A  A  V  F  G  T  G  A  T  I  W  A  V  T  V  G  *
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
1351 ATC GCA GCC GTA TTT GGC ACA GGT GCT ACC ATT TGG GCA GTT ACT GTG GGA TGA
```

Figure 5.2: ***TbAATP1* nucleotide sequence.** The nucleotide sequence was obtained by sequencing of the PCR product of the putative gene cloned into a pGEM-T vector. The predicted amino acid sequence is indicated above the nucleotide sequence.

The predicted amino acid sequence of TbAATP1 was then probed against the general databases using a tBlastN search at NCBI. Significant homology scores were found with amino acid transporters from various organisms, among which the highest levels of homology were with mammalian amino acid transporters.

AF075704	Rattus norvegicus neuronal glutamine transporter, score 2e-28
AF298897	Homo sapiens amino acid transporter system A, score 2e-24
NM006841	Homo sapiens system N1 glutamine transporter, score 1e-17

Figure 5.3: Results of a tBlastN search at NCBI for TbAATP1 homologues.

5.2.2. Predictive structural Analysis of TbAATP1

Hydropathy analysis

Transporters are transmembrane proteins consisting of helices of hydrophobic amino acids which are connected by loops of hydrophilic amino acids. The loops can be of variable length, however for amino acid transporters the number of helices varies generally between ten and twelve. The number of transmembrane domains can be predicted using software based on the determination of hydrophobic regions. The predicted amino acid sequence of TbAATP1 was analyzed using Kyte-Doolittle and TMpred hydropathy analysis programs. Eleven transmembrane domains were predicted by both algorithms (Figure 5.4). Four putative glycosylation sites, based on the presence of a consensus sequence Asn-Xaa-Ser/Thr and the predicted folding of the protein were also predicted using PROSITE (Figure 5.5).

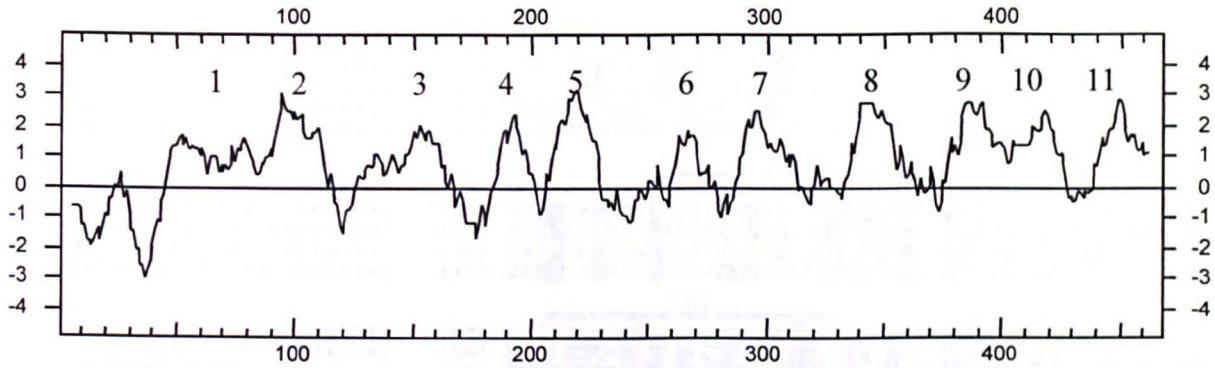


Figure 5.4: **Hydrophobicity plot of TbAATP1**. A Hydrophobicity plot of TbAATP1 was constructed based on Kyte-Doolittle hydropathy analysis. Hydophobic peaks corresponding to putative membrane spanning regions are numbered. The abscissa indicates amino acid numbers and the ordinate is ahydropathy index.

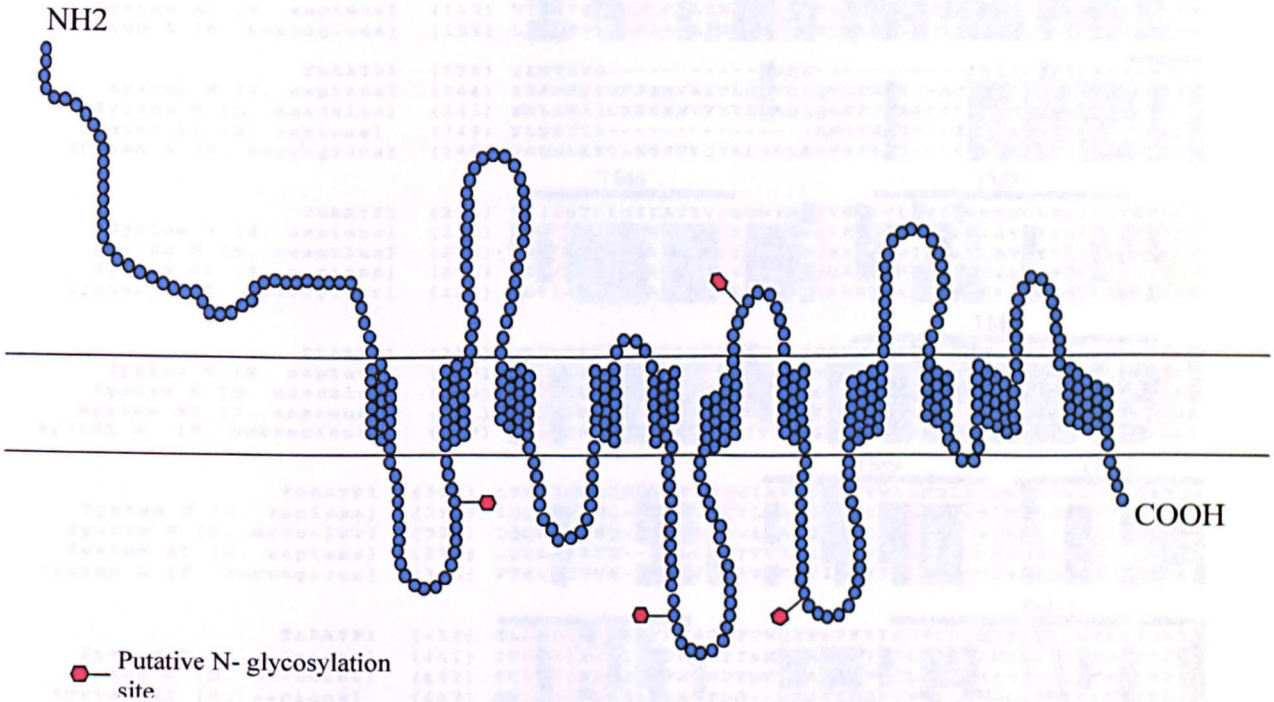


Figure 5.5: **Schematic representation of the predicted topology of TbAATP1**. The topology was predicted by a Kyte-Dolittle hydropathy analysis including an extracellular N terminus and 11 transmembrane domains. Blue circles represent each individual amino acid. Amino acids bearing a red label represent putative glycosylation sites.

The amino acid sequence alignments of TbAATP1 with the mammalian amino acid transporter sequences reveals 11.1 % of identity with conserved regions scattered along the sequence (Figure 5.6).

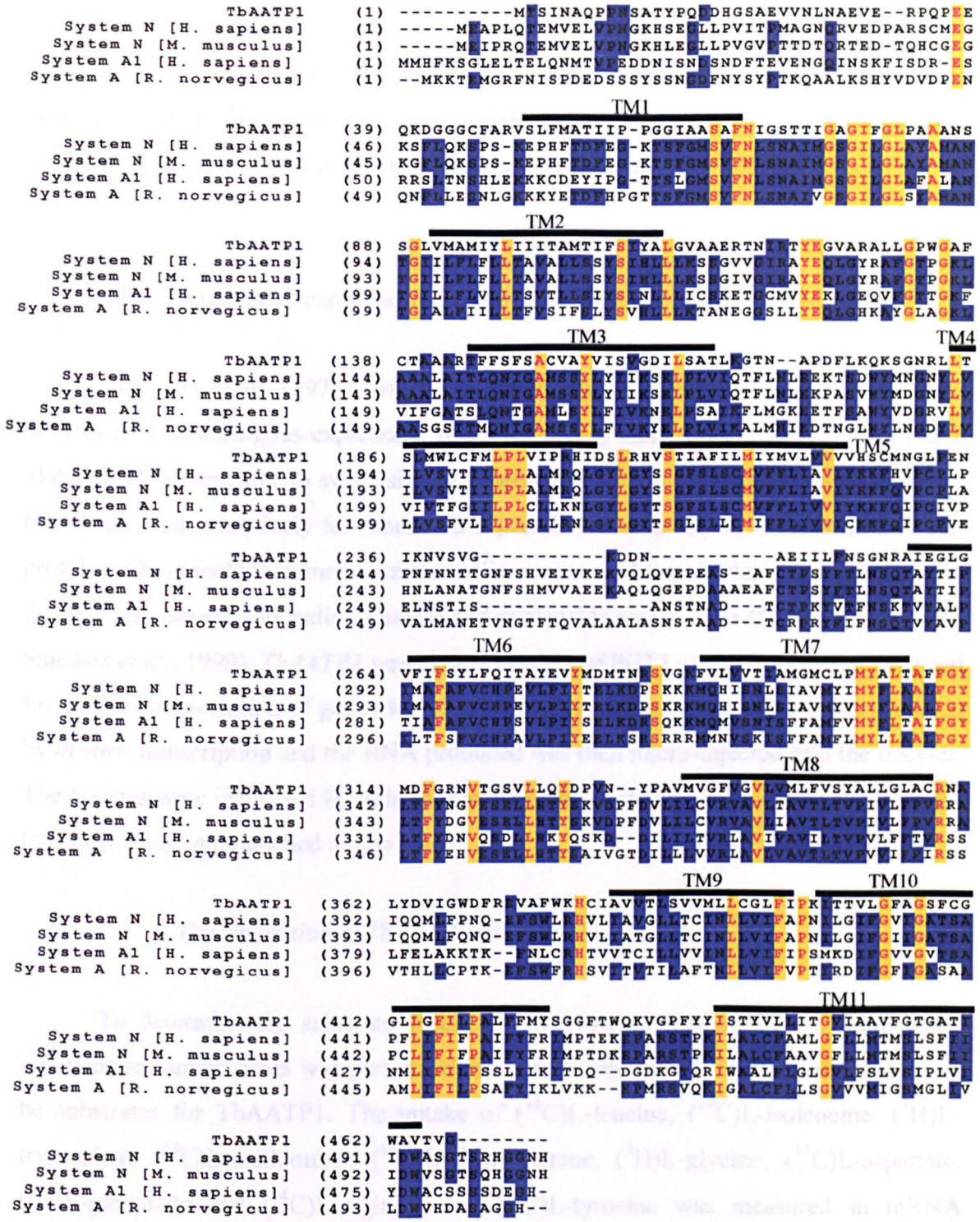


Figure 5.6: Amino acid sequences alignments of mammalian system A and N with TbAATP1. The predicted amino acid sequence of TbAATP1 was aligned with system N and system A from *Homo sapiens* and *Rattus norvegicus* based on the ClustalW algorithm. The positions of predicted transmembrane domains for TbAATP1 are indicated by the solid lines above the sequence. Identical amino

Predictive structural analysis of TbAATP1 along with sequence similarity to other amino acid transporters indicates that this sequence presents features of an amino acid transporter. However, this assumption needed to be assessed by functional expression. *Xenopus laevis* oocytes were chosen as an expression system.

5.2.3. Functional expression of *TbAATP1* in *Xenopus laevis* oocytes.

Gurdon *et al.* (1976) first demonstrated the possibility of using of *Xenopus* oocytes as a heterologous expression system, showing that oocytes injected with rabbit globin mRNA were able to synthesized a large amount of globin. *Xenopus laevis* oocytes have been used extensively for functional expression of a variety of membrane transport proteins. These include numerous mammalian amino acid transporters as well as several *T. brucei* transporters including glucose and nucleoside transporters (Barrett *et al.*, 1995, Sanchez *et al.*, 1999). *TbAATP1* was cloned into the pSPGT1 vector which was designed for *in vitro* transcription of genes for oocytes expression. A capped cRNA was obtained by *in vitro* transcription and the RNA produced was then micro-injected into the oocytes. The oocytes were incubated for at least three days after injection before being assayed for transport using radiolabelled amino acids.

Determination of TbAATP1 substrate specificity

To determine the substrate specificity of TbAATP1, a pilot experiment using radiolabelled amino acids was performed in order to determine which amino acids could be substrates for TbAATP1. The uptake of (^{14}C)L-leucine, (^{14}C)L-isoleucine, (^{3}H)L-tryptophan, (^{14}C)L-methionine, (^{14}C)L-phenylalanine, (^{3}H)L-glycine, (^{14}C)L-aspartate, (^{3}H)L-glutamate and (^{14}C)L-arginine and (^{14}C)L-tyrosine was measured in mRNA injected oocytes and compared to control oocytes. The results are shown figure 5.7. In this pilot experiment L-glutamate was the only amino acid to give an uptake significantly higher in the TbAATP1 RNA injected oocytes compared to the controls indicating that glutamate was a substrate for this transporter (Figure 5.7)

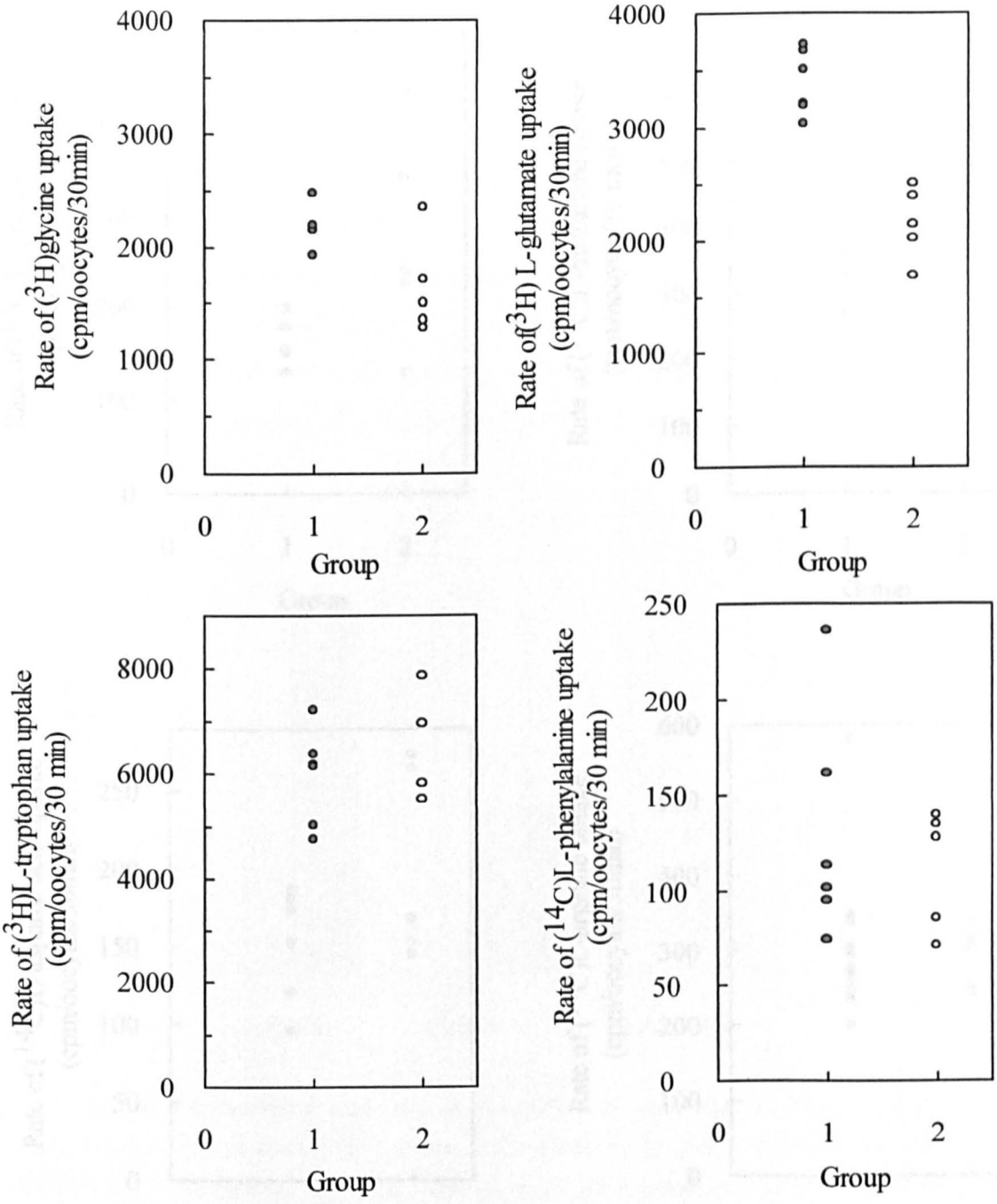


Figure 5.7: Pilot experiment measuring the rate of uptake of (^{3}H)L- tryptophan, (^{14}C)L-phenylalanine, (^{3}H)glycine, (^{3}H)L-glutamate by TbAATP1 injected oocytes (20ng /oocyte) (grey circles) compared to water injected controls (open circles) over a 30 min time period.

