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Mercaptopyruvate Sulfurtransferase and Cysteine Biosynthetic Pathways in *Leishmania*

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Declaration

The results in this thesis are my own work except for the following; Circular dichroism UV studies (Chapter 3), the X-ray analysis and the HPLC studies (Chapter 7). These studies were carried out in collaboration with Profs. N. Price, (University of Glasgow), W. Hunter (University of Dundee) and A. Fairlamb (University of Dundee) respectively.

Roderick Williams

Abstract

Coping with oxidative stress is vital for survival of the intracellular parasite *Leishmania,* but the complex biochemical mechanisms involved are not fully understood. This study focused on enzymes of cysteine metabolism in *Leishmania* and the parts they play. Mercaptopyruvate sulfurtransferase (EC 2.8.1.2) of *Leishmania major* and *L. mexicana* and serine acetyltransferase (EC 2.1.3.30), cysteine synthase (EC 4.2.99.8) and cystathionine β-synthase (EC 4.2.1.22) of *Leishmania major* have been cloned, expressed as active enzymes in *Escherichia coli*, and characterized.

The leishmanial mercaptopyruvate sulfurtransferase is structurally peculiar in possessing a C-terminal domain of some 70 amino acids. Homologous genes of *T. cruzi* and *T. brucei* encode enzymes with a similar C-terminal domain, which suggests that the feature, not known in any other sulfurtransferase, is a characteristic of trypanosomatid parasites. Short truncations of the C-terminal domain resulted in misfolded, inactive proteins, demonstrating that the domain plays some key role in facilitating correct folding of the enzymes.

The recombinant sulfurtransferase exhibit high activity towards 3-mercaptopyruvate and catalyze the transfer of sulfane sulfur to cyanide to form thiocyanate and sulfide. The sulfide can react with *O*-acetyl serine to yield cysteine through the action of cysteine synthase. They also use thiosulfate as a substrate and mercaptoethanol, glutathione, cysteine or reduced thioredoxin as the accepting nucleophile, the latter being oxidized. Mercaptopyruvate sulfurtransferase and cysteine synthase are expressed in all life cycle stages of *Leismania* and the expression levels are increased under hypo-sulfur stress. The expression level of mercaptopyruvate sulfurtransferase is also increased under oxidative stress whereas overexpression of serine acetyltransferase, cysteine synthase and cystathionine beta-synthase in *Leishmania* promastigotes produced cell lines resistant to the oxidants hydrogen peroxide (0.5 mM), *tert*-butyl hydroperoxide (10 μM) and cumene hydroperoxide (10 μM).

These results are consistent with these enzymes having an involvement in the synthesis of sulfur amino acids *per se* and the parasite's management of oxidative stress.

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List of Abbreviations

APS	Adenosine 5-phosphsulate
ATP	Adenosine triphosphate
CBS	Cystathionine beta-synthase
CBL	Cystathionine beta-lyase
C-DES	Cysteine desulfurase
CGL	Cystathionine gamma-lyase
CGS	Cystathionine gamma-synthase
CL	cutaneous leislmaniasis
CS	Cysteine synthase
DALYs	Disability Adjusted Life Years
DCL	Diffuse Cutaneous Leishmaniasis
MAPK phosphatase	Mitogen activated protein kinase phosphatase
MCL	Mucocutaneous leishmaniasis
MGL	Methionine gamma-lyase
MST	Mercaptopyruvate sulfurtransferase
OAS	*O*-acetyl serine
PAPS	Phosphoadenosine 5-phospsulfate
PLP	Pyridoxial 5-phosphate
SAM	S-adenosyl methionine
SAT	Serine acetyltransferase
TST	Thiosulfate sulfurtransferase
VL	Visceral Leishmaniasis

Chapter 1

1.0 Introduction

Parasitic protozoan diseases are of major public health significance. Despite the revolution in medical and health care in the late 20th century, these diseases play critical roles in the economic development of the third world (Table 1.1). They cause a constant drain through chronic sickness, loss of productive labour and premature deaths thus creating an urgent need for new anti-parasitic drugs (Croft, 1997).

Malaria, is the foremost amongst them and infects some 300 million people worldwide, causing several million deaths each year (Table 1.1). The causative agent, *Plasmodium*, belongs to a phylogenetic group known as apicomplexan parasites. This group also includes the human parasites *Toxoplasma gondii* and *Cryptosporidium parvum* and the chicken and cattle parasites *Eimeria tenella* and *Theileria annulata*, respectively.

A second 'group' of parasitic protozoa, based on similarity due to convergent evolution, are the anaerobic or microaerophilic organisms: *Entamoeba histolytica, Giardia lamblia* and *Trichomonas vaginalis*. They show some similar biological characteristics, but are phylogenetically quite diverse.

The third 'group' of parasitic protozoa are the trypanosomatids: *Trypanosoma brucei, T. cruzi, Leishmania spp*. They cause the diseases Sleeping Sickness, Chagas' disease and Leishmaniasis, respectively.

Table 1.1 Some Parasitic Protozoa of Public Health Significance

Disease and causative agent	vector/intermediate host	people infected (millions)	Disease cases/year (millions)	Deaths/year (millions)	At risk (millions)	Distribution	Main control problems
African sleeping sickness (African trypanosomiasis) *Trypanosoma brucei brucei, T. b. gambiense, T. b. rhodesiense*	Tsetse fly	unknown	0.25-0.3	0.23 - 0.27 invariably fatal if untreated (90% of cases)	55	36 countries of Sub Saharian Africa	-Insufficient medical surveillance -Premature cessation of control programmes -Drug toxicity
Chagas Disease (American trypanosomiasis) *Trypansoma cruzi*	Triatome bug	16-18	6-10 million suffer chronic disease	unknown but infection is fatal in 10-40% of chronic cases	90-100 25% of South and Central America	Southern USA, Central + South America	-Zoonotic infections -Blood transfusions
Leishmaniasis *Leishmania spp.* (21 species)	Sandfly	12-15	2	unknown	350-370	88 countries of North Africa, Middle East + South America	-Increase in vector populations as malaria vector control programmes are wound down -Immunosuppressive effects of HIV -Prolonged drug treatment/hospitalization required -Emerging drug resistance
Malaria *Plasmodium spp.* (4 species)	Mosquito	unknown	300-500	1.5-2.7	25,000 (40% global population)	98 countries, mainly in Sub Saharan Africa also Arabia, Latin America + South-east Asia	-Drug resistance -Insecticide resistance -Migration and travel

The success of these parasites is due to their sophisticated means of evading the mammalian host's immune system (Damian, 1997), meaning that very few effective vaccines are currently available. Also, the limited success in controlling the vectors transmitting the diseases coupled with the resistance of these vectors to known insecticidal agents, makes chemotherapy the main weapon in controlling these diseases. A number of useful anti-protozoa drugs were developed during the first half of this century, but, compared with the number of new anti-bacterials, very few have emerged since then. Moreover, the toxicity of existing drugs and resistance of parasites to these drugs mean that current chemotherapy is far from satisfactory.

Phylogenetics suggests that trypanosomatids are relatively early branching eukaryotes whose cell organization differs considerably from that of mammalian cells. Biochemical pathways present in trypanosomatids but absent from their host should, in theory, provide excellent targets for rational design. These provide some optimism for the future rational development of the drugs needed.

1.1 Leishmaniasis

1.1.1 Leishmania Taxonomy

The genus Leishmania belongs to the family, Trypanosomatidae, of the order Kinetoplastida (the kinetoplastid flagellated protozoa). The kinetoplastid protozoa belong to the Phylum Sarcomastigophora, together with the euglenoid protozoa (Table 1.2). Currently, more than 20 species of *Leishmania* that cause human disease, have been identified, each of which has specific mammalian reservoirs - the majority being rodents.

Table 1.2. Taxomony of *Leshmania spp* in Humans

Kingdom	Protista						
Subkingdom	Protozoa						
Phylum	Sarcomastigophora						
Subphylum	Mastigophora						
Class	Zoomastigophora						
Order	Kinetoplastida						
Suborder	Trypanosomatina						
Family	Trypanosomatidae						
Genus	*Leishmania*						
Section	Peripylaria		Suprapylaria				
	L. brasiliensis complex: *L. braziliensis* *L. panamensis* *L. guyanensis*	*L. peruviana*	*L. donovani* complex: *L. donovani* *L. infantum* *L. chagasi*	*L. major*	*L. tropica*	*L. aethiopica*	*L. mexicana* complex: *L. mexicana* *L. amazonensis* *L. pifanoi* *L. garnhami* *L. venezuelensis*
host	mammals, including humans						
disease pattern in humans	CL (Pian Bois), MCL (Espundia)	CL (Uta)	VL (Kala Azar), CL, PKDL	CL (oriental sore)	CL (oriental sore), VL	CL, DCL	CL (Chiclero's ulcer), DCL

CL, cutaneous Leishmaniasis; MCL, mucocutaneous Leishmaniasis; VL, visceral Leishmaniasis; PKDL, post-Kala-Azar dermal Leishmaniasis; DCL, diffuse cutaneous Leishmaniasis

The human pathogens are *L. aethiopica*, *L. braziliensis* (complex), *L. donovani* (complex), *L. major*, *L. mexicana* (complex), and *L. tropica* (Lainson and Shaw, 1979).

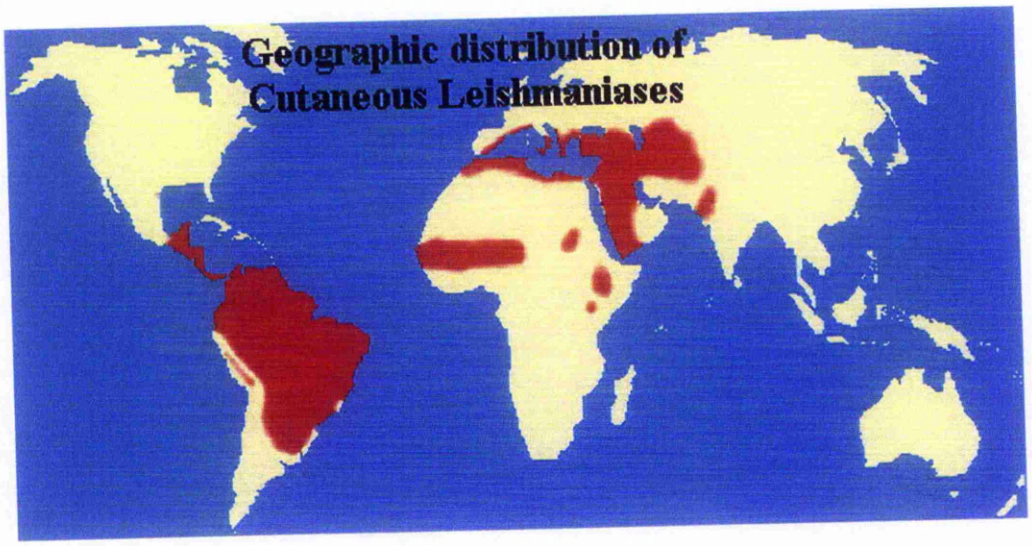
1.1.2 Geographic distribution and estimated number of cases

Leishmania is endemic in areas of the tropics, subtropics in settings ranging from rain forests to deserts, and rural to periurban areas (Figure 1.1). These include 80 countries in South and Central America (from Argentina to Southern Texas, USA but not including Uruguay, Chile or Canada), Southern Europe, Asia (not South East Asia), the Middle East and Africa (particularly East and North), with sporadic cases elsewhere. It is absent in Australia and Oceania.

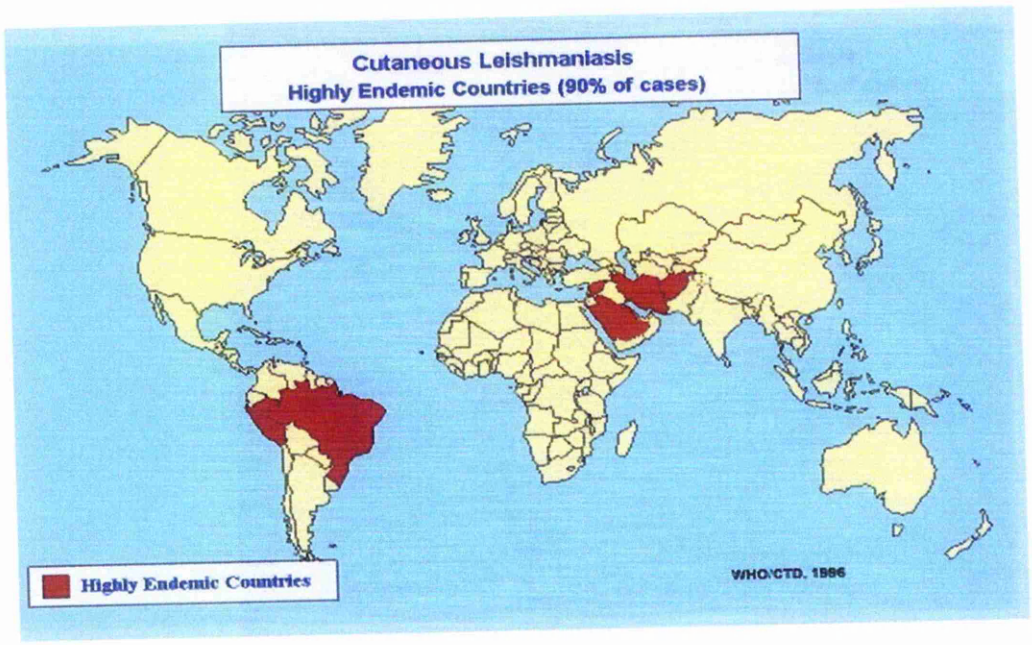
Over the past 20-30 years, there has been a dramatic increase in the number of reported human leishmaniasis cases - a trend that shows no sign of abating. Currently, in terms of global disease burden, leishmaniasis is the third most important vector-borne disease (after malaria and lymphatic filariasis). It is responsible for an estimated 1.8 million disability adjusted life years (DALYs), 57,000 deaths (Reithinger and Davies, 2002) and affects 2 million people (Croft and Yardley, 2002) annually (Table 1.1). A total of 350 million people are at risk. The estimated annual number of new cases of VL is 500,000 with over 90% of world-wide cases being in Bangladesh, north-east India, Nepal, and Sudan (old world) and north-east Brazil (new world) (Figure 1.1c). For CL, the estimated new cases annually is 1.5 million with over 90% of world-wide cases being in Afghanistan, Algeria, Iran, Iraq, Saudi Arabia and Syria (old world), Brazil and Peru (new world) (Figure 1.1 a and b).

During the last 10 years there have been extensive epidemics of the visceral form of the disease, which is also emerging as an important opportunistic infection in immuno-compromised patients - especially those co-infected with human immunodeficiency virus (HIV) (Figure 1.1d). The geographic distribution of cases documented in the developed world reflects travel and immigration patterns.

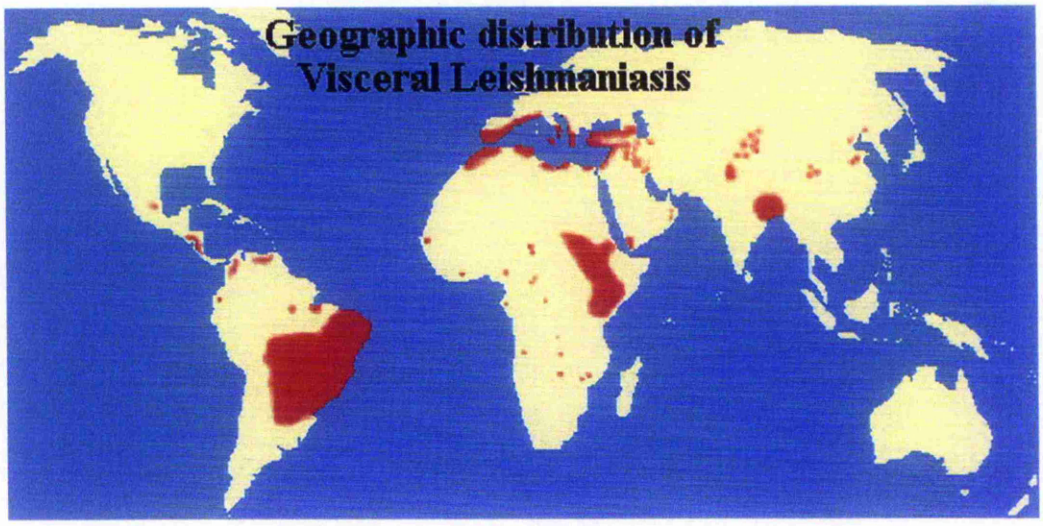
(a)



(b)



(c)



(d)

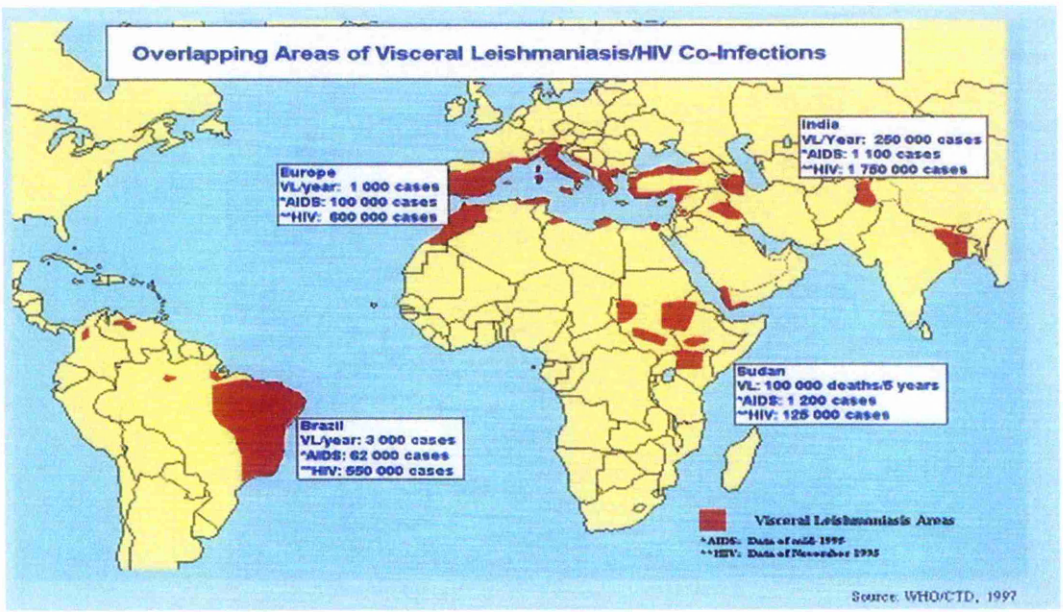


Figure 1.1 Maps showing the geographic distribution of Visceral and Cutaneous Leishmaniasis. (a) Cutaneous Leishmaniasis (CL), (in red); (b) Highly endemic regions of CL, (in red); (c) Visceral Leishmaniasis (VL), (in red); (d) Overlapping areas of VL and HIV co-infection.

Source:http://www.icp.ucl.ac.be/~opperd/parasites/leish4.htm

1.1.3 The disease

Several disease types, with different clinical symptoms are subsumed under the term leishmaniasis. The most notable are as follows:

1.1.3.1 Visceral leishmaniasis

Visceral leishmaniasis (VL) is caused by the species *Leishmania donovani donovani*, *Leishmania donovani infantum* and *Leishmania donovani archibaldi* in the old world. It is prevalent in regions spanning from the Mediterranean region through the Middle East and Central Asia to Northern China, and frequently occurs in rocky areas. In Pakistan, its affects mainly children - hence the name *L. infantum*. On the plains of Northeastern India and Bangladesh, young adults are most at risk from *L. donovani*. *Leishmania donovani chagasi* is the most prevalent species of visceral *Leishmania* in the new world, and is found throughout Central and South America but mainly in Northeastern Brazil. In VL, amastigotes are found in macrophages throughout the reticuloendothelial system. The parasites can invade the cells of the spleen, liver and bone-marrow, resulting in death if there is no treatment (Figure 1.2a).

1.1.3.2 Cutaneous leishmaniasis

Cutaneous leishmaniasis (CL such as oriental sore) is caused mainly by *L. major, L. tropica* and *L. aethiopica* in arid regions of Africa and Asia in the old world. In forests from Mexico to northern Argentina in the new world, the main causative species are *L. guyanensis, L. panamensis* and *L. mexicana.* In cutaneous leishmaniasis (CL), the amastigotes multiply in macrophages at the site of skin inoculation of the parasites. They result in single or multiple lesions, usually on the head and/or neck (Figure 1.2b). The parasites also infect the lymphatic system,

causing skin wounds along the lymphatic ducts. Healing can be spontaneous, between 0.5 to 2 years after infection, in untreated patients.

1.1.3.3 Mucocutaneous leishmaniasis

Mucocutaneous leishmaniasis (MCL, espundia) is caused by the species *L. braziliensis* and *L. panamensis* and is found in South America. The original bite by the infected sandfly which transmits the parasites can be on any part of the body, where it causes a small wound that eventually heals. However, the parasite invades and erodes the cartilaginous tissues of the nose and palate causing facial disfigurement (Figure 1.2c). Healing is never spontaneous and untreated patients can die from secondary infections.

1.1.3.4 Diffuse cutaneous leishmaniasis

Diffuse cutaneous leishmaniasis (DCL) in Africa (Kenya, Ethiopia) is caused mainly by *L. aethiopica* and in Central America and northern South America it is caused mainly by *L. amazonensis*. The disease is characterised by widespread papules or nodules in the skin (Figure 1.2d). It does not heal spontaneously and is difficult to treat.

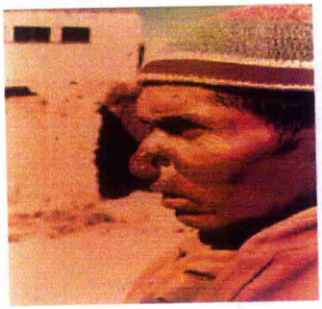
(a)

Visceral Leishmaniasis (VL)

(b)

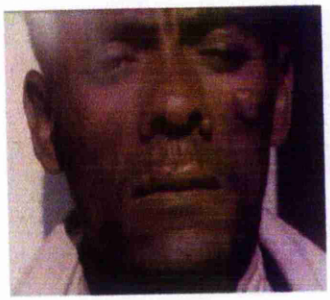
Cutaneous Leishmaniasis (CL)

(c)



Mucocutaneous Leishmaniasis (MCL)

(d)



Diffuse cutaneous Leishmaniasis (DCL)

Figure 1.2. Photographs of people show symptoms of the spectrum of diseases caused by *Leishmania spp.*

Source:- www.icp.ucl.ac.be

1.1.4 Life cycle stages

These various symptoms are caused by a total of 21 species of *Leishmania*. The leishmanial life cycle stages are the round, non-motile amastigotes (3-7 micrometers in diameter), in the macrophages of the vertebrate hosts, and the elongated (10-20 micrometers), flagellated promastigote forms that live in the gut of the insect host where they are regurgitated from the blocked foregut into the wound while the insect feeds.

1.1.5 The vector

The only proven vectors of the *Leishmania* parasites are the blood-sucking female sandflies (Figure 1.3aii) of the genus *Phlebotomus* in the old world and *Lutzomyia* in the new world. Only 30 out of approximately 500 known species of phlebotomine sandflies are known to transmit *Leishmania* parasites. These include *P. argentipes* on the Indian sub-continent, *P. martini* and *P. orientalis* in Africa and the Mediterranean basin, and *P. chinensis* and *P. alexandri* in China. In the new world, *Lutzomyia logipalpis* is the only known vector of *L. d. chagasi* (WHO WWW site, 1997). The insects are 2-3 mm long and are found throughout the tropical and temperate parts of the world. The sandfly larvae require organic matter, heat and humidity for development and so are commonly found in house-hold rubbish, bark of old trees, burrows of old trees and in cracks in house walls. The sandflies are relatively weak flyers with a range of only 50 m from their breeding site. They usually feed at night while the host is asleep (Arias *et al*, 1996). Sandfly numbers are related to natural factors such as rainfall and may increase with global warming.

1.1.6 Leishmania *vertebrate hosts*

The *Leishmania* parasites are digenetic (or heteroxenous) and have both mammals and sandflies as hosts. The mammalian hosts include humans, dogs and some rodents. Wild and domestic animals, such as the fox, jackal, rodents and wolves, serve as reservoir hosts for human infections. Other animals in the surrounding areas can become infected and these are referred to as secondary or incidental hosts. Of all the potential resovoir mammalian hosts, domestic dogs play the most important role by far in harbouring and transmitting the disease to humans due to the close association between humans and dogs as pets (reviewed in a report of the WHO expert committee report, 1991; Arias *et al*, 1996). In anthroponotic VL caused by *L. d. donovani* such as in India and Sudan, humans are the principal reservoir host. Asymptomatic mammalian carriers are a particular source of infection for sandflies (reviewed in a report of the WHO expert committee report, 1991).

1.1.7 Leishmanial life cycle

The digenetic life cycle of *Leishmania* exposes it to contrasting environments, from the macrophages of a mammalian host to the gut of the sandfly vector. They respond and adapt to these dramatic changes with corresponding changes in morphology, metabolism and gene expression.

The sandfly vector becomes infected when it feeds on the blood of an infected individual or an animal reservoir host. The non-motile amastigotes in macrophages ingested by the fly during the blood-meal are released into the abdominal midgut of the insect (Figure 1.3). Almost immediately the amastigotes begin to transform into motile, elongated flagellated promastigotes. The promastigotes then migrate to the

lining of the alimentary tract of the fly, where they live extracellularly and multiply by binary fission. Some species e.g. *Leishmania garnhami* grow in the malpighian tubules of an infected vector sandfly (*Lutzomyia longipalpis* and *Lu. townsendi*) (Anez *et al.*, 1985). Their developmental pattern differs from that of other Suprapylaria, e.g. *L. mexicana* and *L. donovani* complexes because it invades the hind triangle and also from the species of the Peripylaria e.g. *L. braziliensis* complex which invades the hind gut (Anez *et al.*, 1985). Four to five days after feeding, the promastigotes move forward towards the oesophagus of the insect and become metacyclic promastigotes. When the sandfly next feeds on a mammalian host, the metacyclic promastigotes are transferred to the mammal. Once in the host, the inoculated metacyclic promastigotes are phagocytosed by macrophages, where they lose their locomotary flagella and rapidly revert to the multiplicative amastigote form. Infected macrophages then burst, releasing their amastigotes to infect other macrophages and so the cycle continues. Ultimately, all the organs containing macrophages and phagocytes, especially the spleen, liver and bone marrow, can become infected. The subsequent fate of the amastigote depends upon parasite and host factors which are poorly understood. The *Leishmania* are able to resist the microbiocidal action of the host acid hydrolases in the parasitophorous vacuole in which they reside.

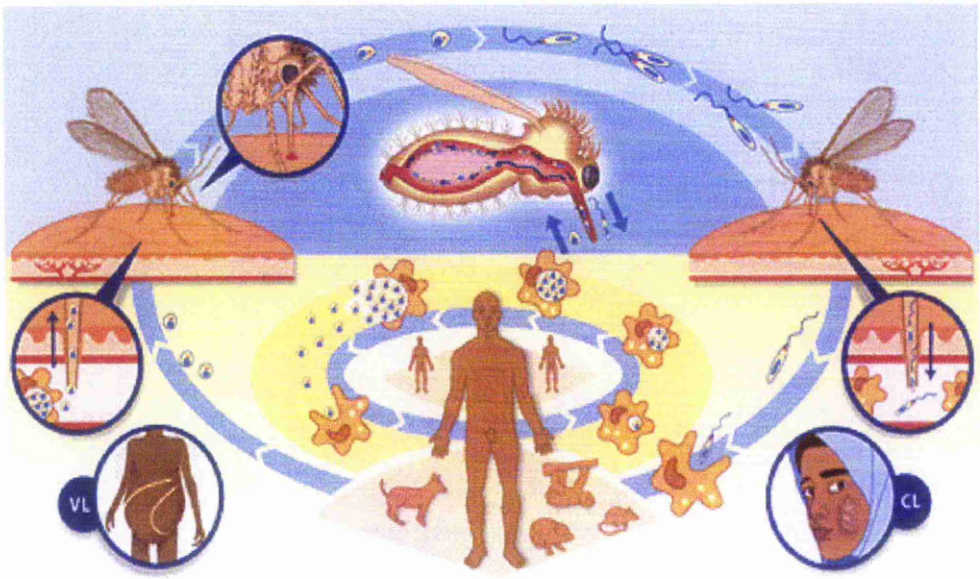


Figure 1.3. Life cycle of *Leishmania spp.*

Source:- http://www.who.int/tdr/diseases/leish/lifecycle.htm

In vitro, the *Leishmania* life cycle stages can be maintained in culture. A normal culture at early log phase comprises procyclic promastigotes apparently equivalent to the form that multiplies in the sandfly midgut, while the stationary phase culture has a combination of metacyclic promastigotes and procyclic promastigotes. The active swimming metacyclics of some species can be isolated by the method described by Sacks *et al.*, 1992. The metacyclic forms recovered by this method was 45% of the total population (Sacks *et al.*, 1992). Metacylogenesis in *L. major* culture is favoured by a down regulation of tetrahydrobiopterin levels (H_4B) encoded by the pteridine reductase 1 (PTR1) gene (Cunningham *et al.*, 2001). Metacyclics can be induced to form and maintained as amastigotes by either inoculating in BALB/c mice and other strains of mice or, with some species, by growing axenically in an acidic medium, as described by Bates *et al.*, (1992). Amastigotes can be harvested from lesions in BALB/c mice (Hart *et al.*, 1981). In particular, the amastigotes of *L. mexicana* can be purified from lesions in mice to a high level of purity.

1.2 Control measures

1.2.1 Elimination of sandfly vectors

In endemic areas where leishmaniasis co-occurs with other parasite infections, an integrated control programme is adopted. In Bangladesh and India, sandfly control is often combined with malaria control and in Brazil it is combined with malaria and Chagas' disease control programmes (WHO WWW site, 1997). This is a cost-effective method given the expense of mass spraying with chemicals such as DDT, Malathion, Fenitrothion, Propoxur and Diazinon. This has to be an on-going process and continued surveillance is required to keep the vector populations low (WHO WWW site, 1997). In epidemics, large scale spraying is required with particular attention to animal shelters, the inside of buildings and the immediate surrounding areas. Self protection is important, and several methods are available to avoid being bitten by sandflies including repellents such as diethyltoluamide (DEET) applied to the exposed areas of the body and clothing. Fine mesh screens (less than 16-mesh) can be applied to doors and windows and bed nets should be used impregnated with insecticides such as permethrin and deltamethrin. Mosquito nets are, however, ineffective since sandflies can get through the holes.

1.2.2 Elimination of reservoirs of the parasites

In areas of anthroponotic foci of VL, active case detection and treatment are imperative - especially asymptomatic, seropositive patients. A study in Israel showed that the asymptomatic or sub-clinical cases are 4-30 times greater in number than those with the VL symptoms (Ephros *et al*, 1994). The immunofluoresent antibody test (IFAT), enzyme linked immunosorbent assay (ELISA), direct agglutination test (DAT), the western blot and the leishmanin skin test have all been useful in detecting

seropositive cases of VL (Marty *et al.*, 1994; El-Harith *et al.*, 1995; Rab and Evans, 1995; Shiddo *et al.*, 1995). In endemic areas of zoonotic VL, elimination of wild animals and stray dogs can be carried out by shooting and by using poisoned baits impregnated with strychnine. Screening and treatment of domestic animals, especially dogs, are recommended. Diagnostic techniques include the IFAT, DAT and ELISA tests (Marty *et al.*, 1994).

1.2.3 Leishmania *chemotherapy; Traditional drugs*

It is evident that VL, CL and MCL, if clinically evident but not treated, cause life-threatening infections, chronic skin sores and facial disfigurement, respectively, to infected patients. No effective vaccines exist for leishmaniasis and though chemotherapy is available it is inadequate and expensive. The current regimens used are geared to prevent death from VL and morbidity from CL and MCL.

1.2.3.1 Pentavalent antimonials

Since the 1940's, the pentavalent antimony compounds, sodium stibogluconate (Pentostam, Glaxo Wellcome Foundation, UK) containing 100 mg Sb ml^{-1} and meglumine antimoniate (Glucantime, Rhone Poulenc Rover, France) containing 85 mg Sb ml^{-1}, have been the first-line chemotherapeutic agents against all forms of leishmaniasis. The two compounds are derivatives of stibonic acid in which antimony is joined through oxygen atoms to the carbon chain(s) of glucose (Hashim *et al*, 1995).

Although these drugs are generally safe, being rapidly excreted by the kidney, and highly effective, one disadvantage is cumulative toxicity, perhaps due to the

production of a slowly excreted trivalent metabolite in proportion to dose and duration (Bryceson, *et al.,* 1985). The most common drawbacks include parenteral mode of administration, long duration of therapy, sub-optimal effectiveness in some settings, nausea, fatigue, body aches, vomiting, diarrhoea, electrocardigraphic (ECG) abnormalities (Franke *et al.*, 1990), raised aminotransferase levels, chemical pancreatitis, and convulsions (Bryceson, *et al.*, 1985).

Sodium stibogluconate is usually administered at a dose of 20 mg kg^{-1} body weight for 20-40 days, however, due to wide-spread antimony resistance, treatment over four months is recommended (Hashim *et al*, 1995). Experimental data suggest that parasite resistance to antimony emerges as a result of discontinous drug exposure or inadequate dosage (Grogl *et al.,* 1989). Should this fail, as seen in epidemic situations in poor countries, a number of other drugs can be employed, depending upon the species of *Leishmania* concerned and the resources available.

1.2.3.2 Pentamidine

Pentamidine (an aromatic diamidine) is recommended only as second choice because of its toxicity and potential side effect. e.g. renal impairment by binding to the kidneys. Pentamidine is administered parenterally and is generally effective against all forms of leishmaniasis. The toxic side effects of pentamidine include a sensation of burning, headache, tightness of the chest, dizziness, nausea, vomiting, hypotension and hypoglycaemia (reviewed in a report of the WHO expert committee report, 1991). Hyperglycaemia and acute pancreatitis can lead to diabetes.

Single doses of 2-4 mg kg^{-1} body weight are usually recommended 1-3 times a week. Doses of 2 mg kg^{-1} body weight may be effective and better tolerated. In cases of VL

in Kenya, thrice-weekly injections of 4 mg kg^{-1} body weight are necessary and these avoid serious toxicity. A short course of 10-20 days treatment followed by a second course after an interval of 10-15 days helps reduce the relapse rate in VL cases (reviewed in a report of the WHO expert committee report, 1991). However, a relatively high proportion of patients with VL have failed to respond to pentamidine in Kenya, India and China due to resistance to the drug (Dietze *et al*, 1995; Thakur *et al.*, 1995). Toxicity will always remain a constraint on its use. Its mode of action is poorly understood, although it is known to damage the kinetoplast DNA-mitochondrial complex. It does not potentate the action of antimonials (Bryceson *et al.*, 1985) and no logical drug combinations have been suggested.

1.2.3.3 Amphotericin B

Amphotericin B desoxycholate, a polyene microlide antibiotic, is another second choice drug. It binds preferentially to 24-substituted sterols, e.g. the major cell membrane sterol, ergosterol, of *Leishmania spp*, but to a lesser extent to the human membrane sterol cholesterol (Steck, 1974). This latter activity underscores the drug's toxicity to humans (Olliaro and Bryceson, 1993; Guerin *et al.*, 2002). The drug is formulated as a colloidal suspension which is administered as a slow intravenous infusion of 2 mg kg^{-1} body weight on alternate days for 20-40 doses (Olliaro and Bryceson, 1993). However, in India, Mishra *et al.*, (1992) used lower doses (0.5 mg kg^{-1} body weight on alternate days) for a shorter duration (14 days) and achieved 100% cure with very little toxicity in patients suffering from VL (Olliaro and Bryceson, 1993; Guerin *et al.*, 2002).

Common side effects include anaphylaxis, thrombocytopenia, flushing, generalised pain, chills, fever, phlebitis, anaemia, convulsions, anorexia, decreased renal tubular

and glomerular function and hypokalaemia in about one third of the patients treated (Guerin *et al.*, 2002). Amphotericin B remains a promising drug because of its specific mode of action towards leishmanial cell membrane sterols. Recent research has orientated towards reducing its toxicity.

1.2.3.4 Miltefosine

Some oral formulations have been developed; miltefosine, has been licensed in India, but sitamaquine are in the clinical phases of development (Sherwood *et al.*, 1994). To date, little is known about its efficacy and toxicity or prospect of eventual availability. Miltefosine, being an analogue of lethicin, has hexadecylphosphocholine as its active ingredient. Its mode of action involves the interaction with cell signal transduction pathway by decreasing phospholipid and sterol biosynthesis involved in cell membrane formation. When administered for 3-6 weeks at 50-200 mg/d, success rate (approximately 98%) is independent of the patients reaction to conventional pentostam therapy. Side effects are negligible, but motion sickness has been reported. Miltefosine is promising as a drug because of its low cost and storage stability. Apart from these compounds, no novel compound is at an advanced stage in the pipeline.

1.2.3.5 New formulations of old drugs.

The usefulness of the traditional agents mentioned above is now being increased with the benefit of new formulations or dosage regimens for use in some settings. Moreover, other drug formulations with some useful efficacy have been discovered.

The new drug formulations currently under clinical evaluation are:

1.2.3.5.1 Aminosidine

Aminosidine (Paromomycin), is an antibiotic of the aminoglycoside family. It is not absorbed when administered orally but an injectionable formulation can be administered intramuscularly or by intravenous infusion or the agent can be applied topically as an ointment. Experiments using mouse peritoneal macrophages have shown that injectionable aminosidine is highly effective against VL and old world CL species and susceptibility varied according to the species of new world CL. Aminosidine and antimony have approximately equal potency against some antimony-susceptible strains of *L. aethiopica* (ED_{50}, 27 mg kg^{-1}) with the same dose of aminosidine fully active against antimony-resistant strains, but aminosidine was 6-fold and more than 600-fold more active than sodium stibogluconate against *L. mexicana and L. major,* respectively (Teklemarian *et al.*, 1994). Aminosidine in combination with sodium stibogluconate show potentiation in treating unresponsive visceral leishmaniasis in India and Kenya (Chunge *et al,* 1990; Thakur *et al,* 1992; Guerin *et al.,* 2002). Aminosidine (15%) prepared with 12% methylbenzthonium chloride (MBCl), when applied topically as an ointment, produced a 77% cure rate in a double-blind placebo-controlled cross-over trial (El-On *et al.,* 1992). Its potency in the treatment of CL has been hampered becauses MBCl causes severe inflammation in some patients (Neal *et al.*, 1994). Currently, clinical trials are underway with a preparation in which MBCl has been substituted with urea in Iran and Tunisia. Preliminary evaluation, however, suggests that new formulations may be needed to improve penetration into the lesion (Bryceson *et al.*, 1994).

1.2.3.5.2 Purine analogues

Leishmania, like other haemoflagellates, are unable to synthesize purines *de novo* and must salvage bases and nucleosides. Purine analogues, e.g. allopurinol, exploit the inability of *Leishmania* to synthesise purines. Allopurinol riboside, an analogue of inosine, when incorporated into leishmanial RNA instead of ATP, interferes with normal protein synthesis and kills the parasite (Marr, 1991; Berman, 1997). Allopurinol on its own is potent (Jha, 1983) but has a synergistic effect with sodium stibogluconate and has proved useful in treating five antimony-resistant cases of VL in Kenya (Chunge *et al,* 1985; Llanos-Cuentas *et al.*, 1997). The biology of the synergy is unknown as there has been no formal trial of combinations (Bryceson, 2001).

1.2.3.5.3 Lipid-associated Amphotericin B

The concept underlying lipid-associated amphotercin B is that the lipid component will find receptors on desired cells only, especially macrophages, and so the drug will be sequestered at the site of the intracellular infection - leaving more drug to interact with the parasite's ergosterol and less to react with the host cholesterol, thereby increasing the therapeutic index (Berman, 1997).

1.2.3.5.3.1 Ambisome

Ambisome (AmBisome, Vestar, San Dimas, CA, USA) is amphotericin B incorporated into liposomes made of phosphatidyl choline, cholesterol and disteroyl phosphatidylglycerol. It has considerable potential for leishmaniasis (Berman, 1997) and has been used successfully to treat VL patients unresponsive to standard drugs (Croft *et al.*, 1991; Guerin *et al.,* 2002) causing rapid clinical recovery (fall in splenic parasite density). The minimum dose necessary to provide 95% cure rate was 6

$mg.kg^{-1}$, 14 $mg.kg^{-1}$ and 21 $mg.kg^{-1}$ in India, Kenya and Brazil, respectively. Hashim *et al.*, (1995) reported of two Post Kala Azar Leishmaniasis (PKDL) patients successfully treated with Ambisome without toxic side-effects. The Ambisome was administered at 3 mg kg^{-1} body weight daily for one month intravenously in a 5% glucose solution as a slow drip.

1.2.3.5.3.2 Amphocil

Amphocil (Liposome Technology Inc, Menlo Park, CA, USA) is a colloidal suspension of Amphotericin B cholesterol sulphate. In hamsters infected with *L. donovani*, Amphocil was 4-15 times as effective as conventional Amphotericin B (Guerin *et al.,* 2002). Trials on VL patients have shown promising results. Amphocil was used to treat Kala Azar in Brazil at a dose of 2 mg kg^{-1} body weight daily for 7 days. Side effects included chills, fever, increased respiratory rate and acute febrile reaction in 80% of the patients during infusion. This was overcome by administration of an anti-inflammatory agent, diclofenac potassium, every day before each infusion of Amphocil. Because of these potentially dangerous adverse reactions, Amphocil is only recommended for adult use while Ambisome can be used for all ages (Dietze *et al*, 1995).

1.2.3.5.3.3 Amphotericin B lipid complex

Amphotericin B lipid complex (Bristol Meyers Squibb, Princetown, NJ, USA) have been tried in India (Sundar *et al.*, 1999) and Brazil (Berman *et al.*, 1992) for treatment of VL leishmaniasis. The Amphotericin B lipid complex contains the lipids dimirystoyl phosphatidylglycerol and dimirystoyl phosphatidyl choline.

The relative efficacy and toxicity of these three drug formulations have yet to be determined fully.

1.2.3.5.4 Immunotherapy

Immunotherapy has been used to improve antileishmanial drug action. One of the methods employed is the use of interferon gamma (IFN-γ) for VL. Its use is based on the finding that the cell mediated immune system fails to recognize leishmanial antigens as measured by T-cell blastogenesis and production of interleukin (IL-2) and INF-γ during the acute phase of the disease (Carvalho *et al.*, 1985). INF-γ enhances the intracellular killing of the *Leishmania* parasites (Badaro *et al.*, 1990) and reduces the dose of antimony required for inhibition or killing of *Leishmania* in mice (Murray *et al.*, 1988). In Brazil, a combination of sodium stibogluconate (20 mg/day for 10-28 days) and recombinant INF-γ (35-500 microgrammes) has been used to successfully treat 6 out of 8 patients with refractory VL (Chance, 1995). An earlier study in Brazil, reported a 46% success rate and another one in Indian Kala Azar patients produced a 69% cure rate (Sundar *et al*, 1994). Treatment with INF-γ is known to produce only minor side effects, which are usual for cytokine therapy, including fever and general flu-like symptoms, but these are easily alleviated by treatment with antipyretic drugs (Sundar *et al*, 1994).

1.3 The problem of drug resistance

The emergence of drug resistance in *Leishmania* parasites is a major obstacle to their control. The lack of response to pentavalent antimonials in VL has been a problem for many years and is increasing world-wide, occurring in 5-70% of the patients in some endemic areas (Croft and Yardley, 2002). Relapse after inadequate treatment with a

single drug selects resistant mutants which are recycled in anthroponotic foci with high rates of transmission (Ouellette and Papadopoulou, 1993). In Bihar, India, up to 65% of new parasites show primary unresponsiveness which is correlated with *in vitro* evidence of antimony resistance (Ouellette and Papadopoulou, 1993). Elsewhere in India and in Africa and Brazil, primary unresponsiveness is still rare (Ouellette and Papadopoulou, 1993). Use of the drugs by unqualified quacks and unrestricted availability has lead to misuse of the compounds. Moreover, sub-optimal doses, variations in drug quality from lot to lot, and low immunological incompetence of the host, have all contributed to treatment failure.

1.3.1 Possible mechanism of action of antimonials and resistance to them in Leishmania spp.

The mode of action of SbV drugs in *Leishmania* is poorly understood although correlation between susceptibility to SbV and inhibition of glycolytic enzymes and of fatty acid β-oxidation has been described (Berman *et al.*, 1988). Current evidence suggests that SbV is likely to be a prodrug and that antileishmanial activity is effected by its more toxic trivalent derivative (SbIII). Trivalent species, of Sb and the related metal arsenic, are sulphdryl-reactive agents and have the potential to interact with cysteine residues in proteins and low molecular weight thiols causing enzyme inhibition and disrupting redox balance. Some studies have indicated that Pentostam may be converted to its toxic form Sb(III) within the macrophage (Goodwin *et al.*, 1995).

More recently it has been shown that amastigotes of *L. donovani* are able to catalyse the reduction of SbV to SbIII directly (Shaked-Mishan *et al.*, 2001) and this raises the

possibility that amastigotes may contain an enzyme homologous to the As(V) reductase present in yeast and bacteria (Ji and Silver, 1992; Gladysheva *et al.*, 1994; Oden *et al.*, 1994). The activity appears to be absent from promastigotes of the species which would account for their relative insensitivity to SbV (but not to SbIII) drugs. One Pentostam-resistant line of *L. donovani* was found to be deficient in reductase activity suggesting that down-regulation of this activity may constitute one mechanism of resistance (Shaked-Mishan *et al.*, 2001).

In other lines, resistance has been shown to be associated with increased efflux of the drugs (Dey *et al.*, 1994). This efflux appears to be effected by anion pumps of the ABC family which are overexpressed in As- and Sb-resistant lines (Dey *et al.*, 1996; Mukhopadhyay *et al.*, 1996; Haimeur *et al.*, 2000). In general these pumps recognise compounds conjugated to GSH, and accordingly, antimonial-resistant lines are also found to have increased levels of GSH and TSH and raised levels of several enzymes involved in their synthesis (Haimeur *et al.*, 1999). By analogy with arsenite-resistant lines from other species (Lee *et al.*, 1989), it has been hypothesised that a glutathione S-transferase may be required to mediate efficient conjugation between the Sb and thiol species. Clearly a trypanothione-S-transferase could be involved.

1.4 The need for new drugs

With no real promising prospects of an effective vaccine in the near future, there is an urgent need to develop new and better drugs. No novel compound except amphotercin B, miltefosine, paromomycin and sitamaquine is in the pipeline. Despite the urgent need for new effective chemotherapy, research and development has been neglected because leishmaniasis is a disease that mainly affects the poor and so ranks as a low

priority in the private sector. Moreover, the public sector currently struggles to undertake the development of drugs and diagnostics in the absence of adequate funds and infrastructure. These problems put in context the search for new anti-parasitic agents. Anti-parasitic agents can be found by searching for novel biochemical targets that could provide the basis for rational drug development. One aspect of parasite biochemistry that has attained increasing attention in recent years is sulfur amino acid metabolism. Sulfur in its different oxidation states represents one of the most vital elements in biology. Information on its general relevance in *Leishmania* is unclear, as progress was for many years hampered by biochemical inaccessibility to many of the relevant enzymes. The recent application of molecular genetic 'tools' has triggered a rapid flow of information on long-standing physiological and biological questions in sulfur metabolism.

1.5 Cysteine Biosynthesis

1.5.1 *Functions of L-cysteine*

L-Cysteine is one of the twenty canonical amino acids in proteins and over 200 non-protein amino acids. In organisms, it acts as the central precursor of all organic molecules containing reduced sulfur, ranging from amino acids (methionine) to peptides (glutathione, phytochelatin), proteins, vitamins, cofactors S-adenosyl methionine (SAM) and hormones. The reactive sulfhydryl (CH_2**SH**) functional group of cysteine performs a variety of functions such as stabilising a protein's overall structure through disulfide bond formation, binding cofactors for enzymatic reactions, e.g. iron-sulfur clusters, direct involvement in catalysis, and tolerance to stress produced by reactive oxygen species and metals.

1.5.2 Cysteine biosynthetic pathways

Two pathways for cysteine biosynthesis in organisms have been documented. The sulfydrylation pathway catalysed by serine acetyltransferase (SAT) and cysteine synthase (CS) (Morzycka *et al.*, 1979) and the reverse trans-sulfuration pathway catalysed by cystathionine β-synthase (CBS) and cystathionine γ-lyase (CGL) (Morzycka *et al.*, 1979) (Figure 1.4).

1.5.3 Plants and bacteria

Plants and the Bacteria synthesize cysteine via the sulfydrylation pathway, but the soil-dwelling genus *Rhizobium leguminosarum* is thought to have an alternative pathway for cysteine biosynthesis which avoids the use of CS (Parker *et al.*, 2001). The filamentous bacteria (actinomycetaceae) use the reverse trans-sulfuration pathway (Nagasawa *et al.*, 1984) for cysteine biosynthesis.