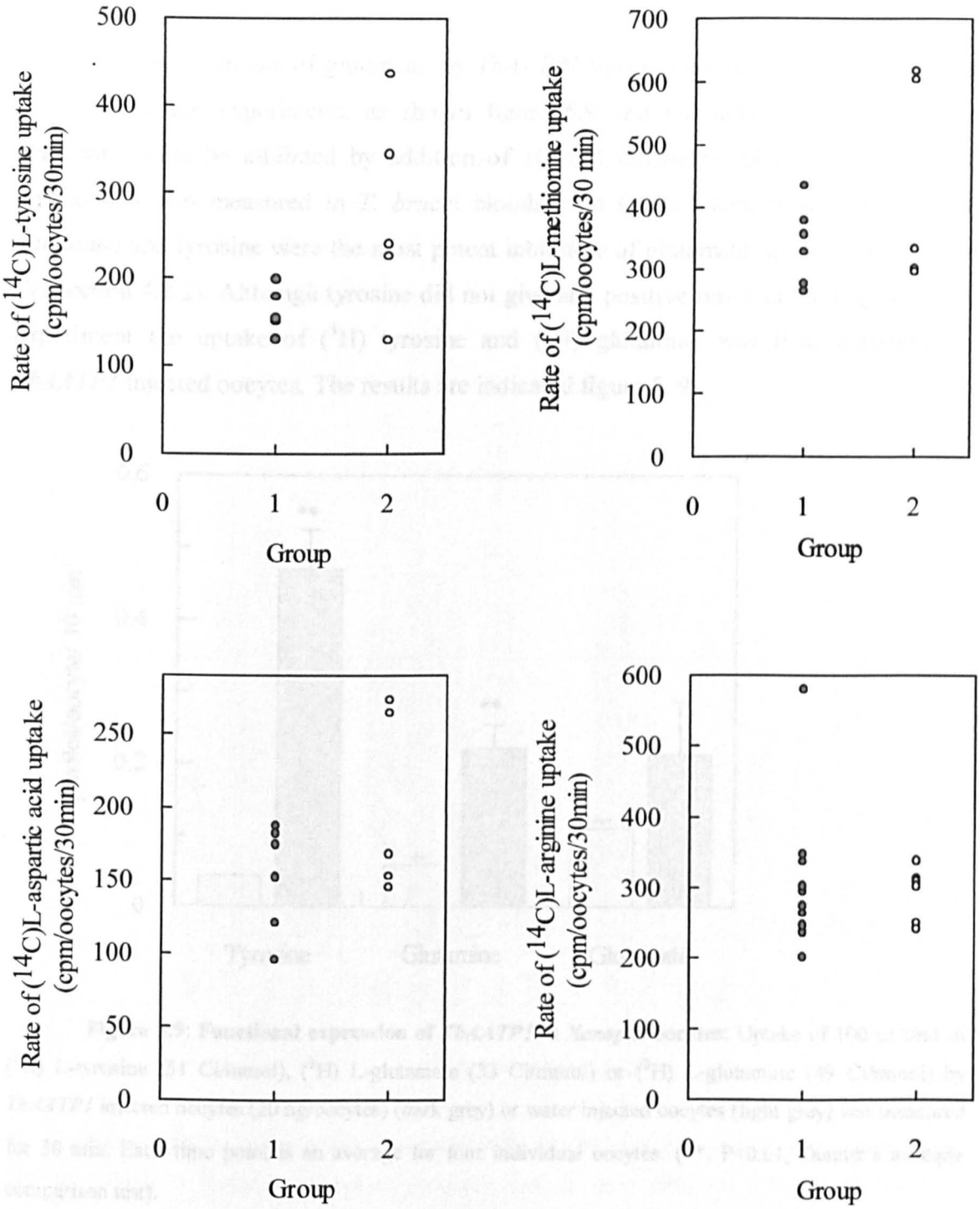


Figure 5.8: Pilot experiment measuring the rate of uptake of (^{14}C)L-methionine, (^{14}C)L-aspartate, (^{14}C)L-arginine and (^{14}C)L-tyrosine by *TbAATP1* injected oocytes (20ng/oocyte) (grey circles) compared to water injected controls (open circles) over 30 min time period.

Enhanced uptake of glutamate by *TbAATP1* injected oocytes was reproduced in five independent experiments as shown figure 5.9 and the uptake of radiolabelled glutamate could be inhibited by addition of 10 mM unlabelled glutamate. Glutamate uptake was also measured in *T. brucei* bloodstream forms where it was shown that glutamine and tyrosine were the most potent inhibitors of glutamate uptake (c.f. Chapter IV, section 4.2.2). Although tyrosine did not give any positive result in the original pilot experiment the uptake of (^{3}H) tyrosine and (^{3}H) glutamine was then measured in *TbAATP1* injected oocytes. The results are indicated figure 5. 9.

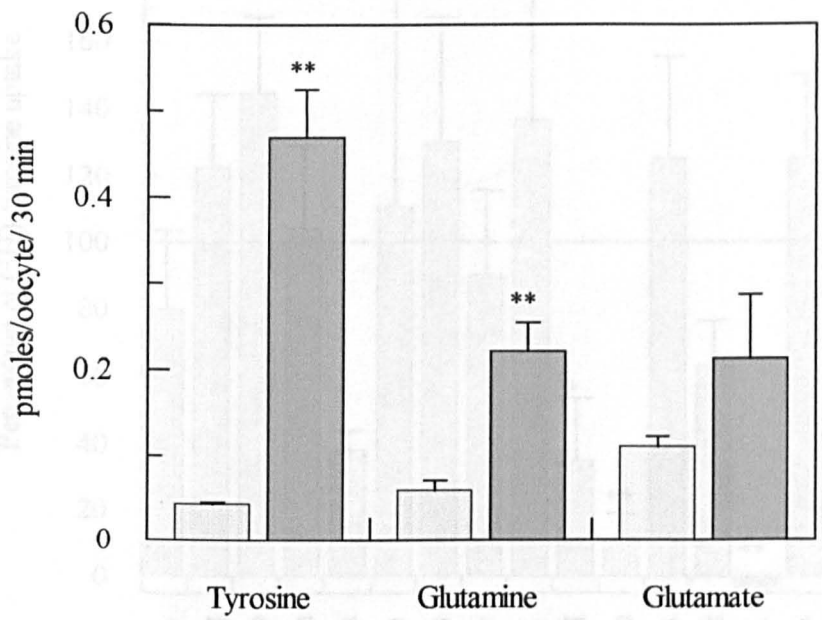


Figure 5.9: Functional expression of *TbAATP1* in *Xenopus* oocytes: Uptake of 100 µCi/ml of (^{3}H) L-tyrosine (51 Ci/mmol), (^{3}H) L-glutamate (33 Ci/mmol) or (^{3}H) L-glutamine (49 Ci/mmol) by *TbAATP1* injected oocytes (20 ng/oocytes) (dark grey) or water injected oocytes (light grey) was measured for 30 min. Each time point is an average for four individual oocytes. (**, P<0.01, Dunnet's multiple comparison test).

In conclusion L-glutamate, L-glutamine and L-tyrosine are transported by TbAATP1.

TbAATP1 substrate specificity was further examined by measuring tyrosine uptake in the *TbAATP1* injected oocytes in the presence of 5 mM of non radiolabelled amino acids. The results presented in figure 5.10 revealed that tyrosine transport was strongly inhibited by L-phenylalanine and L-leucine. A weaker inhibition was observed with L-cysteine, L-methionine, L-threonine, L-isoleucine and L-glutamine.

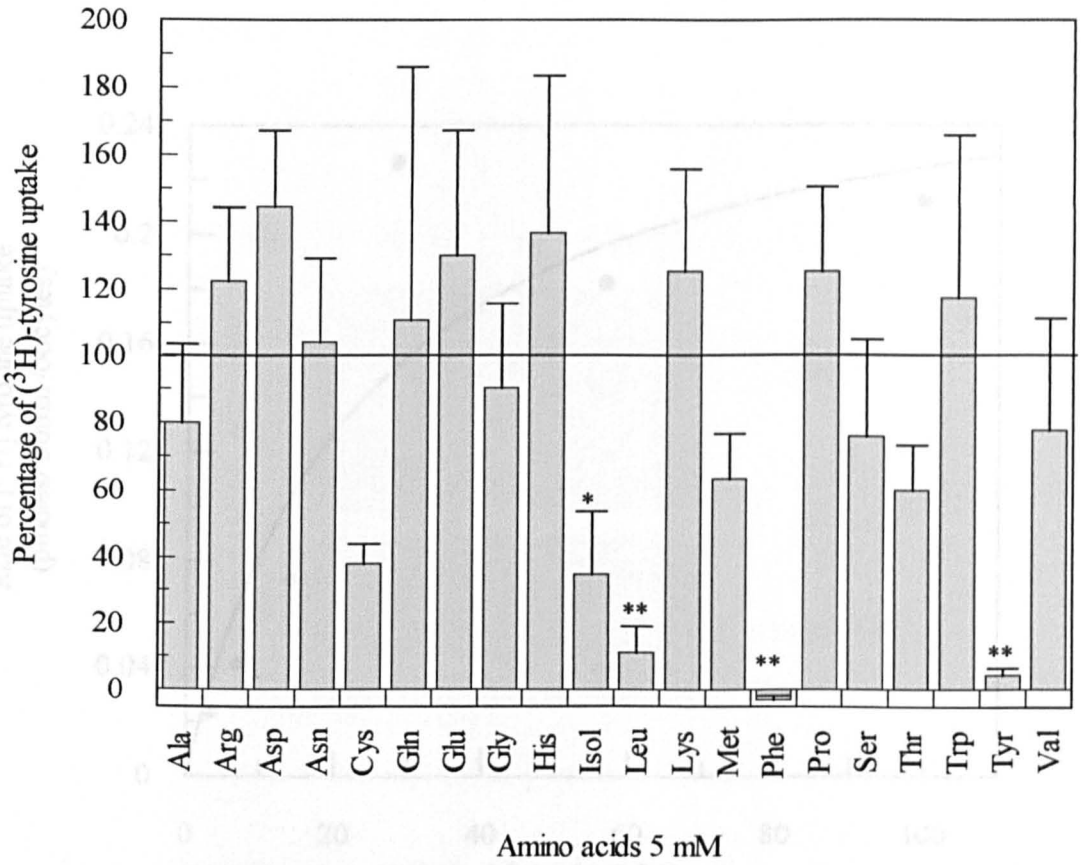


Figure 5.10: Substrate specificity profile of TbAATP1 mediated transport. Uptake of 40 nM of (^{3}H)L-tyrosine by *TbAATP1* injected and control oocytes was determined over a 30 min time period in the presence of 5 mM of each amino acid. Tyrosine is insoluble at 5 mM and was used at 100 μM. Results are expressed as a percent of uptake compared with tyrosine uptake in the absence of amino acids. (**, $P<0.01$, *, $P<0.05$; Dunnet's multiple comparison test).

Kinetics of tyrosine uptake in TbAATP1 injected oocytes.

L-tyrosine appeared to be the preferred substrate for TbAATP1 hence the kinetics of tyrosine uptake were measured in the presence of an increasing concentration of tyrosine in *TbAATP1* injected oocytes and compared to the control oocytes. The results displayed a typical Michaelis-Menten curve and a K_m value was calculated at 25 μM. (Figure 5.11)

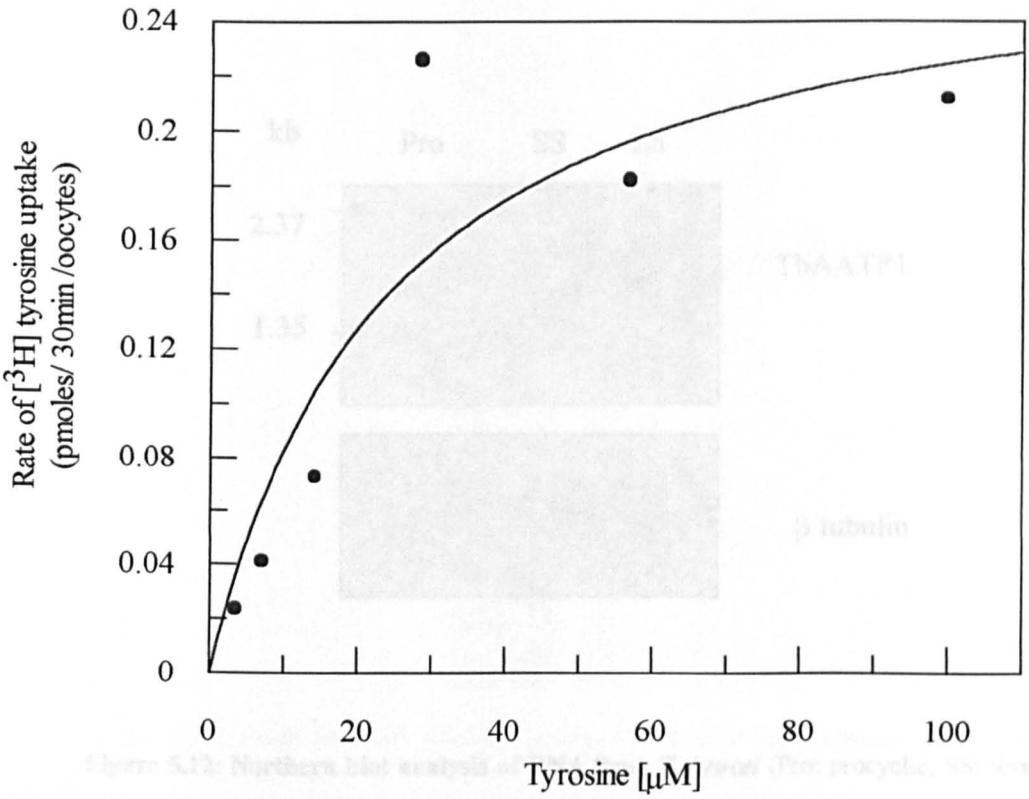


Figure 5.11: Kinetics of L-tyrosine uptake mediated by TbAATP1. Uptake of (^{3}H)L-tyrosine by injected oocytes was determined over a 30 min time period using a range of concentrations from 3.125 μM to 100 μM. The kinetic constants were determined by non linear regression analysis using the Michaelis-Menten equation.

5.2.4. Differential expression of *TbAATP1*:

The level of steadystate RNA from *TbAATP1,* in different the life cycle stages of the parasite was examined by probing a Northern blot containing RNA from procyclic and bloodstream forms with *TbAATP1* (entire ORF). The same blot was also probed with a β-tubulin gene probe, which is constitutively expressed throughout the life cycle. The results shown figure 5. 12 indicate that *TbAATP1* is expressed in bloodstream and procyclic form parasites.

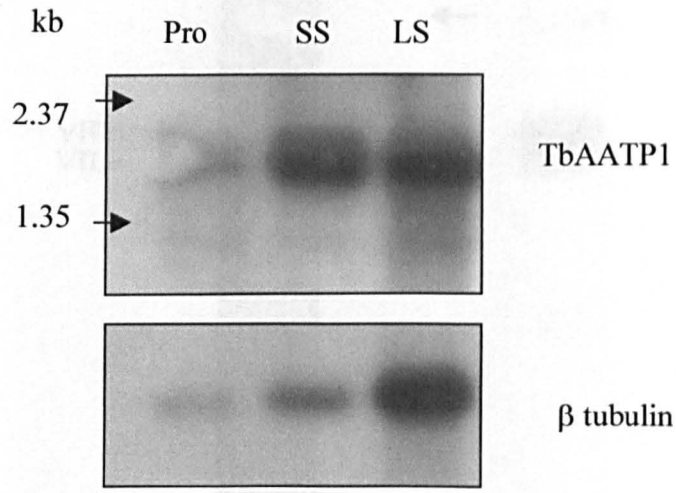


Figure 5.12: Northern blot analysis of RNA from *T. brucei* (Pro: procyclic, SS: short stumpy, LS: long slender) was performed and probed with *TbAATP1* (entire ORF) and with β tubulin as a control.

5.2.5: Gene organization and localization:

The chromosomal position of *TbAATP1* was determined by probing a membrane of a gel on which chromosomes from *T. brucei* had been separated by pulsed field gel electrophoresis. This work was done by the service offered by Dr Melville's group at the *T. brucei* genome project (University of Cambridge). The results are shown Figure 5.13 and indicate the presence of *TbAATP1* on chromosome VIII.

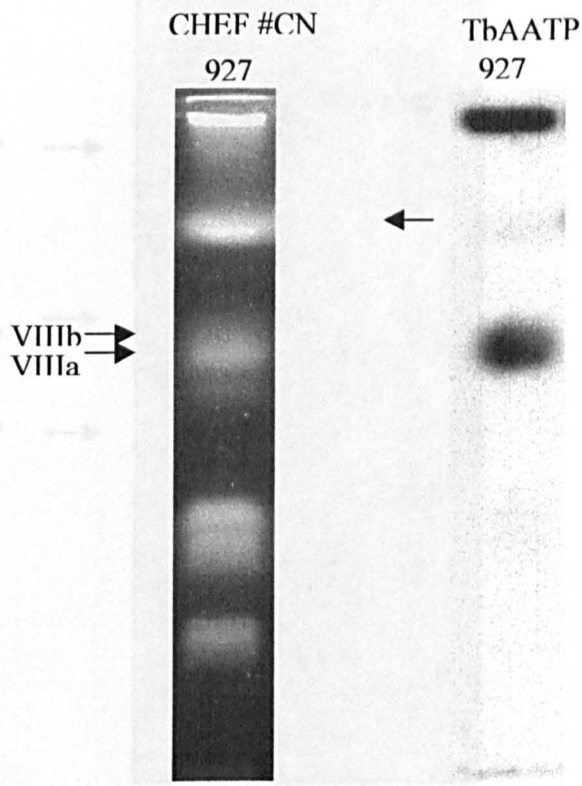


Figure 5.13: Pulse field gel electrophoresis of *T. brucei* chromosomal DNA probed with *TbAATP1*.

High stringency Southern blot analysis of *T. brucei* genomic DNA digested with a restriction enzyme cutting once within the gene (*KpnI*) displays two bands while DNA cut by *Apa*I, *Eco*RI, *Bam*HI, enzymes cutting outside the gene showed the hybrisation of only one band. This indicates that *TbAATP1* is a single copy gene. Low stringency Southern blotting was then used to look for the presence of other related sequences.

Figure 5.15 revealed the presence of multiple hybridizing bands indicative of the presence of a putative family of amino acid transporters.