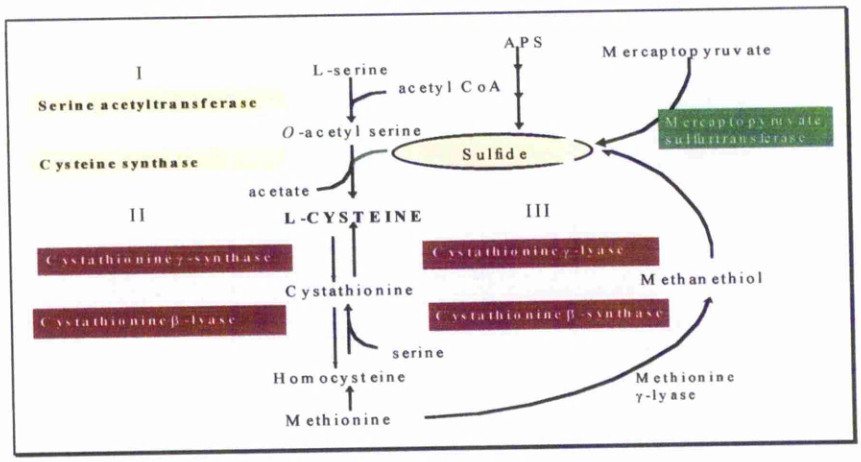


Figure 1.4 Overview of cysteine biosynthesis. The scheme shows the enzymes, serine acetyltransferase (yellow) and cysteine synthase (yellow) involved in the two-step *de novo* synthesis or sulfydrylation pathway (I) of cysteine from serine via the intermediate *O*-acetylserine (OAS). OAS condenses with sulfide from a variety of sources: the sulfate assimilatory pathway with (APS) being the initial substrate; methanethiol produced by the action of methionine γ- lyase on homocysteine; and the action of mercaptopyruvate sulfurtransferase on mercaptopyruvate. The trans-sulfuration pathway that inter-converts cysteine and methionine is also shown. Enzymes involved in this pathway are shaded dark red. The left panel (II) represents reaction of the forward trans-sulfuration pathway occurring in bacteria, plants and fungi; the right panel (III) shows the reverse trans-sulfuration pathway found in fungi and mammals

1.5.4 Fungi

Fungi can synthesize cysteine using the sulfydrylation pathway and the reverse trans-sulfuration pathway. *Saccharomyces lipolytica* utilizes both pathways for cysteine biosynthesis, but a cysteine auxotroph cell line with both pathways simultaneously impaired has been described (Morzycka *et al.*, 1979). The yeasts *Saccharomyces pombe* (Brzywczy *et al.*, 1993, 2002), *Pichia membranofaciens* and *Candida valida* (Piotrowska and Paszewski, 1990; Piotrowska, 1993) lack cystathionine β-synthase and cystathionine γ-lyase, indicating that these organisms must synthesize cysteine through sulfydrylation. In *Neurospora crassa* and *Aspergillus nidulans,* despite the existence of both pathways potentially able to synthesize cysteine, only the sulfydrylation pathway is physiologically operational, while in *Saccharomyces cerevisiae* the reverse trans-sulfuration pathway is the important one (Cherest *et al.*, 1992, 1993). However, when the operational pathway was impaired (e.g. through a mutation) (Paszewski *et al.*, 1974; 1984), the dormant pathway became operational.

1.5.5 Parasitic organisms: protozoa and nematodes

Information on cysteine biosynthesis in parasitic organisms is limited (Walker and Barrett, 1997). The amoebae, *Entamoeba histolytica* and *Entamoeba dispar* (Nozaki *et al*, 1998) and the trichomonad, *Trichomonas vaginalis* (Goodall, 2001) are like the plants and prokaryotes in having the sulfydrylation pathway for cysteine biosynthesis. The trypanosomatid *T. cruzi* mimics the fungi in having two independent pathways for cysteine production (Nozaki *et al.,* 2001). The nematode, *Panagrellus redivivus* (Papadopoulos *et al.*, 1996) and the fluke, *Fasciola hepatica* (Bankov *et al.*, 1996) are believed to have a functional reverse trans-sulfuration pathway. Although some genomes have been sequenced to date, the data analysis so far adds very little to our

understanding of cysteine biosynthesis. ORFs of cystathionine β-synthase from *Leishmania tarentolae* and *C. elegans* have been annotated in the NCBI database (http://www.ncbi.nlm.nih.gov), but little else.

1.6 Enzymes involved in cysteine biosynthesis

1.6.1 Serine acetyltransferase (SAT) (EC 2.3.1.30)

SAT catalyses the acetyl CoA-dependent acetylation of serine giving *O*-acetyl serine, which serves as the acceptor for sulfide in the biosynthesis of cysteine catalysed by cysteine synthase. Kinetic analysis of all purified SATs have shown that their k_m range from 0.1 mM to 5.1 mM for serine and from 0.1 mM to 0.56 mM for acetyl CoA. The pH optimum for SAT ranges from 7.5-8.5 (Kredich and Tomkins, 1966; Smith and Thompson, 1971; Burnell and Whatley, 1977). SAT-encoding genes are found in higher plants (Saito *et al.*, 1995), eubacteria (Kredich and Tomkins, 1966), protozoa (Nozaki *et al.*, 2001), fungi (Grynberg *et al.*, 2000), and algae (Toda *et al.*, 1998) but not in archeae or higher animals. In bacteria and protozoa, only one isoform appears to exist, but multiple isoforms exist in plants, localized in the cytoplasm or organelles (mitochondria and chloroplast) (Ruffet *et al.*, 1995).

The molecular mass of the monomeric unit of SATs ranges from 30-35 kDa. SAT consists of two independent domains that coincide with the α-helix and β-pleated sheet secondary structures of the protein that define the regions of protein contact. The α-helix cluster mediates the interaction of one SAT with another SAT molecule to form higher oligomeric forms of the protein (homotetramers). The β-pleated cluster interacts with CS to form a heterotetrameric bi-enzyme complex (Zhu *et al.*, 1998; Hindson *et al.*, 2000) that stabilises and activates SAT. SAT from *Escherichia*

coli (Hindson *et al.*, 2000) is a hexamer composed of a dimer of trimers joined at the SAT-SAT domain of the protein (Bogdanova and Hell, 1997).

In plants the formation of a bi-enzyme (SAT-CS) complex was primarily involved in the efficient production of cysteine by channelling the thiolation of *O*-acetyl serine with sulfide (Cook and Wedding, 1976; Wirtz *et al.*, 2001) while in bacteria, complex formation is predominantly involved in the activation of the inactive SAT enzyme. OAS once formed is released to the cytosol (Leu and Cook, 1994). Site-directed mutations at the interface of the SAT/CS domain produce an inactive SAT (Hell, 1997), which demonstrates, that protein-protein interaction of SAT with itself and CS was important for its activity and subsequently the synthesis of cysteine (Hell, 1977). Further, complex formation decreased CS activity to 5% while the activity of SAT remained unaltered (Mino *et al.*, 1999). The dissociation of the SAT/CS complex with 1mM *O*-acetyl serine (Mino *et al.*, 1999) was shown to restore CS initial activity. It is now known that complex formation increases the stability of SAT from cold inactivation and proteolysis attack by the proteases, α-chymotrypsin and V8 protease (Mino *et al.*, 2001).

SATs from bacteria, plants and the protozoon *T. cruzi* are feedback inhibited by cysteine (Kredich and Tomkins, 1966; Nozaki *et al.*, 2001). The feedback inhibition site for cysteine is located at the C-terminal end of the protein. This phenomenon has no effect on bi-enzyme complex formation with CS (Mino *et al.*, 1999), suggesting that the two events are independent of each other. *O*-acetyl serine and SAT are rate-limiting and also regulatory in the synthesis of cysteine *in vivo* (Harms *et al.*, 2000). When OAS is limiting, SAT and cysteine induce sulfur transport enzymes (Leu and

Cook, 1994) and co-activate the expression of proteins and enzymes involved in the assimilation of sulfur via the assimilatory sulfate reduction pathway (Kredich, 1971).

The over-expression of SAT in tobacco leads to a 2-fold increase in cysteine and glutathione in the cell (Takagi *et al.*, 1999) and a subsequent resistance to oxidative stress produced by 1 M H_2O_2 (Blaszczyk *et al.*, 1999). The feedback inhibition of SAT by cysteine was also significantly affected (Harms *et al.*, 2000).

1.6.2 Cysteine synthase (CS) (EC 4.2.99.8)

CS is a pyridoxal phosphate-dependent enzyme, found in bacteria, (*Salmonella typhimurium* (Becker *et al.*, 1969, Kredich *et al.*, 1969), *E. coli* (Flint *et al.*, 1996), *Rhodospirillum tenue* (Hensel and Truper, 1983), and *Paracoccus denitrificans* (Burnell and Whatley, 1977)), plants (*Spinacia oleracea* (Murakoshi *et al.*, 1985; Droux *et al.*,1992; Yamaguchi *et al.*, 1998), *Capsicum annuum* (Roemer *et al.*, 1992), *Citrullus vulgaris* (Ikegami *et al.*, 1988), *Pisum sativum* (Ikegami *et al.*, 1987), *Phaseolus* (Bertangnolli *et al.*, 1977), and *Datura inoxia* (Kuske *et al.*, 1994)), algae (*Chlamydomonas reinhardtii* (Leon *et al.*, 1987)), fungi (*Cephalosprium acremonium* (Dobeli and Nuesch, 1980)), and protozoa (*E. histolytica* (Nozaki *et al.*, 1998), *E. dispar* (Nozaki *et al.*, 1998), *T. cruzi* (Nozaki *et al.*, 2001) and *T. vaginalis* (Goodall, 2001)).

It catalyses the formation of cysteine and acetate from *O*-acetyl serine and sulfide via a bi-bi ping-pong reaction. Cysteine formation is a two-step reaction mechanism consisting of:

(a) a reversible interaction of *O*-acetyl serine and pyridoxal phosphate resulting in the β-elimination of acetate to form the α-aminoacrylate (Cook and Wedding, 1976). This is the overall rate-limiting step in the formation of cysteine from *O*-acetyl serine and sulfide (Woehl *et al.*, 1996) and it proceeds at a rate near its diffusion limit (Tai *et al.*, 1993).

(b) sulfide reacts with the α-aminoacrylate to form cysteine (Cook and Wedding, 1976). Kinetics analyses of all purified CSs have k_m ranging from 0.02 mM to 2.7 mM for sulfide and from 1.25 mM to 50 mM for *O*-acetyl serine. The dimeric CS enzymes from *S typhimurium* (cysM; Kredich *et al.*, 1969), *S. oleracea* and *P. haseolus* (Bertagnolli *et al.*, 1977) exhibit positive co-operativity e.g. *D. inoxia* with respect to *O*-acetyl serine and sulfide, respectively (Cook and Wedding, 1976; Kuske *et al.*, 1994) as both substrates compete for the same site in the second monomeric init of the dimeric CS molecule (Burkhard *et al.*, 1998; Tai and Cook, 2000).

All organisms with the *de novo* cysteine biosynthetic pathway, except apparently *T. cruzi* and *T. vaginalis*, have multiple isoforms of CS. The molecular mass of its monomeric unit ranges from 30 to 35 kDa. CS is usually a homodimer, except those from *D. inoxia* and *T. cruzi* where several higher oligomeric forms have been identified (Kuske *et al.*, 1994; Nozaki *et al.*, 2001). *T. cruzi* CS exists as a dimer, trimer and oligotetramer (Nozaki *et al.*, 2001).

Cysteine is the major source of organic sulfur within the cells of organisms. Cysteine in turn plays an important role in the assimilation of sulfide during sulfur starvation. When sulfur is limiting the assimilation of sulfides is aided by a up-regulation of CS

(Hell *et al.*, 1994; Barroso *et al.*, 1995; Nicholson *et al.*, 1995). Saturating amounts of cysteine, sulfide or thiosulfate in the growth mediun of bacteria repress CS (Hensel and Truper, 1976).

The catalytic capacity of plant CS enzyme reflects the multiple roles it plays within the organism (Youssefian *et al.*, 1993). Such roles involve: (a) the detoxification of gaseous pollutants (SO_2, H_2S and HCN) (Ikegami *et al.*, 1988). Tobacco plants transformed with the CS gene are resistant to toxic levels of H_2S (Youssefian *et al.*, 1993). Overexpression of the chloroplast-targeting CS had no effect on cysteine and glutathione biosynthesis (Saito *et al.*, 1994), but overexpression of the cytosolic form resulted in a 6-fold increase in cysteine and glutathione levels and the mutants were resistant to sulfite toxicity; (b) formation of selenocysteine (Burnell and Whatley, 1977); (c) formation of beta-substituted alanines (Murakoshi *et al.*, 1985; Ikegami *et al.*, 1987; 1988); (d) formation of *L*-quisqualic acid; (e) formation of *L*-mimosine (Ikegami *et al.*, 1987); and (f) CS from *Phaseolus*, *E. coli* and *T. cruzi* are also capable of catalysing the desulfuration of cysteine, though this reaction is 100-fold slower than cysteine biosynthetic reaction (Bertangnolli *et al.*, 1977; Flint *et al.*, 1996).

1.6.3 Cystathionine β-synthase (CBS) (4.2.1.22)

CBS catalyses the formation of cystathionine from serine and homocysteine. It is the first and rate-limiting step of the reverse trans-sulfuration pathway in mammals (Roper and Kraus, 1992). CBS has been purified from fungi (Ono *et al.*, 1994), protozoa (Nozaki *et al.*, 2001), nematodes (Walker and Barrett, 1991a, 1992;

Papodopoulos *et al.*, 1996), helminths (Walker and Barrett, 1991b) and mammals (Kraus *et al.*, 1978; Kery *et al.*, 1994).

The human CBS consists of a heme domain, a 'catalytic core' with a pyridoxal 5' phosphate (PGGSVKD) motif, and a regulatory domain composed of two 'CBS domains' (Bateman, 1977). CBS from yeasts and *T. cruzi* lack the heme motif (Jhee *et al.*, 2000; Maclean *et al.*, 2000, Nozaki *et al.*, 2001) but possess the CBS domains. Results are conflicting as to whether the CBS domain in yeast contain the AdoMet regulatory motif (Ono *et al.*, 1994; Jhee *et al.*, 2000).

Mammalian CBSs are heme-dependent enzymes. Heme is incorporated into the protein during folding and was previously thought to be essential for the activity of the enzyme as its incorporation was a prerequisite for pyridoxal phosphate binding (Kery *et al.*, 1994). Further, it had been suggested that the heme plays a catalytic role, by activating homocysteine (Taoka *et al.*, 1999), as well as regulating the redox potential within the cell (Taoka *et al.*, 1998). The heme-independent enzyme from yeast is thus believed to bind homocysteine in a different manner, although the mechanism is as yet unknown (Jhee *et al.*, 2000). The C-terminal CBS domain is the site of S-adenosylmethionine binding (Taoka *et al.*, 1999), an allosteric activator of the mammalian and possibly the fungal enzymes (Jhee *et al.*, 2000; Ono *et al.*, 1994). Binding of S-adenosylmethionine to the enzyme decreases the activity with homocysteine eight-fold (Roper and Kraus, 1992). The mammalian CBS is a homotetramer with subunit size ranging from 55 to 63 kDa. However, proteolysis of C-terminal CBS domains produced a more active dimer unresponsive to allosteric regulation with a subunit ranging from 39 to 48 kDa (Jhee *et al.*, 2000).

CBS has been extensively studied in humans because of their vital role in the detoxification of homocysteine in cells. Marked deficiency of CBS activity is the most common cause for homocystinuria, which causes dislocation of the optic lenses, vascular disorders, skeletal abnormalities and mental retardation. CBS and methionine synthase are the only two pathways known for human cells to decrease the concentration of the toxic metabolite homocysteine. Both enzymes are redox-sensitive, with methionine synthase activity being enhanced at lower cellular redox potentials, while CBS activity is increased under oxidizing conditions. Increased levels of CBS activity leads indirectly to an increase in the thiol, cysteine and the antioxidant glutathione (Taoka *et al.*, 1998) and a subsequent resistance to oxidative stress (Taoka *et al.*, 1998).

The activity and expression of CBS does not change in response to sulfur starvation in *Neurospora* (Flavin *et al.*, 1964) and *S. cerevisiae* (Ono *et al.*, 1994). Moreover, cysteine does not repress the level of activity in *A. nidulans* (Paszewski and Grabski, 1974). The enzymic activity of CBS has several metabolic outcomes, hence it is a multi-activity enzyme. CBS and CS seem to be evolutionary related, and still retain evolutionarily vestigial catalytic activities (Ono *et al.*, 1994). Thus CBS of *S. cerevisiae* and *N. crassa* both had CS activity (Ono *et al.*, 1994). In addition, the human (Kraus *et al.*, 1978), rat (Gomez-Bautista and Barrett, 1988), and *S. cerevisiae* (Ono *et al.*, 1994) CBS catalyse the formation of cysteine from serine and sulfide. Another metabolic outcome of the enzymatic activity of CBS is the desulfurization of cysteine; cysteine desulfurase (C-DES), serine sulfhydrase (SS), and activated serine sulfhydrase (ASS). These reactions involve the production of sulfide from cysteine

(Eto and Kimura, 2002; Gomez-Bautista and Barrett, 1988; Stipanuk and King, 1982). Most studies have been devoted to the pathway in which CBS catalyses the reaction with substrates homocysteine to produce cystathionine, but sulfide production by CBS has been studied in the nervous system of rats where it is believed to act as a neuromodulator (Eto and Kimura, 2002). A model illustrating how the CBS molecule performs CBS and C-DES activities has been proposed (Shan *et al.*, 2001). The CBS/SAM domain in the C-terminal region regulates the two reactions. During C-DES activity, the CBS/SAM domain bends over to cover the catalytic domain, impairing access to the active site of the enzyme thus suppressing enzymatic CBS activity. CBS activity is initiated by a conformational change which frees the catalytic domain (Shan *et al.*, 2001). This is supported by an increased CBS activity in a mutant protein without the CBS/SAM domain (Kery *et al.*, 1998).

1.7 Cysteine sources in protozoa

E. histolytica possesses the cysteine biosynthetic pathways and enzymes involved in the sulfur assimilatory pathway (Nozaki *et al.*, 1998a; 1998b). However, the medium used for *in vitro* cultivation of *E. histolytica* contains high levels of cysteine, and the organism is known to be dependent on this exogenous cysteine for growth and survival in culture (Gillin and Diamond, 1980; 1981). *T. brucei* bloodstream forms also depend on an extracellular source of *L*-cysteine, but not *L*-cystine or any reducing agents, and cysteine has been shown to be essential for growth of the parasite (Duszenko *et al.*, 1985, 1992). In *T. cruzi*, both pathways exist but it is not known whether they are dependent on an exogenous sulfur source. It is anticipated that trypanosomes require cysteine not only for protein biosynthesis, but also for glutathione and trypanthione biosynthesis, which are present in these parasites at high

concentrations (Fairlamb and Cerami, 1992). To satisfy their sulfur requirement to synthesize these thiols, multiple cysteine-acquiring pathways are likely to be vital to ensure that cysteine is always available in the cell. But this does not address the dependence of all of these parasites on an exogenous sulfur source in their medium to aid growth and survival. Possible explanations for this have been documented. (a) *E. coli*, like the protozoon *E. hisolytica,* is able to produce cysteine from sulfate but also salvage extracellular cysteine (Beilan *et al.*, 1983). The *in vivo* synthesis of cysteine within the bacteria is regulated by extracellular amino acid concentration (Beilan *et al.*, 1983). (b) The observed requirement of *Giardia* for extracellular *L*-cysteine in the culture system (Gillin and Diamond, 1980; 1981) suggests that *de novo* synthesized *L*-cysteine may not be efficiently utilised for the maintenance of the redox state of the surface thiol molecule (Gillin and Diamond, 1984); (c) *L*-cysteine biosynthesis may be biologically important in *Entamoeba* due to its reliance on iron-sulfur proteins (Weinbach *et al.*, 1980; Reeves *et al.*, 1980). It will be interesting to determine whether extracellular *L*-cysteine is indeed incorporated in the amoeba and used for the synthesis of iron-sulfur proteins or solely serves to maintain surface thiol molecules in the reduced state.

1.8 Origin of sulfur atom used in cysteine biosynthesis

Like carbon and nitrogen, sulfur is made available to organisms largely in the form of inorganic compounds, principally sulfate. Sulfur metabolism in organisms includes uptake of the macronutrient sulfate from the environment, assimilation into organic compounds, and channelling into proteins and secondary substances. Cysteine, methionine, and sulfur-containing vitamins such as biotin or thiamin are essential in the nutrition of animals and plants, and among the various sulfur-mediated functions

are electron transport in Fe/S-clusters, structural and regulatory roles via protein disulfide bridges, and catalytic centres (Hell, 1997). My research has focussed on the source of sulfide used in the thiolation of *O*-acetyserine to form cysteine by CS.

1.9 Pathways for sulfide production

Several pathways have been documented to be the source of sulfur and in these the sulfur atom is derived from sulfide. In mammalian tissues, H_2S is produced from *L*-cysteine through at least five pathways.

In the first pathway, *L*-cysteine is hydrolysed by CBS producing equimolar amounts of H_2S and *L*-serine. In the second pathway, two *L*-cysteine molecules dimerize to form cystine, which is transformed into thiocysteine, pyruvate and NH_3 by CGL. The thiocysteine can then undergo one of two reactions to form H_2S. CGL can catalyse the reaction of thiocysteine with other thiol compounds (e.g. glutathione or cysteine) to form H_2S and CysSR, or thiocysteine can form cysteine and H_2S, possibly non-enzymatically. In the third pathway, cysteine aminotransferase (EC 2.6.1.3) catalyses the reaction of *L*-cysteine with a ketoacid (e.g. α-ketoglutarate) to form 3-mercaptopyruvate and an amino acid (e.g. *L*-glutamate). The 3-mercaptopyruvate can then be desulfurated by 3-mercaptopyruvate sulfurtransferase to form H_2S and pyruvate, or, in the presence of SO_3^{2-}, thiosulfate and pyruvate. In the 'thiosulfate cycle', the thiosulfate reacts with reduced glutathione to produce H_2S. In the fourth reaction, cysteine lyase (EC 4.4.1.10) can convert *L*-cysteine and sulfite to *L*-cystine and H_2S. The cofactor pyridoxial-5'-phosphate is required by cysteine lyase. A fifth production pathway, termed the activated *L*-serine sulfhydrase reaction (Braunstein *et al.*, 1971), has been identified in some microfauna (Walker and Barrett, 1997), including some nematodes (Walker and Barrett, 1992), parasitic protozoa (Thong and Coombs, 1987), and cestodes (Gomez-Bautista and Barrertt, 1988). In this pathway, a

serine sulhydrase catalyses the reaction of L-cysteine with a second thiol compound (e.g. 2 mercaptoethanol) to form the respective cysteine thioester and H_2S. The serine sulfhydrase has yet to be fully characterized, but in at least some nematodes it appears to be a variant form of CBS (Walker *et al.*, 1992). Although low levels of activated *L*-serine sulfhydrase activity were initially reported for highly purified mammalian hepatic CBS (Braunstein *et a*l., 1971), subsequent studies have failed to detect it (see Thong and Coombs, 1987, Gomez-Bautsa and Barrett, 1988; Walker and Barrett, 1997).

1.10 Sulfur assimilation

The utilization of sulfate via sulphide assimilatory pathways requires metabolic activation to a form that can readily undergo reduction. The process for sulfate assimilation is largely confined to plants and bacteria, and the end product of reduction is hydrogen sulfide (H_2S). In plants, bacteria and the enteric protozoon *E. histolytica*, sulfate activation and reduction into sulfide is achieved via four enzymatic steps in which 3 high energy phosphate bonds are broken, and 4 NADPH are oxidised (Nozaki *et al.*, 1998). Extracellular sulfate is first imported into the cell by specific transporters. Sulfate reduction is mediated by four enzymes (Figure 1.5). First, sulfate is adenylated by ATP sulfurylase to form adenosine-5'-phosphosulfate (APS). 3'-phosphoadenosine 5'-phosphosulfate (PAPS) is later formed through an ATP-dependent phosphorylation of the 3'-hydroxyl group of APS, catalysed by APS kinase. PAPS is subsequently reduced to sulfite by PAPS reductase with thioredoxin serving as the electron donor. APS kinase in some organisms acts on APS to form sulfite as well. Sulfite is finally reduced to sulfide by sulfite reductase using NADPH as electron donor (Kredich, 1971). The combined action of the four enzymes takes sulfur from the +6 to the -2 oxidation

state. Assimilation to cysteine occurs when sulfide reacts with O-acetyl serine catalysed by by serine acetyltransferase. OAS is formed by SAT on serine and acetyl-CoA (Figure 1.4).

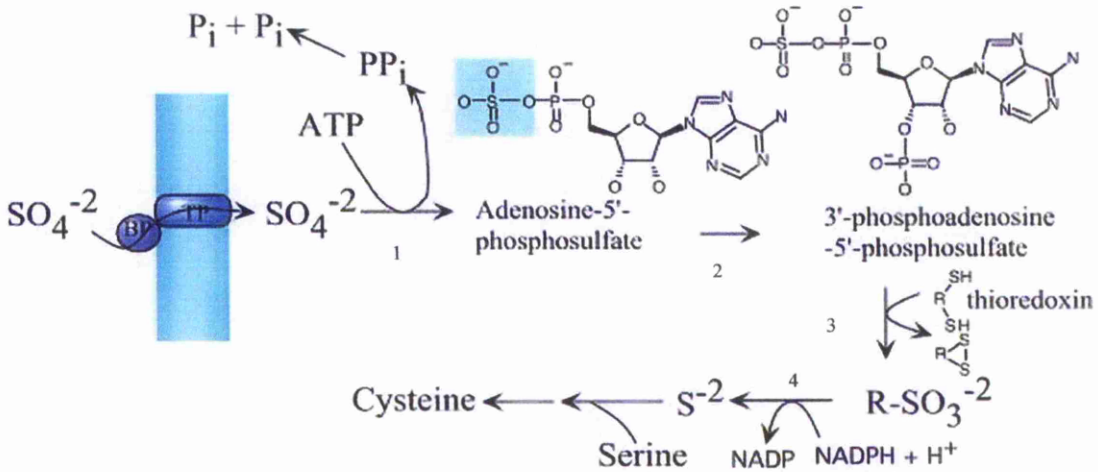


Figure 1.5 The sulfate assimilation pathway showing the transport and reduction of sulfate. Arrows indicate that the assimilation pathway in plants. Enzymes: 1, ATP sulfurylase (EC 2.7.7.4); 2, APS kinase (EC 2.7.1.25); 3, APS reductase (EC 1.8.4.9); 4, Sulfite reductase (EC 1.8.7.1). BP, binding protein, TP, transport protein, R, alkyl group. Source:- www.icp.ucl.ac.be

1.11 Methionine degradation

The methionine degradation pathway as a sulfide source for cysteine biosynthesis is hypothetical. It involves the enzyme methionine γ-lyase (MGL) that catalyses the α,γ-elimination and γ-replacement reactions of methionine and its analogues homocysteine and S-methylcysteine. MGL degrades methionine to α-ketobutyrate, ammonia and methanethiol (Tanaka *et al.*, 1985). Methanethiol is believed to form sulfide though several but unknown pathways. In *P. putida*, MGL is up-regulated (10-fold) when grown in a methionine-rich medium. The methanethiol produced, is

converted to methanesulfonate and subsequently to yield sulfite (Figure 1.6), which enters the sulfur assimilation pathway (Vermeij and Kertesz, 1999).

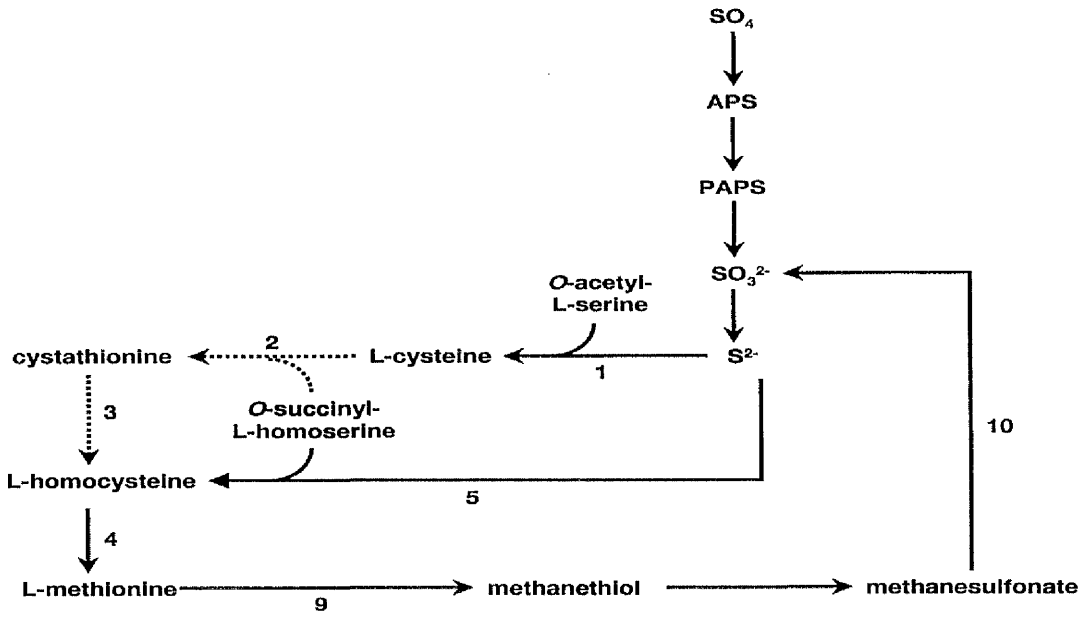


Figure 1.6 Proposed pathways for the biosynthesis of cysteine and methionine in *P. putida* S-313. The conversion of methanethiol to methanesulfonate is still hypothetical. Dotted arrows indicate that the trans-sulfuration pathway is not very active in *P. putida* S-313. Enzymes: 1, *O*-acetyl-L-serine sulfhydrylase; 2, cystathionine γ-synthase; 3, cystathionine β-lyase; 4, methionine synthase; 5, O-succinyl-L-homoserine sulfhydrylase; 9, methionine-lyase; 10, methanesulfonatase (SsuED/MsuED). APS, adenosine-5'-phosphosulfate; PAPS, 3'-phosphoadenosine-5'-phosphosulfate

MGL has also been characterized in the trichomonad *T. vaginalis*. MGL from this organism breaks down methionine to form methanethiol, ammonia, and α-ketobutyrate (Thong and Coombs, 1987; Lockwood and Coombs, 1991). Homocysteine is broken down to H_2S, ammonia and and α-ketobutyrate. The fate of the sulfide produced has not been investigated, but could be used by CS (Goodall, 2001).

1.12 The role of sulfur-transfering enzymes

In plants, C-DES activities cannot alone adequately account for the high concentration and apparent K_m (1000-fold higher, in the μM range) of free sulfide in cell free systems. This apparent discrepancy is yet to be resolved. Furthermore, the catalytic capacity of cysteine synthase exceeds sulfate assimilation needs by a factor of several hundreds casting doubts on the sulfur assimilation pathway as the sole source of sulfide in organisms, for the synthesis of cysteine. It has been speculated that C-DES or/and sulfurtransferase could transfer sulfide from the corresponding reductase to the cysteine synthase, changing the known possible substrates and catalytic properties (Schmidt and Jager, 1992). Enzymes catalysing the cysteine desulfurase reactions e.g. CS, CBS, *Nif*S PLP-dependent enzymes and sulfurtransferases, catalyse the removal of sulfide from cysteine (Lacourciere and Stadtman, 1998). *Nif*S (Zheng *et al.*, 1993) and their homologues have been identified in *E. coli* (Mihara *et al.*, 1997), *S. cerevisiae* (Kolman and Soll, 1993), and also *T. vaginalis* (Tachezy *et al.,* 2001). NifS has been shown to catalyse the formation of FeS clusters in apoenzymes such as nitrogenase to create a functional enzyme (Zheng *et al.*, 1993; 1994). Experiments have shown that the plant-type cysteine synthase will not accept sulfurtransferase as sulfur donor nor was formation of *S*-sulfo *L*-cysteine from OAS detected in algae and higher plants (Schmidt and Jager, 1992). However, coupling of thiosulfate with thiosulfate reductase and cysteine synthase readily demonstrated the formation of cysteine in a plant's cell-free system (see Schmidt and Jager, 1992).

1.13 Sulfurtransferases

Sulfurtransferases (EC 2.8.1.1 - EC 2.8.1.5) are widely distributed enzymes of prokaryotes and eukaryotes, with cytosolic and mitochondial isoenzymes being known (Koj *et al.*, 1975; Wrobel and Czubak, 2000; Nakamura *et al.*, 2000).

The sulfurtransferases thiosulfate sulfurtransferase or rhodanese (EC 2.8.1.1 (TST)) and mercaptopyruvate sulfurtransferase (EC 2.8.1.2 (MST)) are widespread and the most well characterized of the sulfurtransferases. MSTs display up to 66% sequence homology with rhodanese (Nagahara *et al.,* 1995), and groups of both enzymes catalyse the transfer of sulfane sulfur from a donor molecule, such as thiosulfate and mercaptopyruvate, to a nucleophilic acceptor substrate, such as cyanide and mercaptethanol (Sorbo, 1957; Westley, 1973, 1981).

1.14 Biophysical characterization of sulfurtransferases

1.14.1 Rhodanese is comprised of two similar domains

The crystal structures of bovine and *Azotobacter vinelandii* rhodaneses and *E. coli* SseA have been solved (Ploegman *et al.*, 1978; Hol *et al.*, 1983; Spallarossa *et al.*, 2003). The three proteins display very similar three-dimensional (3-D) conformation even though they have only limited identity (22%). The 3-D conformation shows that each protein is composed of two identically-folded domains (each approximately about 120 amino acids long, but with low sequence identity to one another) arranged in tandem (Figure 1.7). Each of the known structures of rhodanese domains displays a typical α/β topology made of five α-helix and four β-pleats, respectively (Figure 1.7).

In TSTs and MSTs, the domain at the C-terminal end of the enzyme is the 'catalytic active' domain and invariably houses the putative catalytic cys residue, whereas the N-terminal end is the 'inactive' domain, in which the cys residue is often replaced by asp or gly. The asp residue forms multiple hydrogen bonds with the main side chain amide groups of the active site loop (Pleogman *et al.*, 1978, 1979; Bordo *et al.*, 2000),

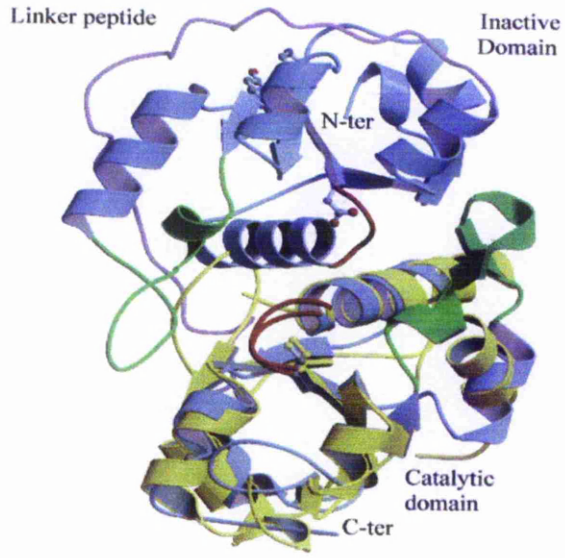


Figure 1.7 Three-dimensional structure of *A. vinelandii* RhdA and of human Cdc25A phosphatase, superimposed on their respective catalytic domains. RhdA and Cdc25A are shown in blue and yellow, respectively. Active-site loops and the structurally equivalent loop on the RhdA N-terminal domain are shown in red. The catalytic cys and the asp counterpart on the inactive domain are represented in ball-and-stick. The linker polypeptide connecting the two RhdA domains is shown in pink. The C–C loops involved in RhdA in interdomain interaction (Bordo *et al.*,2000) are shown in green. Source Bordo and Bork, 2002.

which makes it unavailable for substrate or ion binding. The recent structural characterization of GlpE, a TST from *E. coli* composed of just a single domain (Ray *et al.*, 2001; Spallarossa *et al.*, 2001), indicates that the catalytically inactive N-terminal domain found in TSTs and MSTs is not essential for catalysis.

1.14.2 Rhodanese domains form two distinct subfamilies

Phylogenetic analysis using neighbour-joining methods shows that the N- and C-terminal domains of sulfurtransferases comprise two distinct subfamilies (Bordo and Bork, 2002). Each subfamily also contains the previously characterized MST sequences of *Rattus norvegicus*, *Arabidopsis thaliana* and *E. coli* (Nagahara *et al.*, 1995; 1996; Papenbrock and Schmidt, 2000; Colnaghi *et al.*, 2001). As each of the clusters of the N- and C-terminal domains of TST/MST subfamilies contain eukaryotic, bacterial and archael sequences, it seems that the gene duplication event that is presumed to have given rise to the tandem architecture found in MSTs and TSTs must have occurred very early during evolution - probably before the diversification of living organisms into three major types.

1.14.3 Rhodanese homology domains also occur in Cdc25 and other proteins

Completion of the sequencing of various genomes has shown that multiple ORFs coding for rhodanese-like proteins exist in the same genome, for example the GlpE, PspE, (Adams *et al.*, 2002) and YgaP of *E. coli* K12 genome (Blattner *et al.*, 1997; Tatusov *et al.*, 2000). These observations indicate that rhodanese domains are likely to be involved in several distinct biological functions (Bordo and Bork, 2002).

The structural characterization of the catalytic domain of the human Cdc25 phosphatases, Cdc25A and Cdc25B, involved in cell cycle control revealed that they also have the active site cys residue at the same location as rhodanese and also display the rhodanese-like 3-D fold (Fauman *et al.*, 1998). This suggests that the two enzymes families may have stemmed from a common ancestor (Hofmann *et al.*, 1998). The most relevant structural difference between rhodanese and Cdc25 enzymes is a wider catalytic pocket for Cdc25 to accommodate the larger

phosphorous atom. Sulfur is a smaller atom hence the narrow catalytic pocket for rhodanese.

The rhodanese domain also occurs as a single domain protein in the senescence-associated protein *SenI* from *Arabidopsis thaliana* (Oh *et al.*, 1996), the sulfide dehydrogenase (*Sud*) from *Wolinella succinogenes* (Bordo and Bork, 2002), and *Ntdin* from *Nicotiana tabacum* (Table 1.3).

Further, the 'catalytic' or 'inactive' rhodanese domains are also found associated with other protein domains. Associated in tandem repeat with the catalytic C-terminal domain are the SseA and YnjE of *E. coli* (Ray *et al.*, 2000), and also mitogen-activated protein kinase (MAPK)-phosphatases (Keyse *et al.*, 1993; Fauman *et al.*, 1998; Hofmann *et al.*, 1998). The dual phosphatases and ubiquitin hydrolase proteins are found associated with the inactive domain proteins of the sulfurtransferases (Table 1.3).

1.14.4 Characterization of the active site in the catalytic domain

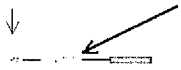
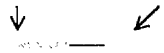
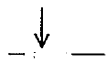
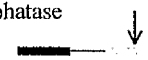
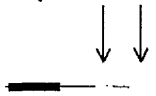
The active-site amino acid sequences differ between TSTs and MSTs. They have the motifs CRXGX[R/T] and CG[S/T]GVT, respectively. The active-site loop of known TSTs always contains two basic residues (i.e. if the last loop residue is Thr, a basic residue is observed at one of the X positions), whereas no charged residues are observed in biochemically characterized MSTs. This difference may relate to the distinct ionic charges of the respective *in vitro* substrates, thiosulfate (2-) and 3-mercaptopyruvate (1-). These active site motifs of the sulfurtransferase family are found the C-terminal 'catalytic' domain. The **CGT**GVTA, and **CGS**GVTA motifs

are found in plants and animal sulfurtransferases (which are specific for mercaptopyruvate) while the **CRK**GVTA motif is present in animal rhodaneses (which are specific for thiosulfate).

The residues arginine (R) and lysine (K) of the vertebrate TST and their corresponding glycine (G) and threonine (T) of *A. vinelandii* in the sulfurtransferase family domain determine the sulfur donor selectivity and recognition in their respective organisms. These catalytic residues fold in a cradle-like structure near the interface between the two domains defining the enzyme catalytic pocket (Westley, 1973; Pleogman *et al.*, 1978; 1979; Hol *et al.*, 1983; Kohanski and Heinrikson, 1990; Pallini *et al.*, 1991; Weiland and Dooley, 1991; Miller-Martini *et al.*,1994).

Sulfurtransferases catalyse the transfer of sulfane sulfur from a sulfur donor to a nucleophilic acceptor by the double replacement reaction. In the double replacement reaction, the enzyme acts a sulfur carrier. The thiolate anion of the cys residue and the positive charges of arg and lys of the enzyme bind thiosulfate (Luo and Horowitz, 1994), polarising the scissile S-S bond of the substrate, and thereby catalyse the transfer of the sulfur atom to nucleophilic acceptors such as cyanide (Westley, 1973; Luo and Horowitz, 1994) to yield sulfite and thiocyanate.

Table 1.3 Co-occurrence of rhodanese in association with other protein domain. Domains are schematised as coloured boxes. Catalytic rhodanese modules with six amino acid active site loop are shown in red, and inactive rhodanese modules are displayed in black. Rhodanese modules with a seven amino-acid active-site loop are displayed in yellow. Other protein domains are shown in grey. Active-site loop positions displaying 80% amino acids conservation within each subfamily are shown as conserved motif.

Modules	Active site loop motif	Species distribution	Observed instances
Thiosulfate:cyanide sulfurtransferase (TST)	**CRXGX[R/T]**	**Eukaryota Bacteria Archaea**	**25**
3-mercaptopyruvate sulfurtransferases	**CG[S/T]GVT**	**Eukaryota Bacteria**	**24**
ThiF	**C[R/K]XGX[R/D]**	**Eukaryota Bacteria**	**19**
THUMPdomain ThiI 	**CX(M)GX(M)**	**Bacteria Archaea**	**11**
Cdc25 regulatory phosphatases domain 	**CE[Y/F]SXXR**	**Eukaryota**	**39**
YceA	**CXGGXR**	**Eukaryota Bacteria**	**8**
Arsenate resistance Acr2	**CXXSXXR**	**Eukaryota (yeast)**	**3**
HTH ArsR motif 	**CRGXYCX**	**Bacteria**	**4**
Dual-specific phosphatase 	**EXXXXX**	**Eukaryota Bacteria**	**48**
Ubiquitin C-ter hydrolase 2 	**TXXXXX**	**Eukaryota**	**8**

1.14.5 The C-terminal domain and retention of the protein in mitochondria

A characteristic feature of the 'catalytic' domain of the mitochondrial rhodanese from bovine liver and hamster ovary is a repetitive signal sequence $(Gly\text{-}Lys\text{-}Xx)_2$ for retention of the enzyme in the mitochondrial matrix (Miller *et al.*, 1991; Trevino *et al.*, 1995). The mature enzyme lacks the last three amino acids (Gly-Lys-Ala) at the C-terminus (Waltner *et al.*, 1996). How and when these are cleaved from the protein is not known. The role of the tripeptide has been looked at by deleting, replacing, or extending the C-terminal end of mammalian rhodanese and analysing the effect of this in relation to catalytic function, structural stabilization (Trevino *et al.*, 1995), and protein folding. In the chinese hamster ovary rhodanese, replacing the cleavable tripeptide with 13-28 residues of unrelated sequence produced an enzyme with similar activity to the wild-type enzyme, whereas addition of a single codon for serine, but not arginine, resulted in a loss of enzymatic activity (Kramer *et al.*, 2001). It is thought that the added serine may have formed bonds with other parts of the molecule. This is thought to have had an unfavorable effect on the conformation of the C-terminal part of the protein, which has been shown to be loose and not closely associated with the otherwise compact C-terminal domain of the molecule (Ploegman *et al.*, 1978). Interestingly, mammalian rhodanese with its C-terminal extended with 23 –106 unrelated amino acids can fold into an enzymatically active conformation either when while bound to bacterial ribosomes (Kudlicki *et al.*, 1995), while the wild type protein and one with a 13 amino acid extension were only active after termination and release from the ribosomes (Kudlicki *et al.*, 1995).

1.14.6 The N-terminal domain and mitochondrial import

The mitochondrial rhodanese from bovine and rat rhodanese are thought to have a mitochondrial targeting sequence consisting of 23 amino acid residues. These amino acid sequences contain a hydrophobic α-helix (residues Ser 11 to Gly 22) (Ploegman *et al.*, 1978; Hol *et al.*, 1983; Gliubich *et al.*, 1996) critical for transport across the mitochondrial membranes (Hammen *et al.*, 1994; Waltner *et al.*, 1996). Site-directed mutagenesis (Luo and Horowitz, 1993) and systematic shortening of the α-helix (Waltner *et al.,* 1996) prevented mitochondrial import as well as decreasing the stability of the enzyme - as shown by urea denaturation, proteolytic degradation *in vitro* (Luo and Horowitz, 1993; Trevino *et al.*, 1998), and decreased chaperone-assisted folding (Trevino *et al.*, 1998; 1999). The loss of interaction near the N-terminus produced by deletion of residues of the α-helix does not distort the folded native structure but does facilitate the transition in solution to a molten globule state – which, among other things, can interact with molecular chaperones at a very slow rate.

Arabidopsis thaliana sulfurtransferase has a longer N-terminal domain than the sequences from other species. The function of this domain is yet to be characterized, but the N-terminal domain expressed by itself is known to increase the sulfurtransferase activity of a separately prepared C-terminal domain (Burow *et al.*, 2002).

1.14.7 The interdomain region

The two globular domains of sulfurtransferase are connected by a short linker sequence (Bordo and Bork, 2002). In plant MSTs, this linker sequence is much longer

than in the proteins from other species and shows strong homologies to several stress and senescence-induced proteins isolated from *Raphamis sativus* and *Arabidopsis* (Papenbrock and Schmidt, 2000). A mutant lacking this domain is *Arabidopsis thaliana* has been produced. The activity of a sulfurtransferase derivative with a shortened linker sequence was reduced by more than 60% of the wild-type activity, probably because the protein's stability was drastically reduced (Burow *et al.*, 2002). Reduced protein stability had been reported previously when either of two amino acid residues (val or asp) were added to the 16 amino acid tether residues (Luo *et al.*, 1995). These results suggest that mutation in the interdomain tether can have drastic effects on the stability and conformational transitions of rhodanese.

1.15 Physiological functions of sulfurtransferases

The physiological functions of the sulfurtransferases family of proteins *in vivo* is uncertain, but has received considerable attention – especially the rhodanese of mammals (Dooley *et al.*, 1995; Aita, *et al.*, 1997; Nandi *et al.*, 2000; Wrobel and Czubak, 2000).

1.15.1 Cyanide detoxification

The first elucidated role of mitochondrial bovine liver rhodanese was the detoxification of cyanide to form thiocyanate, which is harmless and excreted by the kidney (Sörbo, 1955). This role could be important especially in the epithelial cells lining the gut (Picton *et al.*, 2002), but is thought not to account for the distribution of sulfurtransferases so widely and in different cell types (Aminlari *et al.*, 2000; Al-Qarawi *et al.*, 2001).

1.15.2 Iron-sulfur centre formation

Proteins containing iron-sulfur cluster prosthetic groups play a large number of roles in biology e.g. electron transport (Spiro, 1982), catalysis (Flint *et al.*, 1993), stabilization of protein structure (Grandoni *et al.*, 1989; Kuo *et al.*, 1992), metabolic regulation (Bernlohr and Switzer, 1981; Rouault *et al.*, 1991) and the formation of radicals (Frey and Reid, 1993). In spite of the importance of these roles, little is known about how iron-sulfur clusters are made *in vivo* and the role, if any, sulfurtransferases might play. However, it has been shown that some sulfurtransferases provide sulfane sulfur for the formation and regulation of the iron-sulphur centres of proteins, notably respiratory proteins (Bonomi *et al.*, 1977; Pagani *et al.*, 1987; Ogata and Volini, 1990). Sulphane sulphur is the precursor of the sulphur that becomes incorporated into Fe-S proteins and it has long been known that sulfurtransferases such as rhodanese could potentially be the sulphane carriers (Bonomi *et al.*, 1977; Pagani *et al.*, 1983). Interestingly, NifS and NifU and their homologues (IscS, IscU in bacteria, Nfs1p and Isu in yeast, and C-DES in *Synechocystis*), which have become the prototypes of the sulphane sulphur carrier protein implicated in Fe-S cluster synthesis (Kato *et al.*, 2000; Yuvaniyama *et al.*, 2000), have some sequence homology to the sulfurtransferases. Related studies have shown that sulfur transferred by rhodanese activates the electron transport protein succinate dehydrogenase (Bonomi *et al.*, 1976, 1977, 1982), and NADPH dehydrogenase (Pagani *et al.*, 1983), and can be used in the synthesis of biotin, thiamine and lipoate via biotin synthase, thiamine synthase and lipoate synthase, respectively (Tse Sum Bui *et al.*, 1999; 2000). It can also be used by other enzymes of oxidative metabolism e.g. xanthine oxidase (Nishino *et al.*, 1983) and NADH nitrate reductase (Nishino *et al.*, 1983). All of these effects probably arise through the

interaction of rhodanese with iron-sulfur centres via their ability to mobilize sulfur from cysteine and thiosulfate (Tse Sum Bui *et al.*, 2000).

1.15.3 Rhodanese acts an anti-oxidant

Sulfurtransferases may also play a part in the management of the cytotoxicity of reactive oxygen species in aerobic tissues (Nandi *et al.*, 2000). Although bovine rhodanese is mostly known for its ability to catalyse the formation of thiocyanate, it has been shown to have a thousand-fold higher affinity for the reduced form of thioredoxin than for cyanide. Thus it has been postulated that it may function in peroxide detoxification (Nandi *et al.*, 2000). The oxidation of reduced thioredoxin by sulfurtransferases could play other parts in controlling the levels of this key regulatory molecule (Ray *et al.*, 2000).

1.15.4 Providing sulphide for cysteine biosynthesis

A role for sulfurtransferases in assimilatory sulfate reduction by transferring a molecule of sulfide to *O*-acetyl serine (OAS) in the synthesis of cysteine has been postulated (Schmidt and Jager, 1992).

1.15.5 Inhibition and inactivation of rhodanese

Inhibition studies of enzymes can be exploited in various ways, including chemotherapeutic purposes. Unfortunately, no specific inhibitor has been identified for sulfurtransferases. Sulfurtransferases are known to be inhibited by compounds that can oxidize the sulfydryl group of the active site cys (otherwise used for the formation of a persulfide group with sulfur from the donor molecule) to a sulfenyl group (-S-OH) (Weng *et al.*, 1978; Cannella and Berni, 1983; Cannella *et al.*, 1986; Miller-

Martini *et al.*, 1994). Saturating amounts of molecules such as thiosulfate can react with the persulfide group and so maintain the enzyme in the stable sulfur-containing form, which then is resistant to inactivation by oxidizing agents (Horowitz and Criscimagna, 1982).

1.15.6 Rhodanese as a model of protein folding kinetics

Mammalian rhodanese has been studied extensively as a model for protein folding (Bhattacharyya and Horowitz, 2002). However, refolding of rhodanese is relatively difficult because of the presence of multiple disulphide-linked, misfolded states and the ability of folding intermediates to form aggregates (Panda and Horowitz, 2000). Thus rhodanese cannot be refolding unassisted, either a chaperone or a detergent is required for success (Mendoza *et al.*, 1991; Panda and Horowitz, 2000; Krause *et al.*, 2002). The refolding kinetics of rhodanese follows the molten globular hypothesis (Horowitz and Criscimagna, 1990; Tandon and Horowitz, 1990). During enzyme refolding, rhodanese undergoes fast collaspe to an intermediate state (A) which partitions to at least two states (A' and A"). A" is able to refold to the native enzyme but A' is in equilibrium with A and forms aggregates. Biophysical analysis of the nature of the intermediates shows an extensive exposure of hydrophobic surfaces. The intermediates can be stabilised against the irreversible aggregation by glycerol, temperature, (Gorovits and Horowitz, 1998) detergents (Mendoza *et al.*, 1991; Krause *et al.*, 2002) and chaperones (Panda and Horowitz, 2000). Dissociation of the two structural domains of rhodanese occurs during unfolding.

1.16 *Aims of this study*

The overall aim of this study was is divided into 3 manageable sections:

Section 1: Mercaptopyruvate sulfurtransferase

(a) To clone, express and characterize mercaptopyruvate sulfurtransferase from *L. major* (LmajMST) and *L. mexicana* (LmexMST).

(b) To carry out truncation studies to elucidate the role of an extended C-terminal domain of some 70 amino acids.

(c) To investigate the role of this enzyme in relation to the parasite's resistance to oxidative stress.

Section 2: Cysteine biosynthetic pathway

(a) To identify enzymes involved in cysteine biosynthetic pathway(s) from the gene content of *L. major*

(b) To clone, express and characterize selected enzymes involved in the pathway.

(c) To investigate the role of the enzymes in relation to the parasite' resistance to oxidative stress.

(d) to investigate the role of mercaptopyruvate sulfurtransferase in providing of sulfide for cysteine biosynthesis.

Section 3: The requirement for exogenous cysteine

(a) Use feeding experiments and growth studies to investigate the need of an exogenous cysteine source for the growth of *L. major in vitro* and the importance of cysteine biosynthesis by the parasite.

Chapter 2

2.0 MATERIAL AND METHODS

2.1 Molecular Biology methods

2.1.1 Primer design

DNA oligonucleotides were synthesised by MWG-biotech (Germany). Primers used in this study were designed to the N- and C-termini of the DNA fragment or gene to be amplified. Care was taken to make sure such restriction enzyme sites were not present within the DNA fragment of interest. Details of the primers are provided in the appropriate chapters.

2.1.2 Polymerase chain reaction (PCR)

PCR was used in the amplification of DNA fragments for subsequent cloning and analysis. Amplification was performed with 2 U of *Pwo/Taq* polymerase (Roche) in a 100 µl reaction containing 5-10 ng DNA as template, 5% (v/v) DMSO, 10 ng primers, 1% 10x expand high fidelity buffer (Roche) in a Perkin-Elmer DNA thermal cycler. The *Taq* provided an adenine overhang residue to the amplified product, which allows direct cloning into T-vectors such as pGEM-T (Promega). Amplification conditions varied and are described where appropriate.

2.1.3 DNA gel electrophoresis

Typical gels containing 0.7% agarose in TBE buffer (45 mM Tris-borate and 1 mM EDTA, pH 8.0) were used for analysis of DNA fragments. Samples were mixed with 10x DNA loading buffer (431 mM Tris-HCl, pH 6.8, 70% (v/v) glycerol, 13.8 % (w/v) SDS, 0.5% (v/v) bromophenol blue, 500 mM DTT) to a final concentration of 1x loading buffer and were electrophoresed at 50-140 V until the dye in the loading

buffer had migrated approximately two thirds of the length of the gel. Generally, ethidium bromide was mixed with the molten gel prior to setting at a final concentration of approximately 0.3 μg/ml. Gels were viewed under UV illumination and images obtained using an Imager™ gel documentation system (Appligene).

2.1.4 DNA ligation

Ligation of DNA fragments with commercially available T-vectors was carried out according to manufacturer's protocols. Ligation into other vectors was carried out as follows: Plasmid and DNA insert were mixed at a ratio 1:3 in a 10 μl reaction containing 1x T4 ligase buffer and 200 U of T4 DNA Ligase (New England Biolabs). Reactions were incubated overnight at 16°C.

2.1.5 Restriction digest

Restriction enzymes were obtained from New England Biolabs and Promega and used according to manufacturer's instructions. A typical plasmid digest was set up as follows: 0.5-1.0 μg of plasmid DNA was digested in a 20 μl reaction containing appropriate restriction enzyme and buffer for 1-3 hours at 37°C or appropriate temperature as specified by the manufacturers. Reactions were halted by the addition of a one tenth volume of DNA loading buffer at 1x (v/v) final concentration and followed by electrophoresis. Occasionally, reactions would be extracted with phenol-chloroform and/or ethanol precipitated to separate restriction enzymes from the digested DNA fragments (see Sambrook *et al.*, 1989).

2.1.6 Phosphatase treatment of digested plasmid

Where plasmid was digested with a single or double restriction enzyme(s) prior to ligation, it was treated with calf intestinal alkaline phosphatase (CIAP) to remove the 5' terminal phosphate. This minimized the chances of plasmid re-ligation. To the 2 μg of plasmid digested (20 μl reaction), 2 U of CIAP (NEB) was added and incubated at 37°C for 15 min.

2.1.7 Purification of DNA from agarose gels

Where purification of a DNA fragment was required, the agarose gel was prepared with TBE buffer (45 mM Tris-borate and 1 mM EDTA, pH 8.0). Following electrophoresis, the gel was visualized under low intensity UV light to minimize the risk of mutagenesis and the band of interest cut from the gel using a sterile scalpel blade. The Qiaquick Gel Extraction Kit (Qiagen) was used to extract the DNA from the gel slice.

2.1.8 Bacterial strains and transformation of competent bacteria

Several strains of bacteria were used throughout this study and these are described in the relevant chapters. Commercially available DH5α library efficient competent cells (Life Technologies), and BL21-RP (DE3) codon plus (Strategene) were transformed by heat shock according to the manufacture's protocols. Transformed bacterial cells were plated onto LB agar (LB medium, 1.5% (w/v) Bacto-agar (Oxoid), pH 7.0) plates with appropriate antibiotics or selection pressure.

2.1.9 Selection of colonies

PCR products were cloned into the pGEM-T vector utilizing the overhanging adenine left at the 3' end of each strand of DNA amplified by *Taq* polymerase. The insert disrupts the plasmid lacZ gene allowing positive colonies to be screened by blue/white selection. The pET21a$^+$ vector has the β-lactamase ampicillin resistance gene as a selective marker. The production of β-lactamase results in ampicillin resistance colonies being produced. White colonies were grown overnight in LB containing appropriate antibiotics at 37°C and subsequently checked for recombinant plasmids.

2.1.10 Bacteria culture methods

Five to ten millilitres of Luria-Bertania medium (LB - 1% (w/v) Bacto-tryptone (BBL), 0.5% (w/v) Bacto-yeast extract (Oxoid), 170 mM NaCl, pH 7.0) containing appropriate antibiotics were inoculated using sterile techniques. Cultures were grown in a rotatory incubator (250 rpm). Growth times and temperature were dependent on requirements. Stationary phase cultures of both small scale and large-scale plasmid preparation were grown overnight, while growth times of cells for overexpression of exogenous protein were chosen as appropriate (see relevant results chapters).

2.1.11 Plasmid purification

Several different kits were used to purify plasmids from the overnight bacterial cultures, depending on requirements. If plasmids were to be sequenced or used for sub-cloning, the Qiagen Tip-20 miniprep was used according to manufacture's instructions (high quality DNA is generally obtained with this kit). If large quantities of DNA were required, as in the construction of the knockout cassette and re-

expression vector, the Qiagen Midi-prep was used. Plasmid quantity and yield were assessed by spectrophotometry. An absorbance reading of 1 at a wavelength of 260 nm is equivalent to 40 μg/ml of double stranded DNA (Sambrook *et al.*, 1989).

2.1.12 Plasmid verification

Correct ligation of a DNA fragment of interest in a plasmid was verified by either carrying out a diagnostic double digest with appropriate restriction enzymes or by sequencing of the plasmid using appropriate oligonucleotide primers. Samples were analysed by the University of Glasgow Molecular Biology Support Unit (MBSU) or by MWG Biotech.

2.1.13 Long-term storage of bacteria (glycerol stocks)

Cells were plated on LB agar plates with appropriate antibiotics and incubated overnight at 37°C. A single colony was used to inoculate 5 ml of LB supplemented with appropriate antibiotics and cells were grown overnight at 37°C. Five hundred microlitres of the overnight culture was mixed with an equal volume of 40% (v/v) glycerol and cells were stored at -70°C.

2.2 **Leishmania** *species culture methods*

2.2.1 Cell lines

Wild type *L. mexicana* (MNYC/BZ/62/M379) and *L. major* (MHOM/IL/80/Friedlin) cell lines were used throughout this research. All newly produced cell lines were derived from these parent lines.

2.2.2 Cultivation of L. major *in semi-solid medium*

HOMEM plates (MEM salts, 10 ml/L 50x MEM amino acids, 10 ml/L 50 x MEM non essential amino acids, 5 mM glucose, 3.6 mM sodium bicarbonate, 1% sodium pyruvate, 8 μM p-aminobenzoic acid, 0.4 μM biotin, 20 mM HEPES, pH 7.0) were made by a 1:1 dilution of 2x HOMEM with 2% (w/v) bactoagar (Oxoid) containing appropriate antibiotics (J Mottram per. Comm). Plates were allowed to dry in a sterile air-flow for 15 min and then pre-warmed to 25°C for 30-60 min before transfected cells were spread over the solid medium. Liquid was allowed to absorb into the plate for 5 min before plates were sealed with parafilm and incubated at 25°C.

2.2.3 Cultivation of L. major *and* L. mexicana *in liquid medium*

2.2.3.1 Promastigotes

Promastigotes of *L. major* and *L. mexicana* were normally grown at 27°C in HOMEM medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Brooks *et al.,* 2001). *L. major* that had undergone electroporation or grown as colonies on semi-solid medium were first seeded (using a sterile, disposable Pasteur pipette) into 35-mm diameter wells of a 96-well culture plate and cultivated overnight in M199 (Life Technologies) with 20% (v/v) FCS without any selection drug at 27°C. They were subsequently transferred to 100 μl of M199 and 2 x drug added. Cells were grown until densely packed, and later transferred to a 1 ml culture, then to a 5 ml culture and grown at 25°C. For maintenance of parent and transgenic lines, late log or early stationary phase cells (approximately 1-2 x 10^7 cells/ml) were inoculated into fresh HOMEM medium at approximately 1 x 10^6 cells/ml with appropriate antibiotics added if required.

2.2.3.2 Metacyclics

Metacyclic promastigotes of *L. major* were purified from a stationary phase population of cells using the agglutination assay described by Sacks *et al.*, (1985).

2.2.3.3 Amastigotes

Amastigotes of *L. mexicana* were purified from infected BALB/c mice or grown axenically as described previously (Hart *et al.*, 1981).

2.2.4 Cultivation of T. brucei

Bloodstream forms of *T. brucei* strain 427 were grown in rats and isolated by DEAE ion exchange chromatography as described previously (Lanham and Godfrey, 1970). Procyclics forms of *T. brucei* strain 427 were grown at 27°C in SDM 79 containing 10% (v/v) fetal bovine serum and 3.5 mg/L haemin (Brun and Schonenberger, 1979).

2.3 The effects of stressors on the growth of Leishmania *and expression of genes*

2.3.1 The effects of peroxides on the expression of L. major *genes*

To evaluate the effect of peroxides on the growth of *Leishmania* promastigotes and expression levels of selected genes, parent and transgenic lines were cultured for 96 h from a starting density of 2.5 x 10^5 cells/ml prior to the addition of hydrogen peroxide (10, 3, 1, 0.3 or 0.1 mM), cumene hydroperoxide or tert-butyl hydroperoxide both at 1, 0.3, 0.1, 0.03, or 0.01 mM. The cultures were incubated for 24 h or 6 days in the oxidants for IC_{50} estimations and growth in the presence of peroxides respectively.

2.3.2 The effects of hypo-sulfur stress on the expression of L. major *genes*

To investigate whether *L. major* requires exogenous sulfur amino acids, promastigotes were grown in RPMI 1640 (Life Technologies) or MEM Eagle modified (ICN-biomedical) (which lacks an exogenous sulfur source of glutamine, serine, cysteine, methionine or cystine) supplemented with 10% (v/v) of either dialysed or normal heat-inactivated fetal bovine serum or in the same medium supplemented with the additives:- *L*-cysteine (0.2 mg/L), *L*-methionine (0.2 mg/L), *L*-cystathionine (0.2 mg/L), *L*-homocysteine (0.2 mg/L) and sodium sulfide (0.15 mg/L) singly or in combination.

2.3.3 The effects of an anti-leishmania drug on the expression of L. major *genes*

Promastigotes resistant in 350 mg/ml sodium antimony tartate were prepared by serially sub-passaging promastigotes in increasing concentrations of the drug.

2.3.4 The effects of heavy metals on the expression of L. major *cysteine synthase*

Promastigotes were cultured for 24 h, using standard conditions and from a starting density of 2.5 x 10^5 cells/ml, in the presence of concentrations of cadmium chloride (4.9 mM), potassium arsenate (0.34 mM) and copper sulfate (0.26 mM) that inhibited cell growth by approximately 50%.

2.4 Estimation of cell density

Cell density at the end of an incubation period was estimated either by direct counting of cells diluted with equal volume of 4% (v/v) formaldehyde in PBS in an improved Neubauer haemocytometer (Weber Scientific) under microscopy or by using the acid phosphatase assay (Bodley *et al.*, 1995). A typical phosphatase assay reaction

consisted of 200 µl *Leishmania* culture and 20 µl of 40 mg/ml p-nitrophenyl phosphate in 1 M sodium acetate, pH 5.5, and 1% (v/v) Triton X-100. Samples were incubated at 37°C for 2 h and the absorbance read at 405 nm in a microtitre plate reader spectrometer (Titertek Multiscan® MSs/340, UK). It has been shown previously, that the amount of enzyme (and so product) correlates to the number of promastigotes (H. Denton pers. comm.).

2.5 Harvesting of parasites and lysis

Parasites were harvested by centrifugation at 1500 x g for 10 min at 4°C followed by two washes in phosphate buffer saline (PBS, 0.01 M phosphate buffer, pH 7.4, 0.027 M KCl, 0.137 M NaCl), both followed by centrifugation as before, and then used immediately or resuspended in a new medium or stored at -20°C. Parasite lysates (designated fraction T) were produced by resuspension of parasite pellets in 0.25 M sucrose, 0.25% (v/v) Triton X-100, 10 mM EDTA and a cocktail of protease inhibitors (10 µM E-64, 2 mM 1,10-phenanthroline, 4 µM pepstatin A, 1 mM phenylmethylsulfonyl fluoride). Fraction T was centrifuged at 13,000 x g for 15 min at 4°C and the resulting supernatant (designated soluble fraction) retained for analysis. For sub-cellular localization studies, the lysis buffer lacked the detergent Triton X-100. *L. major* promastigotes were harvested by centrifugation at 2100 x g for 10 min at 4°C, washed twice in 0.25 M sucrose, and lysed in lysis buffer (250 mM sucrose, 20 mM Tris-HCl, pH 7.5, 10 µM E-64, 2 mM 1,10-phenanthroline, 4 µM pepstatin A, 1 mM phenylmethylsulfonyl fluoride) by mixing to a thick paste with alumina (Sigma type 405) that had been acid washed, de-fined and dried. Parasite breakage was confirmed to be greater than 99% as assessed by phase contrast microscopy, whereupon the paste was suspended in approximately 4 volumes of 0.25 M sucrose

and the alumina removed by centrifugation at 180 x g for 5 min. The resultant crude cell homogenate (designated fraction H) was fractionated by differential centrifugation using three sedimentation steps: 2100 x g for 10 min; 25 800 x g for 10 min; 240 000 x g for 1 h. The pellets were re-suspended in 0.25 M sucrose to give fractions P1, P2 and P3, respectively, and the final supernatant was labelled S. The entire procedure, which was carried out at 4°C, was based on the method developed by Coombs *et al.*, (1982).

2.6 Preparation of stabilates for long-term storage of L. major *cell lines*

Five hundred microlitres of a log phase culture was diluted with an equal volume of serum containing 30% (v/v) glycerol in a Cryotube™ vial (Nunc.), mixed and left in ice for 5 min. The culture vials were then temporary stored overnight at -70°C in an isopropan-2-ol bath and subsequently under liquid nitrogen (-140°C) until required.

2.7 RNA and DNA manipulation techniques for L. major *and* L. mexicana

2.7.1 Isolation of total RNA

Total RNA of *L. major* and *L. mexicana* promastigotes was isolated using ready-to-use TRIzol (Life Technologies), which is a monophasic solution of phenol and guanidine isothiocyanate. The method was carried out as recommended by the manufacturers. *L. major* and *L. mexicana* were harvested and pelleted by centrifugation. The pellet was lysed by TRIzol, by adding approximately 1 ml reagent per 10^8 cells and incubating for 5 min at room temperature. Two hundred microlitres of chloroform per 1 ml of TRIzol was then added, followed by centrifugation at 10,000 x g for 5 min at room temperature to separate into aqueous and organic phases. The RNA, which remained exclusively in the aqueous phase, was recovered by

precipitation with 1 ml isopropanol at room temperature followed by centrifugation at 10,000 g for another 5 min. The RNA pellets, visible as translucent gels were washed with several volumes of 75% (v/v) ethanol. The pellets were air-dried briefly and dissolved in RNase-free water.

2.7.2 Preparation of RNase-free equipment and RNase-free water

The introduction of RNase during RNA isolation was prevented by using sterile disposable plastic-ware and commercially available RNase-free tips whenever possible. Non-disposable glassware and plastic-ware were soaked in 0.5 M NaOH for 10 min, rinsed in double distilled water (ddH_2O) and autoclaved. RNase-free pipette tips were prepared in tip boxes by washing first with 3% (v/v) H_2O_2, subsequently adding diethylpyrocarbonate (DEPC) to 0.01% (v/v), leaving overnight and then draining and autoclaving. RNase-free H_2O was prepared by adding 2 drops of DEPC (0.01% v/v per litre), leaving the bottles at 37°C overnight, and then autoclaving. All solutions used for RNA work were either treated in this way or were prepared using DEPC-treated water. Electrophoresis tanks used for electrophoresis of RNA were first cleaned with detergent, dried with ethanol and then filled with a solution of 3% (v/v) H_2O_2. After 10 min at room temperature, the electrophoresis tank was rinsed with water treated with DEPC.

2.7.3 First strand cDNA synthesis

The following protocol was used for the synthesis of first strand cDNA from poly A^+ mRNA contained in the total RNA isolated as described above. Four microgram of total RNA was reverse transcribed with Superscript II reverse transcriptase (Life Technologies) into single-stranded cDNA as described by the manufacturers. Four

micrograms of total RNA was mixed with 0.5 μg oligo $(dT)_{15}$ primer (Promega) in a total volume of 11 μl, incubated at 70°C for 10 min and chilled in ice for 1 minute. To this reaction, 4 μl of 5x First strand buffer, 1 μl 10 mM dNTP's mix, 2 μl 0.1 M DTT and 1 μl RNase OUT were added and the reaction incubated at 42°C for 5 min before 200 units of Superscript II RT was added and incubation continued for a further 50 min. Heating at 70°C for 15 min terminated the reaction. The cDNA was then amplified directly in a PCR reaction or stored at -70°C until needed.

2.7.4 RACE

The 5' and 3' ends of the genes from both *L. major* and *L. mexicana* were amplified using the 5' and 3' RACE system kits (Life Technologies) as detailed by the manufacturers. In both types of RACE, gene-specific primers (detailed in the relevant chapters) were used in conjunction with the 3' and 5' RACE anchor primers, respectively, (Life Technologies) attached to the poly (A) tail in mRNA at the C-terminus end of the mRNA or a spliced leader primer that targets the spliced leader site (Agami and Shapira, 1992) at the N- terminus end of mRNA. Reaction conditions for each gene amplified varied and are described where appropriate.

2.7.5 Isolation of genomic DNA

A mini-prep procedure based on a published method (Medina-Acosta and Cross, 1993) was utilized throughout this project. Up to 2 x 10^8 cells were harvested and resuspended in 400 μl of TELT lysis buffer (50 mM Tris-HCl pH 8.0, 62.5 mM EDTA pH 9.0, 2.5 M LiCl, 4% (v/v) Triton X-100) and incubated at room temperature for 5 min. The TELT lysate was extracted carefully with an equal volume of water-saturated phenol/chloroform (1:1) twice (followed, each time by

centrifugation, 13000 x g, 5 min) and once with chloroform before ethanol precipitation with 2 x 70% (v/v) ethanol washes. The precipitated DNA was allowed to dry for no more than 5 min and resuspended in TE buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA). The DNA was incubated at 37°C for an hour and the quantity and quality of DNA was analysed by agarose gel electrophoresis.

2.8 Preparation of a Knock-out cassette and episomal vector for L. major *transfection*

2.8.1 Production of 'gene'-over-expressing plasmid

The *pGL607* plasmid (kindly provided by Prof. J. C. Mottram, Glasgow derived from *pXG-BSD* (Goyard and Beverley, 2000) was used to create *L. major* cell lines that overproduce the gene products described in the appropriate chapters. The ORFs of the genes were amplified from gDNA by PCR with the Expand High Fidelity system using 94°C, 2 min; 30 cycles of 94°C, 30 s; 65°C, 30 s; 72°C, 3 min; and a final step of 72°C, 7 min. For this, perfect match primers encoding the region directly preceding the native stop codon were used. A *BamH*I restriction site and an ATG initiation codon was added to the 5' end of the oligonucleotide N-terminal primer and a *Sma*I restriction site and a stop codon was added to the C-terminal end to facilitate cloning. The *BamH*I and *Sma*I restriction sites were replaced with *Bgl*II and *EcoR*V, respectively, if the gene of interest contained *BamH*I and *Sma*I restriction sites. The PCR products were cloned into pGEM-T vector (Qiagen) and then used to transform *E. coli* DH5α cells. Plasmids were purified from an overnight culture, digested with appropriate restriction enzymes and ligated into a *Sma*I/*BamH*I-digested pGL607 cassette that contains the ORF of the blasticidin-resistance gene.

2.8.2 Production of a knock-out cassette

Construct *pGL345* carrying the *HYG* gene flanked by *L. mexicana* CPB 5' and 3' flanks was obtained from Prof. J. Mottram, Glasgow. These flanks were removed and replaced with the 5' and 3' flanking regions of the *L. major* genes to be knocked-out. The nucleotide sequences of the flanks of the genes were obtained from either the RACE experiments or from HGTS obtained from the *Leishmania* genome projects. Primers designed and conditions used for the amplification of these flanks are given in the appropriate chapter. The 5' flank had *Hind*III and *BamH*I sites engineered onto the 5' and 3' ends, respectively, whilst the 3' flank had *Sma*I and *Bgl*II sites. Primers out-with the amplified regions were available making it possible to screen for integration at the correct locus by PCR. Integration was also verified using Southern blot analysis.

2.8.3 Preparation of plasmid for transfection

Medium-scale production of episomal plasmids and knock-out cassettes were performed using the Qiagen Tip Midi-Prep kit and Qiagen Gel extraction kits, with 25 ml of *E. coli* overnight cultures. Sixty micrograms of each knock-out plasmid were digested overnight with 4 µl of each *Hind*III (80 U) and *Bgl*II (40 U) in a 200 µl reaction volume. Digests were electrophoresed and purified on 0.7% agarose TBE gels, ethanol precipitated, washed twice in 70% (v/v) ethanol, and dried before being resuspended in 20 µl of sterile water. DNA at 1 mg/ml was used to transform *L. major*.

2.8.4 Electroporation of L. major

Transfections were carried out using the procedure of Coburn and co-workers (Coburn *et al.*, 1991). Cells were grown to late log phase (approximately 1.0 x 10^7/ml), harvested and washed twice in electroporation buffer (EPB - 21 mM HEPES pH 7.5, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 5 mM glucose) before being resuspended at 1 x 10^8 cells/ml in EPB and kept on ice. Four hundred microlitres of this suspension (4 x 10^7 cells) were transferred to a pre-chilled 0.2 cm electroporation cuvette (Biorad), and electroporated with the appropriate DNA construct (knock-out cassette or episomal vector) using a Genepulser II apparatus at 0.45 kV (2.25 kV/cm) and 500 μF capacitance. Cells were allowed to recover for 10 min on ice before being transferred to fresh medium and incubated at 25°C overnight. Cells were then plated on semi-solid HOMEM-agar plates containing appropriate antibiotics at the concentrations described earlier.

2.8.5 Southern blotting of DNA fragments

Five to ten micrograms of genomic DNA were digested with appropriate enzymes overnight and fragments fractionated by agarose gel electrophoreses in TBE buffer. The gel was analysed under UV to allow a scale image of the gel to be obtained (a ruler was placed beside the gel to allow measurement of resulting autoradiograms to be equated with the position of the DNA markers). The gel was nicked, denatured, and neutralized by incubation for 20 min in depurination solution (0.125 M HCl), 30 min in denaturation solution (1.5 M NaCl, 0.5 M NaOH), 30 min in neutralization solution (1.5 M NaCl, 0.5 M Trizma base, pH 7.5) and 10 min in 20 x SSC (3 M NaCl, 0.33 M Tris-sodium citrate). Each wash was interspersed by a 10 min wash in distilled water. The gel was blotted onto a Hybond™-N^+ nylon membrane

(Amersham) overnight (Mottram *et al.,* 1993), by capillary transfer. (Sambrook *et al.*, 1989).

2.8.6 Southern hybridisation with nucleic acid probes

The transferred DNA fragments on Hybond-N were covalently attached to the membrane by UV crosslinking (1200J UV Crosslinker, Stratagene). Filters were pre-hybridised for 4 h in Church-Gilbert hybridisation solution (340 mM Na_2HPO_4, 158 mM NaH_2PO_4, 240 mM SDS, 1 mM EDTA) at 65°C. Thirty nanograms of the nucleic acid probe was prepared from agarose gel-purified restriction endonuclease fragments using the Stratagenes's Prime-It II Random Primer kit (Strategene) according to manufacturer's instruction. Probes were purified on Microspin S-200 HR columns (Amersham Phamacia Biotech) and boiled for 5 min before incubation on ice for 2 min. Filters were hybridised overnight with [α-32] dATP probes added directly to the Church-Gilbert solution/Southern membrane and incubated overnight at 65°C. Filters were washed under high stringency (2 x 5 min in 2 x SSC/0.1% SDS pre-warmed at 65°C followed by 1 x 15 min and 2 x 10 min washes with 1 x SSC/0.1% SDS and 0.1 x SSC/0.1% SDS, respectively). Membranes were sealed in polythene and exposed to X-ray film (Konica Medical Film). Hybridisation signals were detected with a film processor (X-Ograph imaging system Compact X4).

2.9 Biochemical methods

2.9.1 Estimation of protein concentration

Protein concentrations were determined according to the Bradford procedure (Bio-Rad) using bovine serum albumin as the protein standard. For CD analysis, the protein concentration of the native and truncated mutant enzymes were determined by

measuring the UV absorbance at 280 nm and taking into account the content of amino acids (tyrosine (y) and tryptophan (w)) using the equation:

A_{280} (1 mg/ml)= ny(1280) + nw(5690)/molecular mass

Where n=number

The suspension buffer of the protein was used as the blank. The spectroscopic scan profile and the ratio A_{280}/A_{260} (1.7) were used to check if protein samples were free of nucleic acid contamination and/or the presence of a light scattering condition, since nucleic acids absorb strongly at 260 nm.

2.9.2 Preparation of protein for SDS-PAGE analysis

Proteins analysed by SDS-PAGE included isolated recombinant protein, *E. coli* and *Leishmania* lysates. The samples were mixed with equal volumes of SDS-PAGE loading buffer, and boiled for 5 min. Proteins were separated by SDS-PAGE on 10% (w/v) acrylamide mini gels of 0.75 mm thickness, which were prepared and run using a Mini-Protean II slab system (Biorad) according to manufacturer's instructions. Gels were stained to detect total protein using 1% (w/v) Coomassie R_{250} or they were electro-blotted to allow specific-protein analysis.

2.9.3 Western Blotting

Following SDS-PAGE analysis, separated proteins were transferred to Hybond-C nitrocellulose membrane (Amersham) by electroblotting in transfer buffer (20 mM Tris, 0.15 mM glycine, 20% (v/v) methanol) using a BioRad mini transblot cell, according to the manufacturer's instructions. Porceau S stain was added to allow quick assessment of protein transfer to the nitrocellulose membrane. Proteins were transferred at 100 V for 45 min (4°C). The membrane was rinsed in Western blotting

transfer buffer to remove fragments of acrylamide before being transferred to a 50 ml falcon tube and incubated for two hours in blocking buffer (20 mM Tris-HCl pH 7.6, 13.7 mM NaCl (1 x TBS) containing 5% (w/v) low fat dried milk) at 37°C.

2.9.4 Antibody detection of protein

Rabbit polyclonal antibodies were raised against *Leishmania* recombinant proteins by the Scottish Antibody Production Unit (SAPU, Carluke, UK), using standard protocols. Western blot analysis was as described previously (Mottram *et al.*, 1993) with polyclonal immune rabbit serum diluted (1:500) with the blocking buffer (1 x TBS containing 1% (w/v) low fat dried milk and 0.1% Tween-20 (TBSPr)). Primary antibodies were incubated with the membrane in blocking solution (TBSPr), generally for 60-90 min at room temperature or overnight at 4°C. Blots were washed 3 x 10 min with 1x TBS-containing 1% (w/v) low fat dried milk (TBSW) and secondary antibody (anti rabbit IgG conjugated to horseradish peroxidase (HRP) (Promega)) was generally used at 1 in 500 dilution in blocking buffer (200 mM Tris-HCl pH 7.6, 137 mM NaCl containing 1 % (w/v) low fat dried milk (TBSSe)) for 2 hours at room temperature. Blots were washed in TBSW as before. Bound antibody was detected by using horseradish peroxidase-coupled secondary antibodies (SAPU, Carluke, UK) using the Supersignal™ Enhanced Chemiluminescence (ECL) reagents (Pierce) according to manufacturer's instructions. Fluorescence was detected by autoradiography with reflection autoradiography film (NEN™ Life Sciences).

2.9.5 Indirect immunofluorescence, Analysis of subcellular location of sulfurtransferase in L. major

Promastigotes of *L. major* were fractionated as described by Coombs *et al.*, (1982) and the distribution of enzyme activities in the fractions analysed using the standard enzyme assays. Immunofluoresence analysis was carried out as follows. Ten to thirty microlitres of late log/stationary phase culture of *L. major* promastigotes were diluted to 100 µl in PBS and smeared on the surface of a slide pre-cleaned by soaking in 100% ethanol. The slides were allowed to dry for 5 min before cells were fixed and permeabilized in methanol for 15 min at -20°C. After 3 washes in PBS, the slides were blocked for 30 min with 2% (v/v) donkey serum in PBS before being washed in PBS as before. The slides were then incubated in a 1 in 100 dilution of anti-MST antibody for 2 hours to detect MST proteins, and then subjected to three washed in PBS for 10 min. Subsequently, the slides were incubated for 30 min at room temperature with a mixture of secondary antibody, donkey anti-rabbit IgG-FITC conjugate, diluted (1 in 500) in PBS with 2% (v/v) donkey serum. After three washes with PBS, the slides were mounted with 25 µl of anti-quenching agent MOWIOL-DABCO in 50% PBS, 50% glycerol. Fluorescence signals were visualized by UV fluorescence microscope on a Zeiss Axioplan fluorescence microscope with a Hamamstsu Digital Camera using the OpenLab Software (Improvision, University of Warwick).

2.10 Enzyme activity measurements

Sulfurtransferase activity was determined for recombinant sulfurtransferases and soluble extracts of parasites by monitoring thiocyanate or sulphide formation as follows.

The 'rhodanese' assay (Papenbrock and Schmidt, 2000) was based on the formation of thiocyanate from the reaction between cyanide and either mercaptopyruvate or thiosulphate and the detection of the thiocyanate by reacting it with an iron reagent to produce a red complex of FeSCN - which absorbs at 460 nm. The optimised reaction mixture (1 ml) contained 0.1 M Tris-HCl, pH 7.3, 10 mM KCN and enzyme extract. The reaction was initiated by the addition of thiosulphate or mercaptopyruvate (5 mM) and incubation was at 37°C for 10 min. The reaction was stopped by the addition of 500 µl formaldehyde and 1.5 ml iron reagent ($Fe(NO_3)_3$, 50 g/L; 65% HNO_3, 200 ml/L) was added for FeSCN- complex formation. The amount of product formation was quantified using a standard curve of NaSCN dissolved in the buffer used in the assay.

The 'sulfurtransferase' assay (Papenbrock and Schmidt, 2000) was based on the formation of hydrogen sulphide from the reaction between mercaptoethanol and mercaptopyruvate or thiosulphate. This was detected using lead acetate and measuring the production of lead sulphide. The reaction mixture (1 ml) contained 0.1 M Tris-HCl, pH 7.3, 0.2 mM lead acetate, 5 mM mercaptoethanol and enzyme extract. The reaction was started by the addition of 5 mM sodium thiosulphate or 3-mercaptopyruvate. The reaction at 37°C was monitored continuously by detecting the formation of lead sulfide spectrophotometrically at 360 nm. The molar extinction coefficient was taken as 5205/cm/mole (Thong and Coombs, 1987). One unit of the enzymatic activity was defined as 1 µmol of hydrogen sulfide or thiocyanate synthesized at 25°C per min.

The oxidation of reduced thioredoxin by the recombinant sulfurtransferase enzymes was measured by monitoring the reduction of oxidised thioredoxin by NADPH-linked thioredoxin reductase (Nandi *et al.*, 2000). A reaction mixture (1 ml) containing 20 μM NADPH, 2 μg *Trichomonas vaginalis* thioredoxin reductase (Coombs *et al.*, submitted) and 5 μg *T. vaginalis* thioredoxin (Coombs *et al.*, submitted) in 0.1 M phosphate buffer, pH 7.0, was incubated at 37°C for 5 min to allow the thioredoxin to be fully reduced and the reaction rate to stabilise. A constant absorbance, as reported by Nandi *et al.* (2000), was not achieved as the thioredoxin reductase-thioredoxin system used (recombinant *Trichomonas vaginalis* proteins) have a low NADPH oxidase activity (Coombs *et al.,* submitted). One hundred nanograms of recombinant sulfurtransferase was added and the reaction started by addition of 5 mM mercaptopyruvate or thiosulphate. The reaction was incubated at 25°C and the consumption of NADPH was followed at 340 nm.

The enzymatic activity of cystathionine β-synthase was assayed by measuring cystathionine production as described (Kashiwamata and Greenberg, 1970). The reaction mixture (100 μl) contained 50 mM pH 7.3, 10 mM serine, 10 mM homocysteine, 0.25 μM pyridoxal phosphate and 2 μl enzyme. All components were pre-incubated at the reaction temperature (37°C) for 5 min and the reaction was initiated by the addition of the enzyme. The reaction was further incubated for 2-5 min and stopped by adding 50 μl 20% (w/v) TCA, vortexed and set on ice for 10 min. The mixture was centrifuged at 13, 000 x g for 5 min and the supernatant used for cystathionine analysis. To determine the amount of cystathionine formed, 1 ml Ninhydrin reagent (1 g Ninhydrin in 100 ml glacial phosphoric acid protected from

light), and 130 µl of sample were boiled for 5 min, cooled in ice for 2 min and left for 20 min for colour to develop. The absorbance was then measured at 455 nm.

Cysteine synthase was assayed by measuring cysteine production (Nozaki *et al.*, 2001). The standard mixture of cysteine synthase assay was identical to that used for cystathionine β-synthase except that serine and homocysteine were replaced with 10 mM O-acetyl serine and 1 mM sodium sulfide or sulfide from the mercaptopyruvate/LmajMST assay outlined earlier. All components were pre-incubated at the reaction temperature (37°C) for 5 min and the reaction was initiated by the addition of the enzyme. The reaction was further incubated for 2-5 min and stopped by adding 50 µl 20% (w/v) TCA, vortexed and set on ice for 10 min. It was centrifuged and the supernatant used for cysteine analysis. To determine the cysteine amount, 200 µl Ninhydrin reagent (250 mg Ninhydrin in 6 ml glacial acetic acid and 4 ml concentrated hydrochloric acid and incubated at room temperature for one day protected from light), 100 µl glacial acetic acid and 130 µl of supernatant were boiled for 10 min, cooled in ice for 1 min and 600 µl ethanol added. The samples were cooled for 5 min in ice and left for 10 min at room temperature for the colour to develop. The absorbance was then measured at 560 nm.

The enzymatic activity of serine sulfhydrylase (SS) was measured in the same way as described for the cysteine synthase assay except that 0.2 mM serine was used instead of O-acetyl serine.

The rate of cysteine desulfhydration in the β-replacement reaction was monitored by measuring hydrogen sulfide and hence lead sulfide production

(Braunstein *et al.*, 1971) in a mixture of 50 mM Tris-HCl pH 8.0, 10 mM cysteine, 20 mM β-mercaptoethanol, 0.1 mM pyridoxal phosphate, 0.33 mM lead acetate and enzyme solution.

The enzymatic assay of serine acetyltransferase involved monitoring the decrease in absorbance at 232 nm of the thioester bond of acetyl-coenzyme A (CoA) (Baecker and Wedding, 1980) or by colorimetric assay (Nakamura *et al.*, 1983) of cysteine formed in a coupled reaction with the recombinant cysteine synthase from *L. major* (LmajCS). The standard mixture for the serine acetyltransferase assay contained 50 mM Tris-HCl, pH 8.0, 0.1 mM acetyl-CoA, 0.2 mM serine and enzyme solution. The reaction was initiated by the addition of serine and carried out at 25°C for 5-10 min. For the coupled assay, the reactions were carried out in 50 mM Tris-HCl, pH 8.0, 0.2 mM acetyl CoA, 5 mM serine, 1 mM Na_2S, 5 mM dithiothreitol, 0.02 unit of recombinant LmajCS and enzyme solution in a final volume of 100 µl. One (v) unit of enzymatic activity was defined as 1 µmol of thioester bond of acetyl-CoA cleaved or 1 µmole of cysteine, cystathionine or hydrogen sulfide synthesized per min at 25°C.

2.11 Effect of organic antimonials on the activity of leishmanial MST

The organic pentavalent antimonals Pentostam (sodium stibogluconate), glucantime and the trivalent antimonial Triostam (sodium antimonyl III gluconate) (dissolved at 2 mg/ml (2.2 mM), 2.0 mg/ml (6.0 mM) and 0.5 mg/ml (3 mM), respectively) were tested for inhibition of rLmajMST using the sulfulfurtransferase assay. The enzyme was pre-incubated with concentrations of the antimonial at the 25°C for 30 min before enzymatic activity was measured.

2.12 Analysis of LmajMST and its truncated derivatives using circular dichroism (CD) spectroscopy and binding of bisANS

2.12.1 Protein preparation, far and near UV measurements

CD spectral measurements of rLmajMSTΔ338-370, rLmajMSTΔ360-370 and rLmajMST were performed on a JASCO J-600 spectropolarimeter at 20°C. All samples were in 20 mM sodium phosphate buffer, pH 7.4. Protein concentrations for far UV and near UV CD measurements were 0.25 mg/ml and 1.10 mg/ml, respectively and samples were scanned in 0.1 cm (near uv) and 0.02 cm (far UV) path length cells. CD data were calculated in terms of mean molar residue ellipticity (θ) using mean residue weight of 109 for each enzyme (Kelly and Price, 1997; White *et al.*, 1985). Spectra were recorded at 20°C from 250 - 185 nm and were determined as the average of 5 scans. Two methods were used to estimate secondary structure content:

1. CONTIN (Provencher and Glockner, 1981). This method approximated the mean residue ellipticity of the native and truncated proteins at a wavelength by linear superposition (combination) of the spectra of a small set of reference spectra with known conformation, each of which is characteristic of and controlled by a simple constrained statistical regularization procedure (similar to ridge regression).

2. SELCON (Sreerama and Woody, 1993). The spectrum of the protein to be analysed is included in the basis set and an initial guess is made for the unknown structure of the native and truncated proteins as a first approximation. The resulting matrix equation is solved using the singular value decomposition algorithm and the initial guess is replaced by the solution. The process is repeated until self-consistency is attained. The best features of the variable selection and the locally linearized methods are incorporated in this procedure.

2.12.2 BisANS fluorescence

The availability of hydrophobic binding sites on each of the MST proteins was assessed using bisANS (1,1'-bis(4-anilino)naphthalene-5,5'disulfonic acid). Protein samples (50 μg/ml) were mixed with bisANS (to 30 μM) in 20 mM phosphate buffer, pH 7.8 containing 50 mM $Na_2S_2O_3$ and fluorescence monitored (excitation at 395 nm, emission at 500 nm) at 25°C using a LS 55 Luminescence spectrometer (Perkin Elmer Instruments, UK).

2.12.3 Refolding of denatured enzyme

Denaturation with urea and reactivation analyses of rLmajMSTΔ338-370, rLmajMSTΔ360-370 and rLmajMST were based on protocols detailed in Bhattacharyya and Horowitz (2002). Proteins at 1 mg/ml were incubated in 50 mM potassium phosphate buffer pH 7.8, containing 50 mM $Na_2S_2O_3$ and 6 M urea, for 120 min at 20°C. Attempts to reactivate the proteins involved one of two procedures: (a) 25 μl of denatured proteins at 1 mg/ml were diluted and allowed to refold in 75 μl of refolding buffer containing 0.2 M β-mercaptoethanol, 50 mM $Na_2S_2O_3$, 10 mM KCl and 10 mM $MgCl_2$ for 18 h. (b) An aliquot of the denatured sample was dialysed at 4°C overnight against the refolding buffer. Successful refolding and hence reactivation was monitored by assaying 10 μl samples using the standard sulfurtransferase assay detailed above (Section 2.10).

2.13 DNA and protein analysis tools and softwares

All DNA and protein sequence analysis were performed using Vector NTI (v 6.0) software (Informax Inc) (searching for reading frames and restriction sites,

construction of cloning and expression plasmids), Align X (sequence alignment), ContigExpress (align overlapping sequences to form contiguous sequences) and the molecular biology and proteomics programmes available at the Expasy Proteomics tools website (http://www.expasy.ch/tools) and European Bioinformatics Institute (http://www.ebi.ac.uk).

The following web resources were used:

TIGR database: http://www.tigr.org (database)

The Sanger Institute: http://www.sanger.ac.uk (database)

EMBL: http://www.ebi.ac.uk

NCBI: http://www.ncbi.nlm.nih.gov (database)

GeneDB: http://www.genedb.org

SignalP: http://www.cbs.dtu.dk/services/SignalP/ (prediction of the presence of signal peptides)

2.14 Evolutionary and phylogenetic analysis

Multiple alignment of protein sequences was constructed based on the joint neighbourhood method ClusterxTree v.1.5b to build phylogenetic trees to obtain degree of similarity and homology among the selected sequences. Phylogenetic trees were constructed by bootstrap analyses and viewed with Treeview v. 1.6.5 (Page, 1996). Protein sequences used in the analysis were obtained from the GenBank non-redundant (NR) sequence database of protein sequences (National Centre for Biotechnology Information, NIH, Bethesda, MD, USA (www.ncbi.nih.nlm.gov)).

Chapter 3

3.1 Introduction

Sulfurtransferases (EC 2.8.1.1 - EC 2.8.1.5) are widely distributed enzymes with representatives being known in many eukaryotes and prokaryotes, but until now there have been no reports on the enzymes of protozoa except for one study on *Euglena gracilis* (Watanabe *et al.*, 1985). The common characteristic of the sulfurtransferase family is their ability to catalyse the transfer of sulfane sulphur from a donor molecule to a nucleophilic acceptor substrate.

The rhodanese family of sulfurtransferases have received considerable attention, with the mammalian enzymes known as rhodanese being most studied in attempts to understand the mechanism underpinning the correct folding and stability of the enzyme. Rhodanese, like most sulfurtransferases, has two aparently similar domains which presumably arose from gene duplication. The non-catalytic N-terminal and catalytic C-terminal domains are structurally 'similar', but lack significant amino acid sequence similarity. Studies have established the importance of the N-terminal 'structural' domain in folding and stability, although the current evidence suggests that correct protein folding also requires the assistance of a chaperone molecule. The C-terminal domain contains the active site and plays a role in enzyme catalysis. However, an enzyme from *E. coli*, GlpE, has only a single domain, which suggests that the catalytically inactive N-terminal domain found in most sulfurtransferase is not essential for catalysis.

Rhodanese-like proteins, some catalytic and some inactive, appear to be present in all major phyla of living organisms and currently some 150 have been identified (Tatusov *et al.*, 2000; Bordo and Bork, 2002). They are found as:

(a) tandem repeats in which the C-terminal domain hosts the properly structured active-site cys residue e.g. SseA and YnjE of *E. coli* (Ray *et al.*, 2000).

(b) as single domain proteins e.g. the senescence-associated protein *SenI* from *Arabidopsis thaliana* (Oh *et al.*, 1996), the sulfide dehydrogenase (*Sud*) from *Wolinella succinogenes* (Bordo and Bork, 2002), and *Ntdin* from *Nicotiana tabacum;* or

(c) in association with other protein domains e.g. the C-terminal part of *Thi*I, an *E. coli* protein involved in thiamin and 4-thiouridine biosynthesis (Palenchar *et al.*, 2000), in mitogen-activated protein kinase (MAPK)-phosphatase (Keyse and Ginsburg, 1993; Fauman *et al.*, 1998; Hofmann *et al.*, 1998) and in NADH oxidase (NoxA-3) from *Archaeoglobus fulgidus* (Reed *et al.*, 2001).

Completion of the sequencing of various genomes has shown that multiple ORFs coding for rhodanese-like proteins exist in the same genome, e.g. the GlpE, PspE, (Adams *et al.*, 2002) and YgaP of *E. coli* K12 genome (Blattner *et al.*, 1997; Tatusov *et al.*, 2000). These observations indicate that rhodanese domains are likely to be involved in several distinct biological functions.

The structures of rhodanese of bovine liver (Rhobov) (Ploegman *et al.*, 1978), and *A. vinelandii* rhodanese (RhdA) (Bordo *et al.*, 2000) have been solved via crystallographic studies. The overall architecture consists of two identically-folded domains with limited amino acid sequence homology. The structure of the GlpE 12 kDa sulfurtransferase from *E. coli* (Bordo *et al.*, 2000; Ray *et al.*, 2000; Spallarossa *et*

al., 2001) shows that it has only one domain, suggesting either divergence before the ancestral gene duplication event or that intragenic deletion occurred more recently. Each of the known structures of rhodanese domains displays a typical αβ topology. Moreover they are structurally related to the catalytic subunit of the human cell cycle-control phosphatase enzymes Cdc25A and Cdc25B (Fauman *et al.*, 1998), suggesting that the two enzyme families are likely to share a common evolutionary origin.

In this chapter, I describe the cloning, expression and purification of a sulfurtransferase of the protozoan parasites *Leishmania major* and *L. mexicana*. The enzymes are structurally peculiar in possessing an unusual C-terminal domain not found in any other known sulfurtransferase. The necessity of this domain for the correct folding of the protein is described.

3.2 Materials and Methods

3.2.1 Rapid Amplification of cDNA ends (RACE)

The 5' and 3' ends of the gene from both *L. major* and *L. mexicana* were amplified using the 5' and 3' RACE system kits (Life Technologies). Degenerate reverse gene specific primers NT22 (5'ACGCGCACCTTCGATGTGGCC3') and NT 41 (5'ATTTCATCCACAAGGTAGTGATGC3'), based on conserved motif sequences from the alignment of the putative rhodanese of *L. major* (AF 163772) and bacterial homologues in the NCBI database, and the spliced leader primers LMEXSL1 (5'TAACGCTATATAAGTATCAGTTTC3') and LMEXSLII (5'AGTATCAGTTTCTGTACTTTATTG3') were used for the 5' RACE. Perfect match primers NT 21 (5' CACCGCCCGGCATCCGCT ACC 3') and NT 54 (5'ATCGACTGGTGCATGGCGAAC3'), based on consensus sequences of the genes

of the organisms listed above, and an oligo(T) primer with an adaptor attached UAP (5'CUACUACUACUAGGCCACGCGTGGACTAGTAC3') and AUAP (5'GGCCACGCGTGGACTAGTAC3'), were used to amplify the 3' end of the genes. The procedure involved the Expand High Fidelity System Pwo/Taq-polymerase and the following incubations: 94°C, 2 min; 30 cycles of 94°C, 30 s; 65°C (55°C), 30 s; 72°C, 2 min; and finally 72 °C, 7 min (annealing temperature for *L. mexicana* in parenthesis). Both the 5' and 3' ends of the genes were cloned into pGEM-T vector and sequenced (MWG Biotech or University of Glasgow Sequencing Service).

3.2.2 PCR Amplification of MSTs

The ORFs of the genes encoding *LmajMST* and *LmexMST* and four constructs, *LmajMSTΔ300-370, LmajMSTΔ320-370, LmajMSTΔ338-370* and *LmajMSTΔ360-370,* encoding truncated proteins (rLmajMSTΔ330-370, rLmajMSTΔ320-370, rLmajMSTΔ338-370 and rLmajMSTΔ360-370) lacking 70, 50, 32 and 10 amino acids from the C-terminus, were amplified from cDNA by PCR with the Expand High Fidelity system using 94°C, 2 min; 30 cycles of 94°C, 30s; 65°C, 30s; 72°C, 3 min; and a final step of 72°C, 7 min. For this, the 5'-perfect match primer NT 42 (5'CGCTGACATATGTCTGCTCCTGCTGCGCCGAA3') was used for all constructs together with an appropriate 3'-primer encoding the regions directly preceding the native stop codon and amino acids 300, 320, 338 and 360, respectively: NT 55 (5'TTCCTCGAGTGGAGGGGCGGAAGAGGCCGCTGT3') for *LmajMST*, NT 56 (5'TTCCTCGAGCGGCAGCGGCGTCACCGC3') for *LmexMST*, NTMST1 (5'TTCCTCGAGGCTGCGCATTATGG3'), for *LmajMSTΔ300-370,* NTMST2 (5'TTCCTCGAGCTTCGGGTTGTCGCCGA3') for *LmajMSTΔ320-370*, NTMST3 (5'TTCTCGAGCTCCGCATCGGGTCTCTCG3') for *LmajMSTΔ338-370* , and

NTMST4 (5'TTCCTCGAGGACGCGGCCGCTCTTGA3') for *LmajMSTΔ360-370.* A *Nde*I restriction site (underlined) was added to the 5' end of the oligonucleotide NT 42 and a *Xho*I restriction site (underlined) added to NT55, NT56, NTMST1, NTMST2, NTMST3 and NTMST4, to facilitate cloning and purification. The PCR products were cloned into pGEM-T (Qiagen) to generate plasmids designated as *pLmajMST, pLmexMST, pLmajMSTΔ300-370, pLmajMSTΔ320-370, pLmajMSTΔ338-370* and *pLmajMSTΔ360-370,* respectively. Plasmids were propagated in *E. coli* DH5α cells and plasmid DNA was prepared by the Qiagen method and sequenced on both strands (MWG Biotech. or University of Glasgow Sequencing Service).