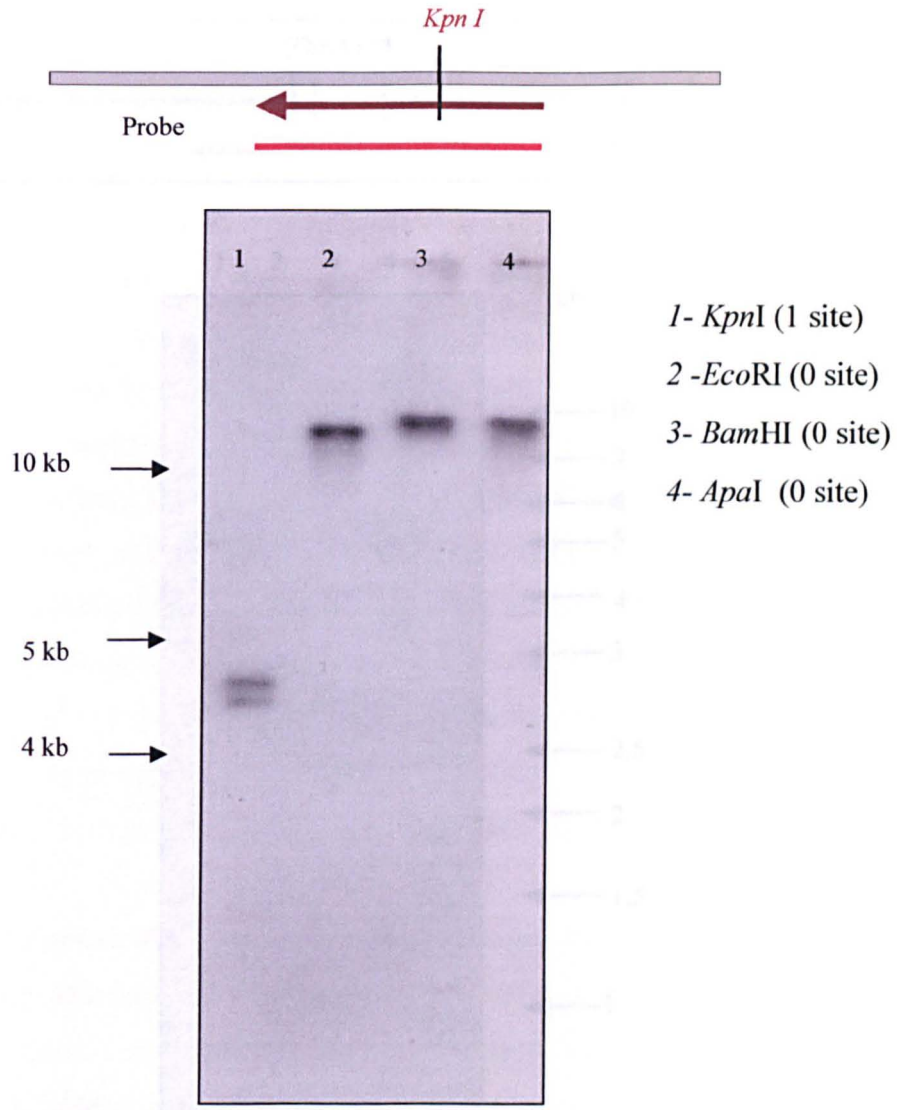
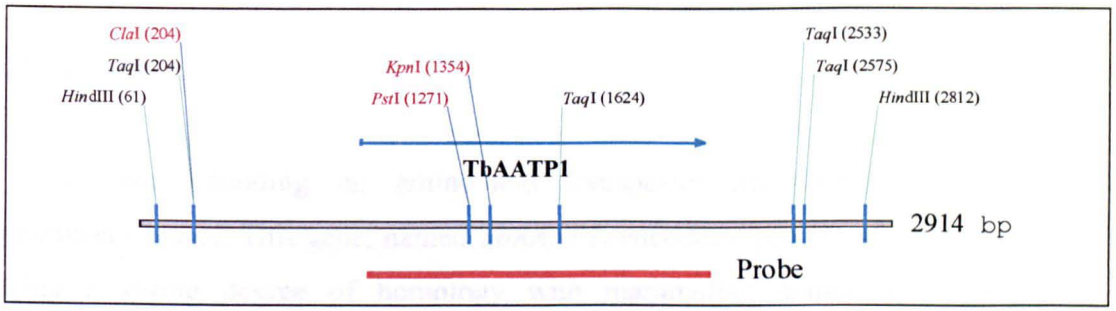
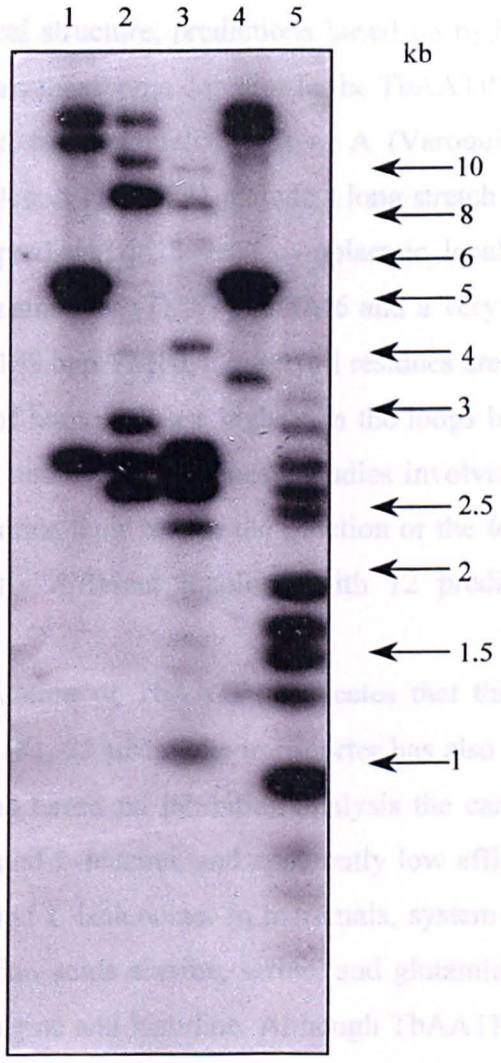


Figure 5. 14: High stringency Southern blot analysis of *T. brucei* genomic DNA digested with *Kpn*I (lane 1), *Eco*RI (lane 2), *Bam*H1 (lane 3), *Apa*I (lane 4) and probed with *TbAATP1* (entire ORF). A schematic representation of *TbAATP1* and its flanking regions is shown above the Southern blot.





Low stringency wash

Figure 5.15: Low stringency Southern blot analysis of *T. brucei* genomic DNA digested with *ClaI* (lane 1), *Hind III* (lane 2), *KpnI* (lane 3), *PstI* (lane 4), *TaqI* (lane 5) and probed with *TbAATP1* (entire ORF). A schematic representation of *TbAATP1* and its flanking regions with the localisation of the restriction enzyme sites for enzymes used is shown above the Southern blot.

5. 3. Discussion

A gene encoding an amino-acid transporter has been identified from *Trypanosoma brucei*. This gene, named *TbAATP1*, encodes a protein of 468 amino acids showing a strong degree of homology with mammalian amino acid transporters especially system A and system N.

In terms of topological structure, predictions based on hydropathy plot analysis suggest the presence of 11 transmembrane domains in the TbAATP1 sequence. A similar topology has been described for mammalian system A (Varoqui *et al*, 2000). Other similarities between system A and TbAATP1 include a long stretch of amino acids at the N terminus of the protein predicted to have a cytoplasmic localization, a long loop between transmembrane domains five (TM5) and TM6 and a very short loop of two to three amino acids between TM9 and TM10. Conserved residues are scattered throughout the protein, however levels of homology are highest in the loops between the predicted transmembrane domains 1-2 and 7-8. Mutagenesis studies involving these amino acids would be of interest to determine their role in the function or the folding of the protein. System N displays a slightly different topology with 12 predicted transmembrane domains.

Functional characterization of TbAATP1 indicates that this transporter carries tyrosine with a high affinity (K_m 25 μM). The transporter has also been shown to carry glutamate and glutamine, and based on inhibition analysis the carrier protein also has affinity for L-phenylalanine and L-leucine, and apparently low affinities for L-cysteine, L-methionine, L-threonine, and L-isoleucine. In mammals, system A is responsible for the uptake of the neutral amino acids alanine, serine, and glutamine while system N is specific for glutamine, asparagine and histidine. Although TbAATP1 and system A and N resemble each other in sequence and topology, they display functional differences that could be exploited in the design of drugs to selectively enter trypanosomes.

The molecular characterisation of *TbAATP1* revealed the presence of a putative family of related genes (this is confirmed in the next chapter).

The characterization of TbAATP1 in *Xenopus* oocytes showed that kinetic characteristics and substrate specificity appear to be similar to those measured for a transporter which carries tyrosine in bloodstream form *T. brucei* (c.f. Chapter IV, section 4.2.5). In procyclic forms, the principle transporter appears to have a substantially higher affinity for substrate, indicating that the main route of entry for tyrosine in the two different forms of the parasite is different. However, interpretation of data on the kinetics of transport obtained by heterologous expression have to be treated with caution. The data obtained are only conclusive of the nature of the transporter and its substrates.

Northern blot analysis indicates that *TbAATP1* mRNA may be present in bloodstream and procyclic form organisms. It is difficult at this stage, however, to draw a definitive conclusion of whether *TbAATP1* is expressed in procyclic form or bloodstream forms or in both forms.

Chapter VI

Identification of a family of amino acid transporter genes in *Trypanosoma brucei*

6.1. Introduction:

A multiplicity of amino acid transporters, distributed in large families, have been shown to be present within most organisms including mammals, yeast, plants and bacteria (c.f. Chapter I, section 2.4). In Trypanosomes the assumption that there are multiple amino acid transporters was originally proposed by Hansen (Hansen, 1979) and the transport studies presented in this thesis on methionine and aromatic amino acids support this hypothesis. A search of the *T. brucei* databases led to the discovery of *TbAATP1* which has been functionally characterised and shown to encode for an amino acid transporter.

The purpose of this chapter is to describe the search of the *T. brucei* databases for putative members of a family of amino acid transporter genes and the use of bioinformatic tools to analyze the structural and phylogenetic characteristics of the different members of the family.

6.2. Results

6.2.1. Identification of a family of amino acid transporter genes from *T. brucei*

TbAATP1 was characterized functionally as being an amino acid transporter (results presented in Chapter V) thus the predicted amino acid sequence encoded by this gene was used to re-screen the *T. brucei* databases using a tBlastN search (which identifies homology to translations of nucleotide sequence). Sequences presenting between 30 and 90 % homology were retrieved from the databases and each seed sequence was used to re-screen the databases using a blastN search in order to find overlapping fragments presenting a minimum of 98 % of identity in the overlapping region). Contiguous sequences with a minimum length of 1.6 kb were searched for the presence of an open reading frame. In this manner ten contiguous sequences, corresponding to ten additional *TbAATP1* homologues, were found. These ten genes

were translated and the predicted amino acid sequence was used to search for homologous sequences in the general databases at NCBI using a tBLASTN search. The ten open reading frames displayed a high homology score with mammalian amino acid transporters and the predicted peptides named **TbAATP2** to **TbAATP11**. The length of the different members varies between 376 and 490 amino acids (Appendix V-1). *TbAATP5* is the shortest open reading frame reported and most probably does not correspond to the full-length gene. However, no sequences with overlapping the upstream region of this putative start codon could be retrieved from any of the *T. brucei* databases. The length of *TbAATP2* (402 amino acids) has also been the subject to detailed investigation but no likely upstream start codon could be identified.

As will be discussed later, these eleven amino acid sequences displayed a strong homology with mammalian amino acid transporters and particularly with system A and N which mainly transport neutral amino acids. The question of whether separate families, with homology to other mammalian amino acid transporter systems, also exist in the *T. brucei* genome was then raised. In order to address this question the *T. brucei* databases were searched with amino acid sequences of members of different families of amino acids including the glutamate family, system ASC and the different members of the cationic amino acid transporter (y+, bo+). This search led to the discovery of a twelfth gene, named ***TbCAATP***. This gene is highly homologous to mammalian cationic amino acid transporters. The predicted amino acid sequence encoded by this gene is of 539 amino acids making TbCAATP the longest member of the family.

System A and N, and the cationic amino acid transporters were the only mammalian families of amino acid transporter showing any significant homologies with sequences in the *T. brucei* databases.

6.2.2. Degree of homology between the *T. brucei* amino acid transporters and members of the amino acid transporter superfamily.

In order to determine the degree of homology between the eleven sequences an alignment of the predicted amino acid sequences from *TbAATP1* to *TbAATP11* was performed using AlignX from the vector NTI suite. This alignment showed a percentage

of strict identity of 10.8 % betwwen the eleven members. The first 50 to 70 amino acids at the amino terminus of the eleven amino acid sequences was revealed to be the most diverse portion of the alignment with no globally identical amino acids while the most conserved domains are found at the carboxy terminus (Figure 6.2). Conserved residues are mainly present in the predicted transmembrane domains (Figure 6.1).

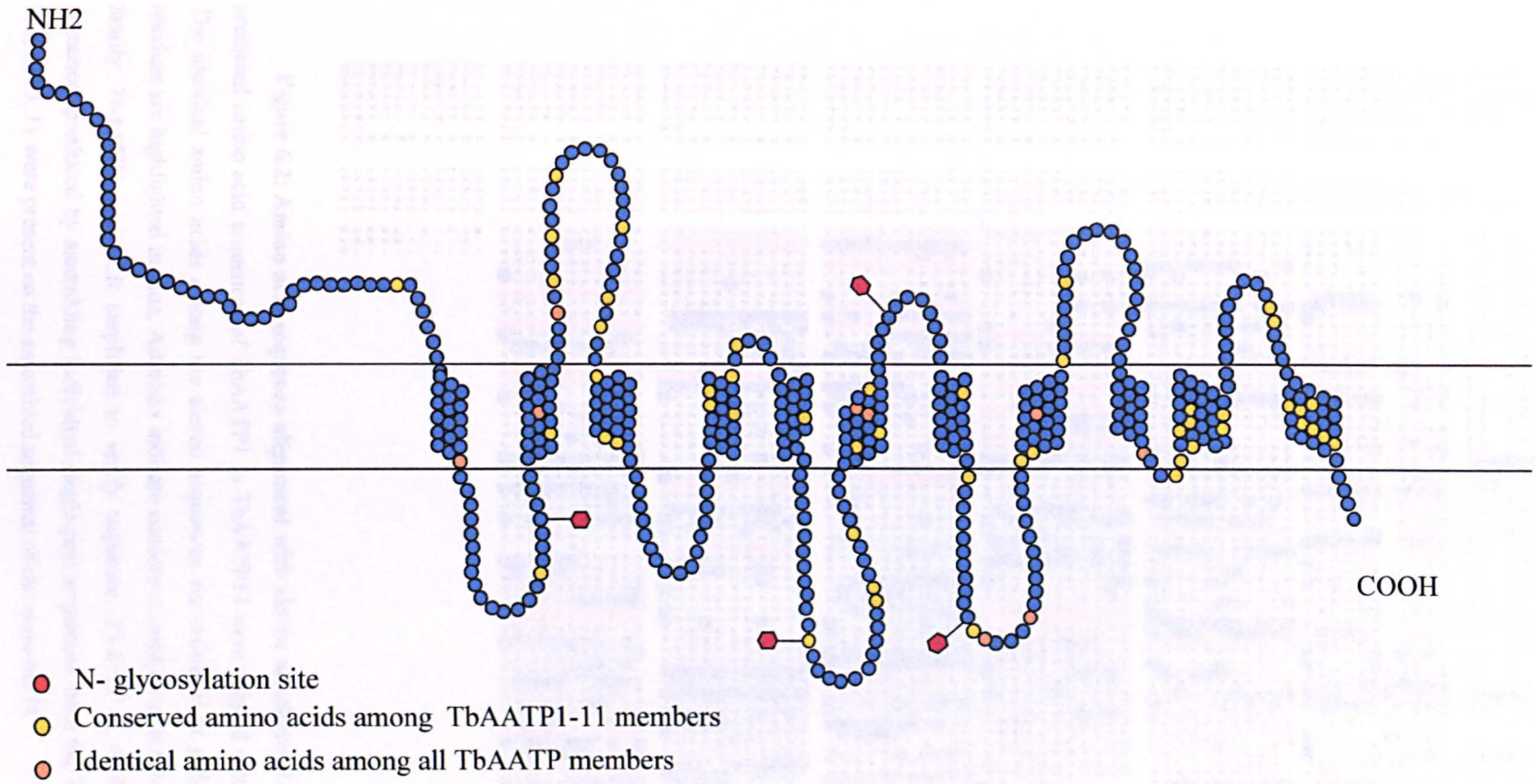


Figure 6.1: **Schematic representation the topology of the predicted structure of TbAATP1** and the conserved amino acid among the different members of the TbAATP family. The yellow circles indicate identical amino acids among TbAATP1 to TbAATP11 family members and the orange circles represent identical amino acids among the entire TbAATP family.

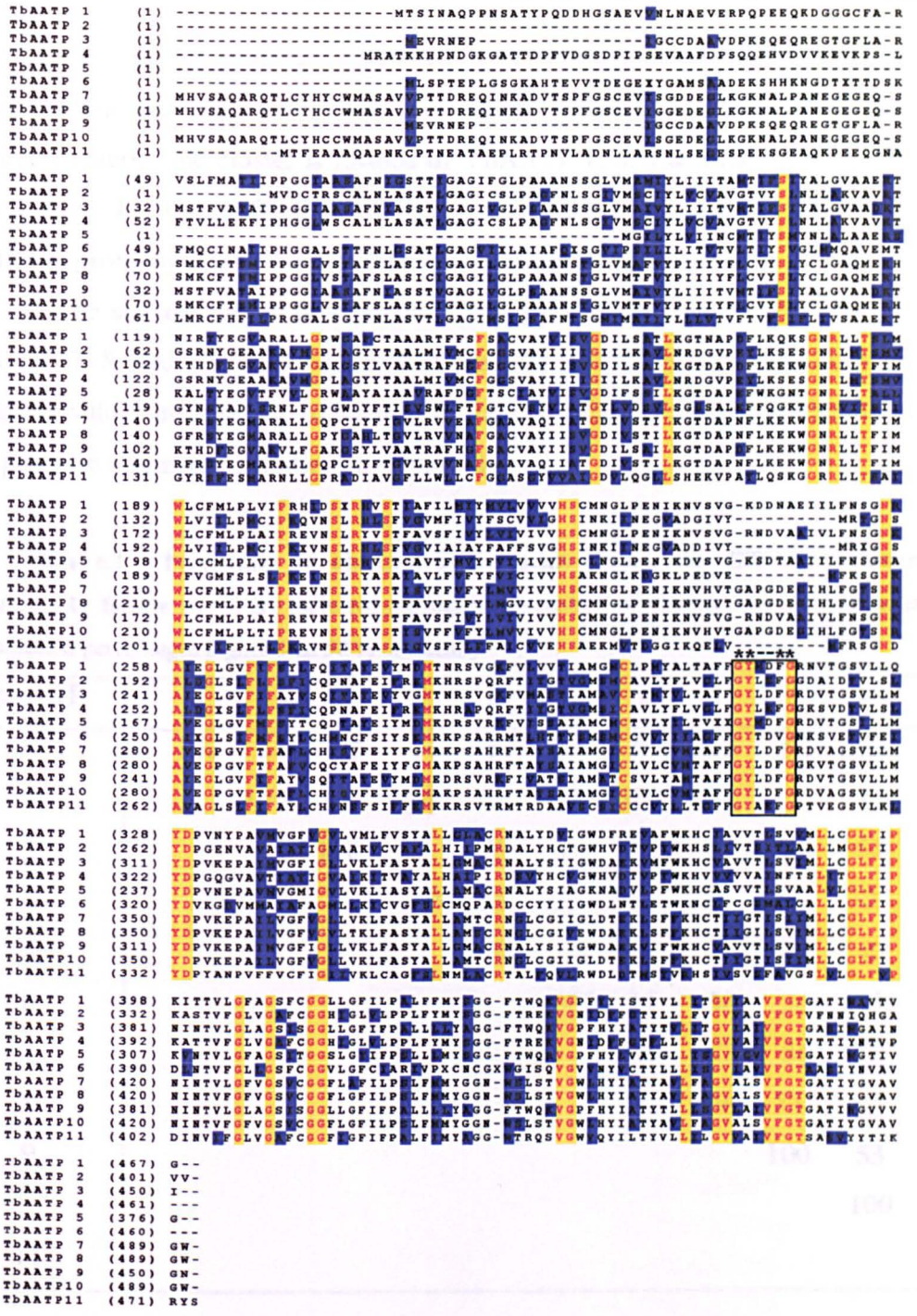


Figure 6.2: Amino acid sequence alignment with eleven members of the TbAATP family. The predicted amino acid sequences of TbAATP1 to TbAATP11 were aligned using the ClustalW algorithm. The identical amino acids among the eleven sequences are indicated in yellow, and blocks of similar residues are highlighted in blue. Asterisks indicate conserved residues of a consensus motif of the AAAP family. *TbAATP1* was PCR amplified to verify sequence. *TbAATP* 2, 4, 5, 6 represent preliminary sequences produced by assembling individual single pass sequences from the TIGR database. *TbAATP* 3, 7, 8, 9, 10, 11 were present on the assembled sequence of chromosome IV.

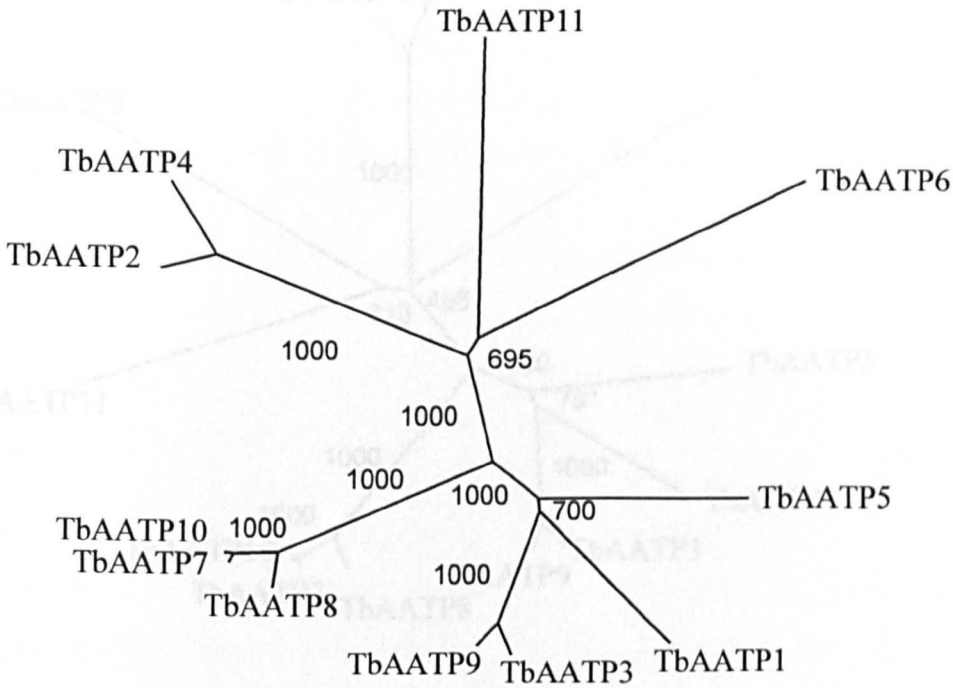
Based on this sequence alignment a neighbor-joining tree including the eleven putative amino acid transporters was constructed (Tree 6.1). The tree reveals the presence of three clusters. One cluster is formed by TbAATP 1, 3, 9 and the other by TbAATP 4, 2, 6, 7, 8, 10, 11. The position of TbAATP5 forming a single branch outside of these two clusters is probably due to the fact that this sequence is incomplete.

The second cluster is subdivided into two sub-groups TbAATP2, 4, 6, 11 and TbAATP 7, 8, and 10. The tree was bootstrapped (based on 1000 random runs) and the bootstrap values indicate the robustness of the tree. The percentage of identity according to a pairwise alignment is indicated Table 6.1.

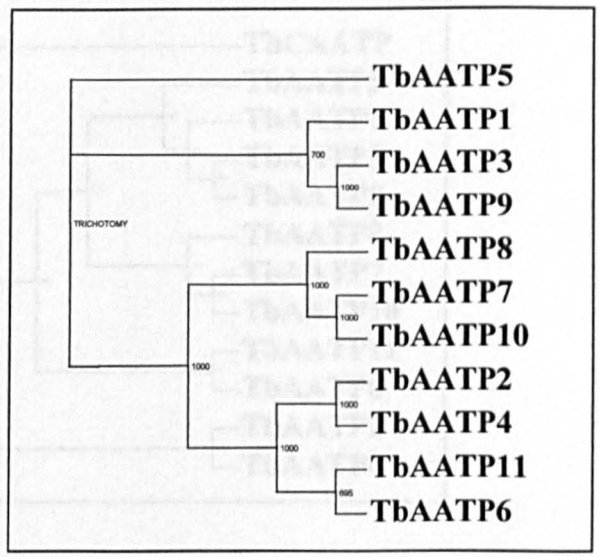
Table 6.1 : Percentage of identical amino acids among the different members of the TbAATP1-11 family. In red are indicated the highest percentage of identity between two genes. In bold are indicated percentages higher than 50 % of identity.

TbAATP	1	2	3	4	5	6	7	8	9	10	11
1	100	34	**67**	36	**53**	34	49	50	**67**	49	37
2		100	35	**74**	36	32	30	32	36	31	37
3			100	35	**55**	33	**53**	**54**	**94**	**53**	37
4				100	35	35	31	34	36	32	39
5					100	28	40	41	**56**	40	28
6						100	34	34	34	35	**41**
7							100	**92**	**54**	**98**	35
8								100	55	**93**	35
9									100	**53**	36
10										100	35
11											100

1-A

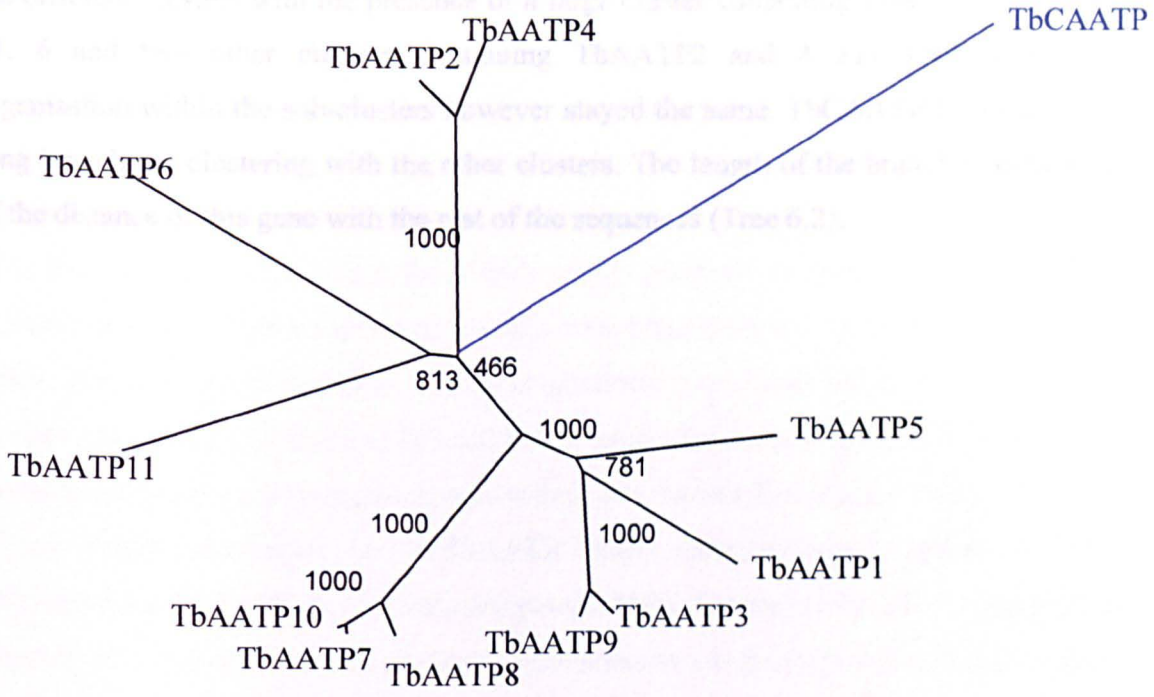


1-B

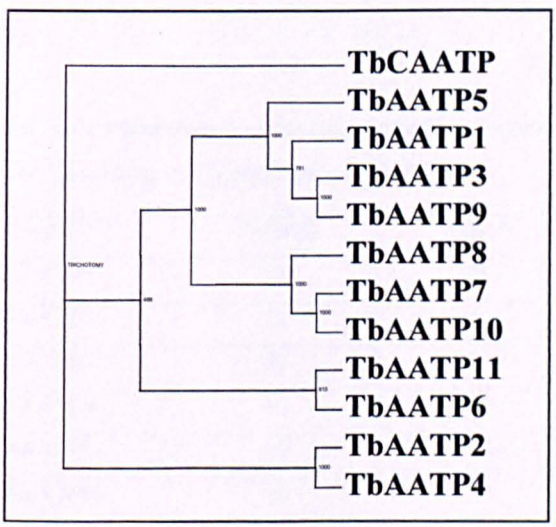


Tree 6.1: Phylogenetic trees for the TbAATP1-11 family members. The trees were generated with the Neighbor-joining program of Saitou and Nei using ClustalX 1.5. Panel 1-A) displays a distal representation, panel 1-B) displays a rectangular cladogram.

2-A



2-B



Tree 6.2: Phylogenetic trees the entire TbAATP family (including the TbCAATP representative) . The trees were generated with the Neighbor-joining program of Saitou and Nei using ClustalX 1.5. Panel 2-A) displays a distal representation of the tree, panel 2-B) displays a rectangular cladogram.

The addition of TbCAATP to Tree 6.1 brought some modifications to the organization of the different clusters with the presence of a large cluster containing TbAATP5, 7, 8, 10, 11, 6 and two other clusters containing TbAATP2 and 4 and TbCAATP. The organisation within the sub-clusters however stayed the same. TbCAATP forms a single long branch not clustering with the other clusters. The length of the branch is indicative of the distance of this gene with the rest of the sequences (Tree 6.2).

6.2.3. Structural analysis of the *TbAATP* family.

The fingerprint of membrane protein structure consists of a helix-loop-helix feature with helices corresponding to hydrophobic regions of the protein spanning through the membrane and loops corresponding to more hydrophilic stretches in contact with the aqueous phase. Therefore, hydropathy profiles of proteins provide strong indications as to whether a polypeptide is a membrane protein. As hydropathy profiles reflect the structure of proteins, which is generally conserved within a family, these profiles represent a tool that can be used beside amino acid alignments, to find structural homologues of a particular membrane protein (Lolkema and Slotboom, 1998).

Structural analysis of the TbAATP family using hydropathy plots based on different algorithms of Kyte Doolittle (Appendix V-2), TMpred and SOSUI revealed the presence of a variable number of putative transmembrane domains from nine to twelve among the different family members. The numbers varied depending on the software package used, and must be interpreted with extreme caution as they represent only predictions. However they indicate that all members of the TbAATP family are transmembrane proteins.

Table 6.2: Number of transmembrane domains predicted within the amino acid sequences of each TbAATP family member according to TMpred and to SOSUI.

TbAATP	TMpred	SOSUI
TbAATP1	11	11
TbAATP2	10	12
TbAATP3	10	11
TbAATP4	10	11
TbAATP5	10	10
TbAATP6	10	9
TbAATP7	9	10
TbAATP8	10	12
TbAATP9	10	11
TbAATP10	9	11
TbAATP11	10	11
TbCAATP	12	11

Amino acid transporters in general have been classified into four different superfamilies among which conserved motifs have been identified and recognized has being signatures for some for these superfamilies. A motif GY-AFG in which the G is conserved, the Y is substitute only by F, the A is not well conserved and the F is substituted only by Y has been described as a signature for the amino acid, auxin family (AAAP) (Young *et al.*, 1999). Analyzing the alignment of the TbAATP family, a corresponding motif was found: **GY- DFG** (Figure 6.1). This motif is localised at the end of the predicted transmembrane helix seven of TbAATP1 which corresponds to the localisation of this motif in the AAAP family described by Young *et al.*

The twelve amino acid sequences were also searched for the presence of peptide signals at the amino terminus using two different software SignalP and TargetP. TbCAATP was the only sequence in which a putative signal peptide sequence possibly involved in targeting for the mitochondria was found. More specifically, some motifs have been published in the literature as being protein targeting motifs in kinetoplastids for the flagellar membrane (Snapp and Landfear, 1999), the flagellar pocket (Hill *et al.*, 1999) and the peroxisome (or glycosome) (Jardim *et al.*, 2000; Chudzik *et al.*, 2000). None of these motifs are found to be present among the TbAATP members.

6.2 4. Gene localization and organization:

The localization of ***TbAATP1*** and ***TbAATP2*** was obtained by probing a membrane on which chromosomes had been separated by pulsed field gel electrophoresis. This was done as part as the service offered by Dr.S. Melville at the *T. brucei* genome project in Cambrige. The two genes were localized on **chromosome VIII** although there is insufficient information to comment on their relative position.

The assembly of sequences of some of the *T. brucei* chromosomes has also been a source of information in terms of gene localization and organization. ***TbAATP3, 7, 8, 9, 10*** and ***11*** appear to form a cluster on **chromosome IV**. The position of each gene on the BAC clone 3M17 (AC079815) is indicated figure 6.3. Based on the percentage of identity at the nucleotide level, two groups of genes presenting more than 95 % identity within each group, could be distinguished within the cluster. Representative members of the two groups show 62% identity to each other. The possibility that these genes are isoforms of two different amino acid transporters can be envisaged but can only be answered by functional expression of each gene. Furthermore, these genes are not organized in two sub clusters of the two putative isoforms but one gene from one putative isoform alternates with a gene from the other putative isoform. In the case of *T. congolense* glucose transporters a similar alternating arrangement is seen. In this later case, however, members of each isoform are identical.

Further analysis of sequence from the 3M17 BAC clone revealed the presence of a cluster of seven genes with a high homology with galactosyl transferase genes, strictly identical to each other, located upstream of the *TbAATP* cluster. The lack of sequences downstream the *TbAATP* cluster on the 3M17 clone did not permit the identification of the nature of the genes situated in this region. However, TbAAP11 and TbAATP10 have been localised to the 3I12 BAC clone (Figure 6.3) and this clone according to Dr El-Sayed overlap with 3M17 on the left hand side (Personal communication).

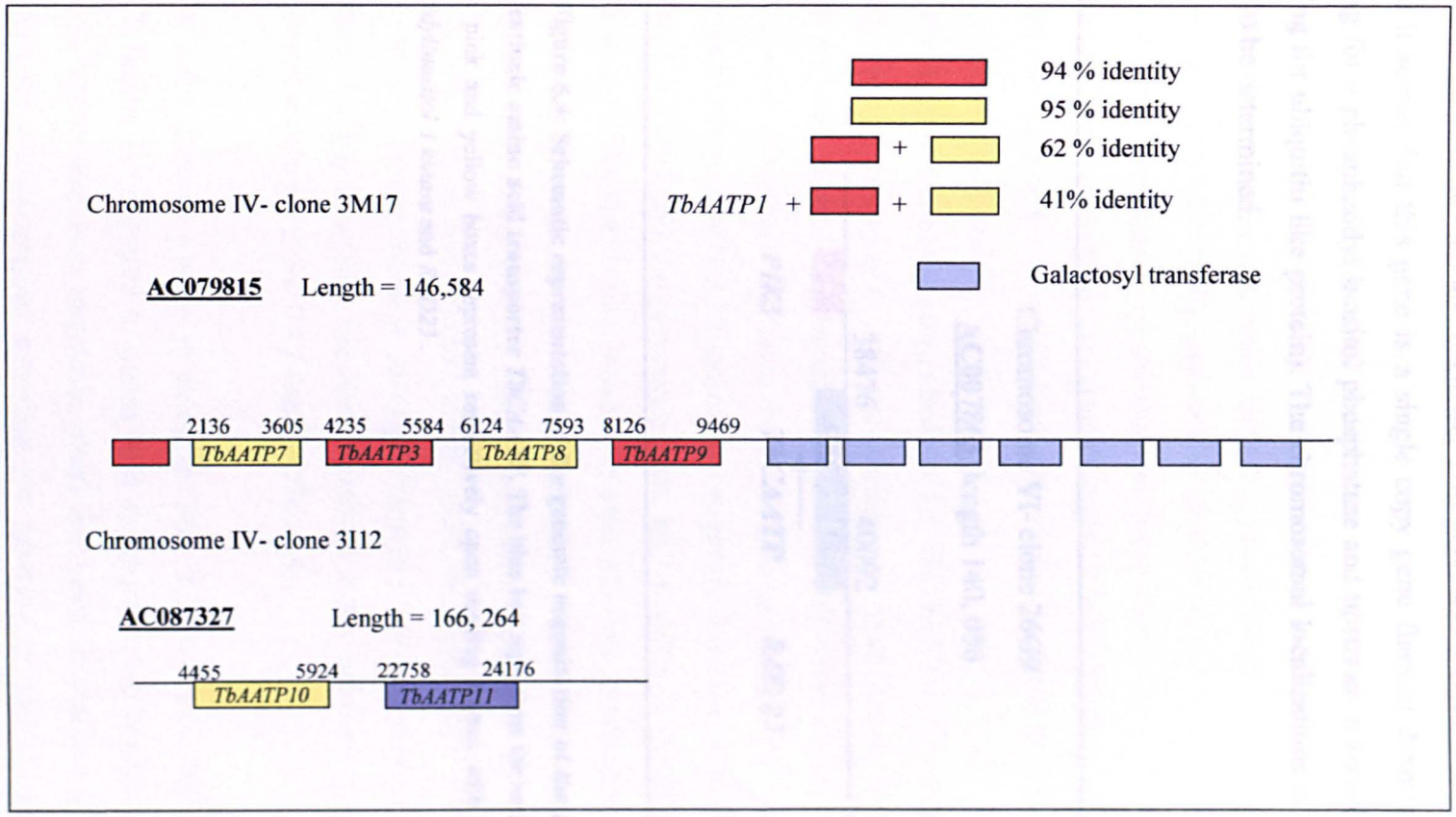


Figure 6.3: **Schematic representation of the localisation of TbAATP7, 3, 8, 9, 10, 11 on chromosome IV**. The two sub-groups are indicated in yellow and red. The blue boxes represent the open reading frame of a sequence of high homology with galactosyl transferase

The localization of ***TbCAATP*** has also been possible by interrogating the databases at the parasite-genome database. This gene was localized to **chromosome VI**. To date it seems that this gene is a single copy gene flanked downstream by a gene encoding for a phosphatidyl inositol phosphatase and upstream a homologue of *RAD23* (encoding for ubiquitin like protein). The chromosomal localizations of TbAATP4, 5, 6 has yet to be determined.