Plasmids *pLmajMST, pLmexMST, pLmajMSTΔ300-370, pLmajMSTΔ330-370, pLmajMSTΔ338-370* and *pLmajMSTΔ360-370* were digested with *Nde*I and *Xho*I and the restriction endonuclease fragments of 1110 bp, 900 bp, 960 bp, 1014 bp and 1080 bp encoding the MST sequences amplified were ligated into *NdeI/Xho*I-digested pET21a^{+} vector (Invitrogen) to produce *pETLmajMST, pETLmexMST, pETLmajMSTΔ300-370, pETLmajMSTΔ320-370, pETLmajMSTΔ338-370* and *pETLmajMSTΔ360-370.*

3.2.3 Expression and Purification of MSTs

The plasmids were used to transform *E. coli* BL21 codon plus (DE3)-RP cells (Stratagene). After testing a variety of culture temperature and induction protocols, the following was found to be optimal for production of soluble, recombinant protein. The bacteria were cultivated in LB medium containing 100 μg/ml ampicillin and 100 μg/ml chloramphenicol at 37°C to an A_{600} of 0.6. Expression was induced by 0.5 mM

IPTG for 5 hours at 15°C. Cells were pelleted and resuspended in 5 ml sonication buffer A (50 mM sodium phosphate, 3 M NaCl, pH 8.0) with 5 mM imidazole, disintegrated by sonication, and the soluble fraction recovered by centrifugation at 13,000 x g for 30 min at 4°C. This was applied to a 13 ml nickel-nitrilotriacetic acid column (bioCAD) pre-equilibrated with sonication buffer A. The column was washed with 60 ml of buffer A containing 5 mM imidazole and then with 300 ml of buffer A containing 60 mM imidazole. The his-tagged recombinant proteins were eluted with 250 mM imidazole in buffer A. A 10 ml/min flow rate was used. The eluant was dialysed with excess 20 mM Tris-HCl, pH 7.9, 1 mM $Na_2S_2O_3$ at 4°C overnight to remove salt and imidazole and then stored at -20°C until analysed. Approximately 25 mg soluble, recombinant proteins (rLmajMST and rLmexMST) were obtained from 1 litre cultures of induced *E. coli*.

3.3 Results

3.3.1 Identification, Cloning and Characterisation of Genes Encoding Sulfurtransferases in L. major *and* L. mexicana

3.3.1.1 Cloning of a partial MST gene sequence using conserved motifs

Searches of *L. major* HTGS sequence in the NCBI database (http://wwwncbi.nih.nlm.gov) revealed a gene (AF 163772 gi:13122208) encoding a protein with similarity to sulfurtransferases. Cloning and expression of the putative rhodanese failed to produce a recombinant protein in the BL21(DE3) expression system, thus an alternative approach was adopted to identify sulfurtransferase genes of *Leishmania.* Alignment of nucleotide sequence of the annotated HTGS sequence with known sulfurtransferase from bacteria produced two conserved motifs (Figures 3.1 and 3.2).

known sulfurtransferase from bacteria produced two conserved motifs (Figures 3.1 and 3.2).

(a)

```
Dr          1 ETDLSGPVQPGGEGGRHPLPDPPVLADWLGAAGIGNGDVVVCYDDPGGGQGFYAARAWWL
Mt          1 EDELSDHTIAGR--GRHPLPSGASLQATVRRCGIRHDVPVVVYDDWNRA-G--SARAWWV
LmajMST     1 DTNLSKLVPTST--ARHPLPPCAEFIDWCMANGMAGELPVLCYDDECGAMG--GCRLWWM

                              NT21

Dr         61 LRWLGHREVYVLDGGWPAYLAAGGEASTEEPHPVP---TTFTPDVQPELVATAQDVQNRE
Mt         56 LTAAGIANVRILDGGLPAWRSAGGSIETGQVSPQLGNVTVLHDDLYAGQRLTLTAQQAGA
LmajMST    57 LNSLG-ADAYVINGGFQACKAAGLEMESGEPSSLPRPATHWPFKTAFQHHYLVDEIPPN-

Dr        118 DGVLLIDSRAPERYRGDSEP--LDKKAGHIPGAVNRPFSAALDEQG--------QFRAGA
Mt        116 GGVTLLDARVPERFRGDVEP--VDAVAGHIPGAINVPSGSVLADDG--------TFLGNG
LmajMST   115 --AIITDARSADRFASTVRPYAADKMPGHIEGARNLPYTSHLVTRGDGKVLRSEEEIRHN

                                                NT22
```

Figure 3.1. Alignment of protein sequences of an annotated HTGS sequence from *L. major* (GenBank Accession no. AF 163772; gi:13122208) and similar proteins from bacteria. Sequences are as follows: Dr, *Deinococcus radiodurans* (GenBank accession no. NP_296251.1; gi:15807515); Mt, *Mycobacterium tuberculosis* (GenBank accession no. AAK46633.1; gi:13882043), LmajMST, *L. major*. These sequences were aligned using Clustal X. Red and green lettering indicate conserved motifs while blue represents similar residues. The motifs identified for sequence specific-primer (NT 21 and NT 22) design are underlined.

(b)

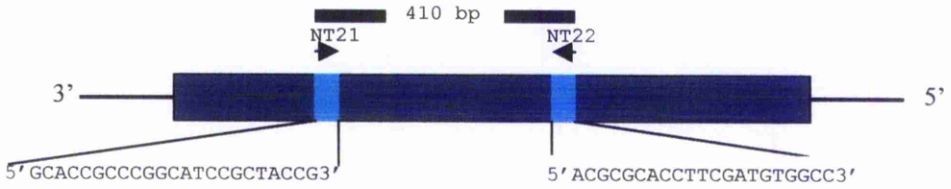
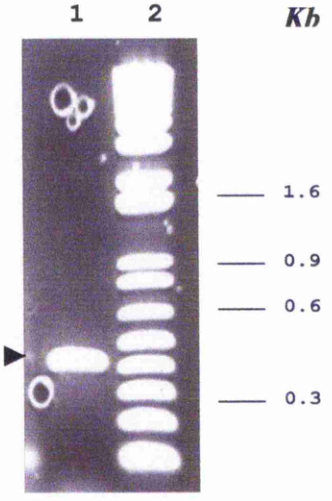


Figure 3.2 Schematic representation of *Leishmania* MST protein highlighting two areas highly conserved in similar proteins from bacteria. Oligonucleotides (NT21 & NT22) designed to these sites amplified a 410 bp internal fragment of the MST gene from *Leishmania*. The direction in which the primers, NT 21 and NT 22, amplified this region is arrowed.

Primers designed (NT21 and NT22) to these motifs (Figure 3.2) amplified a 410 bp of DNA sequence (Figure 3.3a). The fragment was fully sequenced in both sense and anti-sense directions using automated DNA sequencing (Figure 3.3b).

(a)



(b)

```
                NT21
                ======================
  1  CACCGCCCGG CATCCGCTAC CGCCCTGTGC TGAGTTTATC GACTGGTGCA TGGCGAACGG
     GTGGCGGGCC GTAGGCGATG GCGGGACACG ACTCAAATAG CTGACCACGT ACCGCTTGCC

 61  GATGGCGGGA GAGCTGCCAG TGCTCTGCTA CGATGACGAG TGCGGCGCCA TGGGTGGATG
     CTACCGCCCT CTCGACGGTC ACGAGACGAT GCTACTGCTC ACGCCGCGGT ACCCACCTAC

121  CCGCCTGTGG TGGATGCTGA ACTCTCTTGG CGCCGACGCG TACGTGATCA ACGGCGGCTT
     GGCGGACACC ACCTACGACT TGAGAGAACC GCGGCTGCGC ATGCACTAGT TGCCGCCGAA

181  TCAGGCCTGC AAGGCTGCGG GGCTGGAGAT GGAGTCCGGC GAGCCCTCGT CGCTGCCAAG
     AGTCCGGACG TTCCGACGCC CCGACCTCTA CCTCAGGCCG CTCGGGAGCA GCGACGGTTC

241  ACCCGCAACG CACTGGCCCT TCAAGACGGC CTTTCAGCAT CACTACCTTG TGGATGAAAT
     TGGGCGTTGC GTGACCGGGA AGTTCTGCCG GAAAGTCGTA GTGATGGAAC ACCTACTTTA

301  CCCGCCCAAC GCAATCATCA CCGACGCGCG CTCCGCCGAC CGCTTCGCCT CGACAGTACG
     GGGCGGGTTG CGTTAGTAGT GGCTGCGCGC GAGGCGGCTG GCGAAGCGGA GCTGTCATGC

361  ACCTTACGCC GCAGACAAGA TGCCAGGCCA CATCGAAGGT GCGCGT
     TGGAATGCGG CGTCTGTTCT ACGGTCCGGT GTAGCTTCCA CGCGCA
                                      NT22
                                     ======================
```

Figure 3.3 (a) Ethidium bromide stained 0.7% gel showing the PCR product of the internal fragment of *L. mexicana* MST. Lane 1- a 410 bp internal fragment (arrowed); Lane 2, DNA standards. (b) Nucleotide sequence of an internal fragment of the *L. mexicana* MST. Nucleotides are numbered in the 5' to 3' direction, beginning with the first nucleotide of the NT21 primer. Primer sites (NT21 & NT 22) are coloured blue.

3.3.1.2 RACE

To identify the ORFs of the MST genes, nucleotide sequence of the 410 bp internal PCR fragment was used to design gene-specific primers (Table 3.1) which were used in conjunction with a spliced leader primer and an oligo dT primer for 5' and 3' RACE, respectively. These primers amplified distinct band(s) at 650 bp (*L. mexicana*) and 900 bp and 660 bp (*L. major*), in addition to other minor bands, from cDNA for the 5' ends of the gene (Figure 3.4a). The 3' RACE produced major 1110 bp (*L. mexicana*) and 1000 bp (*L. major*) fragments (Figure 3.4 b). Sequencing of the putative *L. major* sulfurtransferase cDNA obtained from RACE, and assembling them together using the Contig Express software (Vector NTI, Informax), revealed that the AG dinucleotide SL addition acceptor site is 354 nt 5' of ATG start codon, a 1160 bp ORF, and a 173 bp 3'UTR followed by a poly(A) tail (Figure 3.5). Using the same methodology to clone the equivalent gene of *L. mexicana* revealed that this gene have a 89 bp 5' UTR, a 1160 bp ORF and a 255 bp 3'UTR followed by a poly(A^+) tail (Figure 3.6). Further, comparison of the nucleotide sequence from this work with the nucleotide sequence of an annotated HTGS sequence of *L. major* rhodanese (GenBank accession no. AF 163772; gi:13122208) revealed 99% identity. However, two nucleotides positioned at 85 nt and 1014 nt from the putative start codon were missing from the annotated HTGS rhodanese sequence (Figure 3.7). The consequence of these additions was a shift in the ORF and the subsequent introduction of stop codons within the apparent ORF of the HTGS sequence suggesting incorrect N- and C- termini of the gene (Figure 3.7).

Table 3.1 Characteristics of PCR primers used in the amplification of *LmajMST* and *LmexMST* from genomic DNA (gDNA) and cDNA

Primer name	Forward (5'-3')	Primer name	Reverse (5'-3')	Comment
NT21	5' CACCGCCCGGCATCCGCTACC 3'	NT 22	5' ACGCGCACCTTCGATGTGGCC 3'	Internal
LMEXSLI	5' TAACGCTATAAGTATCAGTTT 3'	NT 22	5' ACGCGCACCTTCGATGTGGCC 3'	5' RACE
LMEXSLII	5' AGTATCAGTTTCTGTACTTTATTG 3'	NT 41	5' ATTTCATCCACAAGGTAGTGATGC 3'	5' RACE
UAP	5' CUACUACUACUAGGCCACGCGTGGACTAGTAC 3'	NT 21	5' CACCGCCCGGCATCCGCTACC 3'	3' RACE
AUAP	5' GGCCACGCGTGGACTAGTAC 3'	NT 54	5' ATCGACTGGTGCATGGCGAAC 3'	3' RACE
NT 42	5' CGCTGACATATGTCTGCTCCTGCTGCGCCGAA 3'	NT 55	5' TTCCTCGAGTGGAGGGGCGGAAGAGGCCGCTGT 3'	Entire
NT 42	5' CGCTGACATATGTCTGCTCCTGCTGCGCCGAA 3'	NT 56	5' TTCCTCGAGCGGCAGCGGCGTCACCGC 3'	Entire

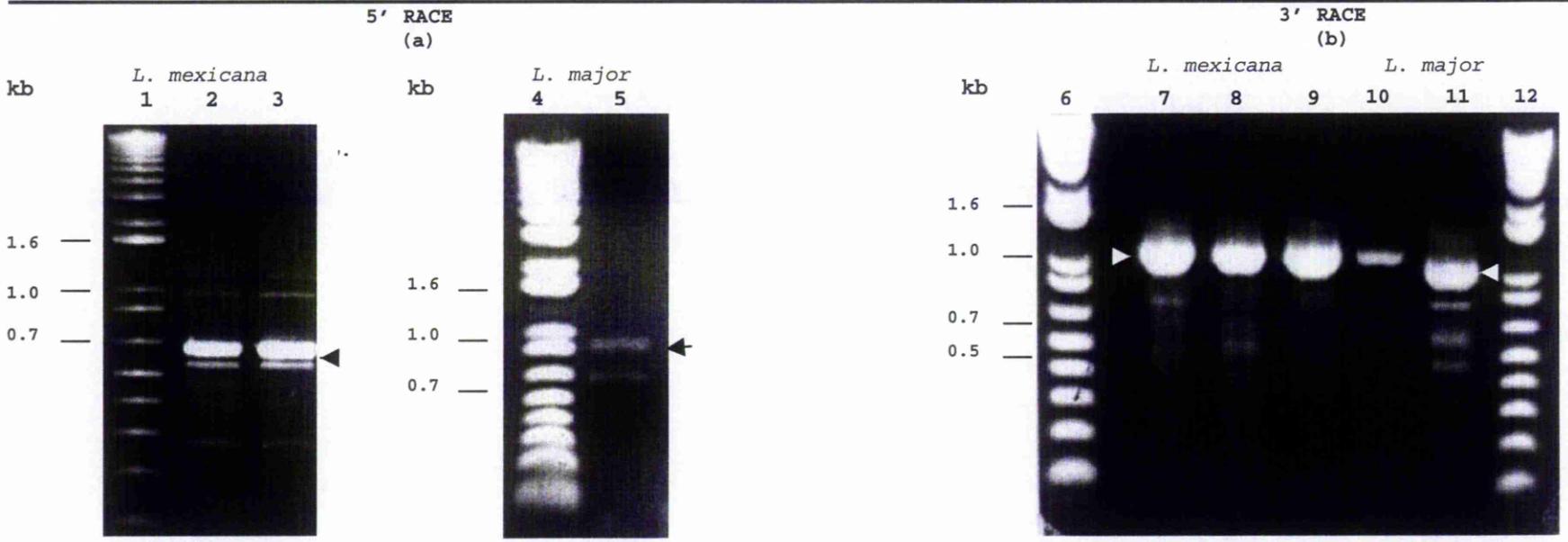


Figure 3.4 Ethidium bromide stained 0.7% gel showing RACE products of MST from *L. major* and *L. mexicana*. (a) Lanes 1 and 4, DNA standards; Lanes 2 and 3, 5'-RACE products from *L. mexicana*; Lane 5, 5' RACE products from *L. major*. (b) Lanes 6 and 12, DNA standards; Lanes 7, 8, and 9, 3'-RACE products from *L. mexicana*; Lanes 10 and 11, 3' RACE products from *L. major*. Arrowed are the fragments of interest amplified by PCR.

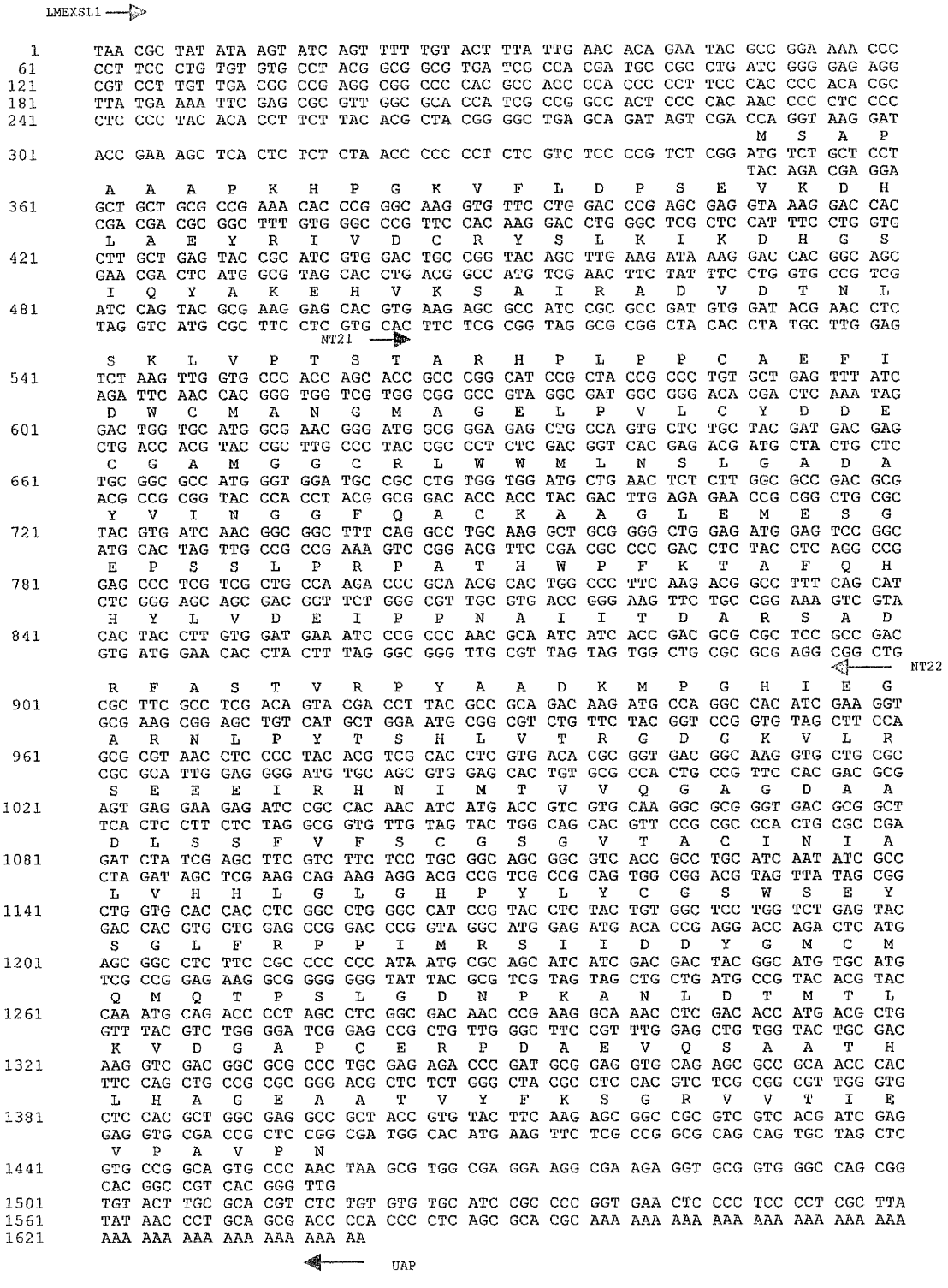


Figure 3.5 Nucleotide sequence of *L. major* MST cDNA and deduced amino acid sequence. Nucleotides are numbered in the 5' to 3' direction, beginning with the first residue at the splice site and ending at the poly(A) tail. The 5' and 3' UTRs nucleotides are coloured blue. The primers, LMEXSL1 & NT 22 and UAP & NT21, used in the RACE to amplify the 5' and 3' ends of the gene respectively, are indicated with red and blue arrows.

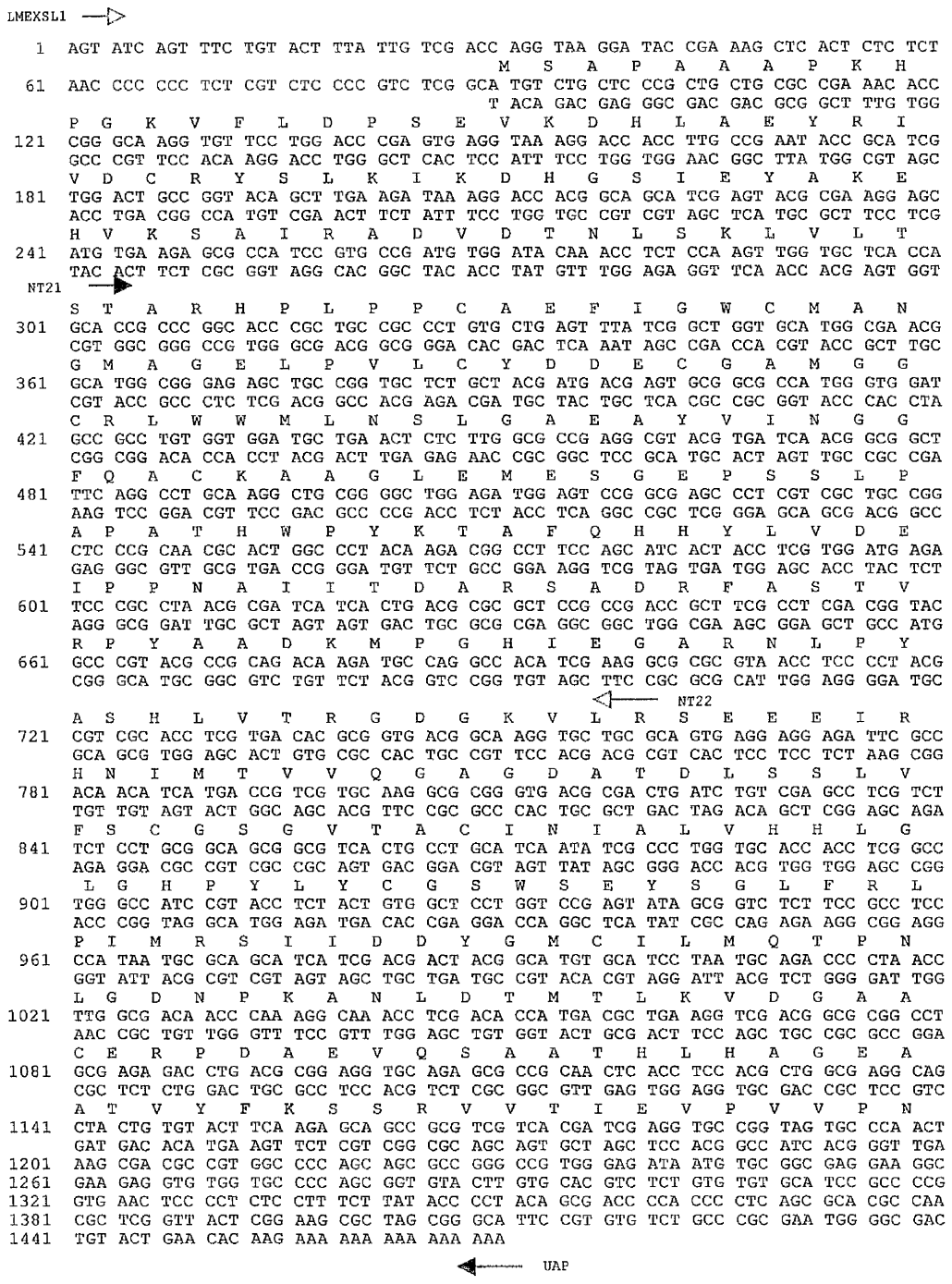


Figure 3.6 Nucleotide sequence of *L. mexicana* MST cDNA and deduced amino acid sequence. Nucleotides are numbered in the 5' to 3' direction, beginning with the first residue at the splice site and ending at the poly(A) tail. The 5' and 3' UTRs nucleotides are coloured blue. The primers, LMEXSL1 & NT 22 and UAP & NT21, used in the RACE to amplify the 5' and 3' ends of the gene respectively, are indicated with red and blue arrows.

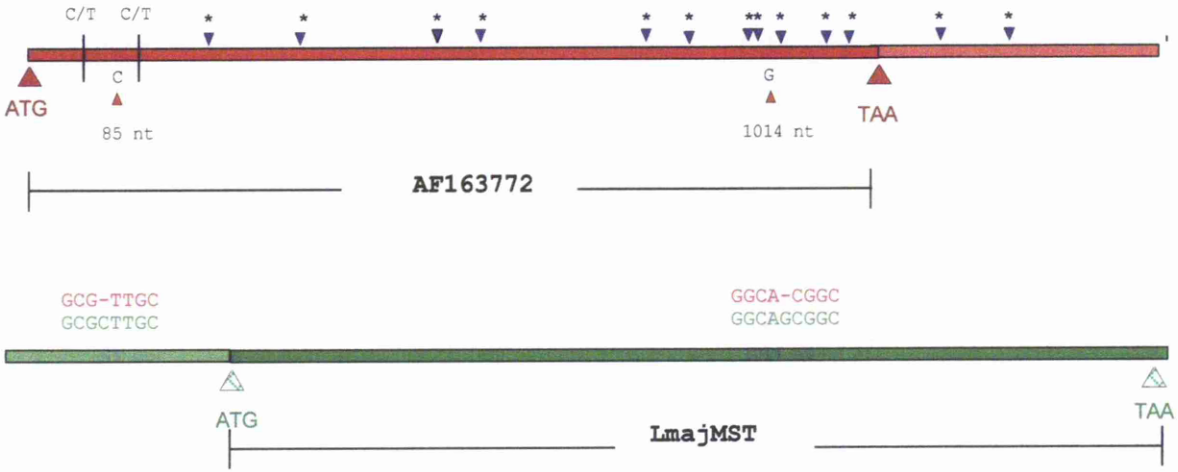


Figure 3.7 Schematic comparison of the putative *L. major* rhodanese gene (red) (GenBank Accession no. AF 163772; gi:13122208) and the *LmajMST* gene (green) (GenBank Accession no. AJ 313201, this study). The ORF ('ATG' start and 'TAA' stop codons highlighted with red arrows) of the putative rhodanese gene shows two nucleotide changes (C/T) and the insertion of two nucleotides (85th and 1014th bp from the putative start included by red arrows) not present in the LmajMST gene, and stop codons (blue arrow and asterisk) arising as a consequence along the ORF. In green is shown the LmajMST ORF derived from RACE (green with 'ATG' start and 'TAA' stop codons highlighted in checked green arrows) and the nucleotides distinguishing the two genes (in red (rhodanese) and green (LmajMST)).

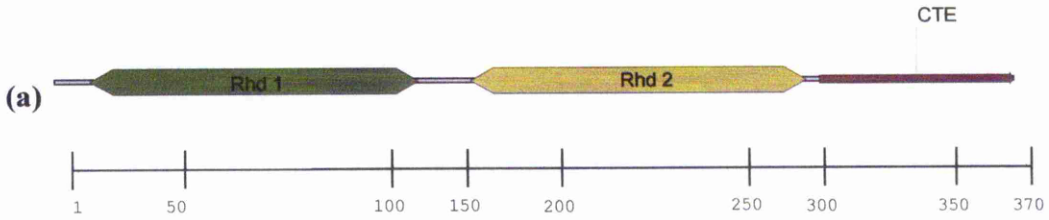
3.3.1.3 Cloning of Leishmania *MST gene*

The 1.1 kb ORFs of *LmajMST* and *LmexMST* were amplified with gene-specific primers NT42 as the forward primer and NT55 and NT56 as reverse primers for *L. major* and *L. mexicana,* respectively (Table 3.1). The predicted amino acid sequences of the sulfurtransferase of each *Leishmania* species comprise 370 residues with a calculated molecular mass of 40.1 kDa (Figure 3.8).

3.3.2 Features of Predicted Amino Acid Sequences of the Sulfurtransferases

3.3.2.1 Sequence alignment and phylogenetic analysis of MSTs amino acid sequences

The deduced amino acid sequences from *L. major* and *L. mexicana* had an internal duplication of the rhodanese domains (Figure 3.8a). Multiple alignments of representative subsets of entries of these two domains detected in genomic databases show them to form two distinct subfamilies (Nagahara *et al.*, 1995; Schultz *et al.*, 1998; Bordo *et al.,* 2000; Papenbrock and Schmidt, 2000, Colnaghi *et al.*, 2001; Bordo and Bork, 2002). Enzymatically active domains (with an active site cysteine) are found in the CDC 25 class of the protein phosphatases, sulfide dehydrogenases, PspE and GlpE in bacteria and arsenate resistance proteins, while inactive domains are common with dual-specificity phosphatases, ubiquitin hydrolases from yeast and in sulfurtransferases. Inactive domains have the key active site cys residue replaced by asp or gly (Ploegman *et al.*, 1978; Bordo *et al.*, 2000). Interestingly, two structural motifs of the superfamily of transfer proteins, designated CH2A and CH2B (Bordo and Bork, 2002), are present in the enzymatically active 'rhodanese' domain of the LmajMST protein (Figure 3.8).



(b)

```
LmajMST     (1) MSAPAAAPKHPGKVFLDPSEVKDHLAEYRIVDCRYSLKIKDHGSIQYAKE
LmexMST     (1) MSAPAAAPKHPGKVFLDPSEVKDHLAEYRIVDCRYSLKIKDHGSIEYAKE

LmajMST    (51) HVKSAIRADVDTNLSKLVPTSTARHPLPPCAEFIDWCMANGMAGELPVLC
LmexMST    (51) HVKSAIRADVDTNLSKLVLTSTARHPLPPCAEFIGWCMANGMAGELPVLC

                 1
LmajMST   (101) YDDECGAMGGCRLWWMLNSLGADAYVINGGFQACKAAGLEMESGEPSSLP
LmexMST   (101) YDDECGAMGGCRLWWMLNSLGAEAYVINGGFQACKAAGLEMESGEPSSLP

                                        ..... CH2A motif .....
LmajMST   (151) RPATHWPFKTAFQHHYLVDEIPPNAIITDARSADRFASTVRPYAADKMPG
LmexMST   (151) APATHWPYKTAFQHHYLVDEIPPNAIITDARSADRFASTVRPYAADKMPG

                .....
LmajMST   (201) HIEGARNLPYTSHLVTRGDGKVLRSEEEIRHNIMTVVQGAGDAADLSSFV
LmexMST   (201) HIEGARNLPYASHLVTRGDGKVLRSEEEIRHNIMTVVQGAGDATDLSSLV

                ..... Active site .....          ..... CH2B motif .....
                2
LmajMST   (251) FSCGSGVTACINIALVHHLGLGHPYLYCGSWSEYSGLFRPPIMRSIIDDY
LmexMST   (251) FSCGSGVTACINIALVHHLGLGHPYLYCGSWSEYSGLFRLPIMRSIIDDY

LmajMST   (301) GMCMQMQTPSLGDNPKANLDTMTLKVDGAPCERPDAEVQSAATHLHAGEA
LmexMST   (301) GMCILMQTPNLGDNPKANLDTMTLKVDGAACERPDAEVQSAATHLHAGEA

LmajMST   (351) ATVYFKSGRVVTIEVPAVPN
LmexMST   (351) ATVYFKSSRVVTIEVPVVPN
```

Figure 3.8 (a) Schematic representation of the inactive (green), and catalytically active (dark yellow) rhodanese domains and the C-terminal extension (CTE) (red) of the MST from *Leishmania*. (b) Alignment of the deduced amino acid sequences of MST from *L. major (LmajMST) and L. mexicana (LmexMST)* with the inactive and catalytically active rhodanese domains and the C-terminal extension present in both proteins shown in green, dark yellow and red, respectively. The positions of the structural motifs of CH2A (blue) and CH2B (light blue), and the active site (black), present in the catalytically active domain of *Leishmania* protein are also shown. Catalytic residues discussed in the text are indicated as 1and 2.

These motifs are vital in protein substrate recognition and protein-protein interactions in tyrosine and dual-specificity phosphatases (Fauman *et al.*, 1998). It is intriguing to

speculate that the CH2A and CH2B motifs of the sulfurtransferases play a similar role in recognition of dithiol protein substrates discussed in Chapter 4.

Alignment of the predicted amino acid sequences of leishmanial MSTs and other sulfurtransferases showed several important aspects of the protein. Intriguingly, both of the leishmanial sulfurtransferases contain an additional C-terminal domain beyond the two rhodanese domains when compared with all other MSTs known (Figures 3.8 and 3.9). The domain, which comprises 70 amino acids compared with the human gene (accession number P25325), has no sequence identity with any other known protein sequence. However, a similar C-terminal domain is also present in an homologous protein of *T. brucei* (accession no. EMBL AC091553, Figure 3.9) and also the protein predicted from contig compiled from GSS gene fragments identified in the genome database for *T. cruzi* (accession no. EMBL:AI667879). Thus it appears that this domain of the protein is a feature characteristic, and perhaps unique, to the MSTs of trypanosomatids. The predicted amino acid sequences of *L. major* and *L. mexicana* were 97 % identical to each other and 47 % identical to a putative *T. brucei* sulfurtransferase identified in the *T. brucei* genome database (accession no. EMBL: AC 091553). Of the other sulfurtransferases known, the most similar are the mammalian and plant mercaptopyruvate and thiosulfate sulfurtransferases, which are approximately 23 % identical within the main domains (excluding the C-terminal extensions of the *Leishmania* sequences). However, other sulfurtransferases (such as *E. coli* and mammalian) are almost as similar based solely on levels of identity (Table 3.2). A phylogenetic analysis of protein sequences from plants, bacteria and vertebrates was used

Table 3.2 Sequence identities and similarities of selected sulphurtransferases to *L. major* mercaptopyruvate sulphurtransferase.

Organism	Similarities (%)	(%)Identities (%)
Arabidopsis thailana MST	21.4	14.0
Arabidopsis thailana TST	24.0	16.0
Distisca glomerta TST	20.4	13.5
Mycobacterium leprae TST	21.1	12.6
Escherichia coli TST	28.1	17.0
Escherichia coli SseA	23.3	15.0
Azotobacter vinelandii	23.5	14.4
Human MST	26.6	17.0
Human TST	25.2	18.3
Mouse TST	25.5	17.5
Bovine TST	21.9	14.7
Rat MST	29.0	19.1
Leishmania mexicana	96.7	96.7

Mercaptopyruvate sulfurtransferase (MST) and thiosulfate sulfurtransferase (TST) sequences were from *L. mexicana* (this study), *Arabidopsis thialiana* MST (accession no. BAA85148 gi 6009981), *Distisca glomerta* TST (accession no AAD19957), rat MST (accession no P97532), mouse MST (accession no P52196), Human MST (accession no P25325), human TST, (accession no. NM 003312.2 gi 17402865), ovine TST (accession no. P00586), *E. coli* TST (accession no. P31142), *Azotobacter vinelandii* (accession no. EMBL AC091553) and *Escherichia coli* SsEA.

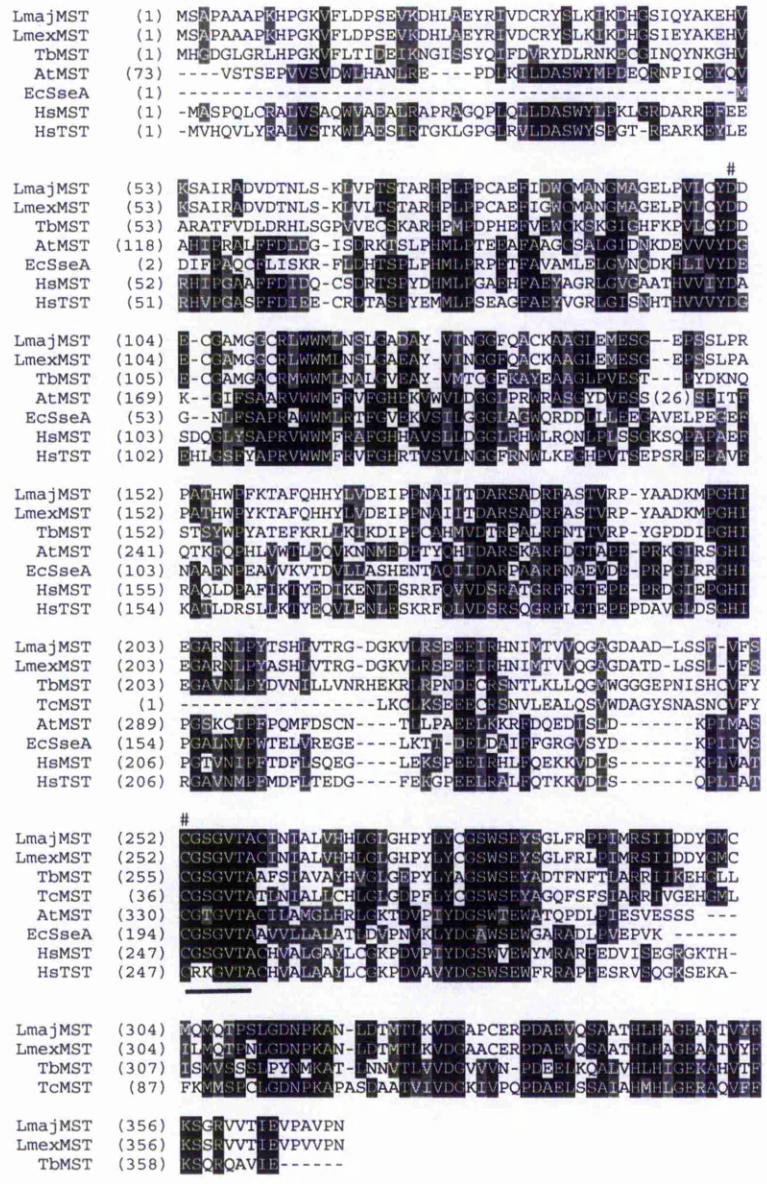


Figure 3.9. Alignment of deduced amino acid sequences of MSTs from *Leishmania* and representative organisms. Sequences are as follows: Lmaj, *L. major* ; Lmex, *L. mexicana* (both this study); Tb, *Trypanosoma brucei* (accession no. EMBL AC091553); Tc, *Trypanosoma cruzi* (accession no. EMBL:AI667879); At, *Arabidopsis thialiana* (accession no. BAA85148 gi 6009981); Ec, *Escherichia coli* SseA protein (accession no. P31142); Hs, human (accession no. P25325); HsTST, human TST (accession no. NM 003312.2 gi 17402865). These sequences were aligned using Align X (VectorNTI). Black shading indicates identical amino acids, grey shading indicates conserved amino acid substitutions. The conserved active site sequence is underlined. The unique N-terminal and hinge domains of AtMST have been omitted, the number of amino acids that have been removed are indicated in parentheses. The *T. cruzi* sequence is incomplete and comprises only the C-terminal segment of the protein. Catalytic residues present in both the N-terminal and C-terminal domain of the protein (discussed in the text) are marked with (#).

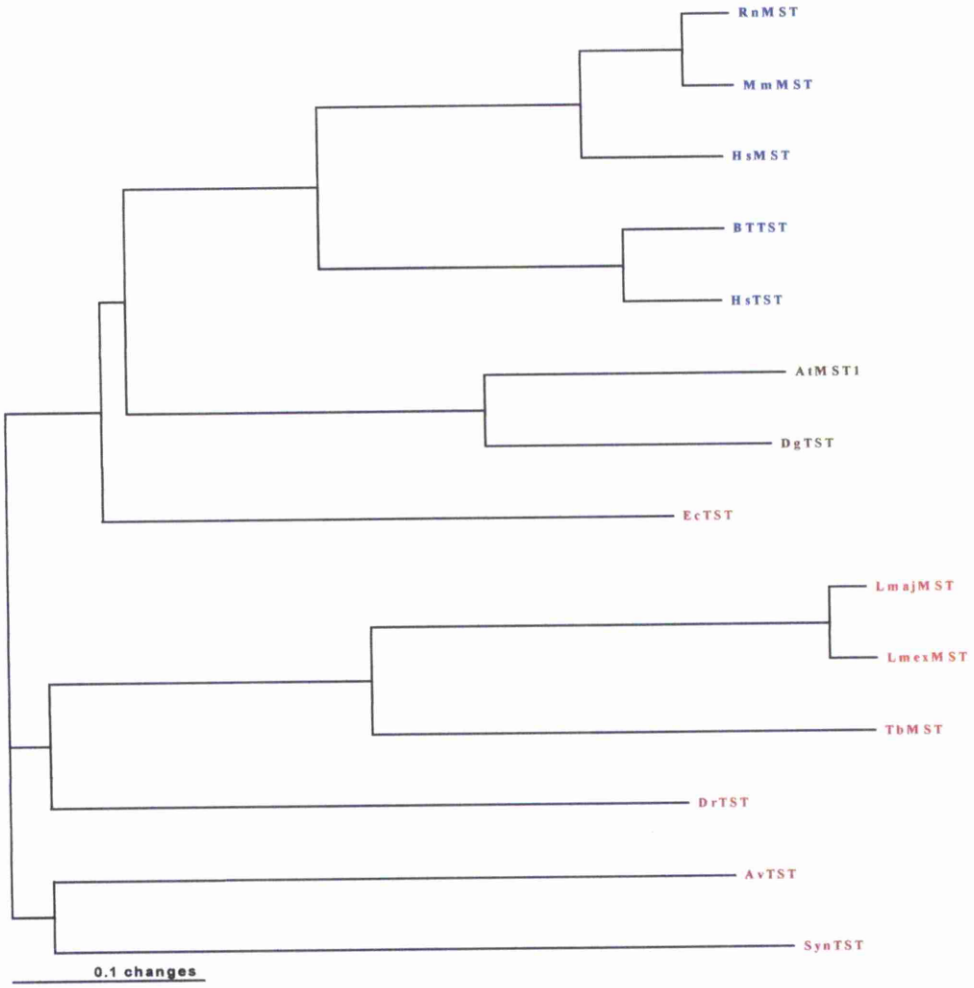


Figure 3.10 Phylogenetic analysis of sulfurtransferase amino acid sequences. The phylogenetic tree was plotted using Treeview (Page, 1996). Sulfurtransferase sequences were from: trypanosomatids (red) (TbMST, *Trypanosoma brucei* (accession no. AC091553); LmajMST, *L. major*; LmexMST, *L. mexicana* (both this study); plants (green) (AtMST, *Arabidopsis thialiana* MST (accession no. BAA85148 gi 6009981); DgTST, *Distisca glomerta* TST (accession no AAD19957); mammals (blue) (RnMST, rat MST (accession no P97532); Mm, mouse MST (accession no P52196); HsMST, human MST (accession no P25325), HsTST, human TST), (accession no. NM 003312.2 gi 17402865); BtTST, bovine TST (accession no. P00586); and bacteria (brown) (EcTST, *E. coli* TST (accession no. P31142), DrTST, *Deinococcus radiodurans* (accession no. NP_296251.1; gi:15807515); Av, *Azotobacter vinelandii* (accession no. EMBL AC091553) and SynTST, *Synechococcus sp* (accession no AAA27364.1 GI:154604). The unique C- and N- terminal extensions of the trypanosomatids and plant proteins were excluded in the development of the tree.

to generate a multiple sequence alignment (Figure 3.10) that might shed some light in the divergent 'function' observed with sulfurtransferases from different organisms. The vertebrate sulfurtransferase which are known to use thiosulfate or mercaptopyruvate as substrates clustered in the same group. The proteins from plants were also clustered close to the vertebrate group. The *E. coli* SseA (TST) is the only prokaryotic protein closely related to the vertebrate sulfurtransferases and it has recently been shown to have MST activity (Colnaghi *et al.*, 2001). The MSTs from the trypanosomatids clustered together, and were more closely related to the bacterial putative MSTs which might suggest similar biochemical and biophysical properties. Papenbrock and Schmidt (2000) produced a similar alignment with completely different sequences, the putative transit peptides. The presence of mitochondrial import sequences in the mammalian TSTs protein sequences showed that they are mainly localised in and imported into the mitochondria, but some cytosol-localised proteins also occur. The MSTs do not show these mitochondrial import sequences and are thus thought to be cytosolic. The PSORT software (http://psort.nibb.ac.jp) predicts that the LmajMST protein lacks a mitochondrial targeting sequence and so is likely to be cytosolic and non-secretory. This was verified using immuno-fluorescence and sub-localization techniques (see chapter 4).

3.3.2.2 Active site motif and catalytic activities

The sequence analysis of the sulfurtransferases from vertebrates, plants, bacteria and protozoa (Figure 3.9) showed a conserved active site domain (underlined in the Figure 3.9). The residues contributing to the active site motif of sulfurtransferases are contained within the second rhodanese domain. The conserved cysteine (cys^{253}) that covalently bonds to the transferred sulfane sulfur atom is conserved and resides at a

comparable site in all six of the sequences aligned in Figure 3.9. The active site motifs of the leishmanial proteins were more similar to other mercaptopyruvate sulfurtransferases (with an active site motif of CGSGVTA/ CGTGVTA) than the eukaryotic thiosulphate sulfurtransferases and rhodaneses (with an active site motif of CRKGVTA). Interestingly, prokaryotic genes thought to encode thiosulfate sulfurtransferases lack the CRKGVTA motif, although in most cases the substrate specificity has not been fully analysed. Bacterial *SseA* proteins from *E. coli* with mercaptopyruvate sulfurtransferase activities have the CGTGVTA active site motif. As the catalytic active site motif CGSGVTA of the *Leishmania* genes (underlined in Figure 3.8) was considered predictive of a mercaptopyruvate sulfurtransferase rather than a rhodanese or thiosulphate sulfurtransferase (Pagani *et al.*, 2000), the genes were designated as *LmajMST* and *LmexMST* (Figure 3.9). In rat MST, this active site motif works in conjunction with two arginines (arg^{187} and arg^{196}). They have been shown to be vital for catalytic activities of MSTs (Figure 3.10) (Weng *et al.*, 1978; Ploegman *et al.*,1978, 1979; Luo and Horowitz, 1994). The positively charged arg^{187} polarizes the carbonyl group oxygen atom of mercaptopyruvate, causing an electron strain in mercaptopurvate which accelerates catalysis by cys^{247} facilitating a nucleophilic attack on the sulfur of mercaptopyruvate. Arg^{187} (Human MST mumbering) was conserved in TSTs and MST while arg196 was unique to MSTs, being absent from TSTs. This residue was not conserved in LmajMST (arg^{185}) (Figure 3.9), although there is an arg at 191.

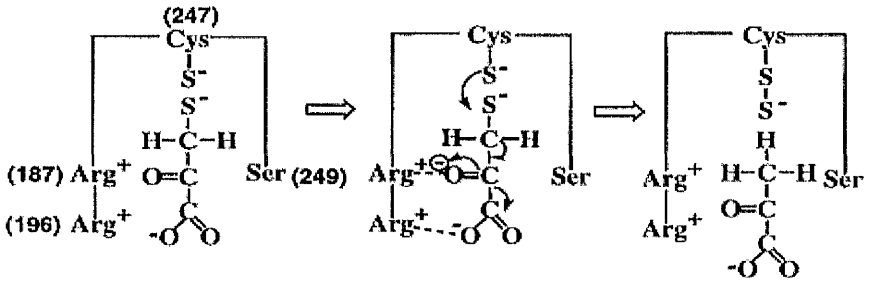


Figure 3.11. Proposed binding site of mercaptopyruvate in rat MST and a possible catalytic mechanism. The positively charged side chain of arg^{187} and arg^{196} interact with and polarize the carbonyl group of mercaptopyruvate which causes an electron strain and facilitates a nucleophilic attack by cys^{247} on the sulfur of mercaptopyruvate. Source:- Nagahara and Nishino (1996)

The active site motif mentioned above is not present in other putative prokaryotic MSTs. These proteins had either the motif CRIGER (*M. leprae* and *M. tuberculosis)* or CQTHHR (*A. vinelandii*). Interestingly, this motif from *A. vinelandii* is present in the putative *L. major* TST (GenBank accession no gi:13122208). Both proteins show a very high degree of similarity (85%) at the nucleotide and amino acid level (Figure 3.12) and have the putative active site cys residue at a strictly comparable site. However, PCR failed to amplify a fragment from the gDNA of *L. major* and so study of the apparent gene was not possible.

Figure 3.12. Alignment of a putative thiosulfate sulfurtransferases (TST) from *Leishmania* and representative organisms. Sequences are as follows: Lmaj, *L. major*; (GeneDB accession no gi:13122208); Av, *Azotobacter vinelandii* (accession no. EMBL AC091553); Ml, *M. leprae* (accession no. EMBL:AI667879). These sequences were aligned using Align X (VectorNTI) and boxshade. Black shading indicates identical amino acids, grey shading indicates conservative amino acid substitutions. The active site sequences are underlined.

3.3.3 Genomic Organisation of the MSTs

3.3.3.1 Genomic analysis of the LmajMST region in chromosome 5

When the regions surrounding the *LmajMST* gene were analysed using the incomplete genomic sequence of the *L. major* Friedlin genome sequencing project, the apparent ORFs of NADH dehydrogenase (EC 1.6.99.3) (GI:13122209) and a probable dipeptidyl-peptidase III (EC 3.4.14.4) were deduced to be positioned 999 bp upstream of the MST start codon and 1234 bp downstream of the MST stop codon, respectively. Within the two MST flanking sequences, are two 439 bp perfect repeats in the same orientation. The repeat regions are located 146 bp upstream and 438 bp downstream from the start and stop codons of the *LmajMST* gene, respectively (Figure 3.13).

Figure 3.13. Genomic analysis of the *LmajMST* region in chromosome 5. Flanking genes (blue), (NADP dehydrogenase (EC 1.6.99.3) (ORF1) at its 5' end and dipeptidyl-peptidase III (EC 3.4.14.4) (ORF2) at its 3' end) and the *LmajMST* gene (orange) showing the 5' and 3' UTRs deduced by RACE analysis. The positions of two 440 nt repeats within the 5' and 3' UTRs are highlighted in red. The positions of the splice site and the poly (A) site are shown.