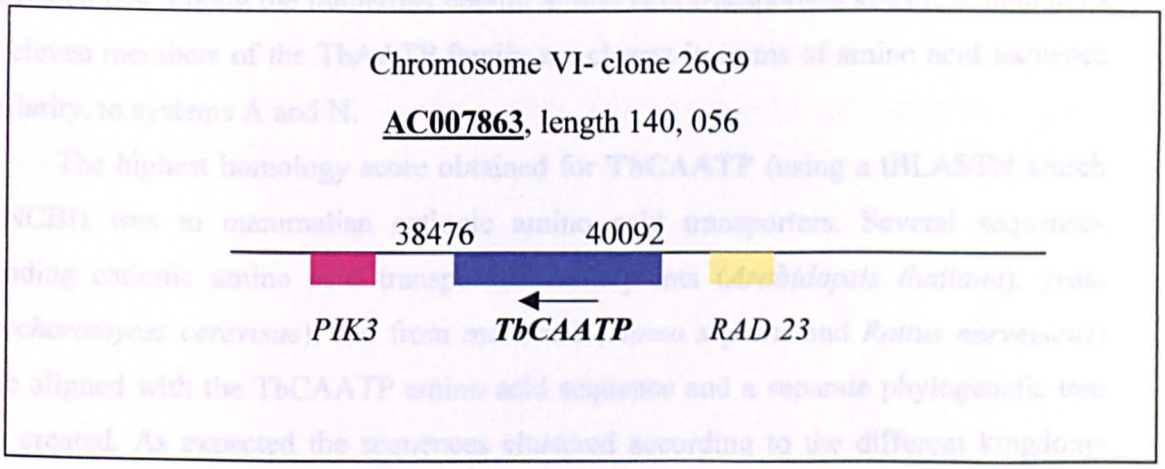


Figure 6.4: Schematic representation of the genomic organisation of the genes flanking the putative cationic amino acid transporter *TbCAATP*. The blue box represents the amino acid transporter gene, the pink and yellow boxes represent respectively open reading frames with high homology to *phosphatidylinositol 3 kinase* and *RAD23* .

6.2.5. Phylogenetic analysis:

In order to relate **TbAATP1-11** family members to known amino acid transporters that have been described in other organisms, a sequence alignment including the TbAATP family members, but excluding TbCAATP, with a large number of amino acid sequences from other organisms including plants, mammals and yeast was created (Tree 6.3). The TbAATP family appeared to be more closely related to the mammalian amino acid transporters than to representatives from plants or yeast. Examination of Tree 4 revealed that among the numerous neutral amino acid transporters known in mammals the eleven members of the TbAATP family are closest in terms of amino acid sequence similarity, to systems A and N.

The highest homology score obtained for **TbCAATP** (using a tBLASTN search at NCBI) was to mammalian cationic amino acid transporters. Several sequences encoding cationic amino acid transporters from plants (*Arabidopsis thaliana*), yeast (*Saccharomyces cerevisae*), and from mammals (*Homo sapiens* and *Rattus norvegicus*) were aligned with the TbCAATP amino acid sequence and a separate phylogenetic tree was created. As expected the sequences clustered according to the different kingdoms and interestingly TbCAATP was found to cluster with the mammalian cationic amino acid transporters. In mammals, four families of cationic transporters have been described: the y+ family, also named the CAT family, which encompasses three members, the y+L family that also carries some neutral amino acids when associated with a heavy chain (4F2hc): the bo+ family which is also associated to a side chain named rBAT and the Bo+ system. Among these four families of cationic amino acid transporters, TbCTAAP seems to be most similar to the CAT family (Tree 6.4).

A more general picture is given in Tree 6.5 in which the **entire putative TbAATP family** is compared to amino acid sequences including neutral and charged amino acid transporters from mammals, plants and yeast. In this tree all the amino acid transporters for *Saccharomyces cerevisae* are grouped in one cluster. The same is observed for those from *Arabidopsis thaliana* while for mammalian amino acid

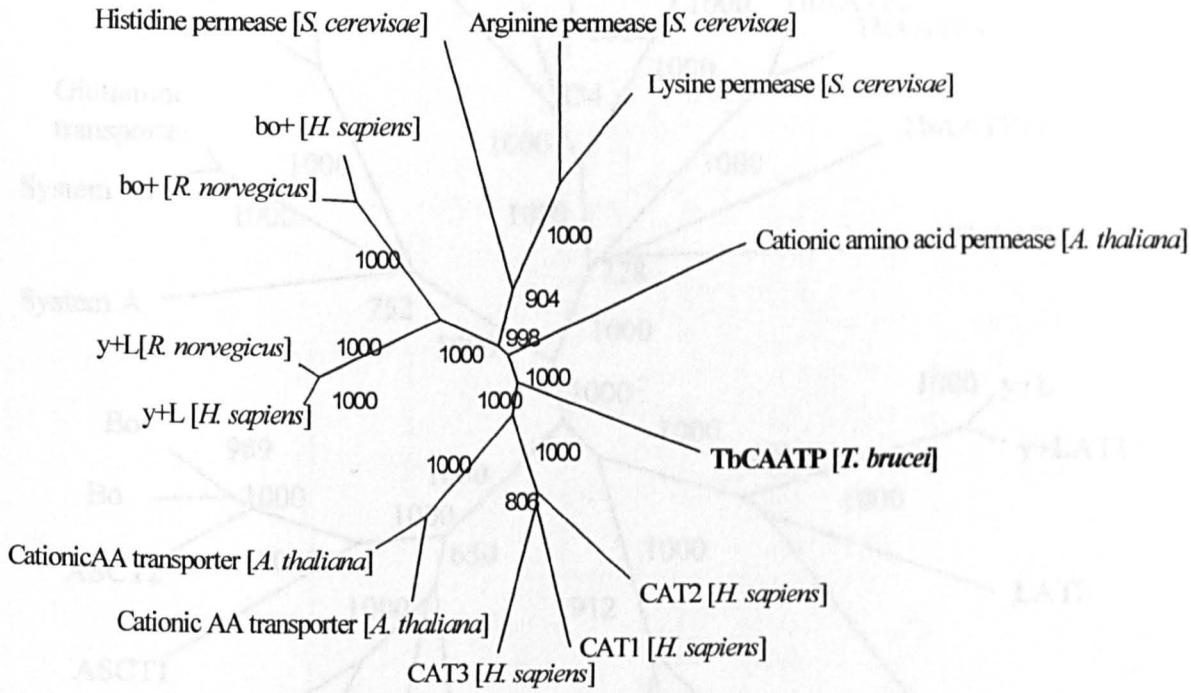
transporters the neutral amino acid transporters form a separate cluster form the charged amino acid transporters. The TbAATP1-11 family forms a cluster related to mammalian system A and N and TbCAATP forms a cluster with mammalian cationic amino acid transporters.

This large tree which provides a position of the TbAATP family among sequences representing each family of known amino acid transporter sequences from three different kingdoms confirms the observations brought out from previous trees

1) the *T. brucei* amino acid transporter family is closer to the mammalian amino acid transporters than to yeast or plants.

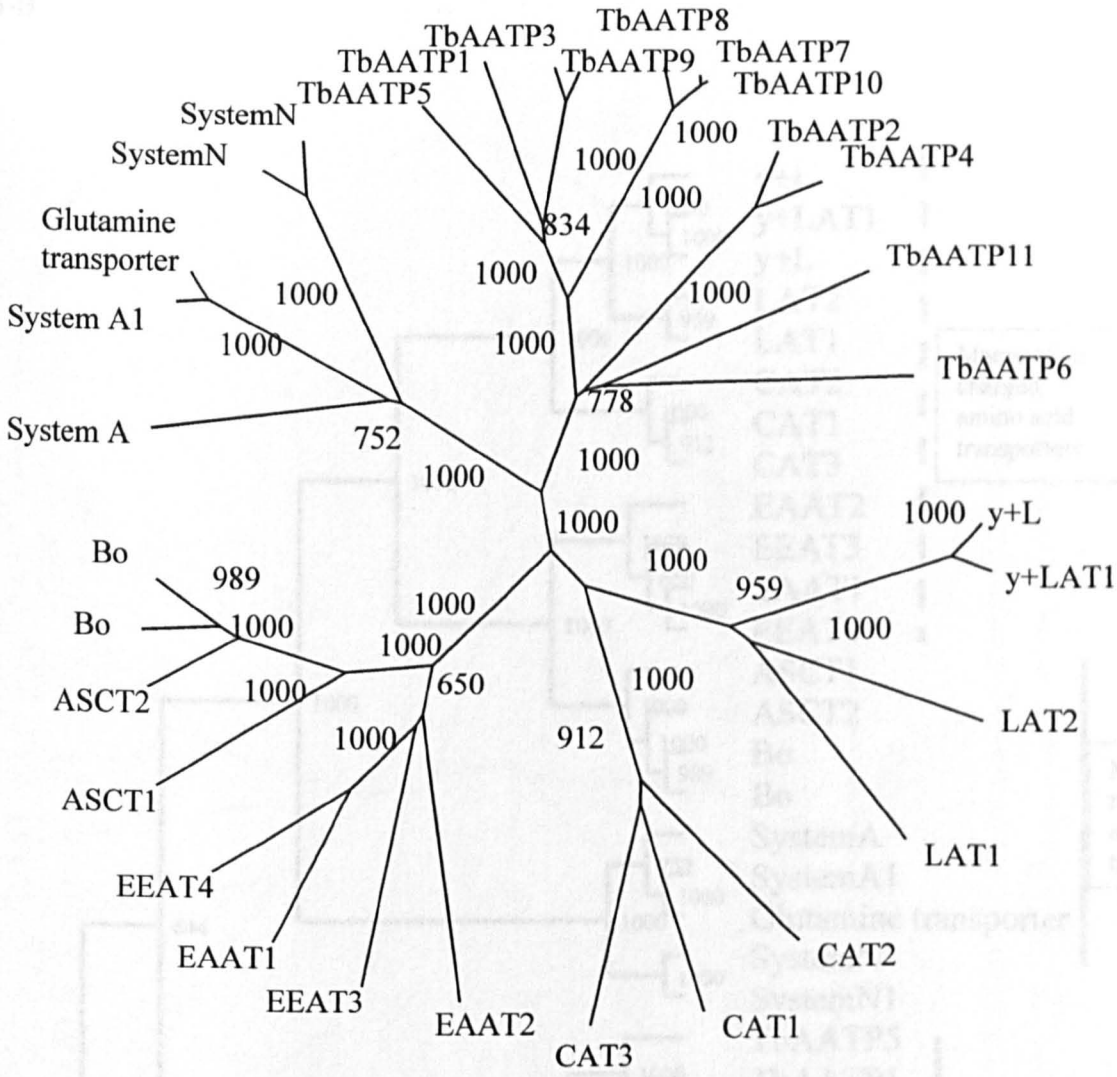
2) the eleven TbAATP members resemble system A and N in mammals.

3) one putative *T. brucei* transporter is significantly different, in terms of amino acid sequence, from the other members of the TbAATP family, and this relates closely the cluster of mammalian cationic amino acid transporters, and in particular with the CAT family.



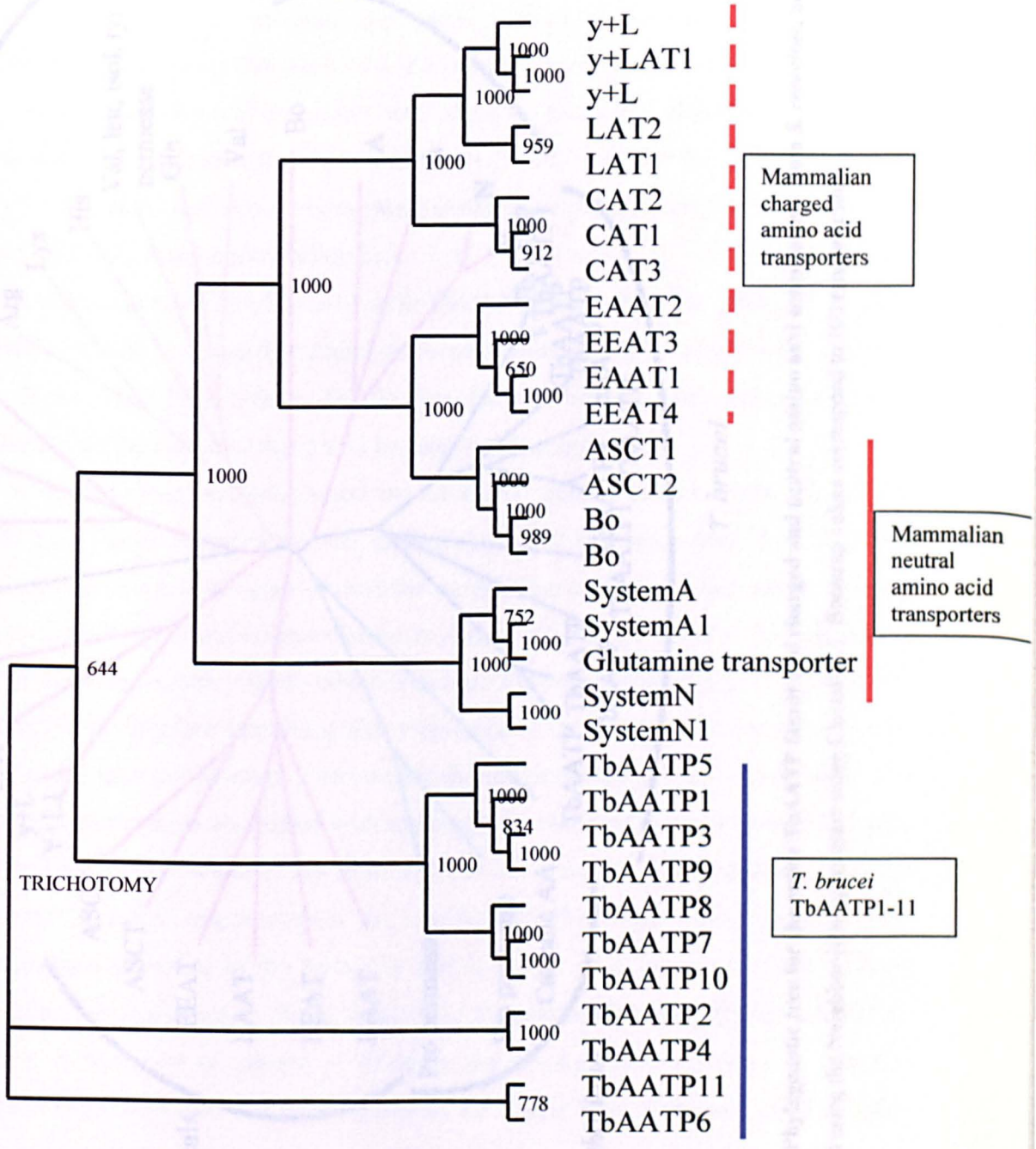
Tree 6.3: Phylogenetic tree for the putative *T. brucei* cationic amino acid transporter TbCAATP and cationic amino acid transporters from *Saccharomyces cerevisae*, *Arabidopsis thaliana*, *Homo sapiens* and *Rattus norvegicus*. The tree was generated using the Neighbor-joining program using ClustalX 1.5. Bootstrap values correspond to 1000 random runs.

3-A

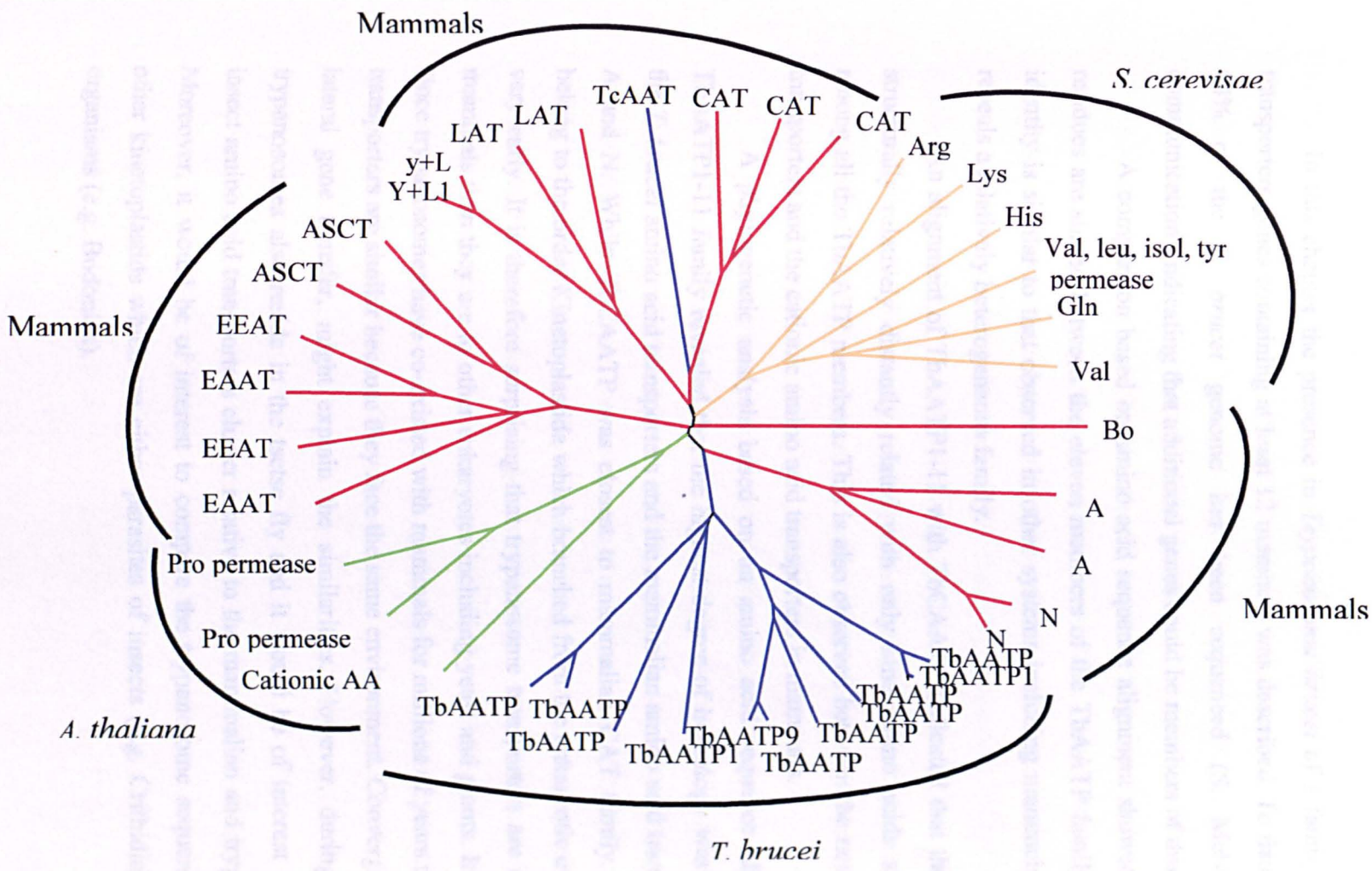


Tree 6.4: Phylogenetic tree for proteins of the TbAATP1-11 family and mammalian neutral and charged amino acid transporters. The trees were generated using the Neighbor-joining program using ClustalX 1.5. Bootstrap values correspond to 1000 random runs. Panel 3-A) diplays a distal representation of the tree, panel B) displays a rectangular representation of the same data

3-B



Tree 6.3 *(continued)*



.Tree 6.5: Phylogenetic tree for the entire TbAATP family and charged and neutral amino acid transporters from *S. cerevisae*, mammals and *A. thaliana*. The tree was generated using the Neighbor-joining program using ClustalX1.5. Boostrap values correspond to 100 random runs.