The 439 bp repeat upstream of the N-terminal end was contained within the 5'UTR obtained from the RACE experiments, while the 438 repeat downstream of the C-terminal end of the gene was outwith the poly(A) site. The repeats do not seem to be replicated at other sites within the genome, as blast searches of the ongoing *L. major* sequencing project detected no other copies. The significance of these repeats is not known, but repeats in the 3' UTRs of trypanosomatid genes have been shown to have regulatory function (Boucher *et al.*, 2002).

To assess the copy number of the leishmanial *MST*s, genomic DNA was digested with five restriction enzymes and analysed by Southern blotting using the *MST* cDNAs as probes (Figure 3.14a).

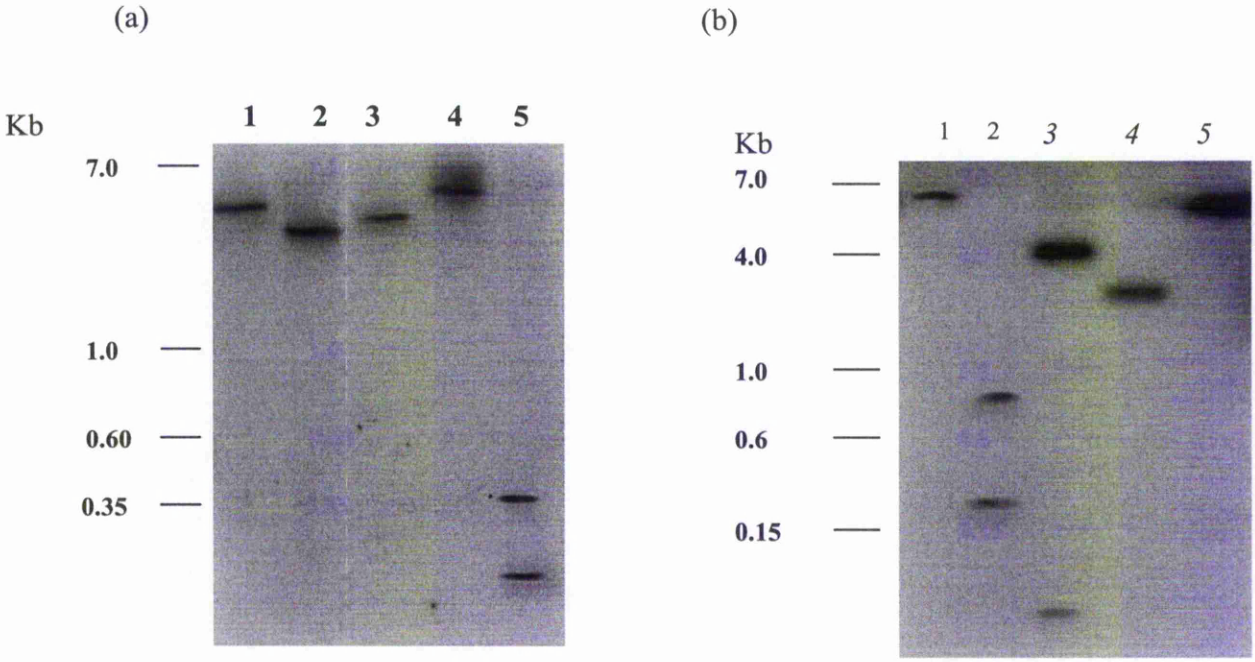


Figure 3.14 Genomic organization of leishmania *MST*s . 5 µg of genomic DNA from *L. major* (bi) and *L. mexicana* (bii) were digested with a variety of restriction endonucleases, separated by agarose gel electrophoresis, transferred onto a nylon membrane, and then hybridised with [^{32}P] dATP-labeled *LmajMST* and *LmexMST* cDNA probes, corresponding to the entire ORFs, respectively. Restriction endonucleases used were: (a) *Pst*1 (lane 1), *Bsa*I (lane2), *Sal*1 (lane 3), and *Eco*RI (lane 4), and *Sau3A*I (lane5) for *L. major*; (b) *Pst*I (lane 1), *Sau*3AI (lane 2), *Bsa*I (lane 3), *Cla*I (lane 4), and *Sal*I (lane 5) for *L. mexicana*. The sizes of DNA standard are indicated in kilobase pairs.

With *L. major*, the enzymes used that do not cut the gene itself, *Pst*I, *Bsa*I, *Sal*I, *Cla*I and *Eco*RI, resulted in a single major hybridising DNA fragment, while *Sau*3A1, which cuts the ORF more than once, resulted in multiple bands (Figure 3.14a). The hybridisation patterns obtained for *L. major* indicate the *LmajMST* gene is probably single copy, although they do not exclude the possibility of two genes in tandem in

single copy, although they do not exclude the possibility of two genes in tandem in the 6 kb fragment shown on Southern. However, the information on genomic environment apparent from the data in the genome database for *L. major*, which show that the gene does not occur in a tandem array, taken together with the Southern data confirm the *LamjMST* is single copy (Figure 3.15). The Southern data for *LmexMST* (Fig. 3.14b) similarly shows that the enzymes *Pst*I, *Sal*I, and *Cla*I did not cut the gene itself and resulted in a single major hybridising DNA fragment, while *Sau*3A1 and *Bsa*I, both of which cut the ORF, resulted in multiple fragments. These data suggest that the gene of *L. mexicana* is also single copy.

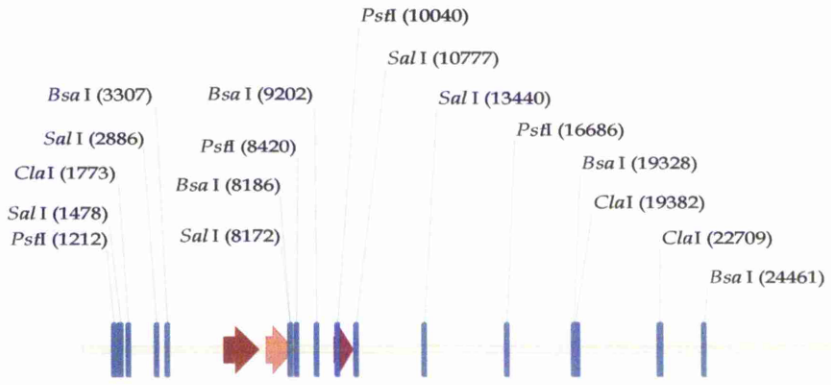


Figure 3.15 The *LmajMST* gene (orange arrow), its flanking genes (red arrows- NADP dehydrogenase (EC 1.6.99.3) at its 5' end and dipeptidyl-peptidase III (EC 3.4.14.4) at its 3' end) and additional nucleotide sequence from chromosome 5 of the *L. major* sequencing project (www.sanger.ac.uk) with the positions of restriction enzyme sites used in the Southern blot shown in (b).

3.3.4 The C-terminal Domain is Required for LmajMST Activity

To investigate the role of the C-terminal domain in the rLmajMST protein, four LmajMSTs were amplified (Figures 3.16 and 3.17).

```
                                                                TMST1 (Δ300-370)
LmexMST (247) SSLVFSCGSGVTACINIALVHHLGLGHPYLYCGSWSEYSGLFRLPIMRSI
LmajMST (247) SSFVFSCGSGVTACINIALVHHLGLGHPYLYCGSWSEYSGLFRPPIMRSI
TbMST   (150) SHCVFYCGSGVTAAFSIAVAYHVGLGEPYLYAGSWSEYADTFNFTLARRI
TcMST    (30) SNCVFYCGSGVTATLNIALLCHLGLGDPFLYCGSWSEYAGQFSFSIARRI

                        TMST2 (Δ320-370)                TMST3 (Δ338-370)
LmexMST (297) IDDYGMCILMQTPNLGDNPKAN-LDTMTLKVDGAACERPDAEVQSAATHL
LmajMST (297) IDDYGMCMQMQTPSLGDNPKAN-LDTMTLKVDGAPCERPDAEVQSAATHL
TbMST   (150) IKEHGLLISMVSSSLPYNMKATLNNV-TLVVDGVVVN-PDEELKQALVHL
TcMST    (80) VGEHGMLFKMMSPCLGDNPKAPASDAATVIVDGKIVPQPDAELSSAIAHM

                        TMST4 (Δ360-370)
LmexMST (346) HAGEAATVYFKSSRVVTIEVPVVPN
LmajMST (346) HAGEAATVYFKSGRVVTIEVPAVPN
TbMST   (150) HIGEKAHVTFKSQRQAVIEAHPRIDT
TcMST   (130) HLGERAQVFF----------------
```

Figure 3.16 Alignment of deduced amino acid sequences of MSTs from trypanosomatids. Sequences are as follows: Lmaj, *L. major*; Lmex, *L. mexicana* (both this study); Tb, *Trypanosoma brucei* (accession no. EMBL AC091553); Tc *Trypanosoma cruzi* (accession no. EMBL:AI667879); These sequences were aligned using Align X (VectorNTI). Blue and red lettering indicate identical amino acids. The conserved active site sequence is bold. Oligonucleotides, TMST1, TMST2, TMST3 and TMST4 designed for amplification of truncated proteins of the C-terminal extension of *L. major* are underlined. These primers were used in conjunction with NT42 primer.

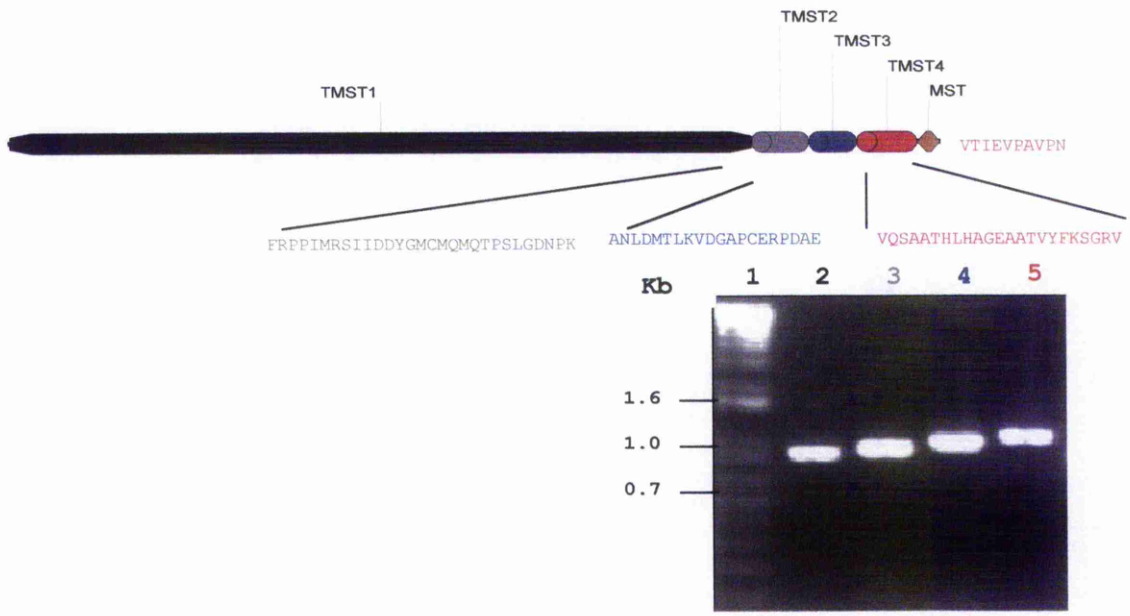


Figure 3.17 Schematic representation of the proteins with truncations of the C-terminal extension of *Leishmania* MST:- LmajMSTΔ300-370 (black) LmajMSTΔ320-370 (grey) LmajMSTΔ338-370 (blue) and LmajMSTΔ360-370 (red), respectively. Amino acid sequences of the C-termini of the soluble proteins and the full length LmajMST (brown) are given and colour coded appropriately. (c) Ethidium bromide stained 0.7% gel showing the PCR products of the truncated MSTs. Lane 1, DNA

and expressed with truncations in the C-terminal domain (Figure 3.18). The choice for the truncations was based on a tertiary structure prediction of the PHD software (Expasy) (Figure 3.19). The reliability of the prediction is questionable, as the proposed model was based on homology to known tertiary protein structures (which is very limited at present). Assuming that the predictions are reliable, the sites chosen for deletion were outwith the α-helices and β-sheets. Although the truncated proteins were expressed in

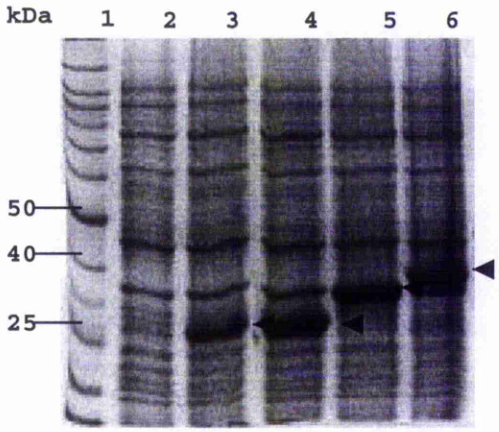


Figure 3.18 SDS-PAGE analysis of *E. coli* expressing *pETLmajMSTΔ300-370*, *pETLmajMSTΔ320-370*, *pETLmajSTΔ338-370* and *pETLmajMSTΔ360-370* plasmids (see section 3.2.2). Expected recombinant protein sizes (arrowed) were 32.18 kDa, 34.71 kDa, 36.75 kDA, and 39.23 kDa. Lane 1, molecular mass standards; lane 2, uninduced *E. coli* with *pETLmajMSTΔ320-370* plasmid; lane 3, LmajMSTΔ300-370; lane 4, LmajMSTΔ320-370; lane 5, LmajMSTΔ338-370; lane 6, pETLmajMSTΔ360-370.

E. coli in similar amounts to the full length rLmajMST (Figure 3.18), removal of C-terminal peptides substantially changed the solubility of the protein. rLmajMSTΔ300-370, the protein lacking the entire C-terminal domain, and rLmajMSTΔ320-370 were expressed entirely in inclusion bodies (Figure 3.20).

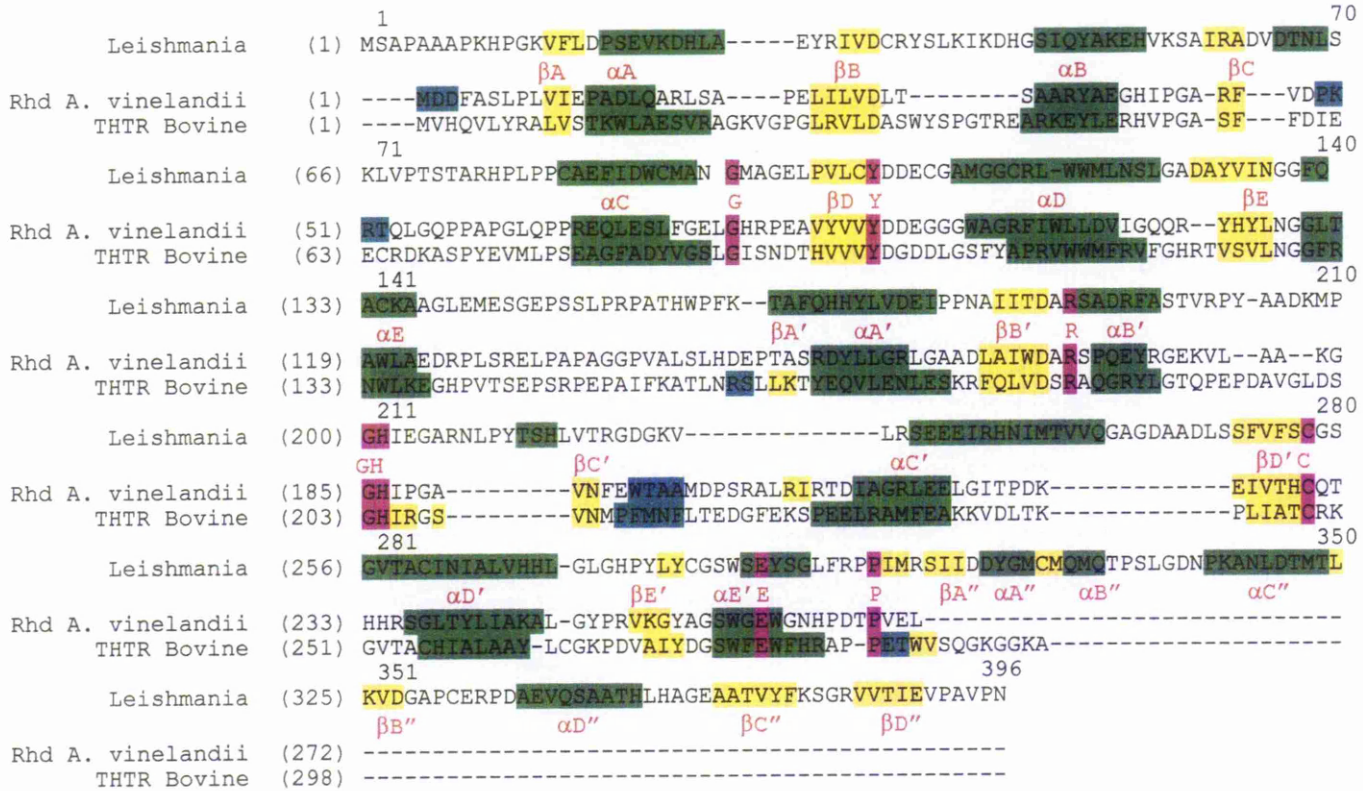


Figure 3.19 Multiple amino acid sequence alignment of known sulfurtransferase sequences. A superimposition of the amino acid sequence of *A. vinelandii* (Rhd A. vinelandi) and bovine (THTR Bovine) rhodanese tertiary strucure showing α-helixes (green) and β-sheets (yellow) (Bordo *et al.*, 2000). The LmajMST 3D structure predicted by the PHD software was superimposed to align with corresponding α-helical and β-strands from the structurally derived Rhd A. vinelandi and THTR Bovine alignment. The nomenclature used to label the α/β topology (in red) of the 3D structure were αA-E and βA-E for the inactive domain, αA'-E' and βA'-E' for the enzymatic active domain. The unusual C-terminal domain of leishmanial MST was labelled αA"-D" and βA"-D". Key residues of the THTR Bovine 3D structure are highlighted in pink.

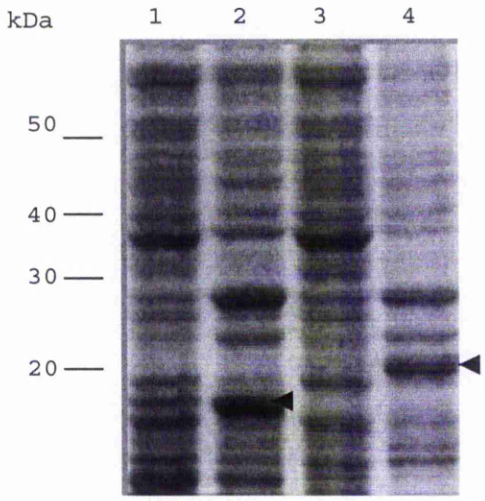


Figure 3.20 SDS PAGE analysis showing the insolubility of rLmajMSTΔ300-370 and rLmajMSTΔ320-370. *E. coli* expressing *L. major* rLmajMSTΔ300-370 and rLmajMSTΔ320-370 proteins were lysed in 250 mM sodium phosphate buffer (pH 8.0), containing 0.3 M NaCl and centrifuged at 13000 x g for 30 min. Lanes 1 & 2, supernatant and pellet of LmajMSTΔ300-370; lanes 3 & 4, supernatant and pellet of LmajMSTΔ320-370.

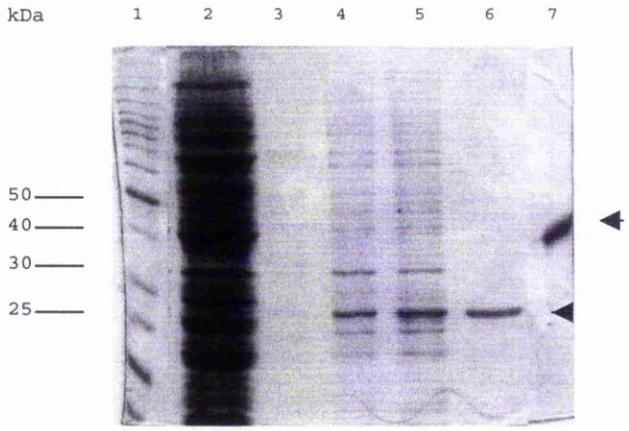


Figure 3.21 Purification of rLmajMSTΔ300-370 under denaturing conditions. The protein in inclusion bodies was solubilised in 250 mM sodium phosphate buffer (pH 8.0), containing 0.3M NaCl (buffer A) and 6 M urea for 2 h., centrifuged at 13000 x g for 30 min, and the supernatant purified on a nickel chelator column. The His-tagged protein was eluted by addition buffer A with 250 mM imidazole. SDS-PAGE analysis of denatured samples are: Lane 1, molecular mass standards; lanes 2 and 3, supernatant and pellet of the crude extract of the recombinant *E. coli* cells, respectively; lanes 4 and 5, column eluants from washes with buffer A with 60 mM imidazole; lane 6 and 7 purified rLmajMSTΔ300-370 (arrowed) and LmajMST (arrowed) eluted from the nicked chelator column with buffer A with 250 mM imidazole. Estimated recombinant protein sizes: 32 and 40 kDa, respectively.

Using different growth conditions that are known to result in a soluble protein in other cases, such as growing and inducing cells under osmotic stress in the presence of sorbitol and glycyl betaine (Blackwell and Horgan, 1991; Barth *et al.*, 2000), lowering temperature (15-37°C) and using different IPTG concentrations (0.1-1mM), all failed to yield a soluble protein. Solubilisation of these proteins using 6 M urea for 60 min and subsequently attempting to refold the enzyme (Figure 3.21) failed to result in active enzyme. Shorter truncations (of 24 or 10 amino acids) yielded some soluble recombinant protein (Figure 3.22).

These rMSTs prepared had greatly diminished MST activity (0.3 and 3 μmol min^{-1} mg $protein^{-1}$, respectively, see section 2.10) compared with the full-length protein (343 μmol min^{-1} mg $protein^{-1}$) (Table 3.3).

Table 3.3 Solubility, specific activity and length of LmajMSTs

Protein	**Solubility**	**Specfic activity (U mg $protein^{-1}$)**	**Length of protein (amino acids)**
LmajMSTΔ300-370	Insoluble	ND	320
LmajMSTΔ320-370	Insoluble	ND	330
LmajMSTΔ338-370	Partially soluble	0.3	338
LmajMSTΔ360-370	Partially soluble	3	360
LmajMST	Soluble	295	370
LmexMST	Soluble	345	370

ND- Not Detected

A plot of velocity versus mercaptopyruvate concentration did not produce a hyperbolic curve when MST activity was measured with mutant MSTs (data not

shown), whereas it did with the native MST. This low activity and deviation from Michaelis Menten kinetics suggested that removal of the end of the C-terminal domain interferes either with protein folding or catalytic activity (full analysis of rLmajMST is described in Chapter 4).

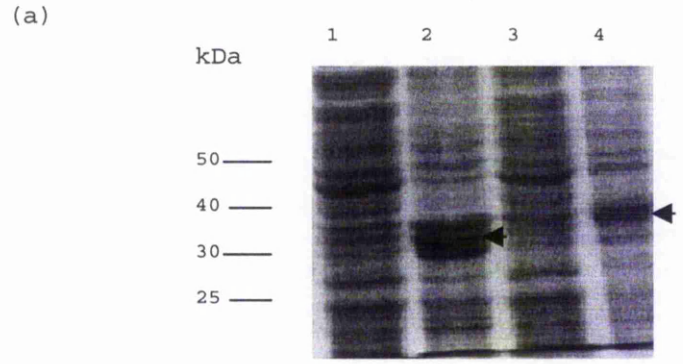


Figure 3.22 SDS-PAGE analysis showing the solubility of rLmajMSTΔ338-370 and rLmajMSTΔ360-370. *E. coli* expressing rLmajMSTΔ338-370 and rLmajMSTΔ360-370 proteins were lysed in 250 mM sodium phosphate buffer (pH 8.0), containing 0.3M NaCl (buffer A) and centrifuged at 13000 x g for 30 min. Lanes 1 and 2, supernatant and pellet of LmajMSTΔ338-370; lanes 3 and 4, supernatant and pellet of LmajMSTΔ360-370. Positions of the truncated MST proteins in the soluble fractions are arrowed.

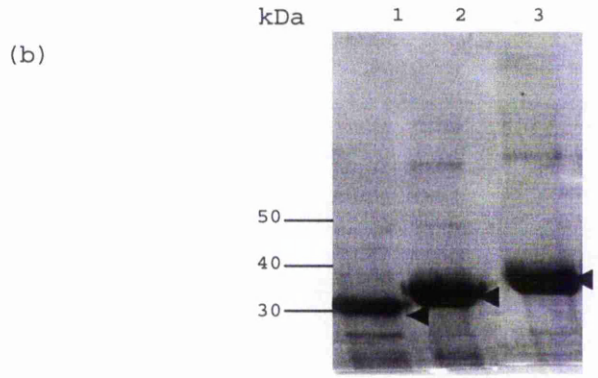


Figure 3.23 SDS-PAGE analysis of the purification of rLmajMSTΔ338-370, rLmajMSTΔ360-370 and rLmajMST. The His-tagged proteins were eluted by addition of 250 mM sodium phosphate buffer (pH 8.0), containing 0.3M NaCl and 25 mM imidazole. Lanes 1, 2 and 3, purified rLmajMSTΔ338-370, rLmajMSTΔ360-370 and rLmajMST eluted from the nicked chelator column. Positions of the purified MSTs proteins are arrowed.

3.3.5 Circular Dichroism and bisANS fluorescence analysis

Circular dichroism was used to investigate the two hypotheses outlined in the previous section. Circular dichroism (CD) analyses of the full length and two soluble, truncated proteins (Figure 3.24) showed that all three had a similar secondary structural conformation (18% α-helix, 26% β-sheet, 23% turn, 33% other) when analysed by the SELCON method (Sneerama and Woody, 1993).

(a)

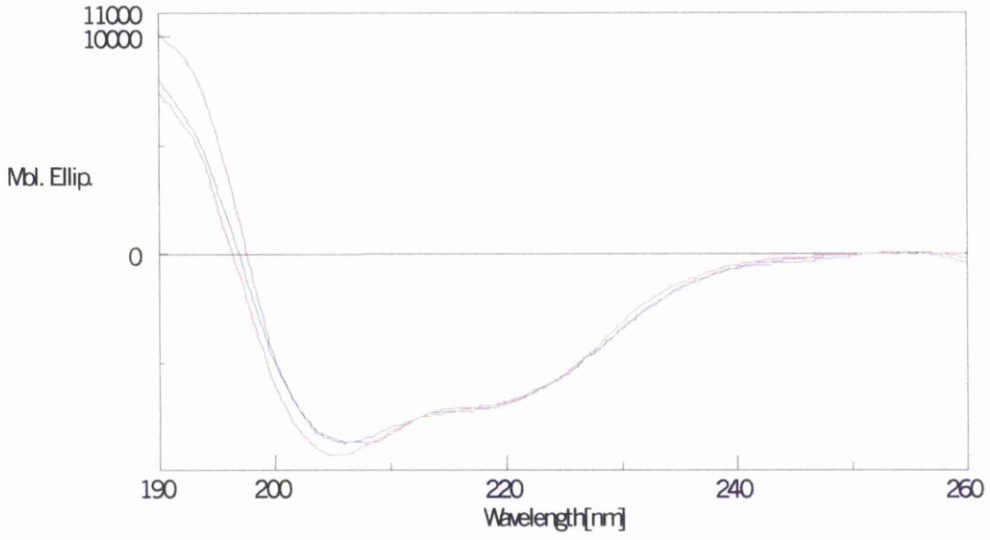


Figure 3.24. CD spectra of rLmajMST proteins. (a) The far UV CD spectra of the full length rLmajMST (pink) did not differ considerably from those of rLmajMSTΔ360-370 (red) and rLmajMSTΔ338-370 (blue). Mol. Ellip.=molecular ellipticity in units of deg.cm^2.dmol^{-1}.

Below 202 nm, there are slight differences at the point where the spectra intercept the x-axis. The differences may reflect very minor perturbations in the alpha helical

regions of the protein as a result of the truncations (Table 3.4). However, there was a marked difference in the near UV spectra, especially in the region between 270 and 290 nm, between wild type protein and the truncated derivatives (Figure 3.25).

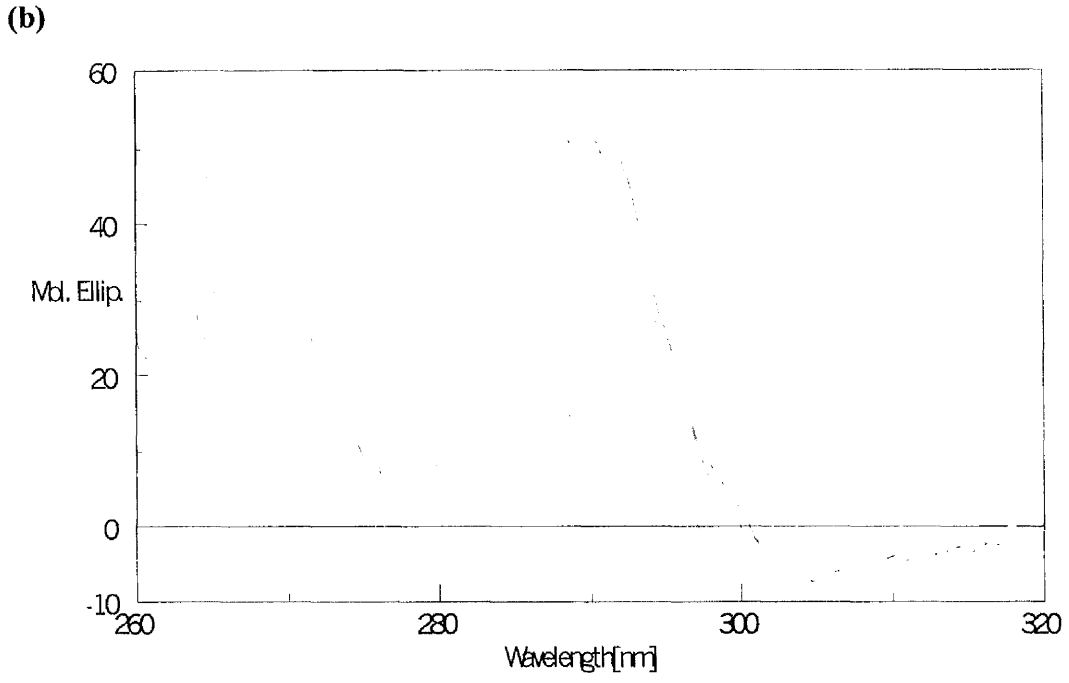


Figure 3.25. CD spectra of rLamjMST and truncated forms. The near UV CD spectra of the full length rLmajMST (pink) differed considerably from those of rLmajMSTΔ360-370 (red) and rLmajMSTΔ338-370 (blue). Mol. Ellip.=molecular ellipticity in units of deg.cm^2.dmol^{-1}.

These results suggested that the consequence of the truncations was a less well-folded protein with significant alterations to the environments of the tyrosine side chains.

Bis-ANS is a sensitive probe that measures the number of hydrophobic sites on protein surfaces and detects partially folded intermediates in protein-folding pathways (Horowitz and Criscimagna, 1990).

Table 3.4. Secondary structure parameters of LmajMST predicted by CD analysis using CONTIN and SELCON

	Protein	**alpha-helix (%)**	**beta-sheet (%)**	**beta-turn (%)**	**remainder (%)**	
CONTIN	LmajMST	13 ± 1.2	50 ± 1.3	22 ± 1.3	15 ± 2.0	-
	LmajMSTΔ360-370	15 ± 0.5	46 ± 0.7	14 ± 0.6	24 ± 0.8	-
	LmajMSTΔ338-370	15 ± 0.4	43 ± 0.6	16 ± 0.5	26 ± 0.7	-
	Protein	**alpha-helix (%)**	**anti-parallel beta-sheet (%)**	**parallel beta-sheet (%)**	**turns (%)**	**others (%)**
SELCON	LmajMST	19.2	21.9	2.3	21.7	33.0
	LmajMSTΔ360-370	17.4	24.3	2.1	22.9	33.1
	LmajMSTΔ338-370	17.1	24	2.3	22.7	32.8

The basis of its application is that less well-folded proteins will have more hydrophobic sites exposed on their surface and so will incorporate more bisANS and give higher fluorescence intensity. Binding of bis-ANS to proteins is non-covalent and involves a combination of electrostatic and hydrophobic modes (Horowitz and Criscimagna, 1990). The truncated proteins exhibited greater fluorescence relative to the native full-length MST (Figure 3.26), which is consistent with hydrophobic residues normally buried in the native enzyme being exposed in the truncated proteins and

supports the conclusions from the CD analyses that the truncated proteins were less well-folded.

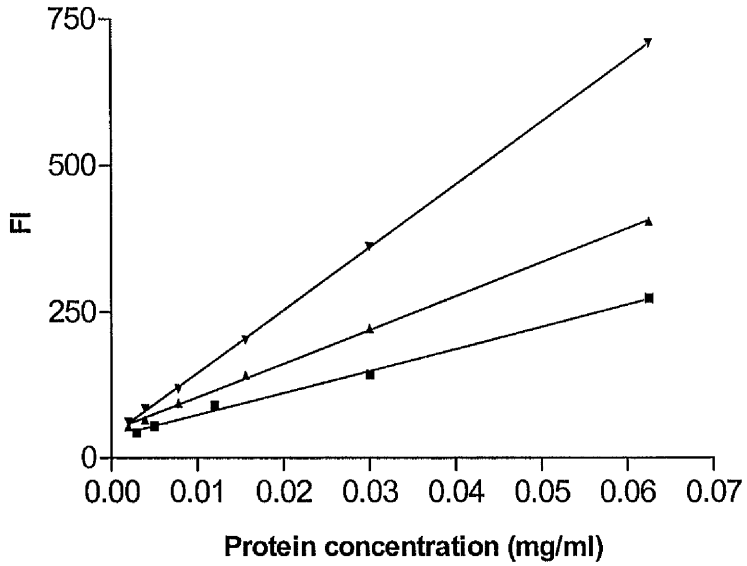


Figure 3.26. BisANS fluorescence of rLamjMST and truncated forms. The full length rLmajMST (closed square) bound less bisANS than did rLmajMSTΔ360-370 (closed triangle) and rLmajMSTΔ338-370 (closed inverted triangle). The fluorescence intensity (FI) is given in arbitary units.

3.3.6 Denatured LmajMST Refolds Unassisted

Mammalian rhodanese has been studied extensively as a model for protein folding (Bhattacharyya and Horowitz, 2002). However, refolding of rhodanese is relatively difficult because of the presence of multiple disulphide-linked misfolded states and the ability of folding intermediates to form aggregates. Thus rhodanese cannot be refolded unassisted, either a chaperone or a detergent is required for success (Mendoza *et al.*, 1991; Panda and Horowitz, 2000). LmajMST denatured using 6 M urea (Figure 3.27) and then either dialysed or simply diluted into a refolding buffer (with excess of the reductant 200 mM β-mercaptoethanol) regained its full enzymatic activity without the need for assistance in form of a chaperone or detergent (Figure

3.28). In contrast, the truncated proteins similarly treated did not even regain the low activity that they originally possessed when they were initially purified from *E. coli*.

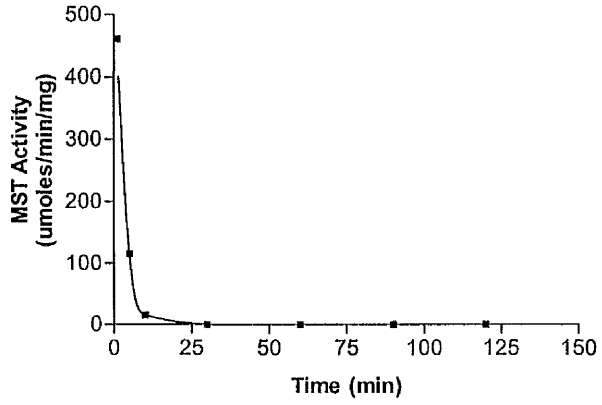


Figure 3.27 Denaturation of LmajMST by 6 M urea. LmajMST (1mg/ml) was incubated for 2 h at room temperature in 6 M urea. Sulfurtransferase activity was determined at fixed time intervals. Aliquots of the denatured sulfurtransferase used for activity determination were diluted 4-fold in refolding buffer (see section . 2.12.3)

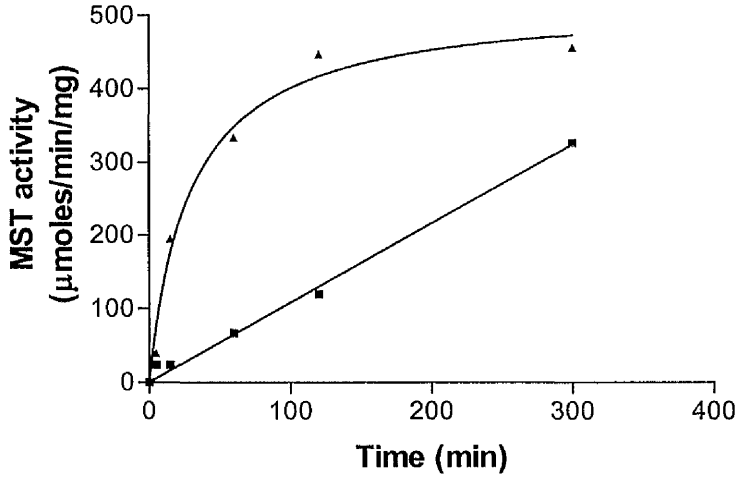


Figure 3.28. LmajMST refolds successfully unassisted. RLmajMST denatured in Figure. 3.21 were diluted in refolding buffer to a protein concentration of 0.25 µg/µl refolded to give active enzyme most efficiently when simply diluted into refolding buffer (closed triangle), with 97% of the original activity being recovered by 2 h. Dialysis (closed square) also yielded active enzyme, but more slowly.

3.4 Discussion

I have characterised at the molecular and biochemical levels a single copy 3-mercaptopyruvate sulfurtransferase (MST) from both *L. major* and *L. mexicana*. The MST gene from *L. major* is located in chromosome 5 (http://www.sanger.ac.uk) flanked by NADP dehydrogenase (EC 1.6.99.3) and dipeptidyl-peptidase III (EC 3.4.14.4) at its 5' and 3' ends, respectively.

Analysis of the recombinant MST enzyme from *Leishmania* has clearly shown that the enzyme prefers 3-mercaptopyruvate to thiosulfate as donor substrate (data in Chapter 4). This substrate preference correlates well with the active site residues (Nagahara *et al.*, 1995, 1996, 1999; Trevino *et al*, 1999; Bordo *et al.*, 2000, 2002) in the C-terminal domain being homologous with other eukaryotic MSTs (CG[S/T]GVTA) rather than rhodaneses (CRXGX[R/T]A). The active site motif of known TSTs always contains two basic residues (i.e. if the last residue is thr, a basic residue is observed at one of the X positions), whereas no charged residues are observed in biochemically characterized MSTs; this may relate to the distinct ionic charge of the respective *in vitro* substrates, thiosulfate (2-) and 3-mercaptopyruvate (1-). The pioneering work of Luo and Horowitz (1994) identified the lysine residue (at the position +2 with respect to the catalytic cysteine) as the key amino acid for binding and catalysis of thiosulfate in bovine rhodanese. The mutagenesis of ser^{240} of SseA (Colnaghi *et al.*, 2001) and rat MST (Nagahara and Nishino, 1996) provided clear evidence that the presence of a hydrophobic residue (ala) did not affect the binding of 3-mercaptopyruvate, but strongly prevented thiosulfate binding. On the contrary, the presence of a positively charged residue (lys) significantly increased the affinity for thiosulfate in rat MST (Nagahara *et al.*, 1995, 1996). It is thus suggestive

that interaction of MST with mercaptopyruvate should be favoured by other ionizable residues (arg^{187} and arg^{196} in rat MST) close to the catalytic cysteine in the three-dimentional structure of the catalytic pocket. However, since no 3D structure of MSTs is to date available, the mechanism of 3-mercaptopyruvate binding and catalysis remains speculative.

The leishmanial proteins are like other MSTs from *Rattus norvegicus* (Nagahara *et al.*, 1995, 1996), *Arabidopsis thaliana* (Papenbrock and Schmidit, 2000), *E. coli* (Colnaghi *et al.*, 2001) and also the Archeal, in having two rhodanese domains. This suggests that the tandem gene duplication architecture of sulfurtransferases may have occurred very early during evolution, probably before the diversification of living organism into the major evolutionary phyla. However the domains do not always occur in pairs. Recent publications and reviews (Bordo and Bork, 2002) and data from complete and on-going genome sequencing projects have shown that distinct proteins with possibly different biological function are associated with one or other of the rhodanese-like domains. For example ThiF (Taylor *et al.*, 1998), ThiI (Palenchar *et al.*, 2000), Cdc25 phosphatase (Draetta and Eckstein, 1997; Forrest and Gabrielli, 2001) and ArsR (Bobrowicz *et al.*, 1997; Mukhopadhyay *et al.*, 2000) are associated with a C-terminal domain, while the dual-specific phosphatase and ubiquitin C-ter hydrolase 2 (Fauman *et al.*, 1998; Hofmann *et al.*, 1998) are associated with a N-terminal domain. The functions of these rhodanese-like domains in association with other protein domains are yet to be determined.

Cdc25 phosphatases have a similar 'rhodanese-like' C-terminal domain with a different motif about the conserved cys active site amino acid (CE[Y/F]SXXR).

Despite the differences, they also play a role consistent with substrate recognition (Bordo and Bork, 2002). In the rhodanese subfamily from *Myobacterium tuberculosis*, *Streptomyces peucetis*, and *Azotobater vinelandii,* this motif is structurally associated with the arsenic resistance operon repressor ArsR. This suggests that this rhodanese motif is able to bind to arsenic, and it is tempting to speculate that they may act as sensory domains modulating ArsR activity. They can bind substrates containing phosphorus or chemically similar arsenic, whereas the similar domain of MSTs and TSTs bind with substrates containing reactive sulfur or, in some cases, selenium (Ogasawara *et al.*, 2001). Considering the very distant relationship between bacterial ArsR proteins and eukaryotic Cdc25/Ars2 proteins, it is likely that these two subfamilies result from two independent mutational events each resulting from a single amino acid insertion in the active-site loop of sulfur-binding rhodanese domains.

The two domain vertebrate rhodaneses have been extensively studied. Studies designed to understand the role of the N-terminal domain in correct folding, stability of the enzyme (Luo and Horowitz, 1993; Trevino *et al.*, 1995, 1998, 1999) and in sub-compartmentalization within the mitochondria (Ploegman *et al.*, 1978; Hol *et al.*, 1983; Miller *et al.*, 1991; Hammen *et al.*, 1994; Gliubich *et al.*, 1996; Waltner *et al.*, 1996) have established its function in the 'structural' stability of the protein. Current evidence suggests, however, that correct protein folding also requires the assistance of a chaperone molecule (Mendoza *et al* 1991; Luo *et al.*, 1993; 1995).

MSTs from the plants *A. thaliana* (Papenbrock and Schmidit, 2000) and *D. glomerata* (GenBank accession no. GI: AAD19957) are unique in having the two rhodanese

domains associated with an additional domain at their N-terminal end. The amino acid sequence of extended N-terminal domain of *A. thaliana* MST has sequence homology with stress proteins involved in the process of leaf senescence (Paperbrock and Schmidt, 2000).

The leishmanial genes also predict an unusual sulfurtransferase that, in comparison with other known sulfurtransferases, has an additional C-terminal domain of some 70 amino acids. The discovery that this also is present in a gene of *T. cruzi* and *T. brucei* suggests that the feature is conserved amongst trypanosomatid parasites and so has some key function. Importantly, the additional C-terminal domain distinguishes the parasite enzymes from those of its mammalian host and so provides some optimism that the enzyme may be exploitable as a drug target. This can be tested by deletion of the gene. The finding that it appears to be a single copy gene means that this should be readily possible.

The effects of an extended C-terminal end of mammalian rhodanese have been looked at in the past in relation to catalytic function, structural stabilization (Trevino *et al.*, 1995), and protein folding. The presence of a chaperone-like molecule at the end of a molecule is rare in nature and not consistent with current knowledge about the kinetics of protein folding. However, mammalian rhodanese with its C-terminal extended with 23 –106 unrelated amino acids can fold into an enzymatically active conformation while bound to bacterial ribosomes (Kudlicki *et al.*, 1995), while the wild type protein and a 13 amino acid C-terminal extension were only active after termination and release from the ribosomes (Kudlicki *et al.*, 1995). The mitochondrial Chinese hamster ovary rhodanese, has a tri-peptide at C-terminal end of

the protein, which is cleaved off the protein once it has been imported into the mitochondrion. Replacing the cleavable tri-peptide with 13-28 residues of unrelated sequence also produced an enzyme with similar activity to the wild-type enzyme, but the addition of a single codon for serine but not arginine resulted in a loss of enzymatic activity (Kramer *et al.*, 2001). It is thought that the added serine may have formed bonds with other parts of the molecule thereby causing an unfavorable effect on the conformation of the C-terminal end of the protein which has been shown to be loose and not closely associated with the otherwise compact C-terminal domain of the molecule (Ploegman *et al.*, 1978).

A possible role of the unusual C-terminal domain of the leishmanial MST was suggested by the finding that the leishmanial MST can refold successfully without a chaperone, which is in contrast to the results reported for sulfurtransferases from other sources (Ellis and Hemmingsen, 1989; Mendoza *et al.*, 1991; Bhattacharyya and Horowitz, 2002). The refolding kinetics of the mammalian sulfurtransferase have been studied in attempts to understand the mechanism underpinning the correct folding and stability of the enzyme. It is known that urea first disrupts the interaction of the tightly bound domains of native rhodanese before domain dissociation (Pleogman *et al.*, 1978; Shibatani *et al.*, 1999). Refolding involves a number of intermediates with characteristics associated with the 'molten globule' hypothesis (Pain, 1987; Tandon and Horowitz, 1990). The refolding of the denatured protein after dilution to reduce urea concentration produced an intermediate as a result of fast compaction (it partially regained structure but was inactive) (Tandon and Horowitz, 1989; Gorovits and Horowitz, 1998) which rapidly partitioned to form intermediates that either slowly regained complete structure and activity or very slowly formed

irreversible aggregates. The latter resulted in lowering the yield of the refolding reaction. The presence of disulfide bonds in the rhodanese hinders this process, but a molecular chaperone, e.g. GroEL, in the refolding buffer interacts with these early folding intermediates of rhodanese to prevent aggregation of the protein occurring. We hypothesised that the unusual C-terminal domain may play some part in the refolding of LmajMST. The finding that the proteins with truncated C-termini were misfolded and showed very little activity is consistent with this postulate.

Very little is known about sulfurtransferases in protozoa, but the findings reported here suggest that further studies to solve the 3D structure of this widespread class of enzymes will yield interesting new insights into its roles, interaction with other proteins, structure and reaction mechanism

Chapter 4

Biochemical Characterisation of Leishmania *mercaptopyruvate sulfurtransferase*

4.0 Introduction

Sulfurtransferases (EC 2.8.1.1 - EC 2.8.1.5) are widely distributed enzymes of prokaryotes and eukaryotes (Nakamura *et al.*, 2000; Bordo and Bork, 2002), with cytosolic and mitochondial isoenzymes being known (Koj *et al.*, 1975; Wrobel and Czubak, 2000; Nakamura *et al.*, 2000). The enzymes catalyse the transfer of sulfane sulfur from a donor molecule, such as thiosulfate or mercaptopyruvate to a nucleophilic acceptor substrate such as cyanide or mercaptoethanol. However, the natural sulfane donors and acceptors and the physiological functions of most sulfurtransferases remain uncertain.

The rhodanese family of sulfurtransferases are thought to occur in the majority of organisms (Bordo and Bork, 2002), with the mammalian enzymes being the most extensively studied (Luo and Horowitz, 1994; Nandi *et al.*, 2000). The first elucidated role of mitochondrial bovine liver rhodanese was the detoxification of cyanide to form thiocyanate, which is harmless and excreted by the kidney. This role could be important especially in the epithelial cells lining the gut (Picton *et al.*, 2002), but is thought not to account for the distribution of sulfurtransferases so widely and in different cell types (Luo and Horowitz, 1994). Another putative function of at least some sulfurtransferases is the provision of sulfane sulfur required for the formation of the Fe-S centres of proteins, notably respiratory proteins (Duin *et al.*, 1997; Ogata and Volini, 1990; Lauhon and Kambampati, 2000; Marquet, 2001).

Sulfurtransferases may also play a part in the management of the cytotoxicity of reactive oxygen species in aerobic tissues (Nandi *et al.*, 2000). Bovine rhodanese has a thousand-fold higher affinity for the reduced form of thioredoxin than for cyanide and may function in peroxide detoxification (Nandi *et al.*, 2000; Ray *et al.*, 2000). It has been reported that MSTs can oxidise thioredoxin, a key intermediate in cellular redox reactions. This may be a physiologically significant mechanism especially in combating oxidative challenges (Nandi *et al.*, 2000). A reaction analogous to that of sulfane-loaded sulfurtransferase with thioredoxin is also thought to be a critical step in the synthesis of thiouridine (Kambampati and Lauhen 2000; Lauhon and Kambampati, 2000; Palenchar *et al.*, 2000; Mueller *et al.*, 2001), and the formation of thiocarboxylate during thiamine biosynthesis by the multidomain protein ThiI of *Escherichia coli* (Palenchar *et al.*, 2000; Lauhon and Kambampati, 2000). Sulfurtransferases have also been implicated in the synthesis of biotin (Tse Sum Bui *et al.*, 2000) and molybdopterin (Leimkuhler and Rajagapalan, 2001). Moreover, a role for sulfurtransferases in assimilatory sulfate reduction, by transferring a molecule of sulfide to *O*-acetyl-L-serine in the synthesis of cysteine has been postulated (Schmidt and Jager, 1992). In this chapter, I will describe the biochemical characterisation of a sulfurtransferase of the protozoan parasites *L. major* and *L. mexicana* and provide data on its possible roles.