6.3. Discussion

In this chapter the presence in *Trypanosoma brucei* of a family of amino acid transporters genes containing at least 12 members was described. To date approximately 84% of the *T. brucei* genome has been sequenced (S. Melville, personnal communication) indicating that additional genes could be members of this family.

A comparison based on amino acid sequence alignment showed ten percent of residues are shared between the eleven members of the TbAATP family. This level of identity is similar to that observed in other systems including mammals and yeast and reveals a relatively heterogeneous family.

An alignment of TbAATP1-11 with TbCAATP indicated that these proteins are structurally relatively distantly related with only nine amino acids strictly identical among all the TbAATP members. This is also observed between the neutral amino acid transporters and the cationic amino acid transporters in mammals.

A phylogenetic analysis based on an amino acid sequence alignment of the TbAATP1-11 family revealed that the highest degree of homology was found between the *T. brucei* amino acid transporters and the mammalian amino acid transporter systems A and N. While TbCAATP was closest to mammalian CAT family. Trypanosomes belong to the order Kinetoplastida which branched from the eukaryotic evolutionary tree very early. It is therefore surprising that trypanosome transporters are more similar to mammals than they are to other eukaryotes including yeast and plants. It is possible that since trypanosomes have co-existed with mammals for millions of years their amino acid transporters are similar because they face the same environment. Convergent evolution or lateral gene transfer, might explain the similarities. However, during its life cycle trypanosomes also reside in the tsetse fly and it would be of interest to know where insect amino acid transporters cluster relative to the mammalian and trypanosome ones. Moreover, it would be of interest to compare the trypanosome sequences to those of other kinetoplastids which are either parasites of insects (e.g. Crithidia) or free living organisms (e.g. Bodonids).

Most of the genes have been localised to particular chromosomes within the genome, with the exception of *TbAATP 4, 5, 6*. They are present on three different chromosomes: IV, VI and VIII. *TbCAATP* was found as a single copy gene on chromosome VI. The clustered organization of *TbAATP 3, 7, 8, 9, 10, 11* on chromosome IV has also been observed for the *THT1* and *THT2* genes encoding glucose transport in trypanosomes. However in *T. brucei* the genes encoding the THT1 isoform are followed by the genes encoding the THT2 isoform, while in *T. congolense* TcoHT1 and TcoHT2 alternate (Bringaud *et al.*, 1998). Seven identical genes encoding for a putative galactosyltransferase were clustered upstream of this cluster. Many other genes in trypanosomes including ubiquitin and tubulins are also present in tandem arrays.

The genomic organization of the flanking region of *TbCAATP* revealed the presence of a phosphatylinositol 3 kinase gene. Interestingly a similar gene was found to be present in the flanking region of the glucose transporter gene cluster. This enzyme is involved in the regulation of the vesicle trafficking and could possibly be involved in the targeting of TbCAATP.

It is most likely that the transport of amino acids occurs at several membranes in trypanosomes including 1) the plasma membrane for the acquisition of amino acids from the host 2) the mitochondrial membrane, since protein synthesis occurs in the mitochondria and amino acids also need to be carried into this compartment 3) membranes of the lysosomal/endosomal system which possess both acquired and endogenously produced proteins which are degradated and released back to the cytosol. The localisation of the different transporters of the TbAATP family within the cell is not known at this point. However, a putative signal peptide for the mitochondrion is found at the amino terminus of TbCAATP sequence.

Chapter VII

General Discussion

7.1- Overview

The aim of this project was to investigate amino acid transport in *Trypanosoma brucei* with a particular interest in transporters with high affinity for substrate. The long-term goal underlying this study was to identify putative gateways for drugs to enter trypanosomes.

The project consisted of two separate approaches: (1) a biochemical approach based on measurements of the uptake of radiolabelled amino acids by bloodstream or procyclic form trypanosomes, (2) a cloning and expression approach of a putative *T. brucei* amino acid transporter gene, identified by searching the *T. brucei* genome databases, using *Xenopus* oocytes.

Using the biochemical approach the uptake of methionine was studied in procyclic and bloodstream forms of *T. brucei.* Methionine appeared to be taken up only via only one transporter and could therefore be studied in detail in the parasites. Methionine transport appeared to be dependent on the proton motive force and the amino acid core is essential for the recognition of substrate by the transporter. Phenylalanine and tryptophan was shown to be transported by at least two different routes. The presence of multiple transporters for the same substrate was an obstacle to the detailed characterization of each individual transporter. This problem highlighted the limitations of this biochemical approach and emphasised the necessity of studying individual transporters in isolation.

Towards this goal, a putative amino acid transporter gene, *TbAATP1,* was cloned and expressed in *Xenopus* oocytes. This gene encodes a protein containing 11 putative transmembrane domains and functional expression of this gene showed that glutamate, glutamine, and tyrosine were substrates for this transporter. Methionine, phenylalanine, tryptophan, leucine, and isoleucine were significant inhibitors of tyrosine uptake which suggest that these amino acids are also possible substrates.

The expression of TbAATP1 in *Xenopus* oocytes was technically difficult because of the irregular quality of the oocytes, and the presence of a relatively high endogenous amino acid uptake. *Xenopus* oocytes possess multiple amino acid

transporters at the plasma membrane and the level of endogenous transport can greatly vary from one batch of oocytes to the other made systematic reproducibility of *TbAATP1* expression.

Other expression systems including mammalian CHO (Chinese Hamster Ovary) cells could have been used but these too would have had endogenous transport activity and presented similar difficulties.

The identification of *TbAATP1* led to the discovery of others genes using the *T. brucei* databases. These genes form a family of twelve members and four of these genes are found in a cluster on chromosome IV. A putative cationic amino acid transporter gene was also found on chromosome VI. The presence of at least twelve genes in the *T. brucei* genome encoding for amino acid transporters is relatively high. However, some of these genes could be isoforms differentially expressed throughout the life cycle of the parasite. Amino acid transporters are also abundant in plants, mammals and yeast (Cf. part 2.4.1 and 2.4.2 of Chapter1).

Phylogenetic analyses comparing TbAATP family members with amino acid transporters from plants (*Arabidopsis thaliana*), yeast (*Saccharomyces cerevisae*) and mammals (*Homo sapiens*) were based on amino acid sequence alignments. This choice of trees based on amino acid sequences and not DNA sequences, is justified for several reasons: 1) the question addressed was a comparison in terms of function and this information is carried by protein sequences, 2) multiple codons encode for the same amino acid and the different codons can be used with a different frequency by different species (known as "codon usage"), 3) The genetic code is based on the use of A, G, C, T while protein sequences are based on 20 amino acids. Thus, the probability of residues randomly aligning is significantly higher with DNA sequences than with amino acid sequences (particularly if gaps are allowed).

This study revealed that *T. brucei* amino acid transporters are more closely related to mammalian amino acid transporters than to yeast or plant amino acid transporters. It is tempting to speculate that this may arise from horizontal gene transfer or of co-evolution due to the fact these transporters face in the same environment.

7.2. Interpretation

7.2.1. A window on trypanosome metabolism

Methionine and aromatic amino acids

The presence of at least three transporters involved in the transport of aromatic amino acids points to the importance of these molecules for parasite metabolism. Other than incorporation into proteins, the main use of aromatic amino acids in trypanosomes appears to be for transamination of α-ketomethiobutyrate into methionine (Berger *et al.*, 1996). Berger suggested that trypanosomes have a high capacity to transport aromatic amino acids and that blocking this uptake could be a putative target for chemotherapy. The data obtained on aromatic amino acid transport in this study confirms the high capacity of trypanosomes to scavenge these molecules. However, the presence of multiple transporters for aromatic amino acids would make the inhibition of their uptake a goal difficult to achieve. It is also interesting to note that methionine and the aromatic amino acids appear to share one transporter. This observation raises questions about the potential of regulating the transport activity according to the metabolic requirements related to methionine cycle. Future studies seeking such regulation would be of interest.

Glutamate and glutamine

In bloodstream form trypanosomes glutamate is abundant (12% of the free pool of amino acids) and represents the second most abundant amino acid after alanine. The data obtained on glutamate transport revealed a relatively poor capacity of trypanosomes to scavenge glutamate and this data suggests that most glutamate is probably synthesised from other amino acids. Glutamine is a candidate and has been shown to be a relatively good substrate of TbAATP1. There is also some evidence of the presence of a glutamate-pyruvate transaminase in bloodstream form trypanosomes, allowing the formation of alanine from glutamate and *vice versa*.

7.2.2. Consequences for the mammalian host

The definition of a parasite is an organism that "eats at the table of another one". In the context of a study on amino acid transporters the question of the impact of the parasite on the level of amino acids in the infected host can be addressed.

A dramatic decrease in tryptophan and tyrosine concentrations has been measured in the serum of mice infected with *T. brucei* (Newport *et al.*, 1977, Vincendeau *et al.*, 1999). However, the presence of a multiple transport system for the aromatic amino acids in trypanosomes is not likely to be directly involved in the decrease because of the regular input from the diet. As suggested by Hall and Seed, this decrease is more likely to be due to a disturbance of the host aromatic amino acid metabolism (Hall and Seed, 1984) with a very limited conversion of phenylalanine to tyrosine in infected *Microtus* (Seed *et al.*, 1982) and an increased urinary excretion of aromatic amino acid catabolites (Hall and Seed, 1984).

Amino acid availability plays an important role in the regulation of neurotransmitter synthesis. Phenylalanine and tyrosine are precursors of the catecholamines: dopa, dopamine, noradrenaline and adrenaline. Tryptophan is the precursor of serotonin. The transport of essential amino acids from blood to brain is regulated at the blood brain barrier (BBB) by the presence of a variety of amino acid transporters. Boado *et al.*, have shown the presence of system L (or LAT) at the BBB with a much higher affinity (1-100 μM) than system L in peripheral tissues (1-10 mM) (Boado *et al*, 1999). Multiple behavioral disorders are associated with African trypanosomiasis: dementia, sleep disorders, and affective disorders are observed in sleeping sickness patients (Keita *et al.*, 1997, Buguet *et al.*, 1999). Studies comparing the level of various neurotransmitters in the brains of mice infected by *T. brucei* and non infected mice showed an alteration in monoamine neurotransmitters. A fall of more than 20% in serotonin levels has been observed in experimental trypanosomiasis (Stibbs, 1984, Stibbs and Curtis, 1987). In this context several hypothesis can be made:

1) the presence of trypanosomes in the brain might deplete the CSF of amino acids crucial for the synthesis of neurotransmitters
2) the parasites could have the ability to deregulate the function of these transporters mammalian transporters
3) the parasites could also affect the metabolism of different neurotransmitters. In infections by *T. cruzi* an alteration of acethylcholine receptors has been shown to occur in the brain as well as an alteration in the activities of choline acetyltransferase and acethylcholine esterase (Tanowitz *et al.*, 1983).

7.3. Future Work

Further characterization of TbAATP1 should consist of a complete investigation of the substrate specificity of TbAATP1 by measuring the kinetics of uptake of radiolabelled phenylalanine, leucine and isoleucine.

Mammalian system A and N have been shown to be dependent onto Na^+ and although none of the nutrient transporters characterized in trypanosomes have shown any Na^+ dependency it would be of interest to investigate the ion dependency of TbAATP1.

In order to determine if TbAATP1 is essential for the parasite a gene knock-out would have to be undertaken. This can be achieved either by replacing both alleles of the gene with antibiotic resistance markers, or using RNA interference technology (Shi, 2000). Uptake of tyrosine, glutamine and glutamate should then be measured in the knock-out mutant and compared to the wild type.

Hydropathy plots of the amino acid sequences of TbAATP1 predicted the presence of eleven transmembrane domains. A more comprehensive topology of the transporter could be determined by assessing the extracellular accessibility of single cysteines to sulfhydyl-modifying reagents (Loo and Clark, 1995). The technique is based on the creation of transporter mutants in which the cysteines have been removed by site-directed mutagenesis and a single cysteine is introduced in different parts of the sequence. The availability of these individual cysteines to impermeant reagents that react with the sulfydryl residue of the amino acid determines its extracellular accessibility.

The role of particular residues in the amino acid sequence of the transporter could be investigated by altering these by site directed mutagenesis and measuring uptake of labelled amino acids and inhibition by various analogues.

The localization and distribution of TbAATP1 could be investigated by the production of polyclonal or monoclonal antibodies against the parts of the protein which are predicted to be extracellular.

TbAATP1 is member of a family of amino acid transporter genes and the *T. brucei* genome project is close to completion. However approximately 20 % of the genome is still unknown, suggesting that other members of the amino acid transporter family could still be found. In order to identify the complete family, the databases should be searched regularly and a systematic cloning and expression approach should be undertaken for all the family members.

7.4. Amino acid transporters and drug design.

Although TbAATP1 possess structural homologies to mammalian amino acid transporters, functional differences between the two systems are indicative of the presence of different recognition motifs.

Using these transporters as gateways for drugs is a long term goal and the first step towards this goal is to characterize this family of transporters in trypanosomes. The role of the P2 nucleoside transporter as a gateway for trypanocidal drugs and its role in the mechanism of resistance to these drugs has set various precedents on features making a transporter a good potential drug carrier. Theoretically, the best strategy in terms of drug design would involve the use of a cocktail of drugs able to enter the cell via different transporters. Practically this requirement would increase the cost of drugs and make them less affordable to the third world countries facing sleeping sickness.

Studies into amino acid transporters will also provide key information about amino acid metabolism in trypanosomes. For example the inhibition of an enzyme involved in the synthesis of one that is not scavenged would represent an ideal target for chemotherapy.

Saccharomyces cerevisiae possesses transporters that are sensors for glucose (Kruckeberg, 1998) and amino acids (Iraqui *et al.*, 1999), the presence of such sensors or of a mechanism of regulation of amino acid transporters in trypanosomes would be of interest to investigate.

Ultimately amino acid transporters in *T. brucei* represent potential gateways for new drugs by fulfilling the criteria set out in part 3 of Chapter I. The identification of a family of genes and establishment of a functional expression system leads to optimism that mechanisms to exploit these molecules in chemotherapy may be found.

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Appendices

Appendix-Chapter I

Amino acid concentrations in human serum

Amino Acid	Concentration	Amino Acid	Concentration
Glutamine	0.7 mM	Histidine	0.09 mM
Alanine	0.3 mM	Arginine	0.08 mM
Cysteine	0.25 mM	Isoleucine	0.07 mM
Valine	0.25 mM	Asparagine	0.06 mM
Glycine	0.23 mM	Tyrosine	0.06 mM
Proline	0.2 mM	Phenylalanine	0.06 mM
Lysine	0.19 mM	Tryptophan	0.04 mM
Threonine	0.14 mM	Glutamic acid	0.04 mM
Leucine	0.13 mM	Methionine	0.02 mM
Serine	0.11 mM	Aspartic acid	0.005 mM

Amino acid concentrations in human serum Amino acids with a concentration above 100 μM are indicated in blue, in red are the amino acids with a concentration below 100 μM (Modified from Brody, 1994).

Proton Motive Force (Equation 1)

$PMF = Vm-(2.3RT/F)(pH_i-pH_o)$

Vm is the membrane potential, T the temperature in degree Kelvin and R is the gas constant

Amino Acids

Name	Abbreviations	*Linear structure formula*
Alanine	Ala A	CH3-CH(NH2)-COOH
Arginine	Arg R	HN=C(NH2)-NH-(CH2)3-CH(NH2)-COOH
Asparagine	Asn N	H2N-CO-CH2-CH(NH2)-COOH
Aspartic acid	Asp D	HOOC-CH2-CH(NH2)-COOH
Cysteine	Cys C	HS-CH2-CH(NH2)-COOH
Glutamine	Gln Q	H2N-CO-(CH2)2-CH(NH2)-COOH
Glutamic ac	Glu E	HOOC-(CH2)2-CH(NH2)-COOH
Glycine	Gly G	NH2-CH2-COOH
Histidine	His H	NH-CH=N-CH=C-CH2-CH(NH2)-COOH
Isoleucine	Ile I	CH3-CH2-CH(CH3)-CH(NH2)-COOH
Leucine	Leu L	(CH3)2-CH-CH2-CH(NH2)-COOH
Lysine	Lys K	H2N-(CH2)4-CH(NH2)-COOH
Methionine	Met M	CH3-S-(CH2)2-CH(NH2)-COOH
Phenylalanine	Phe F	Ph-CH2-CH(NH2)-COOH
Proline	Pro P	NH-(CH2)3-CH-COOH
Serine	Ser S	HO-CH2-CH(NH2)-COOH
Threonine	Thr T	CH3-CH(OH)-CH(NH2)-COOH
Tryptophan	Trp W	Ph-NH-CH=C-CH2-CH(NH2)-COOH
Tyrosine	Tyr Y	HO-p-Ph-CH2-CH(NH2)-COOH
Valine	Val V	(CH3)2-CH-CH(NH2)-COOH

Appendix-Chapter II

BIOCHEMISTRY

BUFFER FOR TRANSPORT ASSAYS

CBSS

Hepes	25 mM
NaCl	120 mM
KCl	5.4 mM
$CaCl_2$	0.55 mM
$MgSO_4$	0.4 mM
Na_2HPO_4	5.6 mM
D-glucose	11.1 mM

Adjust to pH 7.4. Store at –20°C

BUFFER FOR ELUTION OF TRYPANOSOMES FROM DEAE COLUMN

Stock Phosphate-Buffered Saline (PS)

Na_2HPO_4 (anhydrous)	13.48g
$NaH_2PO_4.2H_2O$	0.78g
NaCl	4.25g

Make up to a final volume of 1000 ml in distilled water at pH 8.

Preparation of Phosphate-buffered Saline-Glucose (PSG 6:4) (Separation buffer)

To 6 volumes of PS add 4 volumes of H_2O and 1% of glucose (w/v). Adjust at exactly pH 8.