4.1 Material and Methods

4.1.1 Production of LmajMST over-expressing plasmid

The ORF of the *LmajMST* gene was amplified by PCR (denaturation at 94°C for 2 min, 30 cycles of 15 sec at 94°C, 30 sec at 64°C, 2 min at 72°C and a final extension of 7 min at 72°C), using oligonucleotide primers MSTr1 (5'GGATCCCATATGTCTGCTCCTGCTGCGCCGAA3') and MSTr2 (5'GATATCTTATGGAGGGGCGGAAGAGGCCGCTGT3'), containing the *Sma*I and *BamH*I restrictions sites (underlined) to facilitate subcloning into the re-expression vector, *pGL607*. The amplified *LmajMST* gene was subcloned into the pGEM-T vector (Promega) to give (*pGEMTrLmajMST*) and subsequently into the *Sma*I/*BamH*I-cut *pGL607* re-expression vector to generate the plasmid *pGL607MST* which was subsequently used to transfect *L. major* promastigotes as detailed in Chapter 2. The re-ligated *Sma*I/*BamH*I-cut *pGL607* without any insert acted as a control and was designated *pGL607*.

4.2 Results

4.2.1 Biochemical characterization of recombinant MSTs

The 1.1 kb ORFs of *LmajMST* and *LmexMST* were amplified with gene-specific primers (NT 42 as forward primers and NT 55 and NT 56 as reverse primers) for *L. major* and *L. mexicana,* respectively (Figure 4.1a). The *LmajMST* and *LmexMST* genes were expressed and purified as soluble proteins from *E.coli* (approximately 25 mg.litre^{-1} *E. coli*). The predicted sequences of the MSTs of each *Leishmania* species (without the his-tag) comprise 370 residues with a calculated molecular mass of 40.1 kDa, which was the size of the recombinant protein produced (Figure 4.1b).

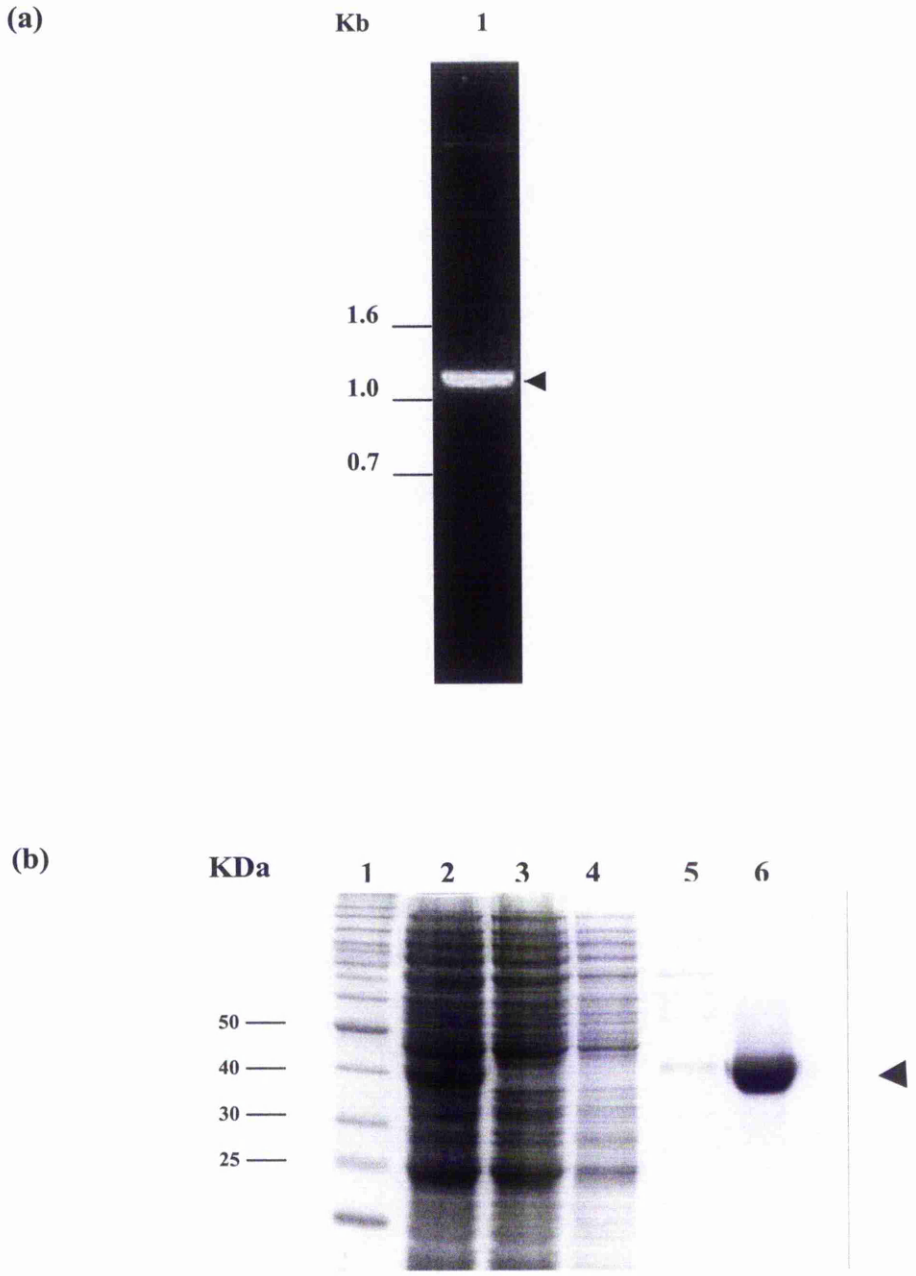


Figure 4.1 PCR amplification and purification of LmajMST. (a) Ethidium bromide stained 0.7% gel showing the 1.1 kb PCR product of the MST of *L. major* (arrowed). (b) Purification of rLmajMST under native conditions. The soluble protein in the supernatant fraction was purified on a nickel chelator column. The His-tagged protein was eluted by addition of buffer A with 5000 mM imidazole. SDS-PAGE analysis of the following samples: Lane 1, molecular mass standards; lanes 2 and 3, supernatant and pellet fractions, respectively, of a crude extract of the recombinant *E. coli* cells; lanes 4 and 5, column eluants from washes with buffer A with 60 mM imidazole; lane 6 purified rLmajMST eluted from the nickel chelator column with buffer A with 250 mM imidazole. Estimated recombinant protein (arrowed) size: 40.1 kDa.

4.2.1.1 Stability upon storage

As the first step, the enzyme assay and storage conditions were optimised and standardised to ensure that the recombinant proteins were in optimal condition during characterisation. Storage conditions investigated reflected published methods described previously for sulfurtransferases (Vachek and Wood, 1972; De Riso *et al.*, 1995; Timasheff *et al.*, 1995; Gorovits and Horowitz, 1998). The stability of the enzyme under these conditions was determined by monitoring sulfurtransferase activity over a period of time. Enzymes stored in the elution buffer lost 90% of original activity overnight (Figure 4.2a). Similar results were observed with enzyme dialysed overnight in 20 mM Tris-HCl, pH 7.9, at 4°C to remove the excess salts (0.25 M imidazole and 0.3 M NaCl) contained in the elution buffer. The cryoprotectant, glycerol, which stabilises proteins in aqueous solution by inducing preferential hydration of the protein molecule (Timasheff *et al.*, 1995; Gorovits and Horowitz, 1998), and the presence of the monovalent cation (0.8 M KCl) with 2 mM β-mercaptoethanol (known to markedly stabilise the enzyme from *E. coli* during storage and purification [Vachek and Wood, 1972]) were ineffective in protecting the enzyme (0.25 $\mu g.ml^{-1}$) at -20°C (Figure 4.2b). The loss of activity of the sulfurtransferase enzyme was also a function of protein concentration. An increase in protein concentration from 0.09 to 0.52 $\mu g.ml^{-1}$ delayed the loss of enzymatic activity by 72 hours (Figure 4.2c). Inactivation of the enzyme is thought to be due to the oxidation of the cysteine to cystine residues or sulfenic acids (Iciek and Wlodek, 2001). Prolonged stability was however, obtained when 50 mM $Na_2S_2O_3$, with or without 40 $mg.ml^{-1}$ BSA, was added to the enzyme after dialysis which was then stored at -20°C

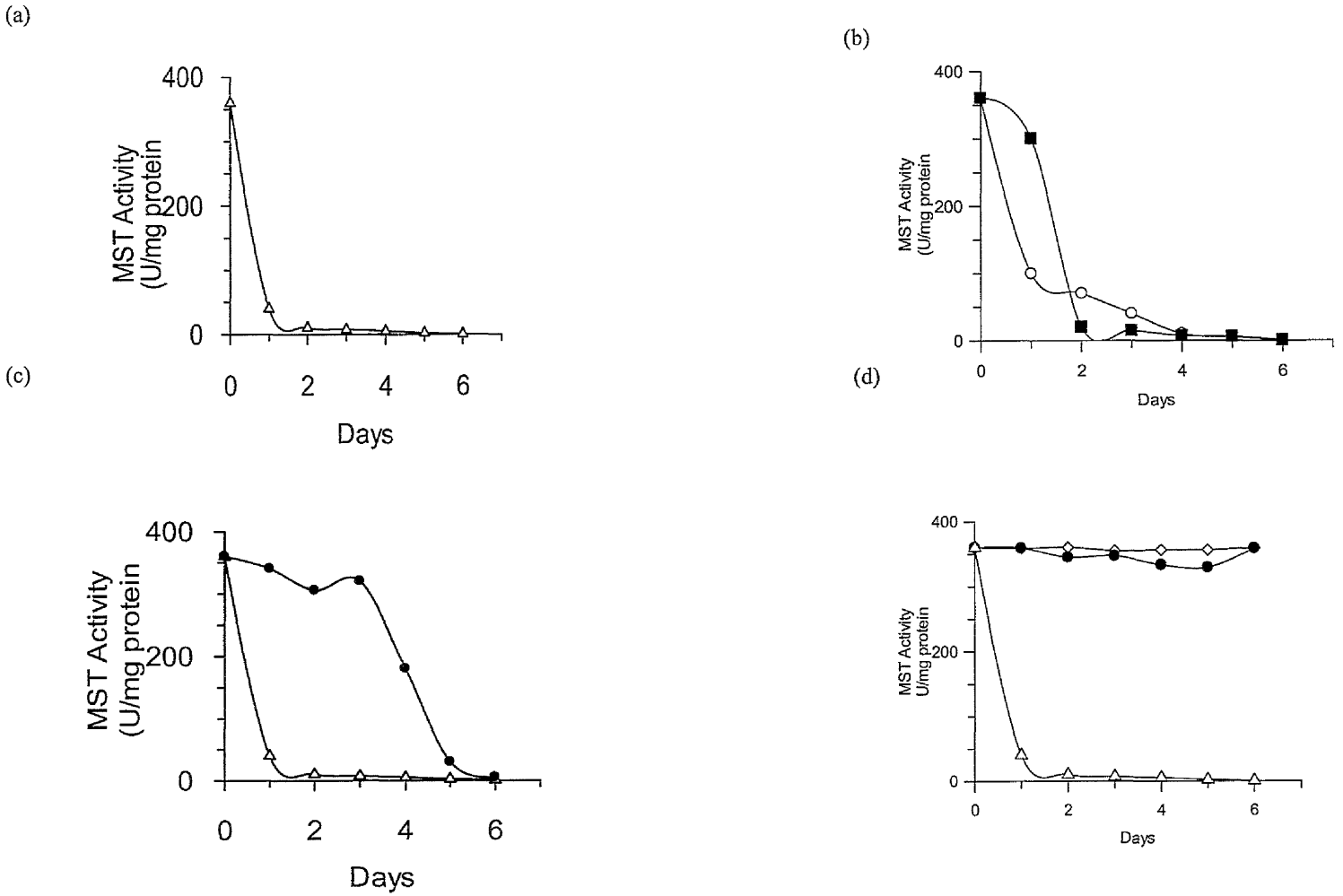


Figure 4.2 MST stability under different storage conditions, all at -20°C. Mercaptopyruvate sulfurtransferase activities recorded over time after storage of rLmajMST in: (a) elution buffer containing 0.3 M NaCl and 0.5 M imidazole in 50 mM phosphate buffer (Δ), (b) 20 mM Tris-HCl, pH 7.9, containing 40% (v/v) glycerol (O), 0.8 M KCl and 2 mM β-mercaptoethanol (■). (c) at protein concentrations 0.52 µg/µl (●) and 0.09 µg/µl (Δ), (d) in 20 mM Tris-HCl, pH 7.9, alone (Δ) and this buffer containing 50 mM $Na_2S_2O_3$ with (◈) and without BSA (●). Specific activities are the means from 2 experiments.

(Figure 4.2d). The enzyme was stable for at least 12 months under these conditions without loss in activity. There was also no loss in activity of the preparation after thawing and freezing or when kept on ice continuously for 1 day. The addition of thiosulfate to the storage buffer is thought to stabilise the enzyme in the sulfur donor form (Wesley, 1973), while addition of BSA was presumably beneficial through the formation of a stable copolymer (De Riso *et al.*, 1995), or stabilizing the protein by acting as a chaperone (Jarabak *et al.*, 1993).

4.2.1.2 Assay optimisation

The enzyme activity using the 'rhodanese' assay and saturating concentrations of substrates was characterised as a function of time and protein concentration. The reaction progressed effectively linearly under these assay conditions for 20 min (Figure 4.3), while doubling the amount of protein in the assay mix approximately doubled the production of thiocyanate (Figure 4.4).

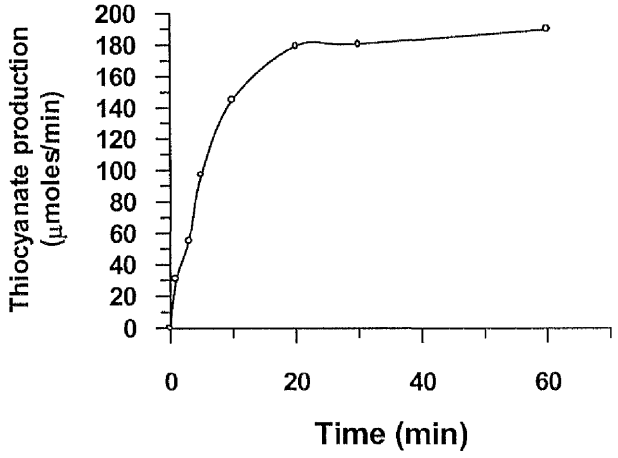


Figure 4.3. Thiocyanate production over time. The thiocyanide produced was measured at fixed time points with 0.016 µg/µl protein. The reaction was approximately linear over a 20 minutes incubation time at 37°C.

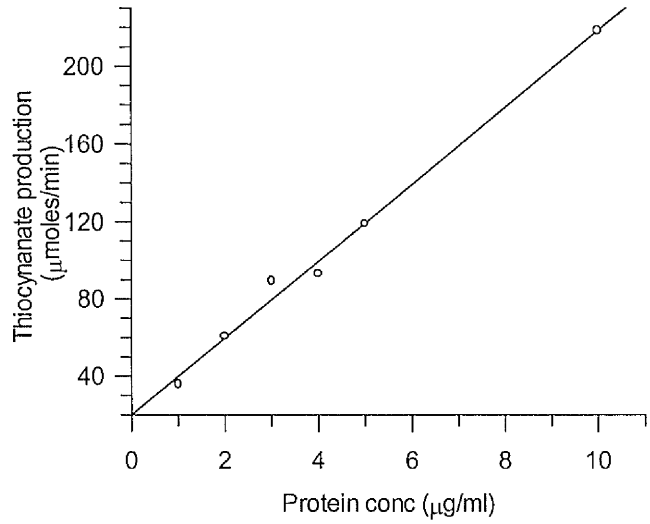


Figure 4.4 The effect of MST concentration on thiocyanide formation. The increase in MST concentration in the rhodanese-like assay incubated for 5 minutes at 37°C was directly proportional to increase in thiocyanide produced expressed as μmoles.min^{-1}. Assay conditions are detailed in section 2.10

The enzyme was most active in the pH range 6.9-7.6 for both substrates, mercaptopyruvate (Figure 4.5) and thiosulfate, when the formation of thiocyanate was monitored. This is in contrast to its bacterial and plant counterparts, with reported pH optima of 9.3 (Vachek *et al.*, 1972) and 9.0 (Papenbrock and Schmidt, 2000), respectively. At pH values greater than 7.5, the non-enzymatic production of thiocyanide was highest. The instability of thiols at elevated pH limits experiments under these conditions. All activity data were subsequently derived from a 5 min assay in Tris-HCl, pH 7.3.

4.2.1.3 Activity analyses

The purified rLmajMST and rLmexMST had activity towards both mercaptopyruvate and thiosulfate using the assays for both 'rhodanese'

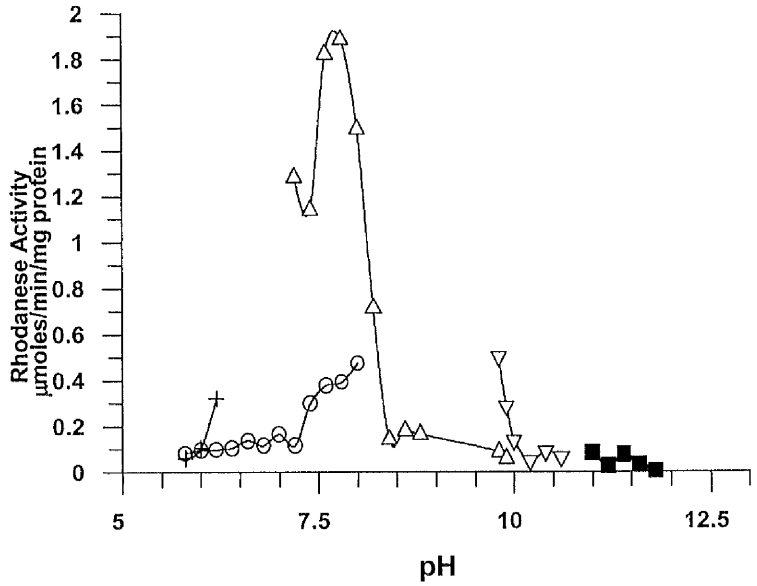


Figure 4.5. pH optimum of MST using the 'rhodanese' activity. The complete system contained (in 1 ml) mercaptopyruvate (5 mM), cyanide (5 mM) and buffers (200 mM) sodium phosphate (O), Tris-HCl (△), sodium carbonate (▽), pottassium phosphate (■) and sodium citrate (+). Data points of activities are mean values of three assays as µmoles.min^{-1}mg protein^{-1}. Protein concentration was 0.05 µg.µl^{-1}.

(Equations 1 and 2; Sorbo, 1957) and 'sulfurtransferase' (Equations 3 and 4; Papenbrock and Schmidt, 2000) activities (Table 4.1).

'rhodanese' assay

$HSCH_2COCOO^- + CN^- = CH_3COCOO^- + \mathbf{CNS^-}$.....1

$S_2O_3^{2-} + CN^- = SO_3^{2-} + \mathbf{CNS^-}$.....2

'sulfurtransferase' assay

$HSCH_2COCOO^- + RSH = CH_3COCOO^- + RSSH$

$RSSH + RSH = RSSR + \mathbf{H_2S}$ 3

$S_2O_3^{2-} + RSH = SO_3^{2-} + RSSH$

$RSSH + RSH = RSSR + \mathbf{H_2S}$.....4

(RSSH, persulfide; RSH, thiol; RSSR, disulfide)

Table 4.1. Activities of the *L. major* and *L. mexicana* rMSTs. Activities are means ± SD from four independent expressions. Sulfurtransferase activity towards 5 mM 3-mercaptopyruvate and 5 mM sodium thiosulfate with 5 mM mercaptoethanol was assayed by monitoring hydrogen sulfide production. Rhodanese activity towards of the same substrates but with 10 mM cyanide was assayed by measuring the amount of ferrous thiocyanide complex formed. For the kinetic analyses, >10 different substrate concentrations were used with at least two replicate assays.

Organism	**Assay**	**Substrate**	**Activity** μmoles/min/mg protein
L. mexicana	Sulfurtransferase activity	Sodium thiosulfate	20.8 ± 0.1
		Mercaptopyruvate	345 ± 1
	Rhodanese activity	Sodium thiosulfate	13.9 ± 1.6
		Mercaptopyruvate	295 ± 6
L. major	Sulfurtransferase activity	Sodium thiosulfate	21.3 ± 0.1
		Mercaptopyruvate	343 ± 1
	Rhodanese activity	Sodium thiosulfate	22.6 ± 0.1
		Mercaptopyruvate	229 ± 2

Organism	**Substrate**	**Kinetics**	
L. major	Sodium thiosulfate	K_m	1.7 mM
		V_{max}	10.9 μmol/min/mg
		k_{cat}	2.73 x 10^3 /min
L. major	Mercaptopyruvate	K_m	0.2 mM
		V_{max}	345 μmol/min/mg
		k_{cat}	1.73 x 10^5/min

However, rLmajMST was considerably more active towards mercaptopyruvate than thiosulfate, with a lower K_m and higher k_{cat} towards this substrate. Unlike reports for bovine liver rhodanese (Aird *et al.*, 1987; Ogata and Volini, 1990), cysteine and homocysteine were not used by rLmajMST as replacements for mercaptopyruvate or thiosulfate. The role of thiols such as mercaptoethanol, homocysteine, cysteine and glutathione as acceptors were analysed using the sulfurtransferase assay involving sulfide trapping with lead acetate. In this assay, the enzyme showed activity in the absence of added β-mercaptoethanol but addition of 5 mM β-mercaptoethanol increased the rate 20-fold to 345 μmoles/min/mg protein. Homocysteine (10 mM), and cysteine (50 μM), but not glutathione (40 μM), also each increased the production of sulfide from mercaptopyruvate, but only by about 10-fold (Figure 4.6).

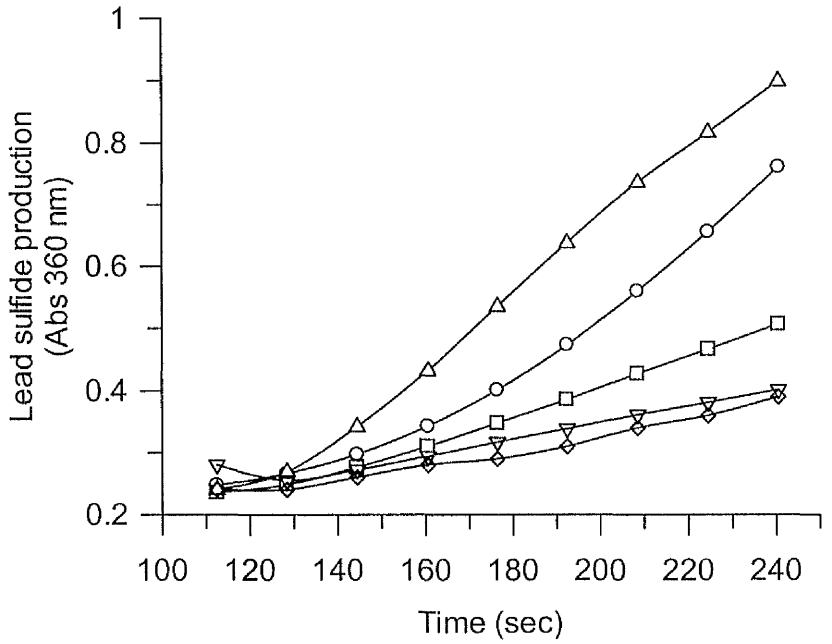


Figure 4.6. Effects of various thiols upon sulfurtransferase activity. MST activity monitored by the production of lead sulfide was increased some 10-fold with 50 μM cysteine (○), 10 mM homocysteine (△) but not 40 μM glutathione(□) or 50 μM N-acetyl cysteine (▽). Lead sulfide production in the absence of an acceptor substrate is represented by (◇)

Table 4.2. Sulfurtransferase activities in *L. major*, *L. mexicana*, *T. brucei*, transgenic strain of *L. major* overproducing MST (*pGL607MST*) and control over-expressing the antibiotic gene only (pGL607). Soluble extracts from the parasite life cycle stages and transgenic strain over-expressing the LmajMST were assayed for their sulfurtransferase activities using two methods: with 5 mM 3-mercaptopyruvate and 5 mM mercaptoethanol as substrates and monitoring the formation of hydrogen sulfide (Sulfurtransferase); with 5 mM 3-mercaptopyruvate and 10 mM cyanide as substrates and measuring the amount of ferrous thiocyanide complex formed (Rhodanese). Activities are means ± SD from triplicate analyses and the number of experiments given in parentheses.

Parasite strain	Stage	Sulfurtransferase μmol min^{-1} mg protein^{-1}	Rhodanese μmol min^{-1} mg protein^{-1}
L. mexicana	amastigote	0.33 ± 0.08 (4)	0.09 ± 0.01 (2)
L. mexicana	stationary phase promastigotes	0.60 ± 0.11 (4)	0.05 ± 0.02 (2)
L. major	log phase promastigotes	0.71 ± 0.02 (3)	0.02 ± 0.01 (2)
L. major	stationary phase promastigotes	0.67 ± 0.05 (3)	0.09 ± 0.05 (2)
L. major	metacyclic promastigotes	0.61 ± 0.07 (3)	0.03 ± 0.01(2)
L. major pGL607	stationary phase promastigotes	0.69 ± 0.09 (4)	-
L. major pGL607MST	stationary phase promastigotes	3.1 ± 0.04 (4)	-
T. brucei	long slender bloodstream forms	0.03 ± 0.00 (1)	0.01 ± 0.00 (3)
T. brucei	procyclic forms	0.19 ± 0.02(2)	0.018 ± 0.06 (2)
C. faciculata		0.87 ± 0.07 (1)	-

4.2.1.4 Expression and localization of leishmanial MSTs

Sulfurtransferase activity was found in extracts of each developmental stage of wild type parasites (Table 4.2). Sulfurtransferase activity of *Leishmania* overexpressing the LmajMST gene (pGL607MST) showed a 4-fold increase in sulfurtransferase activity relative to the control overexpressing the antibiotic marker alone (Table 4.3). When investigated using the same assays, sulfurtransferase activity was also detected in extracts from both bloodstream forms and procyclic forms (insect stages) of *T. brucei* and also *Crithidia fasciculata*. The expression of the leishmanial MSTs was also analysed using polyclonal antibodies raised against rLmajMST. The rabbit antiserum was strongly reactive to purified rLmajMST and also recognized a single 40 kDa protein in soluble extracts of *L. mexicana* amastigotes and promastigotes, and *L. major* log phase and stationary phase and metacyclic promastigotes (Fig. 4.7a).

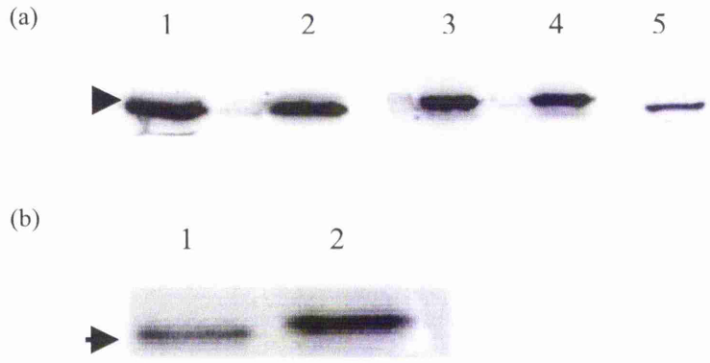


Figure 4.7 Western blot analysis of expression of leishmanial MST. Eighty micrograms of soluble cell extracts were subjected to Western blot analysis using the rabbit α-rMST antiserum. (a) Expression during the life cycle. *L. mexicana* amastigote (lane 1), *L. mexicana* stationary phase promastigotes (lane 2), *L. major* mid-log phase promastigotes (lane 3), *L. major* stationary phase promastigotes (lane 4), *L. major* metacyclic promastigotes (lane 5). (b) *L. major* promastogotes over-expressing of leishmanial MST gene. *L. major* promastigotes with empty vector expressing the blasticidin antibiotic marker (lane 1); *L. major* promastigotes over-expressing the LmajMST gene (lane 2).

Western analysis of the extracts of pGL607MST (the line overproducing MST) also showed an increase in protein expression above that of the control (pGL607) (Figure 4.7b).

The localization of LmajMST was investigated using immunofluorescence microscopy. Labelling was detected throughout the cytoplasm of the cell (Figure 4.8B), suggestive of a cytosolic location for LmajMST. However, the labelling was somewhat spotty, which could suggest otherwise.

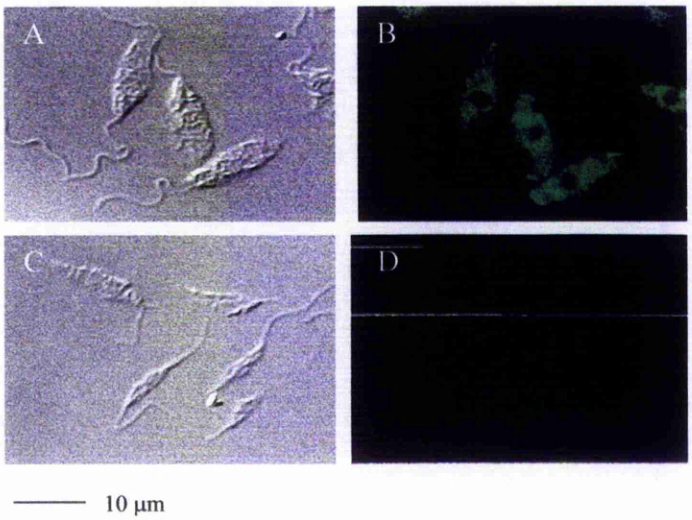


Figure 4.8 Indirect immunolocalization of fixed *L. major* promastigotes. Promastigotes at log phase of growth 5 x 10^6 cell.ml^{-1} were fixed and viewed under phase contrast microscopy before (A and C), and after immunofluorescent staining with (B) and without (D) antibody (anti-MST).

To pinpoint the actual localization of the protein, subcellular localization studies was carried out. Pellets recovered from each differential speed were resuspended in the initial volume (4 volumes) of buffer used to separate lysed cells from alumina. The subcellular distribution of proteins in promastigote

fractions recovered by differential centrifugation (see section 2.9.5), when expressed as a percentage relative to the homogenate (H), was 23%, 18%, 8% and 47% for P1, P2, P3 and S, respectively (Figure 4.9a). Analysis of the subcellular distribution of MST in promastigotes fractions using Western blot analysis showed that the protein was recovered primarily in the cytosolic fraction (Figure 4.9b), whereas antibody raised against the lysosomal CPB cysteine protease of *L. mexicana* (Sanderson *et al.*, 2000) detected protein in the small organelle fraction (P1) as expected (Figure 4.9c). These data together are suggestive that the leishmanial MST is a cytosolic enzyme. This is consistent with analysis using PSORT (http://psort.nibb.ac.jp) which indicated that the leishmanial sulfurtransferases lack any characteristic mitochondrial or other targeting sequence.

4.3 LmajMST is up-regulated in response to oxidative stress

Exposure of *L. major* promastigotes to the oxidants hydrogen peroxide, cumene hydroperoxide and *tert*-butyl hydroperoxide (at 300, 10 and 10 μM, respectively) led to the inhibition of growth by approximately 50% during 24 h incubation. Differing effects upon the expression of MST were noted. The sulfurtransferase activity of parasites exposed to cumene hydroperoxide was increased (to 0.98 ± 0.01 μmole min^{-1} mg $protein^{-1}$) compared with the control promastigotes cultured in HOMEM in the absence of oxidants. In contrast, there were no significant changes in sulfurtransferase activity after exposure to *tert*-butyl hydroperoxide or hydrogen peroxide. Western analysis of the same cells

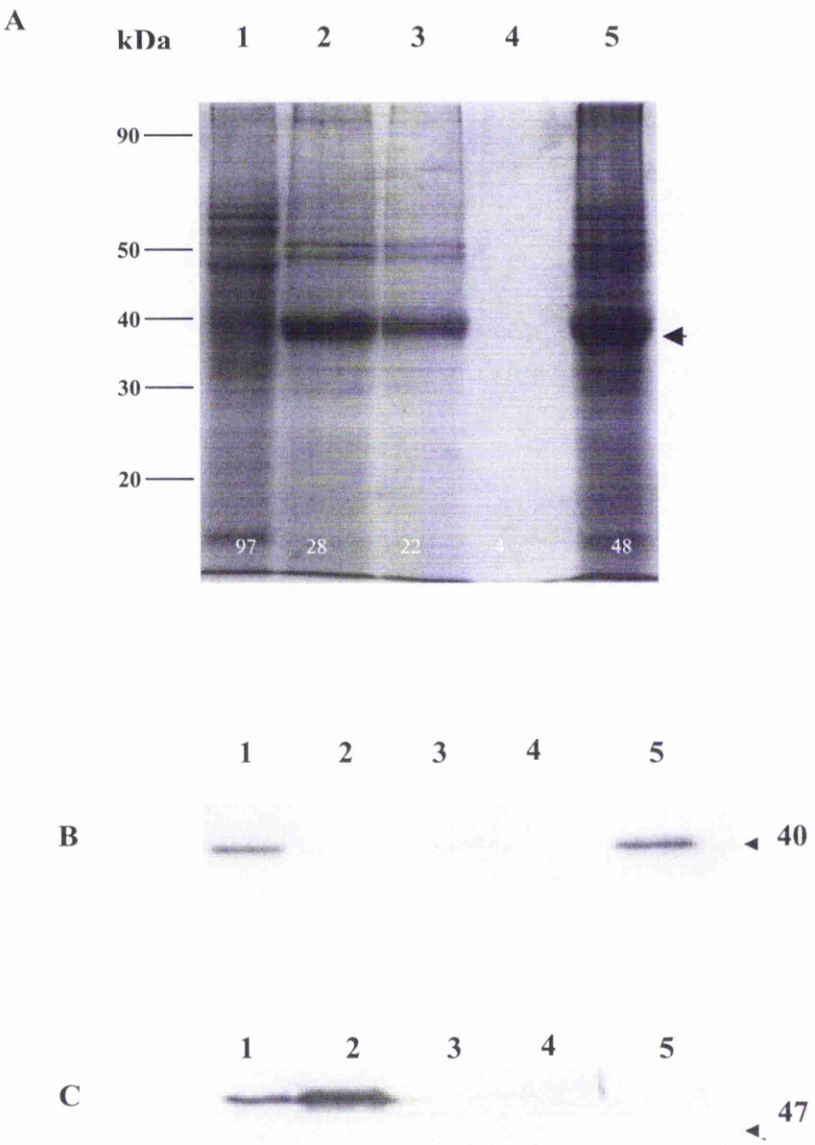


Figure 4.9 (a) SDS PAGE analysis of subcellular distribution of proteins in promastigote fractions derived by differential centrifugation fractionation and the percentage of proteins recovered relative to the Homogenate (H). Lane 1- H, Lane 2- P1, Lane 3- P2, Lane 4- P3, and Lane 5- S. (b) Western blot analysis of 80 μg of cell extracts showed the protein to be recovered primarily in the cytosolic fraction (S). Lane 1- H, Lane 2- P1, Lane 3- P2, Lane 4- P3, and Lane 5- S. (c) Antibodies raised against the lysosomal CPB cysteine proteases of *L. mexicana* detects protein in the small organelle fraction (P1), Lane 1- H, Lane 2- P1, Lane 3- P2, Lane 4- P3, and Lane 5- S of 80 μg of cell extracts.

extracts (Fig. 4.10) also showed an increase above the wild type levels of protein in the parasites exposed to cumene hydroperoxide stress.

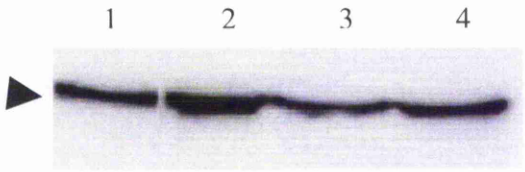
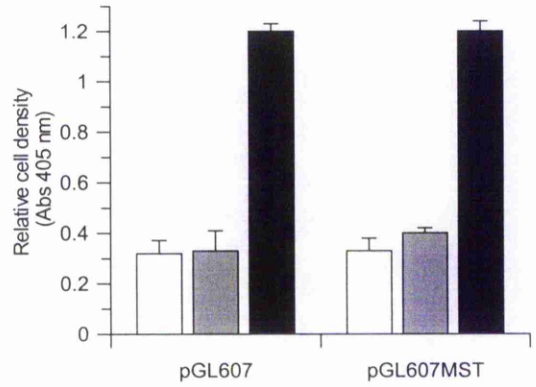


Figure 4.10. Western blot analysis of expression of leishmanial MST in *L. major* promastigotes subjected to oxidative stress. 80 μg of soluble cell extracts were subjected to Western blot analysis using the rabbit α-rMST antiserum. Lane 1, Control; lane 2, cumene hydroperoxide at 10 μM; lane 3, hydrogen peroxide at 300 μM; lane 4, *tert*-butyl hydroperoxide at 10 μM.

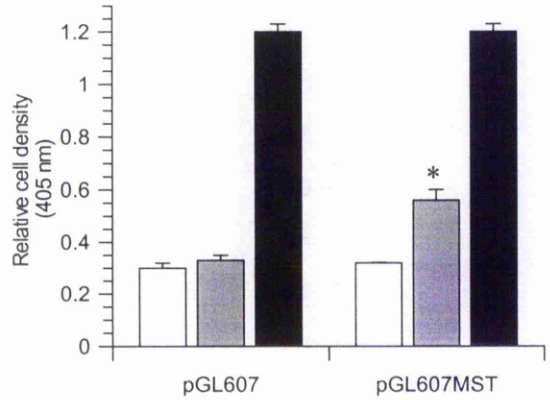
4.3.1 Overexpression of LmajMST confers resistance to oxidative stress

Knowing that *L. major* promastigotes with *pGL607MST* plasmid was overexpressing MST at a higher level relative to the control, the next step was to evaluate whether the overproduction of MST in *L. major* affects the parasite resistance to oxidants. *L. major* overproducing the MST (pGL607MST) and the control cells overexpressing the antibiotic gene only (pGL607) were exposed to H_2O_2 (0.5 mM), or cumene hydroperoxide (10 μM) or *tert*-butyl hydroperoxide (10 μM). These values (except for H_2O_2) were estimated prior to conducting the experiment to be the IC_{50} of wild type parasites. The relative cell densities of the pGL607MST estimated on addition of the oxidants were 0.32 ± 0.01,

(a)



(b)



(c)

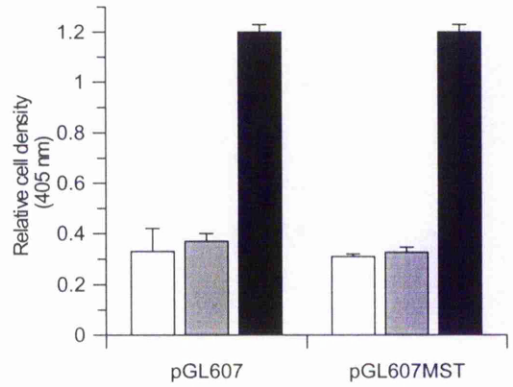
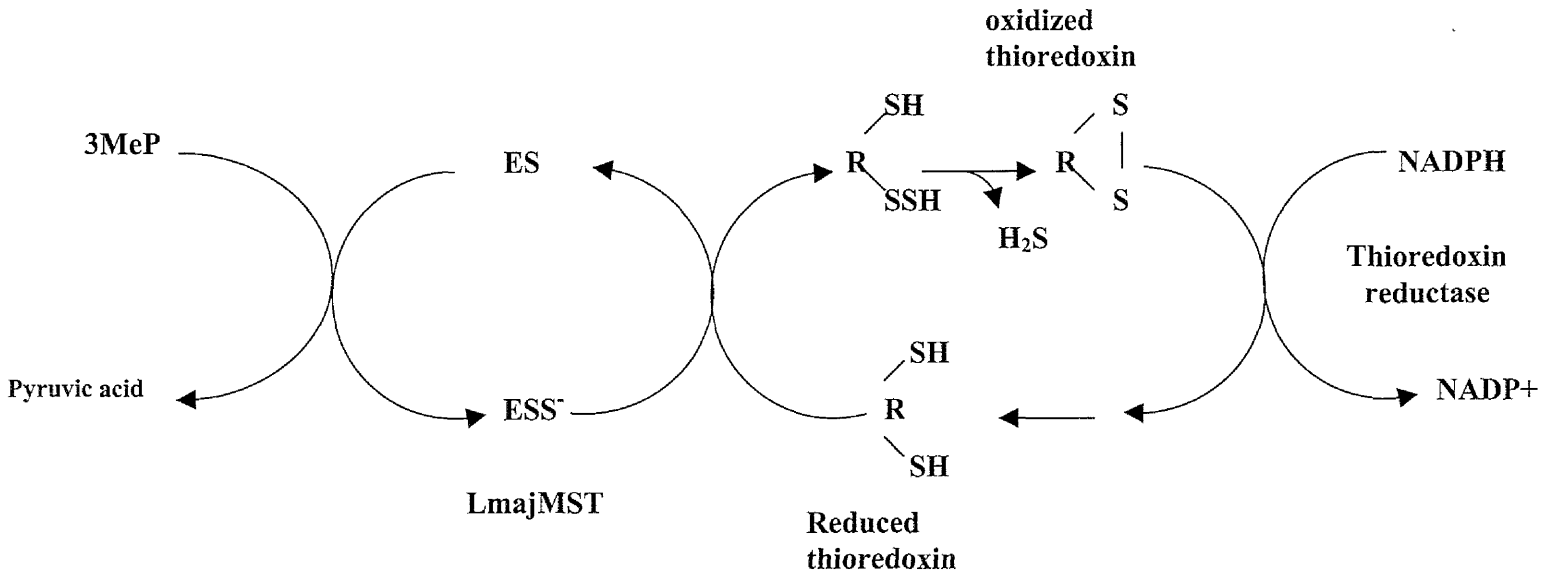


Figure 4.11 Relative cell density of *L. major* promastigotes subjected to oxidative stress. *L. major* wild type and transgenic cell lines promastigotes transformed with *pGL607* and *pGL607MST* were incubated at 27°C for 6 days in the presence (grey) or absence (black) or (a) 10 μM t-butyl hydroperoxide or (b) 10 μM cumen hydroperoxide or (c) 0.5 mM H_2O_2. The cell relative density of viable cells was estimated by the acid phosphatase assay prior to adding the oxidant (open boxes) and at the 6th day (grey and black). Relative cell densities are means from triplicate analysis. Differences considered statistically significant by Student's t test ($p<0.05$) between the pGL607 and pGL607MST transformants are marked with asterisks.

0.31 ± 0.01 and 0.33 ± 0.05 for cumene hydroperoxide (Figure 4.11a, open boxes), H_2O_2 (Figure 4.11b, open boxes) and *tert*-butyl hydroperoxide (Figure 4.10c, open boxes) respectively. The initial relative cell density for the control cells lines (pGL607) were 0.30 ± 0.02, 0.33 ± 0.09 and 0.32 ± 0.05 for cumene hydroperoxide (Figure 4.11a, open boxes), H_2O_2 (Figure 4.11b, open boxes) and *tert*-butyl hydroperoxide (Figure 4.10c, open boxes) respectively. The cell were incubated for 7 days at 27°C, the duration at which wild type cells reaches the late log phase cell density of 7×10^7 cells.ml^{-1}. This value corresponded to an absorbance of 1.20 ± 0.03 at 405 nm (black) with n-nitrophenyl phosphate (acid phosphatase assay). The relative cell densities of pGL607MST estimated by the acid phosphatase assay at the end of the seven days incubation in oxidants were 0.4 ± 0.02 (Figure 4.11a, grey), 0.56 ± 0.04 (Figure 4.11b, grey) and 0.32 ± 0.02 (Figure 4.10c, grey) for *tert*-butyl hydroperoxide, cumene hydroperoxide, and H_2O_2 respectively. The relative cell densities of the control were 0.33 ± 0.02, 0.37 ± 0.03 and 0.33 ± 0.08 for cumene hydroperoxide (Figure 4.11a, grey), H_2O_2 (Figure 4.11b, grey) and *tert*-butyl hydroperoxide (Figure 4.11c, grey) respectively. pGL607MST was resistant to the concentration of cumene hydroperoxide used but not those of H_2O_2 and *tert*-butyl hydroperoxide relative to the control.

(a)



(b)

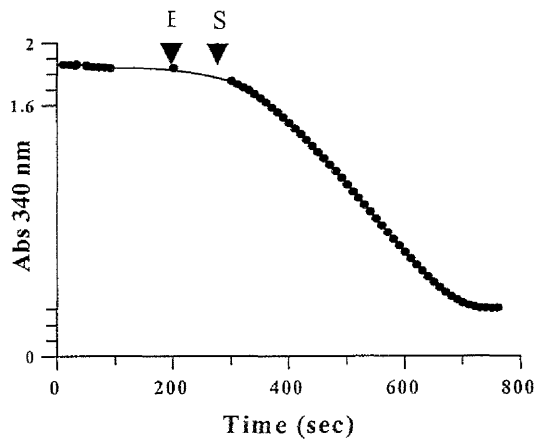


Figure 4.12. **rLmajMST reacts with thioredoxin.** (a) Schematic representation of the reaction mechanism of a coupled assay between MST and the thioredoxin/thioredoxin reductase system from *T. vaginalis*. MST reacts with mercaptopyruvate forms a complex which reacts with reduced thioredoxin to for oxidized thioredoxin via an intermediate. Thioredoxin reductase reduces the oxidized thioredoxin by the reduction of NADH which can be monitored spectrometrically 340nm. Source Nandi *et al.*, 2000 (b) rLmajMST (E) and 3-mercaptopyruvate (S) were added at the times indicated by the arrowheads to the reduced thioredoxin system which was at equilibrium and the subsequent NADPH oxidation followed spectrophotometrically at 340 nm. Experimental procedure is detailed in section 2.10

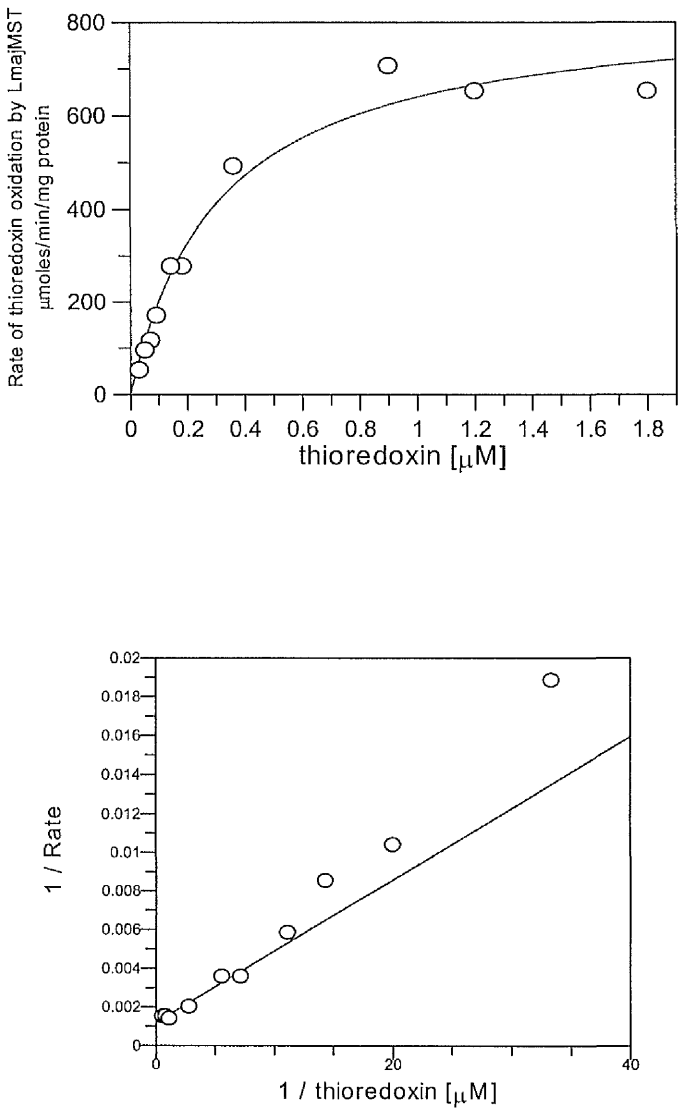


Figure 4.13 Kinetics of rLmajMST oxidation of reduced thioredoxin (Trx) from *T. vaginalis*. The oxidation of Trx by rLmajMST was measured by the reduction of NADPH in the presence of various Trx concentrations at 37°C. Kinetic constant was determined by non-linear regression analysis using Michaelis-Menten equation of Graphit4® (Erithacus Software Limited). (d) A plot of the reciprocal values of the various Trx concentratons and the rate of NADP reduction measured at 340 nm in (c) used to estimate the kinetic parameters (K_m and V_{max}) of Trx oxidation mentioned in the text. The results are the mean of four points from two independent experiments. Experimental procedures are detailed in section 2.10.

4.3.2 LmajMST oxidises reduced thioredoxin

The addition of rLmajMST (E) and mercaptopyruvate (S) to a mixture of thioredoxin reductase, thioredoxin and NADPH that had equilibrated lead to the rapid oxidation of NADPH (Figure 4.12a). Addition of thiosulfate rather than mercaptopyruvate resulted in activity, albeit at a lower level (the specific activities were 37 ± 0.23 and 386 ± 0.01 μmoles min^{-1} mg $protein^{-1}$ towards thiosulfate and mercaptopyruvate, respectively). There was a high affinity for the reduced thioredoxin (K_m, 300 nM)(Figure 4.13). Similar oxidation did not occur if any substrate was omitted or if mercaptopyruvate (S) was replaced by 10 μM cumene hydroperoxide (Figure 4.12a).

4.4 LmajMST is up-regulated in response to hypo-sulfur stress

Promastigotes of *L. major* also responded to hypo-sulfur stress over 24 hours by increasing the expression of MST relative to the control grown in normal medium (section 2.3.2) (Figure 4.14). Sulfurtransferase activity of the lyaste of the same parasites also showed an increase at the activity level of 0.85 ± 0.01 μmole min^{-1} mg $protein^{-1}$ compared with the control (0.67 ± 0.05), in a medium abundant in exogenous sulfur.

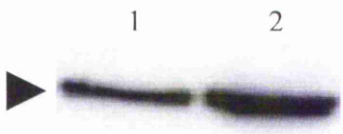


Figure 4.14. Western blot analysis of expression of leishmanial MST in *L. major* promastigotes subjected to hypo-sulfur stress. 80 μg of soluble cell extract was subjected to Western blot analysis using the rabbit α-rMST antiserum. Control, (lane 1); hypo-sulfur stress, (lane 2).

4.4.1 LmajMST and cysteine biosynthesis

To address the question of whether rLmajMST plays a role in the synthesis of cysteine by providing sulfide, the potential use of the sulfide generated by the enzymes' action on mercaptopyruvate was tested *in vitro* using *O*-acetyl serine and the recombinant enzymes cysteine synthase or cystathionine *β*-synthase. The identification, cloning, and characterization of cysteine synthase and cystathionine *β*-synthase from *L. major* is presented in Chapters 5 and 6, respectively. The specific activity of cysteine synthase and cystathionine β-synthase (acting as a cysteine synthase) in this linked assay were 20 and 18 μmole.min^{-1}.mg protein^{-1}, respectively, and the amount of cysteine produced was proportional to the amount of mercaptopyruvate added in the standard assay up to 3 mM mercaptopyruvate when it was saturating (Figure 4.15).

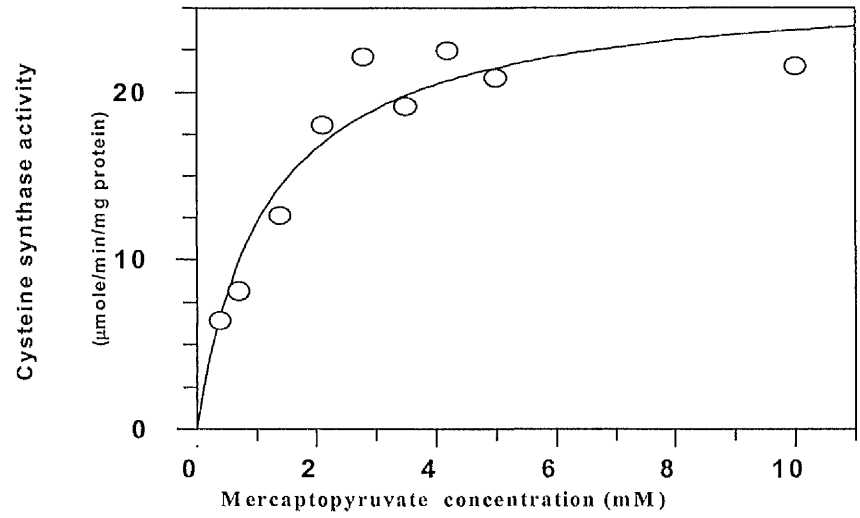


Figure 4.15. Relationship between the cysteine synthase activity and the concentration of mercaptopyruvate. The reaction mixture contained mercaptopyruvate (5 mM), mercaptoethanol (5 mM), *O*-acetyl serine (5 mM) and 0.5 μg of rLmajMST and LmajCS.

4.5 Inhibition of rLmajMST by antileishmanial antimonials

The pentavalent organic antimonials (Pentostam and Glucantime) have been for several decades the first line drugs used clinically to treat leishmaniasis (Berman, 1997). The precise mode of action of these antimonial compounds remains unknown, although it is believed that their activity is mediated via reduction to the trivalent forms and then binding to thiol groups of proteins which are inactivated as a consequence (Shaked-Mishan *et al.*, 2001). Thus a potential interaction of the drugs and Triostam, the trivalent analogue of Pentostam, with rLmajMST was investigated in two ways. Firstly, to check whether the enzyme may be involved in activation of the pentavalent drugs and, secondly, to determine whether the trivalent form of the drug could inhibit the enzyme. Glucantime/Pentostam can be used as substrates for the enzyme (Figure 4.16). The addition of 20 μM sodium stibogluconate (Sb(V)) increased sulfide production significantly. Sodium antimony tartrate (Triostam) at 50 μM inhibited the enzyme's activity, assayed with mercaptopyruvate and β-mercaptoethanol, by approximately 20%. Incubation of the enzyme with varying amounts of sodium antimony tartrate produced an apparent IC_{50} of 10 mM.

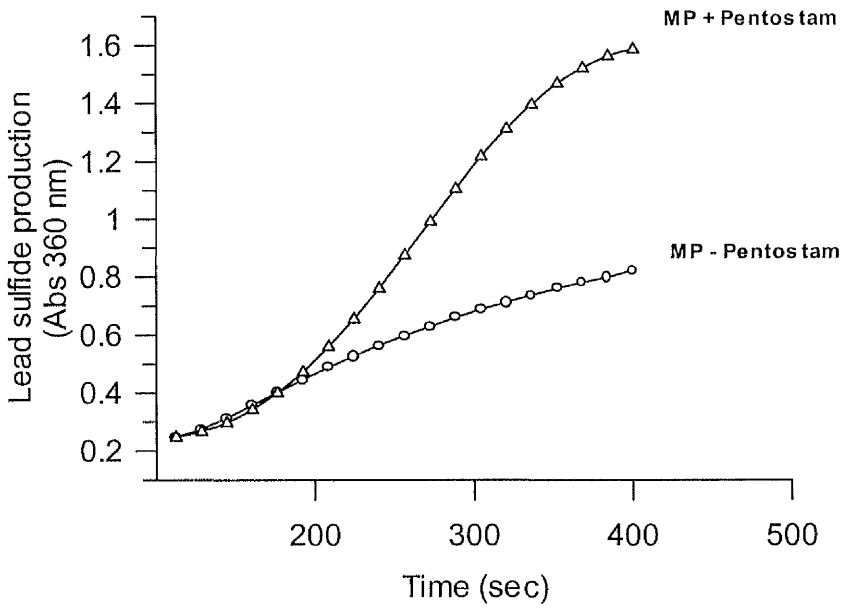


Figure 4.16. Pentostam as a potential substrate for leishmanial MST. Production of lead sulfide is significantly increased in a MST assay reaction (lacking β-mercaptoethanol, see section 2.10) of 5 mM mercaptopyruvate (MP) and 20 μM pentostam in 50 mM Tris-HCl buffer pH 7.4 (Δ) relative to a control assay reaction of 5 mM mercaptopyruvate (MP) in the same buffer but without pentostam and β-mercaptoethanol (O).

4.6 Discussion

The data presented show that mercaptopyruvate sulfurtransferase that is expressed throughout the life cycle of *Leishmania major* and *L. mexicana*. The life cycle stages used reflected the ease at which a reasonably high level of purified samples can be obtained. Currently, metacyclic promastigotes of *L. major* but not *L. mexicana* can be harvested from a stationary-phase culture (Sacks *et al.*, 1992). Likewise, amastigotes from *L. mexicana* but not *L. major* can be harvested from lesions in the BALB/c mice, and purified to a high level of purity. *L. mexicana* and *L. major* mid-log phase cultures (day 4) presumably dominated by procyclic promastigote had the same MST expression levels and activity. Thus one can speculate that the results obtained from life cycle stages of two different species of *Leishmania* might reflect the overall picture in either species.

Analysis of the recombinant enzyme has clearly shown that it prefers mercaptopyruvate to thiosulfate as donor substrate. This substrate preference correlates well with the active site residues being the same as those in other eukaryotic mercaptopyruvate sulfurtransferases, whilst differing from those of rhodaneses. The K_m and V_{max} values obtained compare favourably with those reported for bacterial, plant and vertebrate mercaptopyruvate sulfurtransferases (Bordo and Bork, 2002; Tse Sum Bui *et al.*, 2000). The very high activity towards mercaptopyruvate is suggestive that this could function as a natural

substrate, although currently there is nothing known about the relative levels of this compound in leishmanial parasites or any roles that it may have.

The finding that the leishmanial sulfurtransferases can use thioredoxin as acceptor with a K_m of 300 nM suggests that this could also be a natural substrate. It has been demonstrated previously that thioredoxin from *E. coli* can serve as a sulfur acceptor substrate for the *E. coli* sulfurtransferase when thiosulfate was near its K_m (Ray *et al.*, 2000) and it has also been shown that mammalian sulfurtransferases can utilise thioredoxin (Nandi *et al.*, 2000). Its would be intriguing to speculate that the CH2A and CH2B motifs (mentioned in Chapter 3) flanking the active site, thought to be involved in protein-protein interaction, might be involved in the recognition of this dithiol protein substrate. At one time thioredoxin was considered to be absent from *Leishmania* (see Krauth-Siegal and Coombs, 1999), however a thioredoxin-like gene has been identified in *L. major* (GenBank accession number AAG10802) and *T. brucei* (Reckenfelderbaumer *et al.*, 2000). *Leishmania* contains multiple dithiols that have been characterized e.g. trypanothione and tryparedoxin. Given the overlap demonstrated by the 'dithiol family' of proteins, it is possible that they can also serve as sulfur acceptor substrate for sulfurtransferase. One possible function of the interaction between a sulfutransferase and thioredoxin could be the involvement of the enzyme in reduction and detoxification of peroxides (Nandi *et al.*, 2000). The finding that the leishmanial enzyme cannot catalyse this reaction suggests that it must have some other role. The oxidation status of thioredoxin is thought to be crucial in regulating a number of cellular reactions (Holmgren and Fagerstedt, 1982; Berendt *et al.*, 1995) and so the oxidation by

the leishmanial sulfutransferase of reduced thioredoxin implicates this enzyme in similar processes.

The antioxidant machinery of trypanosomatids has been considered to rely almost exclusively on trypanothione and its associated enzymes (Krauth-Siegel and Coombs, 1999). However, other enzyme systems may well also be involved. The up-regulation of LmajMST when *L major* promastigotes were exposed to the oxidant cumene hydroperoxide shows that MST may be an important additional enzymatic mechanism for protection against oxidative stress.
The up-regulation of MST when *L. major* promastigotes were cultured without an exogenous source of sulfur suggests that the enzyme might also have a physiological role in sulfur amino acid metabolism. The expression of RhdA, a rhodanese-like protein of *Synechococcus* sp strain PCC7942, is also induced by sulfur starvation (Laudenbach *et al.*, 1991). Furthermore, sulfurtranferases have been implicated in the metabolism of sulfur as shown by the formation and regulation of iron-sulfur centres of proteins (Ogata and Volini, 1990). Another possible role of the sulfurtransferase in *Leishmania* is that it functions to provide sulfide for the cysteine synthesis pathway. LmajMST, like the rhodanese from *Thiobacillus novellus,* was capable of providing sulfide from mercaptopyruvate in the absence of an acceptor molecule. The results have shown that LmajMST works in conjunction with both recombinant *L. major* cysteine synthase (GenBank accession number AL 499624) and also recombinant *L. major* cystathionine β-synthase (GenBank accession number AL 499619) to synthesise cysteine *in vitro*. Whether this occurs *in vivo* in *Leishmania* remains to be

determined together with the levels of mercaptopyruvate and also its concentration.

This idea has been tested with the yeast two-hybrid assay with one isoenzyme of each protein involved in the cysteine synthesising pathway of *Arabidopsis* chosen as the putative interacting partner. The results were negative for each single protein (see Papenbrock and Schmidt, 2000). These experiments make the association of MST with the *de novo* synthesis *in vivo* of cysteine unlikely. Nevertheless, disadvantages (such as masking of interactions due to lack of activators necessary for interaction) associated with the yeast two-hybrid system cannot be ignored (Ito *et al.,* 1999).

The first line drugs used against leishmaniasis are the organic pentavalent antimonials Pentostam and Glucantime (Croft, 1997). The mode of action of these compounds is unknown, although the perceived dogma is that they are reduced to the trivalent equivalents, probably within the amastigote stage of the parasite (Shaked-Mishan *et al.*, 2001), and that the resultant trivalent compounds interact with some essential thiol (Haimeur *et al.*, 1999). It was conceivable that the sulfurtransferases could be the target enzymes. Therefore the use of antimonials as both substrate and inhibitor was assessed. Replacing β-mercaptoethanol with the pentavalent antimonial increased sulfurtransferase activity by 50% relative to the assay mixture without an acceptor molecule. This could be intriguing, but the involvement of MST in the reduction of the pentavalent form to the trivalent form needs further investigation. However, no such increase was observed in MST activity with the trivalent equivalent

Triostam. The results obtained showed that although it does inhibit LmajMST, it was not particularly potent. Thus, the possibility that this enzyme is involved in some way in the reduction of the pentavalent drug to its trivalent form within the parasite should not be ruled out yet.

Eukaryotic mercaptopyruvate sulfurtransferases can be cytosolic or mitochondrial (Nagahara and Nishino, 1996; Papenbrock and Schmidt, 2000). Both the immunolocalisation and fractionation data suggest that the MST of *L. major* occurs within the cytosol. The only other report on a sulfurtransferase of a protozoan (Watenebe *et al.*, 1985) suggested that an enzyme in *Euglena gracilis* was also distributed in the cytosol.

The results obtained provide compelling evidence that *Leishmania* contain high activity of a structurally unusual sulfurtransferase that has good activity towards mercaptopyruvate and thioredoxin and plays some role in antioxidant metabolism and in sulfur amino acid metabolism. The finding that the enzyme itself differs from mammalian counterparts structurally (Chapter 3) and is expressed in the mammalian form of the parasite provides optimism that it may have a crucial role in the parasite and so represent a possibly useful drug target. Very little is known about sulfurtransferases in protozoa, but the findings reported here suggest that study of them will yield interesting new insights into the roles and structures of this widespread class of enzymes.

The successfully generation of a null mutant and its characterisation, as well as the characterisation of the transgenic strains over-expressing the LmajMST gene,

will provide insights into the possible role of the parasite with regards to oxidative stress, sulphur metabolism and its involvement in cysteine biosynthesis. Further, an analysis of the thiols, thiosulfate and mercaptopyruvate, levels of both wild type and transgenic derivatives can provide information of potential donors and acceptors molecules *in vivo*.

The stage is also set to look at the interaction of LmajMST with other proteins, e.g. dithiols, believed to be the key molecules in *Leishmania* defence against oxidative stress. Further, sulfane sulfur, the precursor of sulfur incorporated into Fe-S proteins, has long been known to be produced by sulfurtransferases such as rhodanese (Bonomi *et al.*, 1977; Pagani *et al.*, 1983). A possible investigation of the role of this protein in such a process could also be informative and will yield interesting new insights into the roles of this widespread class of enzymes.

Chapter 5

Cysteine biosynthesis via the sulfydrylase pathway in Leishmania major

5.0 *Introduction*

The *de novo* synthesis of cysteine, otherwise called the sulfhydralase pathway, is one of two major routes organisms use to synthesis cysteine in nature. Cysteine is formed from serine in a two-step reaction initiated by serine acetytransferase (SAT) to form an intermediate *O*-acetylserine (OAS) which condenses with sulfide from a variety of sources to form cysteine- a reaction catalysed by cysteine synthase (CS). Plants (Blaszczyk *et al.*, 1999), fungi (Marzluf, 1997), bacteria (Kredich *et al.*, 1969) and protozoa (Goodal, 2001; Nozaki *et al.*, 2001), but not mammals, can fix sulfur and synthesize cysteine using the sulfhydrylase pathway (Figure 5.1). The sulfide required is formed via the sulfate assimilation pathway in bacteria and some protozoa. However, it is speculated that in other protozoa either methanethiol, formed by the action of methionine γ-lyase on methionine in *Trichmonas vaginalis*, or a sulfurtransferase such as rhodanese can provide the sulfide source (Schmidt and Jager, 1992; Nozaki *et al.*, 2001).

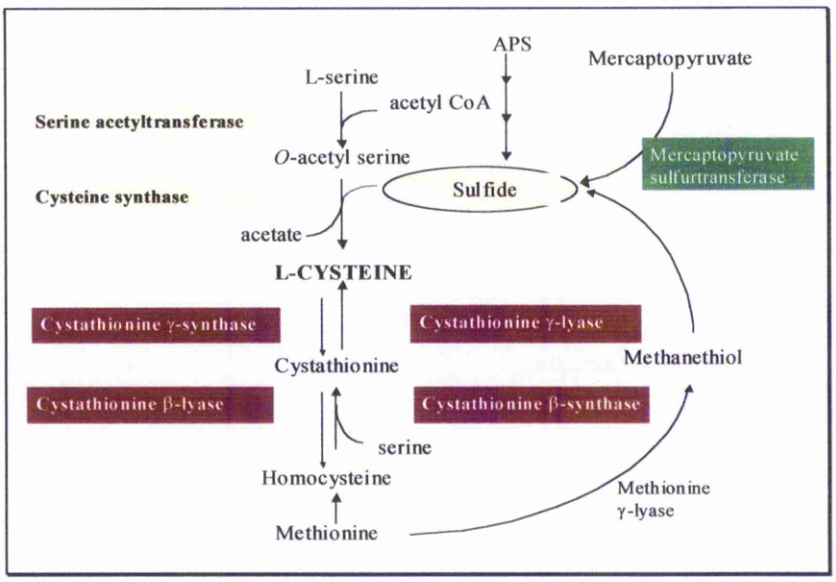


Figure 5.1. Metabolic map of the *de novo* synthesis of cysteine. The scheme shows the enzymes, serine acetyltransferase (yellow) and cysteine synthase (yellow) involved in the two-step synthesis of cysteine from serine via the intermediate *O*-acetylserine (OAS). OAS condenses with sulfide from a variety of sources: the sulfate assimilatory pathway with (APS) being the initial substrate ; methanethiol produced by the action of methionine γ- lyase on homocysteine; and the action of mercaptopyruvate sulfurtransferase on mercaptopyruvate. The trans-sulfuration pathway that inter-converts cysteine and methionine is also shown. Enzymes involved in this pathway are shaded dark red.

SAT forms a complex with CS suggesting an efficient metabolic channelling of the intermediate OAS (Ruffet *et al.*, 1994; Bogdanova and Hell, 1997), while CS occurs either bound or free (Kredich *et al.*, 1969; Nakamura *et al.*, 1987; Droux *et al.*, 1992; Ruffet *et al.*, 1994). Bound CS is inactive, but can regulate SAT activity, while the unbound CS dimer is active and capable of catalysing cysteine formation. SAT coupled with CS is known to be highly active and has a high affinity for its substrates and its action results in the production of OAS. This reaction is rate limiting for cysteine synthesis (Neuenschwander *et al.*, 1991; Saito *et al.*, 1994). It is interesting

to note that free SAT purified from spinach was inactive, and its activation was dependent on a saturating amount of CS (Ruffet *et al.*, 1994). The OAS produced was not channelled within the complex (Cook and Wedding, 1977), but was released into the surrounding solution to react with free CS dimers and sulfide to form cysteine. These findings are not consistent with the report that a bi-enzymatic complex is required to channel the intermediate OAS (Bogdanova and Hell, 1997).

In vitro, the SAT/CS complex is regulated by its substrates as well as cysteine. A high concentration of OAS (Rennenberg, 1983; Neuenschwander *et al.*, 1991; Saito *et al.*, 1994) and sulfide (Kredich, 1987) regulate the dissociation or stabilization of the complex, respectively (Droux *et al.*, 1998; Hell, 1998), while cysteine can feedback inhibit cysteine synthesis (Saito *et al.*, 1995). Thus the evidence that SAT plays an important role in regulating cysteine biosynthesis and sulfate assimilation is considerable. This has been verified by *in vitro* biochemical studies (Bogdanova and Hell, 1997; Droux *et al.*, 1998) and *in vivo* by a genetic approach using the 2-hybrid system (Bogdanova and Hell, 1997).

An intriguing feature of cysteine formation in plants is that the activity of SAT is much lower when compared to the activity of CS (Nakamara *et al.*, 1987; Ruffet *et al.*, 1994). CS activity is usually 10 to 345-fold higher than SAT (Smith, 1971; Ngo and Shargool, 1974; Nakamura *et al.*, 1987; Ruffet *et al.*, 1994). It is thought that such an expression ratio is vital to stabilize the interaction of the two proteins, and allosterically regulate the efficient synthesis of cysteine. However, *in vitro* experiments with protein extracts from plants have shown that this phenomenon is not universal (Kuske *et al.*, 1994).

In plants, homomerization and heteromerization of SAT is attributed to a central SAT-SAT domain, and a C-terminal SAT-CS domain respectively (Figure 5.2) (Bogdanova and Hell, 1997).

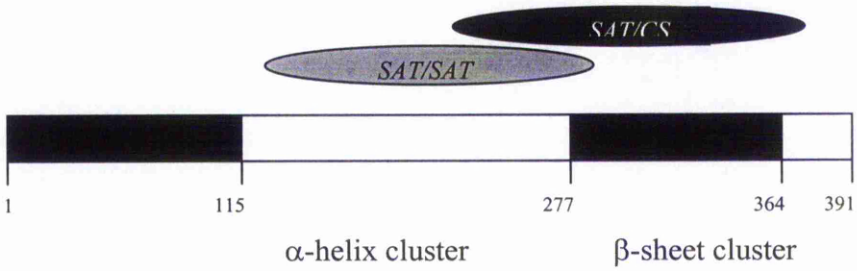


Figure 5.2. Domain structure of SAT. The ORF of *A. thaliana* showing the central SAT/SAT and C-terminal SAT/CS protein interaction domains indicated by grey and black ovals, respectively. The secondary structure of the SAT-SAT (open box) and SAT-CS (black box) domains are shown.

The highly conserved C-terminal domain of SAT has considerable homology to the *O*-acyltransferase protein family (Murray and Shaw, 1997). The carboxyl terminal end of both protein families are composed of imperfect copies of a hexapeptide repeats of acyltransferases (Dicker *et al.*, 1992; Vaara, 1992), described as ([LIV]-[GAED]-X2-[STAV]-X) annotated in the PROSITE database (Bairoch, 1993). Hexapeptide proteins as they are usually called, function in acyltransferase activity, cell wall biosynthesis, amino acid metabolism, and detoxification (Gilvarg *et al.*, 1970; Anderson and Raetz, 1987; Downie, 1989; Kelly *et al.*, 1993; Raetz and Roderick, 1995; Murray and Shaw, 1997). These acyltransferases are dominated by left-handed β-helix structures (Raetz and Roderick, 1995) and are quaternally arranged in homotrimers (Kisker *et al.*, 1996; Beaman *et al,*1997). The three clefts between the three subunits form the catalytic centres in which a histidine residue is

essential for transfer of the acetyl or succinyl moiety from CoA to the second substrate (Kisker *et al.*, 1996; Beaman *et al.*, 1997). The similarity between these two families of proteins could have arisen by the stacking of an 'ancestral' trimer, but such an evolutionary transition may have facilitated the formation of a larger, more stable and efficiently regulated SAT/CS complex. However, in the absence of structural data on SAT (Wigley *et al.*, 1990; Leu and Cook, 1994), the function of its hexapeptide-repeat domain in transferase activity, and oligomerization with CS, remain speculative. In plant, SAT-SAT interaction was adequately provided by a central α-helical domain (Bogdanova and Hell, 1997). The physiological relevance of this is not known.

Cysteine is the major source of fixed sulfur used to synthesize sulfur-containing compounds in both prokaryotes and eukaryotes (Giovanelli *et al.*, 1980). Cysteine, is significantly abundant and is the major thiol in glutathione-lacking protists such as *Entamoeba spp.* (Fahey *et al.*, 1984). These organisms salvage and are dependent on an exogenous source of cysteine despite their capability to synthesize cysteine *de novo* (Tekwani *et al.,* 1999*)*. Cysteine maintains a low redox potential within the cell, aids cell growth, and helps detoxify oxygen reactive species. (Gillan *et al.*, 1980; 1981). *T. brucei* (Duzenko *et al.*, 1992), and *E. histolytica* (Nozaki *et al.*, 1998; 1999) likewise requires cysteine for growth, but catalyse synthesize cysteine *de novo*, and how they satisfy their sulfur requirement remains unknown. In this chapter, the characterization of the enzymes serine acetyltransferase (SAT) and cysteine synthase (CS) involved in the *de novo* synthesis of cysteine in *L. major* is presented. The cysteine requirement for the growth of *L. major* will also be discussed.

5.1 Material and Methods

5.2. PCR amplification of LmajSAT and LmajCS

The ORFs of the genes encoding *LmajSAT, and LmajCS* were amplified from the gDNA by PCR with the Expand High Fidelity system using a thermal cycle of 94°C, for 2 min; 30 cycles of 94°C, for 30s; 65°C, for 30s; 72°C, for 3 min; and a final step of 72°C, for 7 min with appropriate primers detailed in Table 5.1.

Primers used for gene amplification were modified with *Nde*I and *Not*I restriction sites (underlined), to facilitate cloning. The PCR products were cloned into pGEM-T (Qiagen) to generate plasmids designated as *pLmajSAT* and *pLmajCS*. Plasmids were propagated in *E. coli* DH5α cells and plasmid DNA were prepared by the Qiagen method and sequenced on both strands (MWG Biotech. or University of Glasgow Sequencing Service). Plasmids *pLmajSAT* and *pLmajCS* was digested with *Nde*I and *Not*I and the restriction endonuclease fragments of 1410 and 1010 bp, encoding the *LmajSAT* and *LmajCS* gene sequences respectively, were ligated into *NdeI/Not*I-digested pET21a^{+} vector (Invitrogen) to produce *pETLmajSAT* and *pETLmajCS.*

5.3 Expression and Purification of LmajSAT and LmajCS

rLmajSAT and rLmajCS were expressed and purified using the same procedures described for rLmajMST except that: (a) expression was induced by 0.5 mM IPTG for 5 hours at 15°C: (b) cells were pelleted and resuspended in 5 ml of sonication buffer A with 25 μM pyridoxial phosphate (PLP) for CS; and (c) the eluant from the affinity column was dialysed with excess 500 mM Tris-HCl, pH 8.0, with 25 μM PLP at 4°C overnight to remove salt and imidazole and then stored at 4°C until analysed.

Approximately 3 mg and 25 mg soluble, recombinant proteins (rLmajSAT and rLmajCS, respectively) were obtained from 1 litre cultures of induced *E. coli.* No PLP was used in the purification of rLmajSAT.

5.4 Production of LmajSAT and *LmajCS over-expressing and knock-out plasmids*

The ORFs of the *LmajSAT* and *LmajCS* genes were amplified by PCR (denaturation at 94°C for 2 min, 30 cycles of 94°C for15 sec, 64°C for 30 sec, 72°C for 2 min and a final extension of 7 min at 72°C) using oligonucleotide primers detailed in Table 5.1. Primers were modified with the *Sma*I and *BamH*I restrictions sites to facilitate subcloning into the re-expression vector, *pGL607*. The amplified *LmajSAT* and *LmajCS* genes were subcloned into the pGEM-T vector (Promega) to give *pGEMTrLmajSAT* and *pGEMTrLmajCS* and subsequently into the *Sma*I/*BamH*I-cut pGL607 re-expression vector to generate the plasmids *pGL607SAT* and *pGL607CS,* which were subsequently used to transfect *L. major* promastigotes as detailed in Chapter 2. The re-ligated *Sma*I/*BamH*I-cut pGL607 without any insert acted as a control and was designated *pGL607*.

Single-targeted gene replacement constructs were obtained by PCR amplification (denaturation at 94°C for 2 min, 30 cycles of 94°C for 15 sec, 64°C for 30 sec, 72°C for 2 min for 30 cycles and a final extension of 7 min at 72°C) of the 5'- and 3'-untranslated regions (5' and 3' UTR) of *LmajSAT* and *LmajCS* using primers detailed in Table 5.1. The nucleotides used in primer design of the UTRs were obtained from HTGS from the on-going *L. major* sequencing project. The primers (KOSAT1 and KOCS1; KOSAT2 and KOCS2; KOSAT3 and KOCS3; KOSAT4 and KOCS4) were modified with the *Bgl*II, *BamH*I, *Spe*I and *Hind*III restriction sites, respectively. The

Table 5.1 Characteristics of primers used in the amplification of *LmajSAT*, *LmajCS* and *LmajCBS* genes from genomic DNA (gDNA)

Primers	Forward (5'-3')	Primers	Reverse (5'-3')	Comments
SAT1r	5'GGATCCATATGAAGCGCCTTGACCGCATTCGGGA3'	SAT2r	5'CCCGGGTCAGATGCCAGAGCCATC3'	Rexpression
CS1r	5'GGATCCGATATGGCAGCACCGTTCGACAAGTCGAGA3'	CS2r	5'CCCGGGTTCCCTGCAGCTCCGAGGCATCGACTACTG3'	Rexpression
KOSAT1	5'AAGCTTTTCACAGATGGAACTGCTCTTGTGGCCGCTA3'	KOSAT2	5'GTCGACACACATGCACTCACCTCTCGCAGTAGTAGC3'	Knock-out 5' flank
KOCS1	5'AAGCTTCGGCGAAACACGCCAGCCCT3'	KOCS2	5'GTCGACTTCTCGACTTGTCGAACGGTGCTGC3'	Knock-out 5' flank
KOSAT3	5'CCCGGGTGATGCGCGGAAGCGAGCAC3'	KOSAT4	5'AGATCTACCACCGGGATCGATGCGCGCCGAAAA3'	Knock-out 3' flank
KOCS3	5'CCCGGGTGAACTGCCACCGGCCACGT3'	KOCS4	5'AGATCTGTGCTCATACAGTAGTGTAGGCCAAGGAGGAGG3'	Knock-out 3' flank

DNA fragments were subcloned into the pGEM-T vector to give *pGEMTαSAT-5F*, *pGEMTαSAT-3F*, *pGEMTαCS-5F* and *pGEMTαCS-3F* and sequenced. The *Bgl*II/*BamH*I *LmajSAT* and *LmajCS* 5'-UTR PCR DNA fragments, the *Spe*I/*Hind*III *LmajSAT* and *LmajCS* 3'-UTR PCR DNA fragments and the *Spe*I-*BamH*I DNA fragment containing the hygromycin phosphates gene were ligated consecutively into *pGL345* to generate the knock-out plasmids *pGL345αSAT* and *pGL345αCS*. *L. major* promastigotes were transfected with this construct as described in Chapter 2. Positive clones were analysed by southern blotting using the [α-^{32}P] dATP labelled 5'-UTR as probe.

5.5 Cultivation of L. major *in cysteine-free medium*

To determine the cysteine requirements of *L. major* promastigotes, cells to be sub-passage and grown axenically in minimum essential medium (MEM) with Earl salts (ICN-biomedical) but without exogenous sulfur source (none of cysteine, cystine, methionine and serine were present) and supplemented with 20% (v/v) dialysed heat inactivated fetal calf serum (dHiFCS) (Sigma-Aldrich, UK) for 6 days at 27°C. Prior to the introduction into the new medium cells were washed once in phosphate buffer (PBS). To facilitate growth of promastigotes under these conditions, *D*- glucose (1000 mg.L^{-1}), glutathione (0.10 mg.L^{-1}), hypoxanthine (0.70 mg.L^{-1}), thiamine (0.6 mg.L^{-1}), uracil (0.6 mg.L^{-1}), alanine (.50 mg.L^{-1}), aspartic acid (0.06 mg.L^{-1}), glutamic acid (0.14 mg.L^{-1}), glutamine (0.02 mg.L^{-1}), proline (0.08 mg.L^{-1}), haemin (.035 mg.L^{-1}), and folic acid (0.02 mg.L^{-1}) were added to the medium. To investigate the use of sulfur sources by *L. major,* the medium was further supplemented with either methionine (0.2 mg.L^{-1}) or cysteine (0.2 mg.L^{-1}) or homocysteine (0.2 mg.L^{-1}) or

serine (0.2 mg.L^{-1}) or *O*-acetylserine (0.2 mg.L^{-1}) or hydrogen sulfide (0.15 mg.L^{-1}). Cells cultured in medium without an exogenous sulfur source acted as the control in these experiments. Cell densities were determined at days 1 and 6.

5.6 Results

5.6.1 Identification, Cloning and Characterisation of a Gene Encoding SAT in L. major

To identify the SAT gene (E.C 2.3.1.30.) in *L. major*, advantage was taken of the *L. major* sequencing projects. HTGS (accession numbers. LM 34.2 contig 123 and LM34.2 contig 268) containing the N- and C- termini of the *LmajSAT* gene were identified by BLAST searches of the *L. major* sequencing project database (http://www2.ebi.ac.uk/blast2/parasite.html) using the amino acid sequences of *T. cruzi* SAT (TcSAT) as the query. The overlapping HTGS sequences were made into a contig to produce the 1.4 kb ORF of *LmajSAT*. The ORF of *LmajSAT* was amplified with gene-specific primers SAT1 and SAT3 as forward and reverse primers, respectively, after 30 cycles of PCR (Figure 5.3a). Sequencing and BLASTX analysis showed that the nucleotide sequence encoded a protein possessing strong homology to SAT from various organisms. rLmajSAT was expressed and purified as soluble protein from *E. coli* (Figure 5.3b). The predicted amino acid sequence of LmajSAT of *L. major* comprised 411 residues with a calculated molecular mass of 45.5 kDa (Figure 5.4).

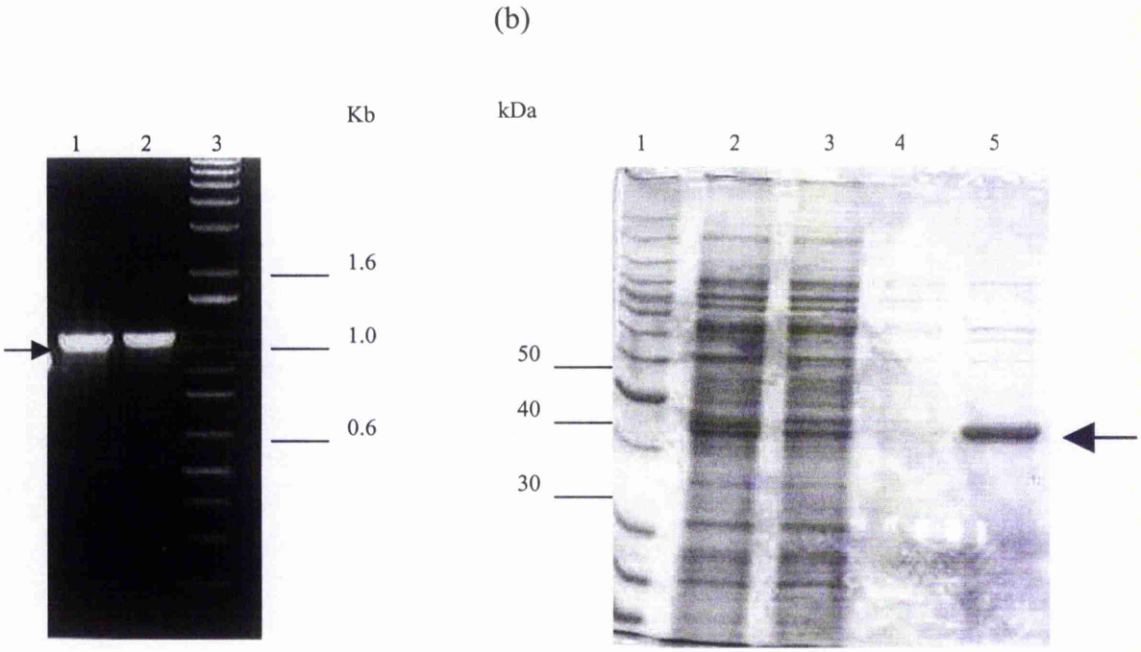


Figure 5.3 (a) Ethidium bromide-stained 0.7% agarose gel showing PCR product of *LmajSAT of Leishmania major*. Lanes 1 and 2, *LmajSAT* (arrowed); Lane 3, DNA standards. (b) Purification of rLmajSAT under native conditions. The soluble protein in the supernatant fraction was purified on a nickel chelator column. The His-tagged protein was eluted by addition of Buffer A with 500 mM imidazole. SDS-PAGE analysis of the following samples: Lane 1. molecular mass standards; Lanes 2 and 3, supernatant and pellet of fractions of a crude extract, respectively, of the *E. coli* cells; Lane 4, wash with Buffer A with 60 mM imidazole; lane 5, purified rLmajSAT eluted from the nickel chelator column with Buffer A with 500 mM imidazole. Estimated recombinant protein size: 45 kDa (arrowed).

```
          M  K  R  L  D  R  I  R  E  A  L  E  Q  L  N  P  Q  R  A  D  S  T  L  P
1         ATGAAGCGC CTTGACCGC ATTCGGGAG GCTCTGGAG CAGCTGAAC CCGCAGCGC GCGGATAGC ACTTTGCCT
          TACTTCGCG GAACTGGCG TAAGCCCTC CGAGACCTC GTCGACTTG GGCGTCGCG CGCCTATCG TGAAACGGA
          T  F  H  P  R  I  S  L  H  K  L  K  E  L  V  E  C  L  F  Y  V  F  F  P
73        ACGTTTCAC CCCCGCATC AGCCTTCAC AAGTTGAAG GAGCTGGTC GAGTGCCTC TTTTATGTC TTCTTCCCA
          TGCAAAGTG GGGGCGTAG TCGGAAGTG TTCAACTTC CTCGACCAG CTCACGGAG AAAATACAG AAGAAGGGT
          E  C  T  N  P  P  S  E  V  T  P  T  F  A  E  A  D  S  L  M  E  W  V  I
145       GAGTGCACG AACCCGCCG TCGGAGGTG ACGCCGACG TTCGCCGAG GCAGACTCG CTGATGGAG TGGGTGATT
          CTCACGTGC TTGGGCGGC AGCCTCCAC TGCGGCTGC AAGCGGCTC CGTCTGAGC GACTACCTC ACCCACTAA
          G  H  I  A  D  I  L  V  T  Q  I  Y  V  A  F  V  L  H  G  D  A  H  A  V
217       GGCCACATT GCAGACATT CTTGTGACA CAGATCTAC GTCGCCTTT GTCTTGCAC GGCGACGCG CACGCGGTG
          CCGGTGTAA CGTCTGTAA GAACACTGT GTCTAGATG CAGCGGAAA CAGAACGTG CCGCTGCGC GTGCGCCAC
          E  D  T  K  P  F  K  P  L  N  T  S  Q  R  T  Q  L  E  W  N  G  Q  R  C
289       GAGGACACA AAGCCTTTC AAGCCCTTG AACACATCG CAACGCACG CAGCTGGAG TGGAATGGG CAGCGCTGC
          CTCCTGTGT TTCGGAAAG TTCGGGAAC TTGTGTAGC GTTGCGTGC GTCGACCTC ACCTTACCC GTCGCGACG
          A  T  L  S  L  L  A  M  E  S  R  S  T  K  G  G  F  A  V  A  A  A  A  E
361       GCCACACTG TCGTTGCTC GCGATGGAG TCGAGAAGC ACAAAAGGT GGATTTGCT GTGGCTGCT GCGGCGGAG
          CGGTGTGAC AGCAACGAG CGCTACCTC AGCTCTTCG TGTTTTCCA CCTAAACGA CACCGACGA CGCCGCCTC
          Q  D  A  L  K  A  S  L  E  E  L  S  C  T  G  N  R  A  R  L  P  S  T  E
433       CAGGACGCT TTAAAGGCA TCGCTTGAA GAGCTGAGC TGCACGGGG AACCGCGCT CGCTTACCA TCGACGGAG
          GTCCTGCGA AATTTCCGT AGCGAACTT CTCGACTCG ACGTGCCCC TTGGCGCGA GCGAATGGT AGCTGCCTC
          V  S  S  K  S  A  A  S  K  A  N  A  M  R  V  T  A  D  I  N  S  S  A  N
505       GTCTCGTCC AAGTCGGCA GCCTCTAAG GCAAACGCC ATGCGTGTC ACTGCCGAC ATCAACAGC AGCGCCAAT
          CAGAGCAGG TTCAGCCGT CGGAGATTC CGTTTGCGG TACGCACAG TGACGGCTG TAGTTGTCG TCGCGGTTA
          Q  D  F  L  T  N  C  K  R  R  A  D  A  I  V  T  K  F  V  V  E  R  L  A
577       CAGGACTTC CTCACCAAC TGCAAGCGC AGGGCAGAC GCGATTGTA ACGAAGTTT GTCGTGGAG CGACTCGCG
          GTCCTGAAG GAGTGGTTG ACGTTCGCG TCCCGTCTG CGCTAACAT TGCTTCAAA CAGCACCTC GCTGAGCGC
          H  V  H  W  L  L  H  T  D  V  E  A  I  F  K  G  D  V  A  A  S  S  P  S
649       CACGTGCAC TGGCTCCTC CACACAGAC GTGGAGGCC ATCTTTAAG GGGGACGTT GCCGCCTCC TCACCGAGT
          GTGCACGTG ACCGAGGAG GTGTGTCTG CACCTCCGG TAGAAATTC CCCCTGCAA CGGCGGAGG AGTGGCTCA
          E  V  V  L  C  Y  P  G  L  R  C  M  V  H  Q  R  V  A  H  Q  L  H  L  L
721       GAAGTGGTA CTGTGCTAC CCAGGACTT CGCTGCATG GTGCATCAG CGCGTGGCA CATCAGCTG CACCTGCTC
          CTTCACCAT GACACGATG GGTCCTGAA GCGACGTAC CACGTAGTC GCGCACCGT GTAGTCGAC GTGGACGAG
          G  V  P  Q  N  F  T  R  M  L  T  E  L  A  H  S  E  T  G  I  D  I  H  P
793       GGAGTCCCA CAGAACTTT ACCCGTATG CTGACAGAG TTGGCGCAT TCAGAGACG GGGATCGAC ATCCACCCG
          CCTCAGGGT GTCTTGAAA TGGGCATAC GACTGTCTC AACCGCGTA AGTCTCTGC CCCTAGCTG TAGGTGGGC
          N  T  A  I  G  H  H  F  F  I  D  H  G  T  G  V  V  I  G  A  T  A  V  I
865       AACACGGCC ATTGGGCAT CATTTCTTC ATTGACCAC GGCACCGGC GTCGTCATA GGGGCGACG GCGGTCATC
          TTGTGCCGG TAACCCGTA GTAAAGAAG TAACTGGTG CCGTGGCCG CAGCAGTAT CCCCGCTGC CGCCAGTAG
          G  N  D  V  S  I  Y  Q  G  V  T  L  G  A  R  S  F  P  T  D  E  K  T  G
937       GGCAATGAC GTCTCCATC TACCAAGGT GTTACTCTT GGTGCGCGG AGTTTTCCG ACGGACGAG AAAACAGGC
          CCGTTACTG CAGAGGTAG ATGGTTCCA CAATGAGAA CCACGCGCC TCAAAAGGC TGCCTGCTC TTTTGTCCG
          E  R  I  R  D  L  P  R  H  P  I  I  E  D  G  V  T  L  Y  A  N  A  V  V
1009      GAGAGGATT CGAGACCTC CCACGACAC CCCATCATC GAGGACGGC GTCACGCTG TACGCAAAC GCCGTTGTG
          CTCTCCTAA GCTCTGGAG GGTGCTGTG GGGTAGTAG CTCCTGCCG CAGTGCGAC ATGCGTTTG CGGCAACAC
          L  G  R  V  T  I  G  E  R  S  T  I  G  G  N  C  W  V  V  R  D  V  P  P
1081      CTGGGTCGC GTGACAATC GGTGAGCGC AGCACCATT GGCGGTAAC TGCTGGGTG GTGCGCGAT GTGCCGCCC
          GACCCAGCG CACTGTTAG CCACTCGCG TCGTGGTAA CCGCCATTG ACGACCCAC CACGCGCTA CACGGCGGG
          F  S  A  I  V  Q  R  S  H  H  V  L  Q  P  H  E  R  M  F  L  E  G  D  G
1153      TTCTCAGCG ATAGTGCAG AGGTCCCAC CACGTACTG CAACCACAT GAGCGCATG TTTCTTGAG GGGGATGGC
          AAGAGTCGC TATCACGTC TCCAGGGTG GTGCATGAC GTTGGTGTA CTCGCGTAC AAAGAACTC CCCCTACCG
          S  G  I
1225      TCTGGCATC
          AGACCGTAG
```

Figure 5.4 Nucleotide sequence of *L. major* SAT gDNA and deduced amino acid sequence. Nucleotides are numbered in the 5' to 3' direction, beginning with the first residue at the start codon and ending at the codon preceding the stop codon.

5.6.2 Features of Predicted Amino Acid Sequence of L. major *Serine acetyltransferase (SAT)*

The deduced amino acid sequence of LmajSAT has significant identities with SAT from *T. cruzi* (TcSAT, 57%), *E. histolytica* (EhSAT, 37%) and *E. dispar* (EdSAT, 37%) as well as SATs from bacterial and plant origin. Among the homologues from

bacteria, two species of Cyanobacteria, *Synechococcus sp.* PCC7942 (accession numbers U23436 and L41665) and *Synechocystis sp.* PCC6803 (accession number D90912), showed the highest identities (47-60%) to *LmajSAT* within the conserved region.

Sequence alignment of LmajSAT with amino acid sequences of SAT from other organisms shows that great diversity exists at the N- and C- termini regions of SAT proteins (Figure 5.5). A comparison of *L. major* and other protozoon, plant and bacterial SAT amino acid sequences reveal that *L. major*, *T. cruzi*, *E. histolytica*, *E. dispar* and *P. yoeli* as well as the *A. thaliana*, *E. coli*, *Synechococcus sp.* and *Synechocystis spp.* carry amino-terminal extensions (NTE). The NTE was absent from SAT of *S. pombe*, *A. thaliana, M. tuberculosis, Nostoc sp. A. vinelandii, L. interrugans, B. subtilis* and *S. mutans* (Figure 5.5). In plants SAT, the NTEs are known to be organelle-targeting sequences.