BUFFERS TO MAINTAIN XENOPUS OOCYTES

Barths

NaCl	88 mM
KCl	1 mM
$NaHCO_3$	2.4 mM
HEPES-NaOH	15.0 mM
$CaNO_3.4H_2O$	0.30 mM
$CaCl2\ .6H_2O$	0.41 mM
$MgSO_4.7H_2O$	0.82 mM
Sodium penicillin	10μg/ml
Streptomycin sulphate	10μg/ml

Barths without Ca^{2+}

NaCl	88 mM
KCl	1mM
$NaHCO_3$	2.4 mM
HEPES-NaOH	15.0 mM
$MgSO_4.7H_2O$	1.64 mM
Sodium penicillin	10μg/ml
Streptomycin sulphate	10μg/ml

SOLUTION FOR DNA ELECTROPHORESIS

50 x TAE

Trizma base	242 g
EDTA	18.6 g

Make up to 800 ml and adjust to pH 8 with glacial acetic acid. Make up to a final volume of 1000 ml. Store at Room temperature.

SOLUTION FOR NORTHERN GEL BUFFER

20 X Northern Gel Buffer

Na_2HPO_4	0.36 M
NaH_2PO_4	0.04 M

1x Northern Gel Buffer

For 500 ml	
20 x Northern Gel Buffer	25 ml
Formaldehyde	44 ml
H_2O	430 ml

SOLUTIONS FOR BLOTTING

Depurination solution

HCl	11 ml
distilled water	989 ml

Store at room temperature

Denaturation buffer

NaCl 87.66 g

NaOH 20 g

Make up to 1000 ml. Store at room temperature.

Neutralization solution

NaCl 87.66 g

Trizma base 60.5 g

Add 800 ml of distilled water and adjust to pH 7.5 with HCl. Make up to a final volume of 1000 ml. store at room temperature.

SOLUTIONS FOR HYBRIDISATION

20 x SSC

Tri-sodium citrate 88.23 g

NaCl 175.32 g

Check the pH is 7-8. Make up to a final volume of 1000 ml. Store at room temperature.

100 x Denhardt's solution

Bovine serum albumin 2 g

Ficoll 400.2g

Polyvinylpyrolidone 2 g

Make up to a final volume of 100 ml. Store at -20 °C.

Luria Bertani medium (LB)

Bacto-tryptone	10 g
Bacto yeast extract	5 g
NaCl	10 g

Make up to 800 ml and adjust at pH 7 with NaOH. Make up to a final volume of 1000 ml. Sterilise by autoclaving.

For solid LB medium add Bacto agar 15g/l.

Ampicillin (Stock solution)

50 mg/ ml dissolved in distilled water. Sterilised by filtration using a syringe filter (0.2 μm). Store at –20 ° C.

X-Gal (Stock solution)

50 mg/ml in dimethylformamide. Store at –20 ° C

IPTG (stock sloution)

0.4 M dissolved in dd H_2O. Store at –20 ° C.

Appendix-Chapter VI

PREDICTED AMINO ACID SEQUENCES OF THE TWELVE MEMBERS OF THE TbAATP FAMILY.

TbAATP1 (467 AA)

MTSINAQPPNSATYPQDDHGSAEVVNLNAEVERPQPEEQKDGGGCFARVSLFMATIIPPGGIAASAF
NIGSTTIGAGIFGLPAAANSSGLVMAMIYLIIITAMTIFSIYALGVAAERTNIRTYEGVARALLGPWGA
FCTAAARTFFSFSACVAYVISVGDILSATLKGTNAPDFLKQKSGNRLLTSLMWLCFMLPLVIPRHIDS
LRHVSTIAFILMIYMVLVVVVHSCMNGLPENIKNVSVGKDDNAEIILFNSGNRAIEGLGVFIFSYLFQI
TAYEVYMDMTNRSVGKFVLVVTIAMGMCLPMYALTAFFGYMDFGRNVTGSVLLQYDPVNYPAV
MVGFVGVLVMLFVSYALLGLACRNALYDVIGWDFREVAFWKHCIAVVTLSVVMLLCGLFIPKITT
VLGFAGSFCGGLLGFILPALFFMYSGGFTWQKVGPFYYISTYVLLITGVIAAVFGTGATIWAVTVG

TbAATP2 (402 AA)

MVDCTRSCALNLASATLGAGICSLPAGFNLSGIVMSCIYLVCVAVGTVYSLNLLAKVAVKTGSRNY
GEAAKAVMGPLAGYYTAALMIVMCFGGSVAYIIIIGIILKAVLNRDGVPEYLKSESGNRLMTSMVW
LVIILPMCIPKQVNSLRHLSFVGVMFIVYFSCVVIGHSINKIINEGVADGIVYMRTGNSALDGLSLFLF
SFICQPNAFEIFREMKHRSPQRFTIYGTVGMSMCAVLYFLVGLFGYLEFGGDAIDTVLSLYDPGENV
AVAIAYIGVAAKVCVAFALHIIPMRDALYHCTGWHVDTVPYWKHSLIVTSITLAALLMGLFIPKAST
VFGLVGAFCGGHIGLVLPPLFYMYSGGFTREKVGNIDFFGTYLLLFVGVVAGVFGTVSTIYNTVP

TbAATP3 (450 AA)

MEVRNEPIGCCDAAVDPKSQEQREGTGFLARMSTFVATAIPPGGIAASAFNIASSTVGAGIVGLPSA
ANSSGLVMAIVYLIIITVMTIFSIYALGVAADKTKTHDFEGVAKVLFGAKGSYLVAATRAFHGFSGC
VAYIISVGDILSAILKGTDAPDFLKEKWGNRLLTFIMWLCFMLPLAIPREVNSLRYVSTFAVSFIVYL
VIVIVVHSCMNGLPENIKNVSVGRNDVAAIVLFNSGNKAIEGLGVFIFAYVSQITAYEVYVGMTNRS
VGKFVMASTIAMAVCFTMYVLTAFFGYLDFGRDVTGSVLLMYDPVKEPAIMVGFIGLLVKLFASY
ALLGMACRNALYSIIGWDAEKVMFWKHCVAVVTLSVIMLLCGLFIPNINTVLGLAGSISGGLLGFIF
PALLLLYAGGFTWQKVGPFHYIATYTVLITGVIAIVFGTGASIWGAINI

TbAATP4 (460 AA)

MRATKKHPNDGKGATTDPFVDGSDPIPSEVAAFDPSQQEHVDVVKEVKPSLFTVLLEKFIPHGGLW
SCALNLASATLGAGICSLPAGFNLSGIVMSCIYLVCVAVGTVYSLNLLAKVAVKTGSRNYGEAAKA
VMGPLAGYYTAALMIVMCFGGSVAYIIIIGIILKAVLNRDGVPEYLKSESGNRLMTSMVWLVIILPM
CIPKXVNSLRHLSFVGVIAIAYFAFFSVGHSINKIINEGVADDIVYMRXGNSALDGXSLFLFSFICQPN
AFEIFREMKHRAPQRFTIYGTVGMSICAVLYFLVGLFGYLEFGGKSVDTVLSLYDPGQGVAVTIAYI
GVAIKITVAYALHAIPIRDSVYHCVGWHVDTVPYWKHVVVVVAINFTSLITGLFIPKATTVFGLVGA
FCGGHIGLVLPPLFYMYSGGFTREKVGNIDFFGTFLLLFVGVVAIVFGTVTTIYNTVP

TbAATP5 (376 AA)

MGILYLVIINCMTIYSMYNLALAAERSKALTYEGVTFVVLGRWAAYAIAAVRAFDGFTSCIAYVISV
GDIFSSILKGTDAPEFWKGNTGNRLLTALLWLCCMLPLVIPRHVDSLRHVSTCAVTFMVYFVIVIVV
HSCLNGLPENIKDVSVGKSDTAAIILFNSGNAAVEGLGVFMFSYTCQDTAYEIYMDMKDRSVRKFV
ISSAIAMCMCTVLYILTVXXGYMDFGRDVTGSILLMYDPVNEPAVMVGMIGVLVKLIASYALLAM
ACRNALYSIAGKNADVLPFWKHCASVVTLSVAALVLGLFIPKVNTVLGFAGSITGGSLGYIFPSLLL
MYSGGFTWQRVGPFHYLVAYGLLISGVVGVVFGTGATIWGTIVG

TbAATP6 (459 AA)

MLSPTEPLGSGKAHTEVVTDEGEXYGAMSAADEKSHHKNGDTXTTDSKFMQCINAIIPHGGALSTT
FNLGSATLGAGVIXLAIAFQXSGVIPSILILITVTVLTIYSVGLMMQAVEMTGYNSYADLSRNLFGPG
WDYFTISVSWLFTFGTCVSYVIATGYLVDSVLSGSSALEFFQGKTGNRVITSIIWFVGMFSLSLPKEIN
SLRYASAIAVLFVFYFVICIVVHSAKNGLKDGKLPEDVEMFKSGNRAIXGLSIFMFSYLCHMNCFSIY
SEMRKPSARRMTLHTTYSMSMCCVVYIIAGFFGYTDVGNKSVETVFEIYDVKGDVMMAIAFAGML
LKICVGFSLCMQPARDCCYYIIGWDLNTLETWKNCLFCGSMALCALLLGLFIPDLNTVFGLLGSFCG
GVLGFCIARIVPXCNCGXWGISQVGVVNYVCTYLLLISGVIAVVFGTAASIYNVAV

TbAATP7 (490 AA)

MHVSAQARQTLCYHYCWMASAVVPTTDREQINKADVTSPFGSCEVISGDDEGLKGKNALPANEGE
GEQSSMKCFTSMIPPGGLVSTAFSLASICIGAGILGLPAAANSTGLVMAFVYPIIIYFLCVYSLYCLGA
QMERHGFRSYEGMARALLGQPCLYFIGVLRVVEAFGAAVAQIIATGDIVSTILKGTDAPNFLKEKW
GNRLLTFIMWLCFMLPLTIPREVNSLRYVSTISVFFVFYLMVVIVVHSCMNGLPENIKNVHVTGAPG
DEGIHLFGTSNRAVEGPGVFTFAFLCHISVFEIYFGMAKPSAHRFTAYSAIAMGICLVLCVMTAFFGY
LDFGRDVAGSVLLMYDPVKEPAILVGFVGLLVKLFASYALLAMTCRNGLCGIIGLDTEKLSFFKHC
TIIGTISIIMLLCGLFIPNINTVLGFVGSVCGGFLAFILPSLFMMYGGNWSLSTVGWLHYIATYAVLFA
GVALSVFGTGATIYGVAVGW

TbAATP8 (490 AA)

MHVSAQARQTLCYHCCWMASAVVPTTDREQINKADVTSPFGSCEVIGGEDEGLKGKNALPANEGE
GEQSSMKCFTSMIPPGGLVSTAFSLASICIGAGILGLPAAANSTGLVMTFVYPIIIYFLCVYSLYCLGA
QMERHGFRSYEGMARALLGPYGAHLTGVLRVVNAFGACVAYIISVGDIVSTILKGTDAPNFLKEK
WGNRLLTFIMWLCFMLPLTIPREVNSLRYVSTFAVVFIFYLMGVIVVHSCMNGLPENIKNVHVTGA
PGDEGIHLFGTSNRAVEGPGVFTFAFVCQCYAFEIYFGMAKPSAHRFTAYSAIAMGICLVLCVMTAF
FGYLDFGGKVTGSVLLMYDPVKEPAILVGFVGVLTKLFASYALLAMSCRNGLCGIVEWDAEKLSFF
KHCTIIGILSVIMLLCGLFIPNINTVFGFVGSVCGGFLGFILPSLFMMYGGNWSLSTVGWLHYIATYA
VLFAGVALSVFGTGATIYGVAVGW

TbAATP9 (451 AA)

MEVRNEPIGCCDAAVDPKSQEQREGTGFLARMSTFVATAIPPGGIAASAFNIASSTVGAGIVGLPSA
ANSSGLVMAIVYLIIITVMTIFSIYALGVAADKTKTHDFEGVAKVLFGAKGSYLVAATRAFHGFSAC
VAYIISVGDILSAILKGTDAPDFLKEKWGNRLLTFIMWLCFMLPLAIPREVNSLRYVSTFAVSFIVYL
VIVIVVHSCMNGLPENIKNVSVGRNDVAAIVLFNSGNKAIEGLGVFIFAYVSQITAYEVYMDMEDRS
VRKFIVATSIAMATCSVLYAMTAFFGYLDFGRDVTGSVLLMYDPVKEPAIMVGFIGLLVKLFASYA
LLGMACRNALYSIIGWDAEKVIFWKHCVAVVTLSVIMLLCGLFIPNINTVLGLAGSISGGLLGFIFPA
LLLLYAGGFTWQKVGPFHYIATYTLLLSGVLAIVFGTGATIHGVVVGN

TbAATP10 (490 AA)

MHVSAQARQTLCYHCCWMASAVVPTTDREQINKADVTSPFGSCEVISGEDEGLKGKNALPANEGE
GEQSSMKCFTSMIPPGGLVSTAFSLASICIGAGILGLPAAANSTGLVMTFVYPIIIYFLCVYSLYCLGA
QMERHRFRSYEGMARALLGQPCLYFTGVLRVVNAFGAAVAQIIATGDIVSTILKGTDAPNFLKEKW
GNRLLTFIMWLCFMLPLTIPREVNSLRYVSTISVFFVFYLMVVIVVHSCMNGLPENIKNVHVTGAPG
DEGIHLFGTSNRAVEGPGVFTFAFLCHISVFEIYFGMAKPSAHRFTAYSAIAMGICLVLCVMTAFFGY
LDFGRDVAGSVLLMYDPVKEPAILVGFVGLLVKLFASYALLAMTCRNGLCGIIGLDTEKLSFFKHC
TIIGTISIIMLLCGLFIPNINTVFGFVGSVCGGFLGFILPSLFMMYGGNWSLSTVGWLHYIATYAVLFA
GVALSVFGTGATIYGVAVGW

TbAATP11 (473 AA)

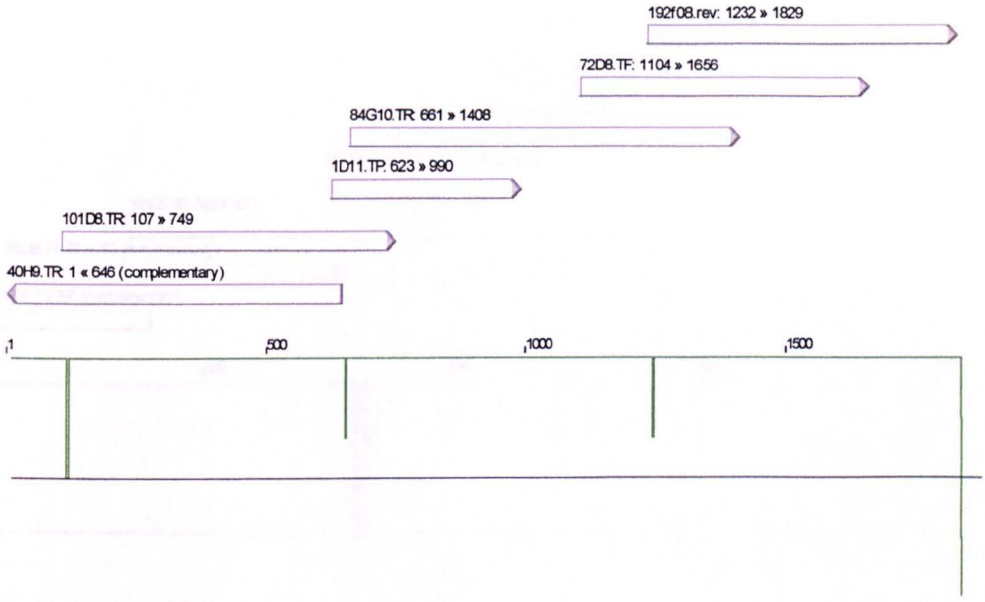
MTFEAAAQAPNKCPTNEATAEPLRTPNVLADNLLARSNLSEGESPEKSGEAQKPEEQGNALMRCFH
FILPRGGALSGIFNLASVTLGAGIMSIPSAFNTSGMIMAIIYLLLVTVFTVFSIFLIVSAAEKTGYRSFES
MARNLLGPRADIAVGFLLWLLCFGGASGYVVAIGDVLQGLLSHEKVPAYLQSKGGRRLLTSAIWF
VFIFPLTLPKRVNSLRYASAIGVSFILFFAICVVEHSAEKMVTDGGIKQELVMFRSGNDAVAGLSLFIF
AYLCHVNSFSIFFEMKKRSVTRMTRDAAVSCSICCCVYLLTGFFGYAEFGPTVEGSVLKLYDPYANP
VFFVCFIGIIVKLCAGFSLNMLACRTALFQVLRWDLDTMSYVRHSIVSVSFAVGSLVLGLFVPDINVI
FGLVGAFCGGFIGFIFPALFIMYAGGWTRQSVGWVQYILTYVLLILGVVAIVFGTSASVYYTIKRYS

TbCAATP (539 AA)

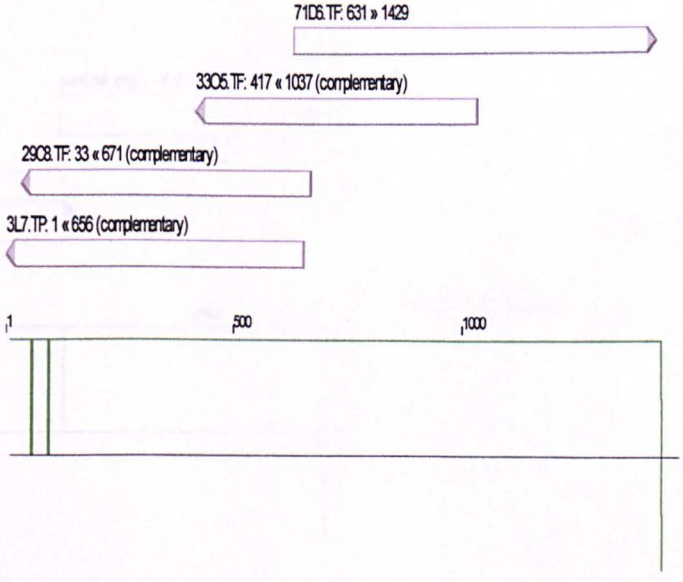
MVSWSRPEFAEAFFGKLVRRRCIYATKAALDSSPFVRVLGLLSLVSLGVGAVVGAGIFVITGQAAA
QYAGPGLTISFVLCMFPCFLTALCYGELAAMLPVAGSAYTHTSVALGEFASWTVAVCMTLECLVS
GCAVSVSWSAYVQAFLKRFSFVLPQPLRKSPIDVVGGRFVLTGSVVNFPAVVITVVCFVVLCLGVE
QTASMNSFFVVVKLAALVCFVFYGIYYSLGNWAEVNANLTPFVPPNDGHFGHFGVSGILRGASVVF
FANVGFDTICASAQECRSPQRDIPRGIILTLLLCSTLYVMVTVSLTGLVKYTELGTDAPVIAALEKVK
APSFLRLFIEVGTVAALSSVCFVSFYAMPRLIMAVAKDGLLPALLTHVHEQFRTPINATIFCGIPATFI
CAVFPLGMLGELISFGTLIALACVCVSMWKIRIDHPEFHRPFVAPLFPYVPILGALLNAAQLFFLPLTT
WRNYFVVMATTSLWYIVYGIRHSTVGEDGITRRPDSLLGTVEPPLCEALEGVQGAGGSLSIELTERY
VHN

TIGR REFERENCE NUMBERS OF OVERLAPPING SEQUENCES USED TO PRODUCE CONTIGUOUS SEQUENCES IN CONTAINING ORF CORRESPONDING TO *TbAATP 2, 3, 4, 5, 6*

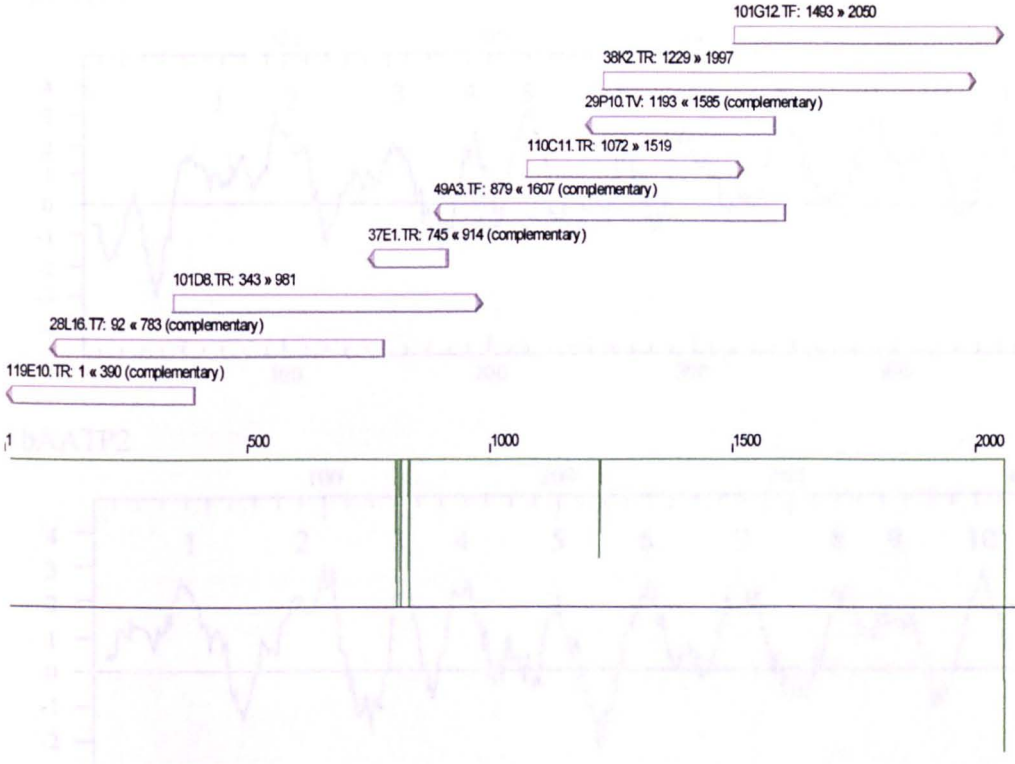
TbAATP2



TbAATP3

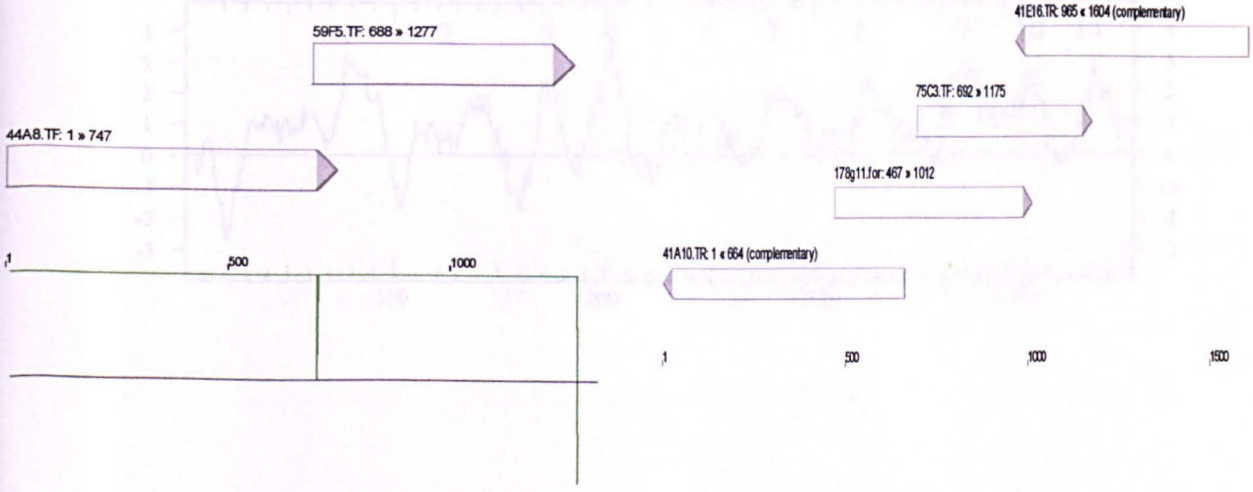


TbAATP4



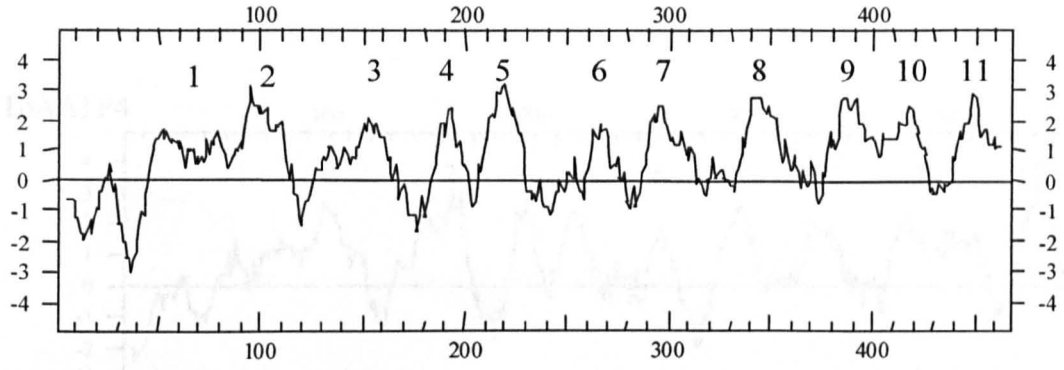
TbAATP5

TbAATP6



HYDROPATHY PLOTS BASED ON KYTE-DOOLITTLE ANALYSIS OF THE TWELVE MEMBERS OF THE PUTATIVE TBAATP FAMILY

TbAATP1



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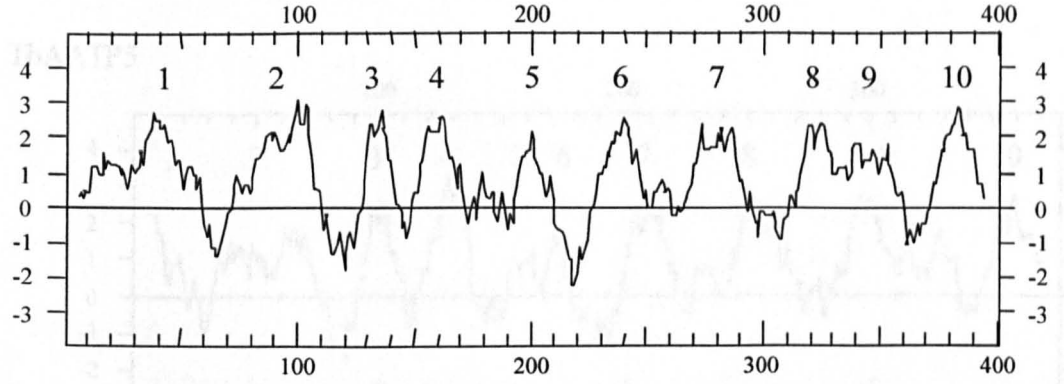

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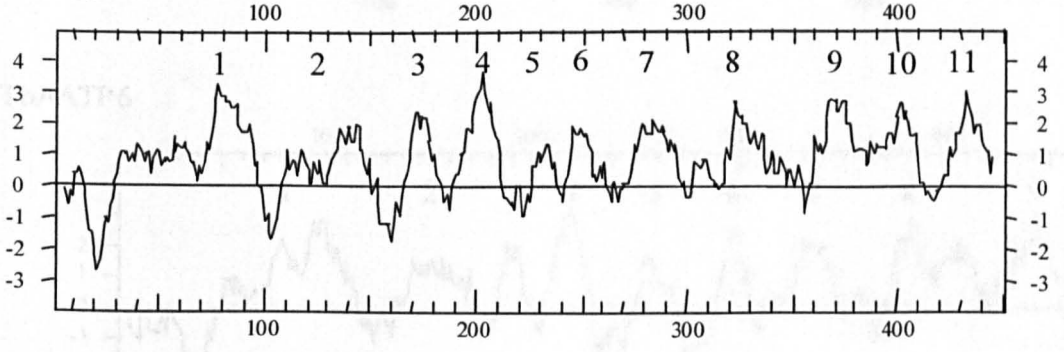

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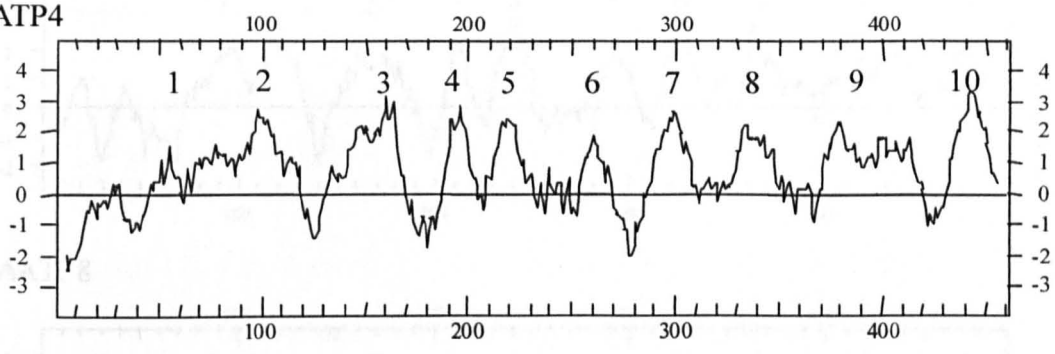

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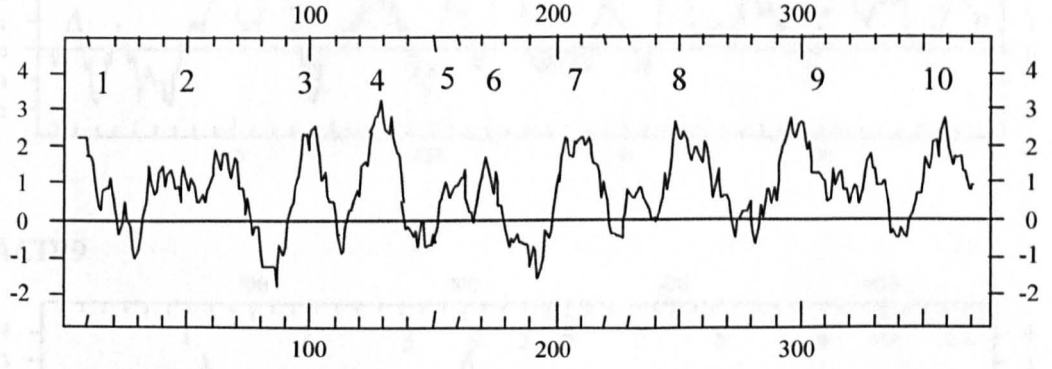

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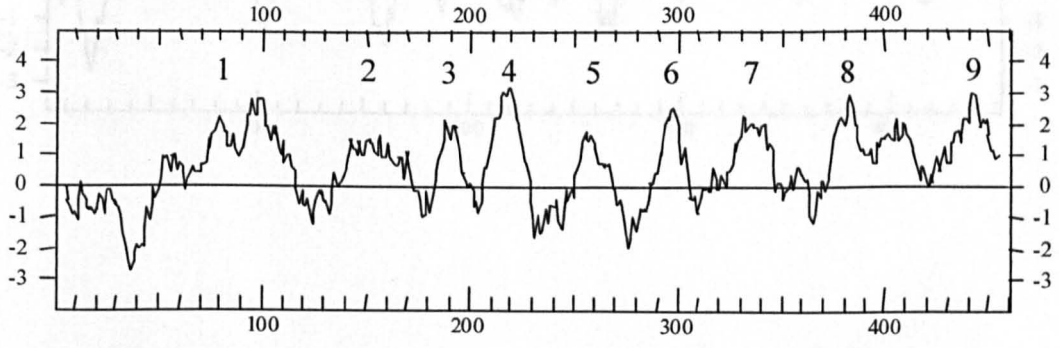

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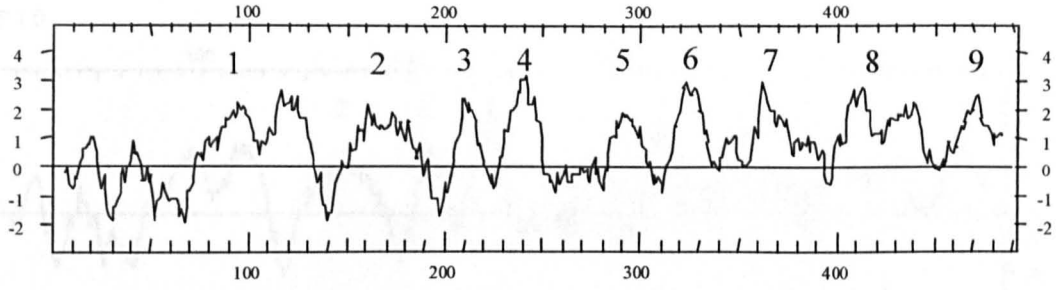

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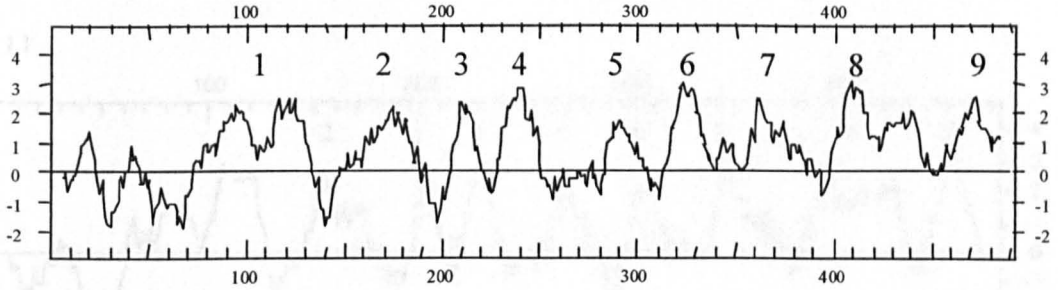

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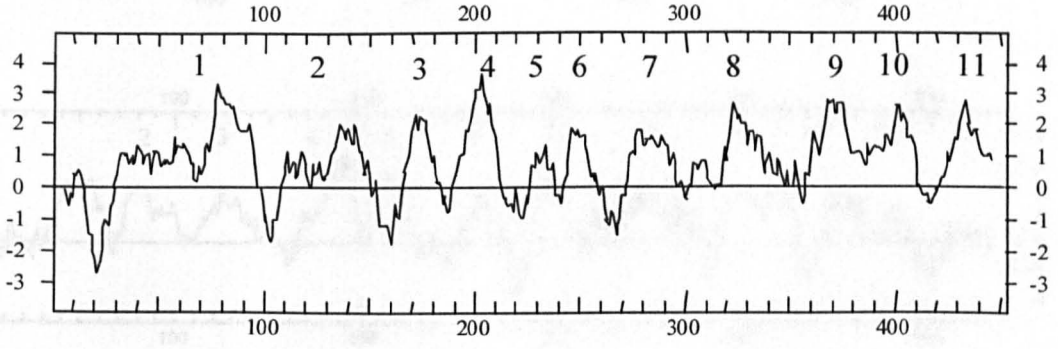

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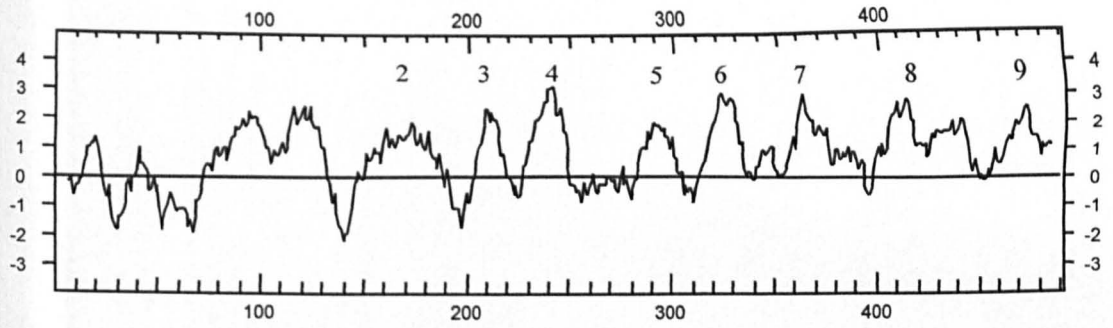

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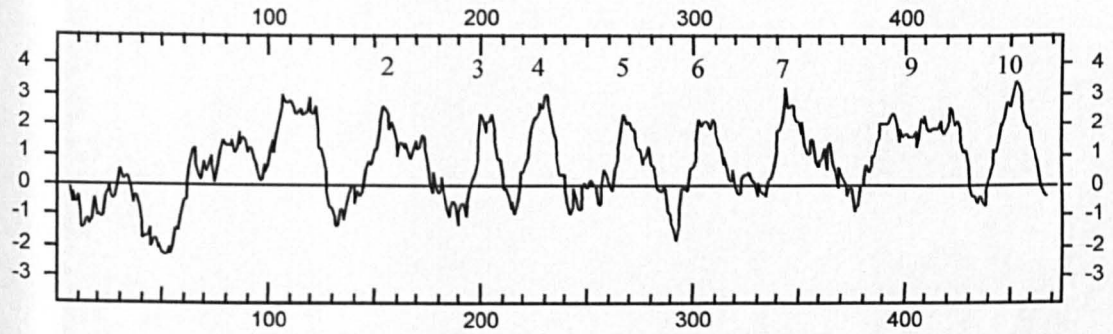

TbCAATP