The most conserved region between all known SATs has been suggested by primary structure comparisons to *E. coli* SAT to confer transferase activity (Vaara, 1992). The LmajSAT protein has the hexapeptide structural domain (Figure 5.6) well conserved at the primary sequence level. The domain consists of β-sheets, T-turns and loops arranged in four coils. Between coil regions 2 and 3 in the LmajSAT protein is an insertion of 17 residues (Figure 5.5). This insertion is also present in SAT from *Entamoeba spp.* (Nozaki *et al.*, 1999), *P. yoeli*, the Cyanobacteria, *Synechococcus spp.* and *Synechocystis spp.*

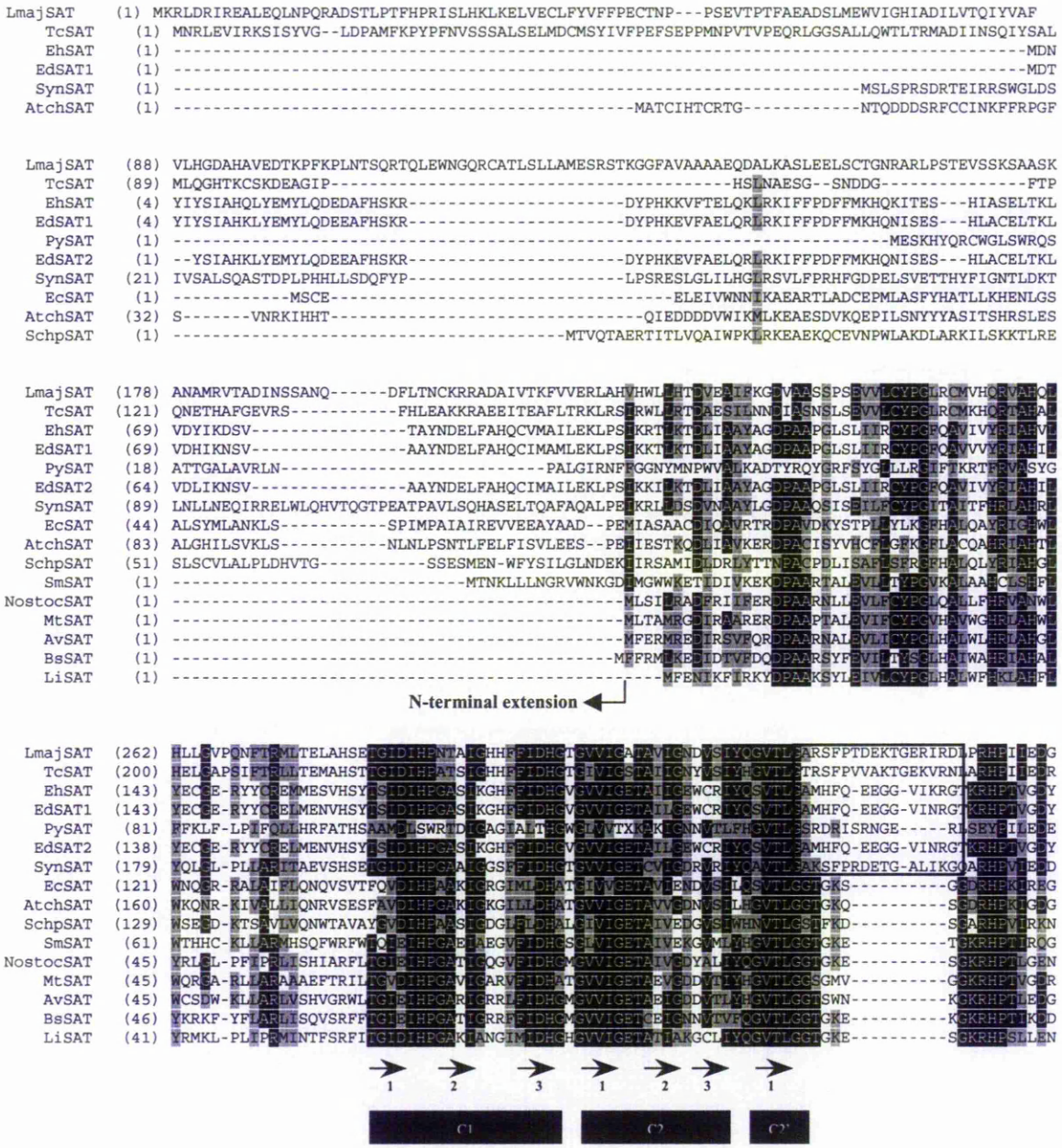

LmajSAT (1) MKRLDRIREALEQLNPQRADSTLPTFHPRISLHKLKELVECLFYVFFPECTNP---PSEVTPTFAEADSLMEWVIGHIADILVTQIYVAF
TcSAT (1) MNRLEVIRKSISYVG--LDPAMFKPYPFNVSSSALSELMDCMSYIVFPEFSEPPMNPVTVPEQRLGGSALLQWTLTRMADIINSQIYSAL
EhSAT (1) ----------MDN
EdSAT1 (1) ----------MDT
SynSAT (1) ----------MSLSPRSDRTEIRRSWGLDS
AtchSAT (1) ----------MATCIHTCRTG----------NTQDDDSRFCCINKFFRPGF
LmajSAT (88) VLHGDAHAVEDTKPFKPLNTSQRTQLEWNGQRCATLSLLAMESRSTKGGFAVAAAAEQDALKASLEELSCTGNRARLPSTEVSSKSAASK
TcSAT (89) MLQGHTKCSKDEAGIP----------HSLNAESG--SNDDG----------FTP
EhSAT (4) YIYSIAHQLYEMYLQDEDAFHSKR----------DYPHKKVFTELQKLRKIFFPDFFMKHQKITES---HIASELTKL
EdSAT1 (4) YIYSIAHKLYEMYLQDEEAFHSKR----------DYPHKEVFAELQRLRKIFFPDFFMKHQNISES---HLACELTKL
PySAT (1) ----------MESKHYQRCWGLSWRQS
EdSAT2 (1) --YSIAHKLYEMYLQDEEAFHSKR----------DYPHKEVFAELQRLRKIFFPDFFMKHQNISES---HLACELTKL
SynSAT (21) IVSALSQASTDPLPHHLLSDQFYP----------LPSRESLGLILHGLRSVLFPRHFGDPELSVETTHYFIGNTLDKT
EcSAT (1) ----------MSCE----------ELEIVWNNIKAEARTLADCEPMLASFYHATLLKHENLGS
AtchSAT (32) S-------VNRKIHHT----------QIEDDDDVWIKMLKEAESDVKQEPILSNYYYASITSHRSLES
SchpSAT (1) ----------MTVQTAERTITLVQAIWPKLRKEAEKQCEVNPWLAKDLARKILSKKTLRE
LmajSAT (178) ANAMRVTADINSSANQ------DFLTNCKRRADAIVTKFVVERLAHVHWLGHTDVEAIFKGGVAASSPSSVVLCYPGLRCMVHQRVAHQL
TcSAT (121) QNETHAFGEVRS----------FHLEAKKRAEEITEAFLTRKLRSLRWLGRTDAESILNNNIASNSLSSVVLCYPGLRCMKHQRIAHAL
EhSAT (69) VDYIKDSV----------TAYNDELFAHQCVMAILEKLPSLKKTLKTDLIGAYAGDPAAPGLSLIIRCYPGFQAVIVYRIAHVL
EdSAT1 (69) VDHIKNSV----------AAYNDELFAHQCIMAMLEKLPSLKKTLKTDLIGAYAGDPAAPGLSLIIRCYPGFQAVVVYRIAHIL
PySAT (18) ATTGALAVRLN----------PALGIRNFFGGNYMNPWVSLKADTYRQYGRFSYGFLLRCIFTKRTFRVASYG
EdSAT2 (64) VDLIKNSV----------AAYNDELFAHQCIMAILEKLPSLKKILKTDLIGAYAGDPAAPGLSLIIRCYPGFQAVIVYRIAHIL
SynSAT (89) LNLLNEQIRRELWLQHVTQGTPEATPAVLSQHASELTQAFAQALPELKRLGDSDVNAAYLGDPAAQSISFLFCYPGITAITFHRLAHRL
EcSAT (44) ALSYMLANKLS----------SPIMPAIAIREVVEEAYAAD--PEMIASAACDIQAVRTRDPAVDKYSTPLFYLKGFHALQAYRIGHWL
AtchSAT (83) ALGHILSVKLS----------NLNLPSNTLFELFISVLEES--PEFIESTKQDLIGVKERDPACISYVHCFYGRKGFLACQAHRLAHTV
SchpSAT (51) SLSCVLALPLDHVTG----------SSESMEN-WFYSILGLNDEKLIRSAMIDLDRLYTTNPACPDLISAFLSFRSFHALQLYRLAHGL
SmSAT (1) ----------MTNKLLLNGRVWNKGDLMGWWKETIDIVKEKDPAARTALGVLFTYPGVKALAAHCLSHFV
NostocSAT (1) ----------MLSIDRADFRIIFERDPAARNLLGVLFGYPGLQALLFHRVANWL
MtSAT (1) ----------MLTAMRGDIRAARERDPAAPTALGVIFGYPGVHAVWGHRLAHWL
AvSAT (1) ----------MFERMREDIRSVFQRDPAARNALGVLIGYPGLHALWLHRLAHGL
BsSAT (1) ----------MFFRMLKEDIDTVFDQDPAARSYFGVIFTYSGLHAIWAHRLAHAL
LiSAT (1) ----------MFENIKFIRKYDPAAKSYLGIVLGYPGLHALWFHKLAHFL
N-terminal extension
LmajSAT (262) HLLGVPQNFTRMLTELAHSETGIDIHPNTAAGHHFPIDHGTGVVIGATAVIGNDVSIYQGVTLGARSFPTDEKTGERIRDLPRHPIIEDG
TcSAT (200) HELGAPSIFTRLLTEMAHSTTGIDIHPATSAGHHFPIDHGTGIVIGSTAIIGNYVSIYHGVTLGTRSFPVVAKTGEKVRNLARHPIIEDR
EhSAT (143) YECGE-RYYCRELMESVHSYTSKDIHPGASAKGHPFPIDHGVGVVIGETAIIGEWCRIYQSVTLGAMHFQ-EEGG-VIKRGTKRHPTVGDY
EdSAT1 (143) YECGE-RYYCRELMENVHSYTSKDIHPGASAKGHPFPIDHGVGVVIGETAILGEWCRIYQSVTLGAMHFQ-EEGG-VINRGTKRHPTVGDY
PySAT (81) FFKLF-LPIFQLLHRFATHSAAMGLSWRTDIGAGIALTHGWGLVVTKKGKIGNNVTLFHGVTLGSRDRISRNGE-----RLSEYPIIEDE
EdSAT2 (138) YECGE-RYYCRELMENVHSYTSKDIHPGASAKGHPFPIDHGVGVVIGETAILGEWCRIYQSVTLGAMHFQ-EEGG-VINRGTKRHPTVGDY
SynSAT (179) YQLGL-PLIARITAEVSHSETGIDIHPGAAKGGSFFIDHGTGVVIGETCVIGDRVRIYQAVTLGAKSFPRDETG-ALIKGQARHPVIEDD
EcSAT (121) WNQGR-RALAIFLQNQVSVTFQVDIHPAAKKGRGIMLDHATGIVVGETAVVGDNVSILHGVTLGGTGKS----------GGDRHPKIRDG
AtchSAT (160) WKQNR-KIVGLLIQNRVSESFAVDIHPGAKKGKGILLDHATGVVIGETAVVGDNVSILHGVTLGGTGKQ----------SGDRHPKIGDG
SchpSAT (129) WSEED-KTSAVLVQNWTAVAYGVDIHPAASIGDGLFLDHALGIVIGETAIVEDGVSIWHNVTLGSIFKD----------SGARHPVIRKN
SmSAT (61) WTHHC-KLLARMHSQFWRFWTQAEIHPGAEIAEGVFIDHGSGLVIGETAIVEKGVMLYHGVTLGGTGKE----------TGKRHPTIRQG
NostocSAT (45) YRLGL-PFIPRLISHIARFLTGIEIHPGATIGQGVFIDHGMGVVIGETAIVEDYALYQGVTLGGTGKE----------SGKRHPTLGEN
MtSAT (45) WQRGA-RLLARAAAEFTRILTGVDIHPGAVVGARVFIDHATGVVIGETAEVGDDVTLYHGVTLGGSGMV----------GGKRHPTVGDR
AvSAT (45) WCSDW-KLLARLVSHVGRWLTGIEIHPGARIGRRLFIDHGMGVVIGETAEIGDDVTLYHGVTLGGTSWN----------KGKRHPTLEDG
BsSAT (46) YKRKF-YFLARLISQVSRFFTGIEIHPGATIGRRFFIDHGMGVVIGETCEIGNNVTVFQGVTLGGTGKE----------KGKRHPTIKDD
LiSAT (41) YRMKL-PLIPRMINTFSRFITGIDIHPGAKIANGIMIDHGHGVVIGETATIAKGCLIYQGVTLGGTGKE----------SGKRHPSLLEN
1 2 3 1 2 3 1
C1 C2 C2'

Figure 5.5. Multiple alignment of the deduced amino acid sequences of SAT from *L. major* and other representative organisms. Sequences are as follows: *Leishmania major*, LmajSAT (this study), *Trypanosoma cruzi*, TcSAT (GenBank accession no. AAK16403.1 GI:13241626), *Entamoeba histolytica*, EhSAT (GenBank accession no. BAA82868.1 GI:5689218), *Entamoeba dispar*, EdSAT1, (GenBank accession no. BAA82869.1 GI:5689220), *Plasmodium yoeli*, PySAT (genebank accession no. EAA 20201 GI:23485088), *Synechococcus spp.* PC7942, SynSAT (GenBank accession no.NP 665778 GI:11954793), *Arabidopsis thaliana*, AtchSAT (GenBank accession no. AAM15485 GI20198259), *E. coli*, EcSAT (GenBank accession no. NP 418064.1 GI:16131478), *Schizosaaccharomyces pombe*, SchpSAT, (GenBank accession no. NP 5949991 GI:19115911), *Streptococcus mutans*, SmSAT (GenBank accession no. NC 004305.1), *Nostoc spp.*, NostocSAT (GenBank accession no. NP 48807 GI:17231529), *Mycobacterum tuberculosis*, MtSAT, (GenBank accession no. NP 336876 GI:158413839), *Azotobacter vinelandii*, AvSAT (GenBank accession no. T 44279 GI:11257527), *Bacillus subtilis*, BsSAT, (GenBank accession no. B53402 GI:541421), *Leptospira interrogans*, LiSAT, (GenBank accession no. NP 7110243 GI:24212762). Black shades represents identical amino acids, grey shades indicate conserved amino acids substitutions. Asterisks indicate amino acids residues known to be important for catalytic activity of *Salmonella sp* SAT (Vaara, 1992). An insertion present only in *Synechococcus* SrpH, *E. histolytica*, *P. yoeli*, and *T. cruzi* SATs is also *boxed*. Structural motifs composed of hexapeptides (coils 1-4) and parallel β-strands, predicted based on homology to UDP-N-acetylglucosamine acyltransferase (LpxA), are depicted below the sequences as filled boxes (C1-C4) and arrows, respectively. The N-terminal extension of plants and protozoon SATs is arrowed.

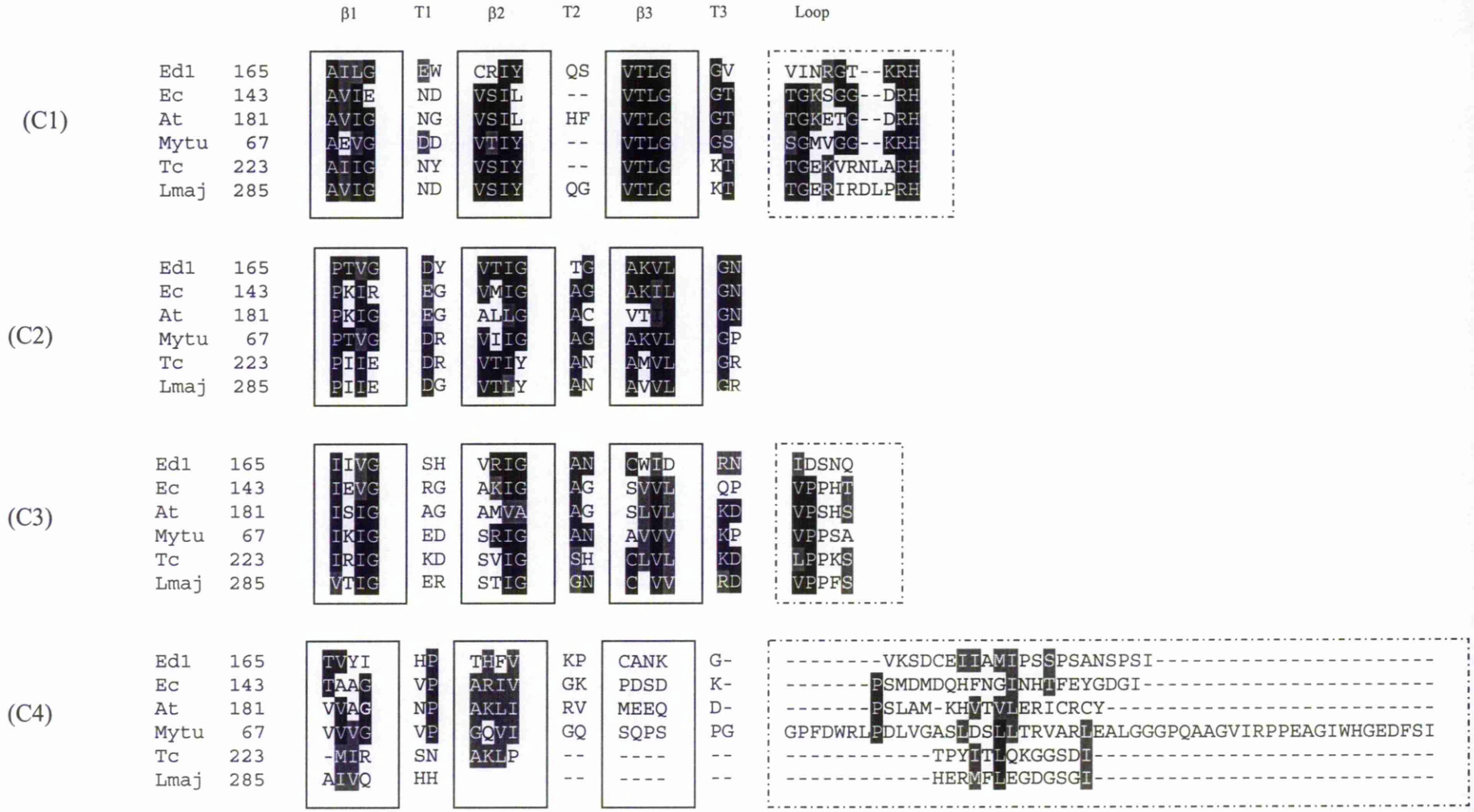


Figure 5.6. Secondary-structure analysis of the C-terminus with hexapeptide-repeat motifs of the acyltransferase family. (b) Alignment of the C-terminal amino acid sequences of SAT from a variety of organisms, showing residues that corresponds to the secondary structure coils (C1-4) of the hexapeptide-repeats of the acyltransferases. Within each coil are shown parallel β-sheets (β1-3, open boxes), T turns (T1-3) and loop extensions (dotted boxes). Black shades represents identical amino acids, grey shades indicate conserved amino acids substitutions. Database accessions of SAT used: *Entamoeba dispar*, Ed1 (GenBank accession no. BAA82869.1 GI:5689220); *Esherichia coli*, Ec (GenBank accession no. NP 418064.1 GI:16131478); *Arabidopsis. thaliana,* At (accession no. U22964); *Mycobacterium tubercolosis*, Mytu (GenBank accession no. NP 336876 GI:15841839), *T. cruzi,* Tc (GenBank accession no. AAK16403.1 GI:13241626); *Leishmania major* Lmaj (this study).

The amino acid residues, ser^{263}, pro^{270}, gly^{277}, pro^{279} (*Arabidopsis* numbering and indicated in Figure 5.7) are involved in binding of the plant SAT protein to acetyl-CoA (Ruffet *et al.*, 1995). Sequence alignment shows that only pro at the 383rd (LmajSAT numbering) position is conserved in LmajSAT. The other three positions are occupied by cys^{376}, gln^{391} and ser^{393} in the LmajSAT (Figure 5.8) instead of ser, gly and pro, respectively. The first serine residue is only conserved in most eubacteria and plants (Figure 5.7) but was replaced with cysteine in SAT proteins from *E. histolytica, E. dispar, T. cruzi, P. yoelii* or alanine in *S. mutans, S. pneumonae, M. leprae, M. tuberculosis, A. vinelandii* SATs or valine in *Synchococcus spp* and *Synechocystis spp* SATs. The other residues, though conserved in SAT from other organisms, were quite variable among the protozoa (Figure 5.8). The existence of such variability is intriguing, and it might be informative to find out whether these residues are vital for activity of the protozoan proteins.

(a)

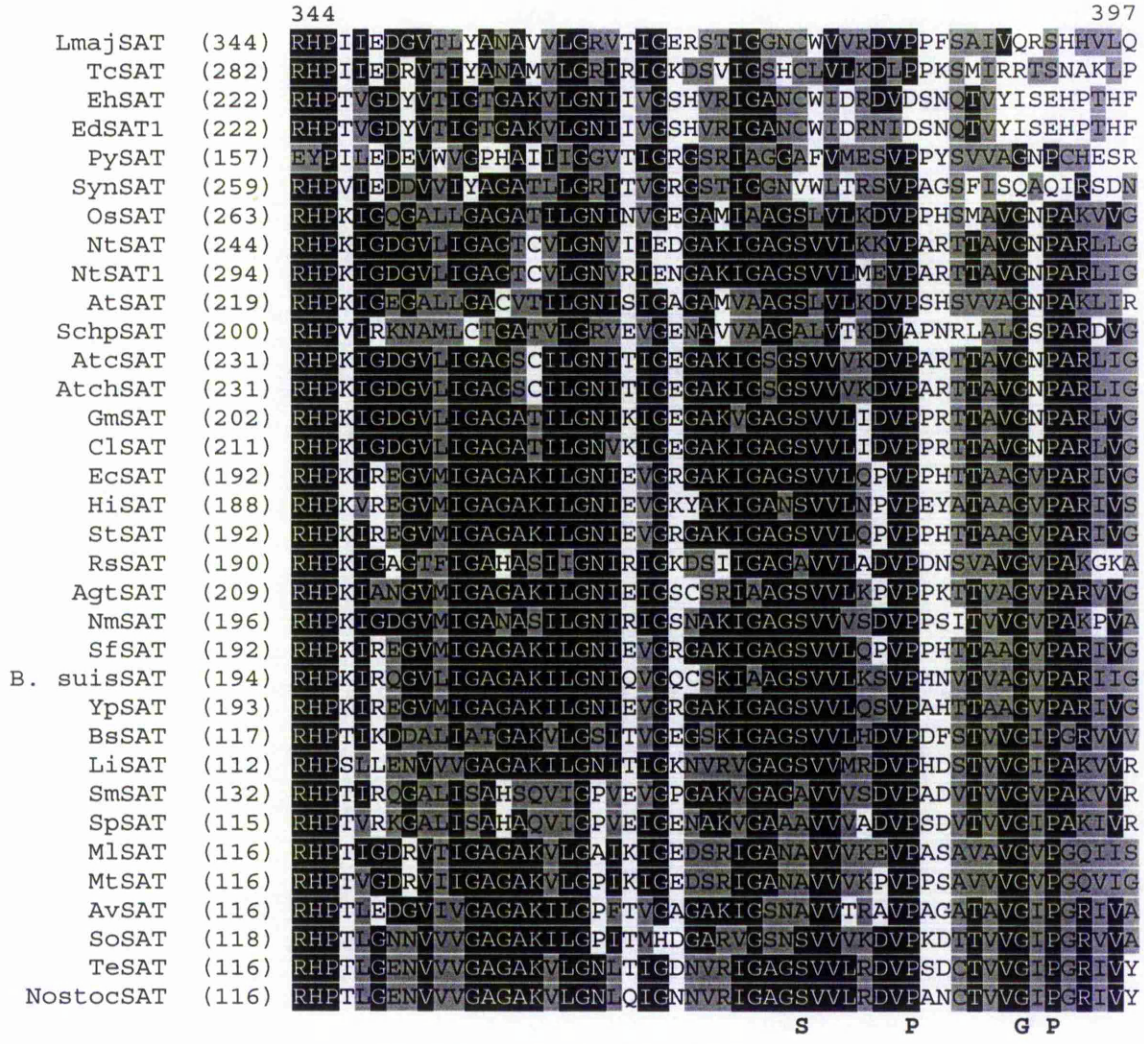
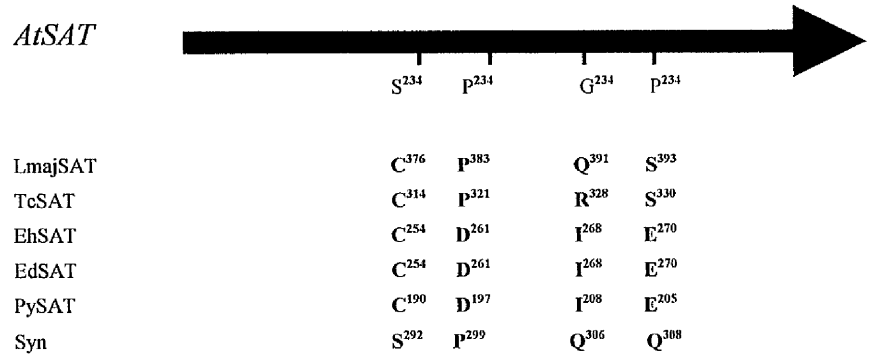


Figure 5.7. Multiple alignment of deduced amino acid sequences of the C-terminal domain of SAT from *L. major* and other representative organisms containing the residues involved in catalysis. Black shades represent identical amino acids, grey shades indicate conserved amino acid substitutions. The residues known to be important for catalytic activity of *Arabidopsis thalaiana* SAT are shown at the bottom of the alignment. Sequences are as follows: *Leishmania major*, LmajSAT (this study), *Trypanosoma cruzi*, TcSAT (GenBank accession no. AAK16403.1 GI:13241626), *Entamoeba histolytica,* EhSAT (GenBank accession no. BAA82868.1 GI:5689218), *Entamoeba dispar*, EdSAT1 (GenBank accession no. BAA82869.1 GI:5689220), *Plasmodium yoeli*, PySAT (GenBank accession no. EEA 20201 GI:23485088), *Synechococcus* strain PCC 7942, SynSAT (GenBank accession no. NP 665778), *Oryza sativa*, OsSAT (GenBank accession no. AAN05506 GI:22758278), *Nicotiana tabacum*, NtSAT (GenBank accession no. CAC88763.1 GI: 23343583), *Arabidopsis thaliana*, AtSAT (GenBank accession no. AAM 15485 GI:20198259), *Schizosaccharomyces pombe*, SchpSAT (GenBank accession no. NP5949991 GI:19115911), *Arabidopsis thialiana* cytosolic form, AtcSAT (GenBank accession no. S67482 GI:2146776), *Arabidopsis thaliana* chloroplast borne isoform, AtchSAT (GenBank accession no. S71181 GI:2146775), *Glyaine max*, GmSAT (GenBank accession no. AAL66292 GI:18252508), *Citrullus lanatus*, ClSAT (GenBank accession no. A57478 GI:1361979), *Escherichia coli*, EcSAT (GenBank accession no. NP 418064.1 GI: 16131478), *Haemophilus influenzae*, HiSAT (GenBank accession no. P43886 GI:1169183), *Salmonella typhimurium*, StSAT (GenBank accession no. NP 462599 GI:16766984), *Ralstonia solanacearum*, RsSAT (GenBank accession no. CAD 18590 GI:17431912), *Agrobacterium tumetaciens*, AgtSAT GenBank accession no. NP 532253 GI:17935463), *Neisseria meningitides*, NmSAT (GenBank accession no. CAB 84026 GI:17379464), *Shigella flexmeri*, SfSAT (GenBank accession no. NP 709386 GI:24114876), *Brucella suis*, B.suisSAT (GenBank accession no. NP 698265 GI:23502138), *Yersinia pestis*, YpSAT (GenBank accession no. NP 667414 GI:22123993), *Bacillus subtilis*, BsSAT (GenBank accession no.B53402 GI: 541421), *Leptospira interrugans*, LiSAT (GenBank accession no. NP 710243 GI:24212762), *Streptococcus mutans*, SmSAT (GenBank accession no. NC 004305.1), *Streptococcus pneumoniae*, SpSAT (GenBank accession no. NP 345102 GI:15900498), *Mycobacterium leprae*, MlSAT (GenBank accession no. NP 301632 GI:15827369), *Mycobacterium tuberculosis*, MtSAT (GenBank accession no. NP 336876 GI:15841839), *Azotobacter vinelandii*, AvSAT (GenBank accession no. T44279), *Shewanella oneidensis* MRI, SoSAT (GenBank accession no. NP 717858 GI:24373815), *Thermosynechoccus elongates*, TeSAT (GenBank accession no. NP 681641 GI:22298394).



(Figure 5.8) Schematic representation of heterogeneity of SAT active site residues. The arrow represents the ORF of AtSAT. The amino acids involved in SAT activity are shown. The heterogeneity of the active site residues of SAT from the protozoa (*Trypanosoma cruzi*, *Leishmania major*, *Entamoeba histolytica, Entamoeba dispar*, *Plasmodium yoelii*), and the cyanobacteria, *Synchoccoccus spp.*, that are distinct from the *Arabidopsis thaliania* SAT are shown. Accession numbers of the sequences used are detailed in Figure 5.7.

5.7 Phylogenetic analysis of SATs

Phylogenetic reconstruction of the domain of high homology in SATs from various organisms in the database with the neighbourhood joining (NJ) method also supports the peculiarity of the LmajSAT (Figure 5.9). The phylogenetic tree demonstrates the existence of three major monophyletic clades separated by a well-supported node of 81% bootstrap proportion (BP) (Figure 5.9). The eubacteria proteins are more closely related and fall into two groups relative to LmajSAT. The first clade grouped together most of the γ-proteobacterial (pink) and all sequences of plants (red) and the only fungal SAT (black) in the database. The second clade comprises the three amoebic sequences (dark red) as well as the LmajSAT and TcSAT (blue). Two cyanobacteria, *Synechococcus sp.* and *Synechocystis sp.* (green) derived from the chromosomal and plasmid-borne SAT gene (Nicholoson *et al.*, 1995) were also part of the protozoa assembly. The third clade holds the remainder of the eubacterial sequences (black). The SAT from *Plasmodium yoelli* (dark yellow) was the most divergent SAT.

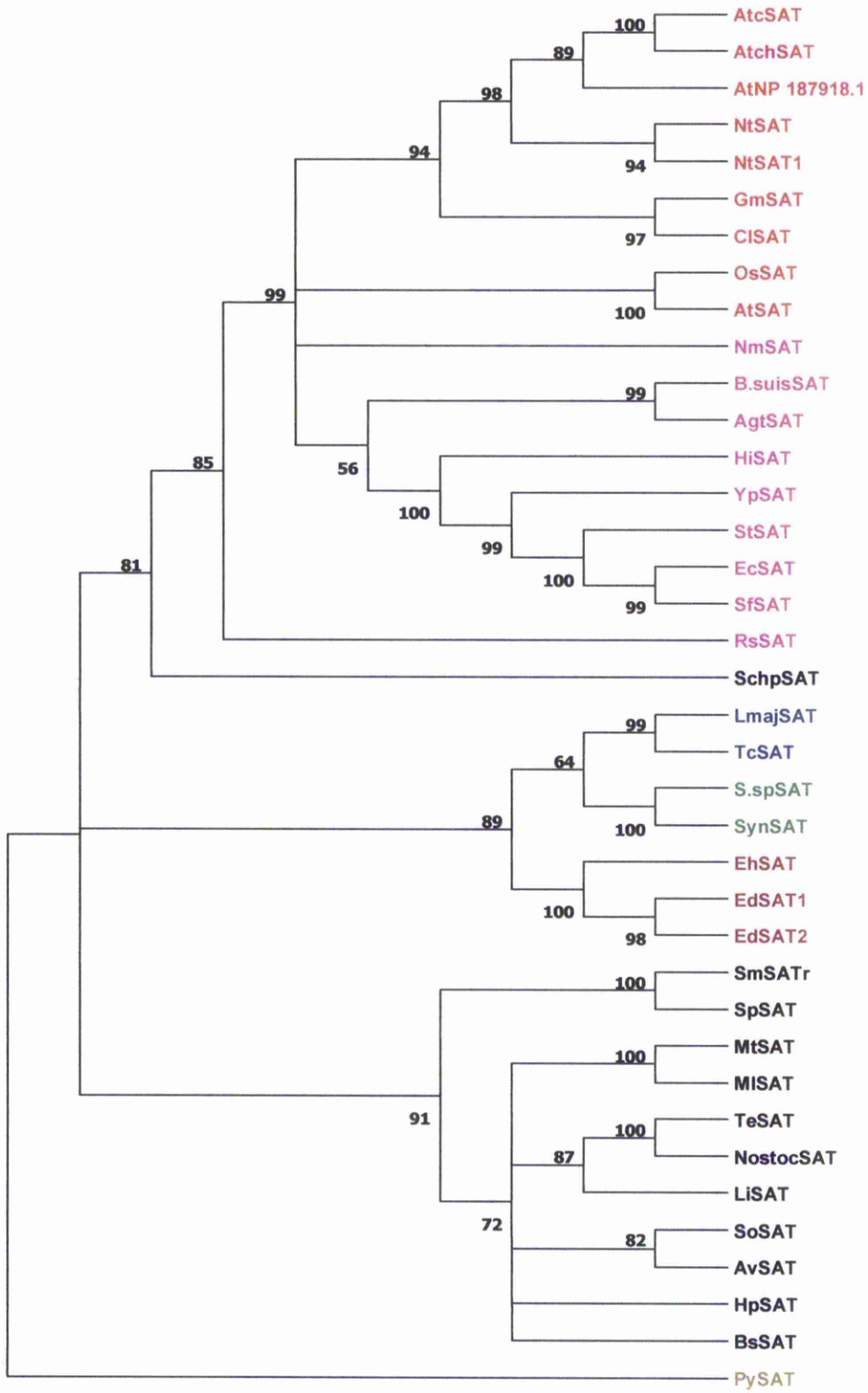


Figure 5.9. Phylogenetic analysis of conserved regions of LmajSAT and SATs from other organisms. Regions showing high homology (residues 218-398, LmajSAT numbering) in the SAT pile-up were used for phylogenetic analysis. Accession numbers of the SAT amino acid residues used are detailed in Figure 5.5a.

Taking a closer look at the phylogenetic tree in relation to the pile-up in Figure 5.5, the homology of group 1 represented by the CysE gene encoding SAT from *E. coli*, and SAT of *S. pombe* starts at position 61 relative to LmajSAT and terminates at a similar point as the leishmanial sequence. Group 3, represented by *M. tuberculosis, Noctoc spp. L. interrugans, A. vinelandii,* and *B. substilis* SATs begin at position 134-136 with respect to LmajSAT, and clearly extend further than the leishmanial sequence at the C-terminus (Figure 5.5). It is interesting that the trend observed with the full-length protein ties up well with the phylogenetic divisions observed with the conserved regions of the protein. Further, since all these proteins are capable of catalysing the same reaction, it seems reasonable to assume the catalytically functional domain is restricted to the central part of the sequence common to all SATs, which also coincides with the region of highest similarity of the trypanosomatid protein with other SATs.

5.8 Genomic Organisation of the SAT

To assess the copy number of the leishmanial *SAT*, genomic DNA was digested with thirteen restriction enzymes and analysed by Southern blotting using the *SAT* ORF as probe (Figure 5.10). Enzymes used that do not cut the gene itself, *Pst*I, *Sal*I, *BamH*I, *EcoR*I, *Hind*III, *Nde*I, *Sac*I, *Xba*I, *Apa*I, *Sac*II, and *Sal*I, resulted in a single major hybridising DNA fragment, while *Sau*3A1, and *Bsa*I which cuts the ORF more than once, resulted in multiple bands (Figure 5.10). The hybridisation patterns obtained and information on the genomic environment apparent from the data in the genome database for *L. major* indicate the *LmajSAT* gene is probably single copy.

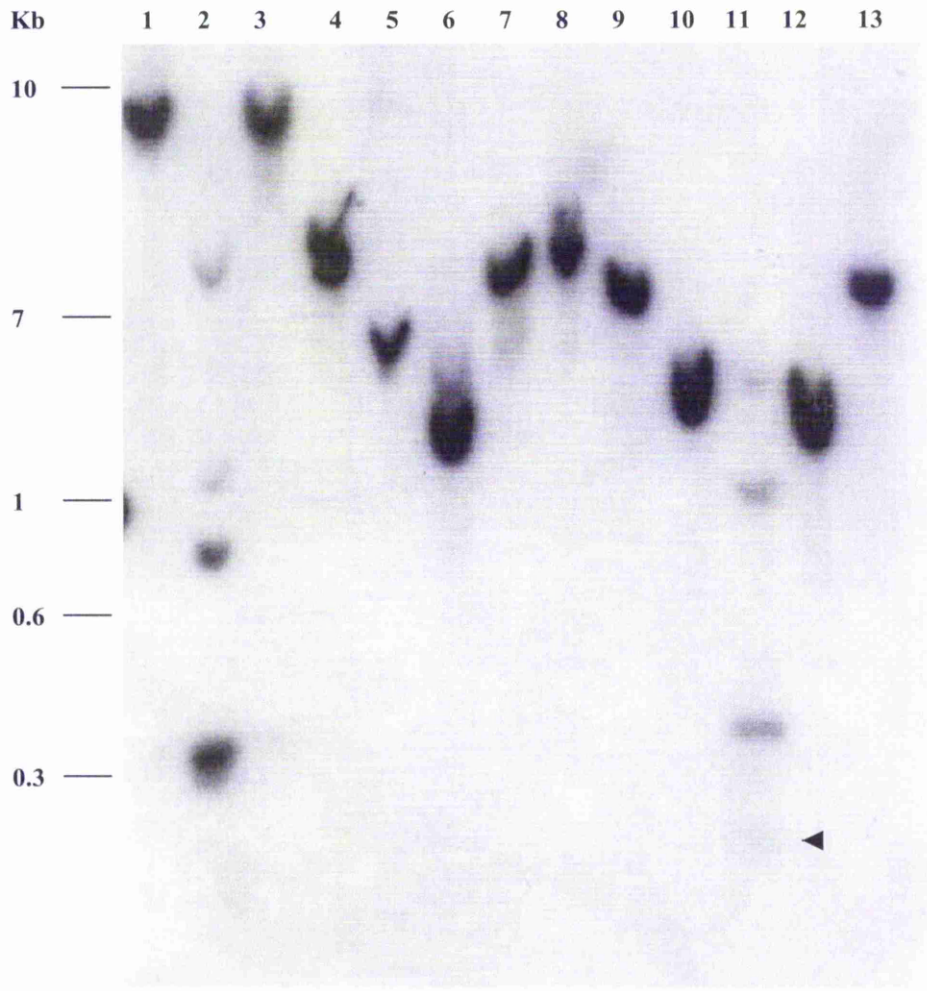


Figure 5.10. Genomic organisation of leishmanial SAT. 5 µg of genomic DNA from *L. major* was digested with a variety of restriction endonucleases, seperated by agarose electrophoresis, transfered onto a nylon membrane, and then hybridized with [^{32}P] dATP-labelled LmajSAT probe corresponding to the entire ORF. The restriction endonucleases used were *BamH*I (lane 1), *Bsa*I (lane 2), *EcoR*I (lane 3), *Hind*III (lane 4), *Nde*I (lane 5), *Pst*I (lane 6), *Pvu*I (lane 7), *Sac*I (lane 8), *Sac*II (lane 9), *Sal*I (lane 10), *Sau*3AI (lane 11), *Pvu*I/*Nde*I (lane 12) and *Xba*I/*Apa*I (lane 13). The sizes of the DNA standards are indicated in kilobase pairs. A weak band is arrowed for clarity.

5.9 Biochemical characterisation of SAT

The purified rLmajSAT was pure as judged by SDS-PAGE analysis (Figure 5.3). An apparent enzyme activity (Section 2.10) was detected initially in pure sample during purification, which this was not reproducible with pure protein from subsequent purification preparations of the rLmajSAT protein. Similarly, no activity could be detected in the crude lysate of *L. major* promastigotes. The possibility that the assay conditions were not optimal could not be excluded at this point as no SAT is commercially available.

5.10 Identification, Cloning and Characterisation of Genes Encoding cysteine synthase (CS) in L. major

The CS gene (E.C 2.3.1.30.) of *L. major* was identified using a similar strategy to that described for the *LmajSAT* gene. Blast searches of the *L. major* sequencing project database (http://www2.ebi.ac.uk/blast2/parasite.html) using CS from *E. histolytica* (EhCS) as query produced an HTGS (EMBL/Genebank accession no. AL499624) containing the *LmajCS* gene. The 1.0 kb ORF of *LmajCS* was amplified using CS1 and CS3 (Table 5.3) after 30 cycles of PCR (Figure 5.11a). LmajCS was expressed and purified as a soluble protein from *E. coli* (Figure 5.11b). The predicted amino acid sequence of *LmajCS* of *L. major* comprised 342 residues with a calculated molecular mass of 35.2 kDa (Figure 5.12).

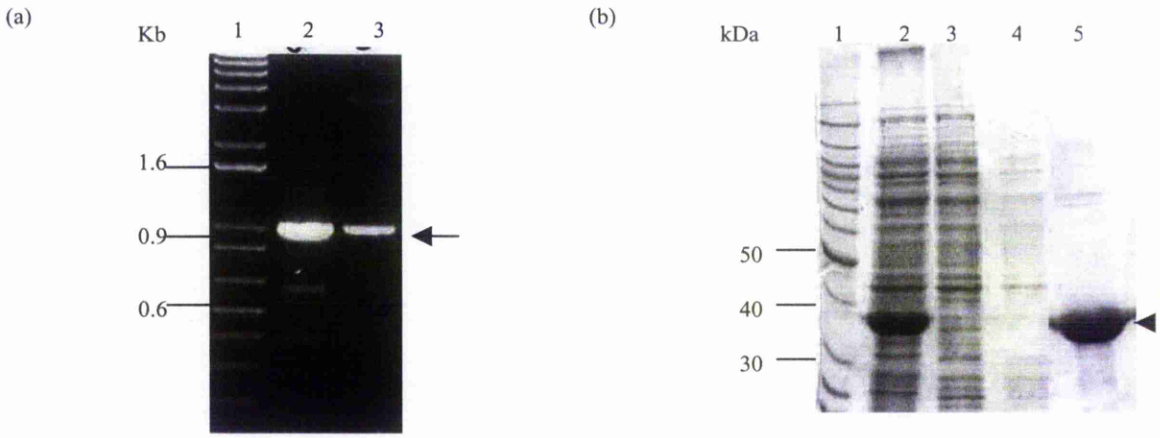


Figure 5.11a Ethidium-bromide stained 0.7% agarose gel showing PCR product of *LmajCS* of *Leishmania major*. Lane 1, DNA standards; Lanes 2 and 3, *LmajCS* (arrowed). (b) Purification of rLmajCS under native conditions. The soluble protein in the supernatant fraction was purified on a nickel chelator column. The His-tagged protein was eluted by addition of Buffer A with 500 mM imidazole. SDS-PAGE analysis of the following samples: Lane 1. molecular mass standards; Lane 2 and 3, supernatant and pellet of fractions of a crude extract, respectively, of the recombinant *E. coli* cells; Lane 4, wash with buffer A with 60 mM imidazole; lane 5, purified rLmajCS eluted from the nickel chelator column with buffer A with 500 mM imidazole. Estimated recombinant protein size: 35 kDa (arrowed).

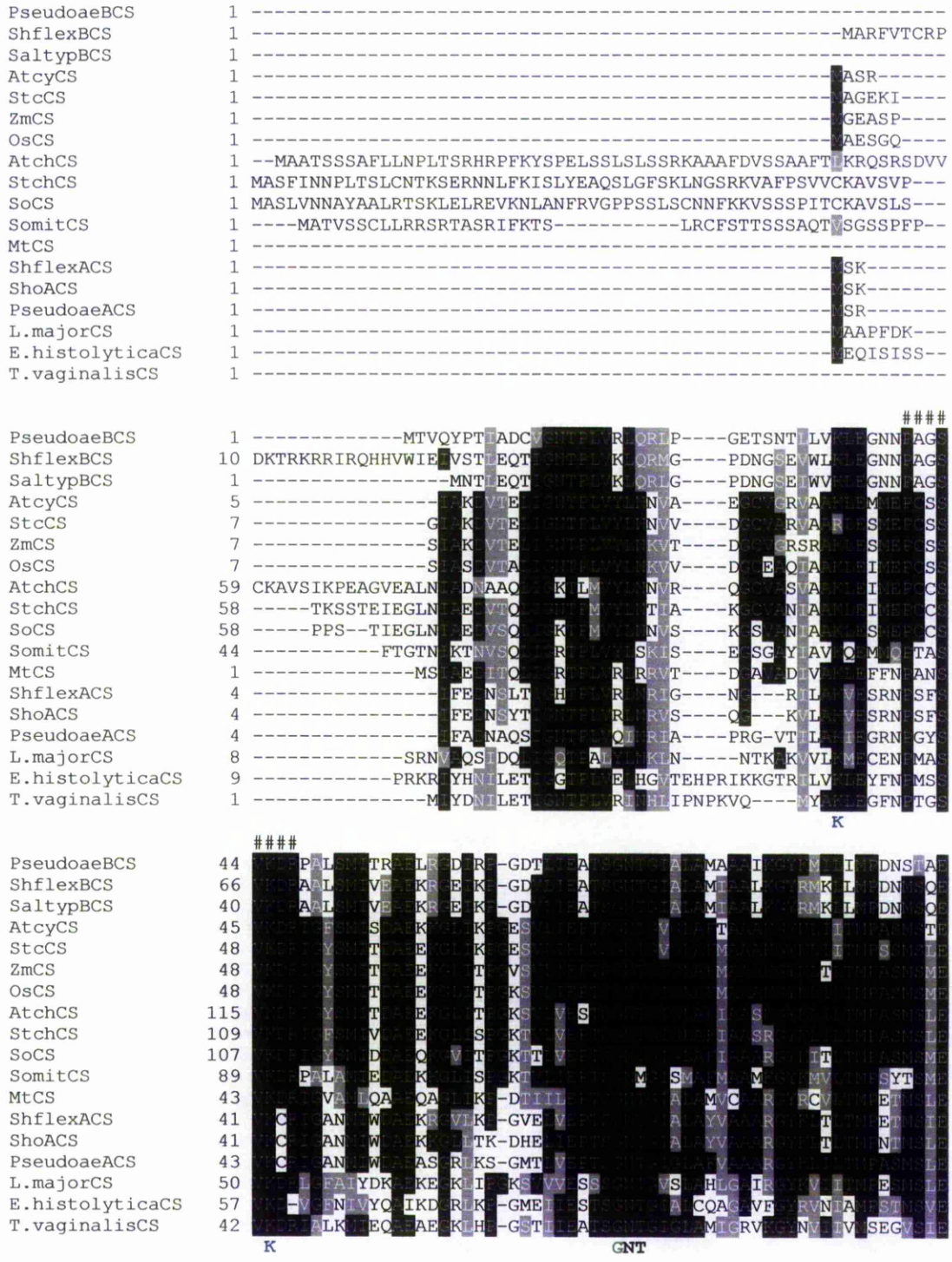
```
          M  A  A  P   F  D  K   S  R  N   V  A  Q  S   I  D  Q   L  I  G   Q  T  P
          A ·
    1     ATGGCAGCAC CGTTCGACAA GTCGAGAAAT GTGGCGCAGT CCATCGATCA GCTGATCGGC CAGACGCCGG
          TACCGTCGTG GCAAGCTGTT CAGCTCTTTA CACCGCGTCA GGTAGCTAGT CGACTAGCCG GTCTGCGGCC
          · L  Y  L   N  K  L   N  N  T  K   A  K  V   V  L  K   M  E  C  E   N  P  M
          ·
   71     CGTTGTACCT AAACAAGCTG AACAATACCA AGGCAAAGGT TGTGCTCAAG ATGGAGTGCG AGAACCCGAT
          GCAACATGGA TTTGTTCGAC TTGTTATGGT TCCGTTTCCA ACACGAGTTC TACCTCACGC TCTTGGGCTA
          · A  S  V   K  D  R  L   G  F  A   I  Y  D   K  A  E  K   E  G  K   L  I  P
  141     GGCGTCTGTA AAGGATCGCC TCGGCTTCGC CATCTACGAT AAAGCGGAGA AGGAGGGCAA GCTGATTCCT
          CCGCAGACAT TTCCTAGCGG AGCCGAAGCG GTAGATGCTA TTTCGCCTCT TCCTCCCGTT CGACTAAGGA
           G  K  S  V   V  V  E   S  S  S   G  N  T  G   V  S  L   A  H  L   G  A  I
          R ·
  211     GGCAAGTCTG TAGTGGTCGA GTCGTCCAGT GGAAACACGG GCGTGTCGCT AGCGCACTTG GGTGCAATTC
          CCGTTCAGAC ATCACCAGCT CAGCAGGTCA CCTTTGTGCC CGCACAGCGA TCGCGTGAAC CCACGTTAAG
          · G  Y  K   V  I  I   T  M  P  E   S  M  S   L  E  R   R  C  L  L   R  I  F
          ·
  281     GTGGTTACAA GGTCATCATT ACGATGCCCG AATCCATGTC TCTCGAGCGC CGCTGCCTGC TGCGTATCTT
          CACCAATGTT CCAGTAGTAA TGCTACGGGC TTAGGTACAG AGAGCTCGCG GCGACGGACG ACGCATAGAA
          · G  A  E   V  I  L  T   P  A  A   L  G  M   K  G  A  V   T  M  A   K  K  I
  351     CGGCGCCGAG GTAATCCTCA CCCCTGCCGC TCTCGGCATG AAGGGTGCCG TGACCATGGC CAAGAAAATC
          GCCGCGGCTC CATTAGGAGT GGGGACGGCG AGAGCCGTAC TTCCCACGGC ACTGGTACCG GTTCTTTTAG
           V  T  A  N   P  N  A   V  L  A   D  Q  F  A   T  K  Y   N  A  L   I  H  E
          E ·
  421     GTGACCGCTA ACCCCAACGC CGTCTTGGCG GATCAGTTCG CAACCAAGTA CAACGCCCTC ATACACGAGG
          CACTGGCGAT TGGGGTTGCG GCAGAACCGC CTAGTCAAGC GTTGGTTCAT GTTGCGGGAG TATGTGCTCC
          · T  T  G   P  E  I   W  E  Q  T   N  H  N   V  D  C   F  I  A  G   V  G  T
          ·
  491     AGACCACGGG GCCTGAGATT TGGGAGCAGA CGAACCACAA TGTCGACTGC TTCATAGCCG GCGTTGGAAC
          TCTGGTGCCC CGGACTCTAA ACCCTCGTCT GCTTGGTGTT ACAGCTGACG AAGTATCGGC CGCAACCTTG
          · G  G  T   L  T  G  V   A  R  A   L  K  K   M  G  S  H   A  R  I   V  A  V
  561     GGGTGGCACG CTGACAGGTG TGGCGCGGGC GCTCAAGAAG ATGGGCAGTC ATGCCCGCAT CGTTGCCGTG
          CCCACCGTGC GACTGTCCAC ACCGCGCCCG CGAGTTCTTC TACCCGTCAG TACGGGCGTA GCAACGGCAC
           E  P  M  E   S  P  V   L  S  G   G  K  P  G   A  H  K   I  Q  G   I  G  P
          G ·
  631     GAGCCGATGG AGTCACCCGT GCTGTCGGGT GGAAAACCAG GCGCGCACAA AATTCAGGGC ATCGGCCCAG
          CTCGGCTACC TCAGTGGGCA CGACAGCCCA CCTTTTGGTC CGCGCGTGTT TTAAGTCCCG TAGCCGGGTC
          · F  V  P   D  V  L   D  R  S  L   I  D  E   V  F  C   V  A  G  D   D  A  I
          ·
  701     GATTTGTGCC TGACGTGCTC GACCGCAGCC TCATCGACGA GGTGTTTTGC GTAGCAGGTG ATGATGCCAT
          CTAAACACGG ACTGCACGAG CTGGCGTCGG AGTAGCTGCT CCACAAAACG CATCGTCCAC TACTACGGTA
          · E  T  A   L  K  L  T   R  S  D   G  V  F   C  G  F  S   G  G  A   N  V  Y
  771     TGAGACGGCA CTGAAGCTCA CGCGCAGCGA CGGGGTCTTC TGCGGCTTCT CTGGCGGAGC CAACGTTTAC
          ACTCTGCCGT GACTTCGAGT GCGCGTCGCT GCCCCAGAAG ACGCCGAAGA GACCGCCTCG GTTGCAAATG
           A  A  L  K   I  A  E   R  P  E   M  E  G  K   T  I  V   T  I  I   P  S  F
          G ·
  841     GCGGCGCTGA AGATTGCGGA GCGTCCGGAG ATGGAGGGCA AGACAATTGT GACGATCATT CCGTCCTTCG
          CGCCGCGACT TCTAACGCCT CGCAGGCCTC TACCTCCCGT TCTGTTAACA CTGCTAGTAA GGCAGGAAGC
          · E  R  Y   L  S  T   A  L  Y  R   S  V  R   D  E  V   S  S  L  P   V  V  D
          ·
  911     GTGAGCGGTA CCTCTCCACC GCGTTGTACA GGAGCGTCCG GGATGAGGTG TCGTCTCTGC CAGTAGTCGA
          CACTCGCCAT GGAGAGGTGG CGCAACATGT CCTCGCAGGC CCTACTCCAC AGCAGAGACG GTCATCAGCT
          · A  S  E   L  Q  D
  981     TGCCTCGGAG CTGCAGGAC
          ACGGAGCCTC GACGTCCTG
```

Figure 5.12 Nucleotide sequence of *L. major* CS gDNA and deduced amino acid sequence. Nucleotides are numbered in the 5' to 3' direction, beginning with the first residue at the start codon and ending at the codon preceding the stop codon.

5.11. Features of Predicted Amino Acid Sequences of the cysteine synthase

The deduced amino acid sequence of LmajCS was similar to other CSs and its conserved region (corresponding to residues 190-311 in LmajCS) had 37% and 33% identity to the CS from *E. histolytica* (EhCS) and *E. dispar* (EdCS), respectively.

Sequence alignment of LmajCS with amino acid sequences of other CS reveals the following:- The absence of an NTE (typical of plants organellar CSs) in the LmajCS protein suggesting that it has a cytosolic localization within the parasite (Figure 5.13). The primary sequence of LmajCS deduced from the crystal structures of *S. typhimurium* and spinach CSs (Burkhard *et al.*, 1998) shows that residues of the leishmanial CS (15-319) has a PLP motif (PXXSVKDRA) (Byrne *et al.*, 1998). The PLP motif is the key residues vital for binding to the co-factor PLP and thereby facilitating catalysis of OAS and sulfide to form cysteine. The PLP motif has a reactive lysine involved in catalysis at the 52^{nd} position (LmajCS numbering) as the key residues for binding to the co-factor PLP. Three other lysine residues deduced from the plants CS as playing a role in catalysis corresponds to positions 40, 66 and 200 in the LmajCS protein (blue in Figure 5.13). Further, the residues, involved in co-factor binding are also conserved in the LmajCS protein asn^{82}, thr^{174}, thr^{187} and ser^{274} (green in Figure 5.13). This similarity of key residues in LmajCS and spinach CS suggests a similar reaction mechanism.


PseudoaeBCS
ShflexBCS
SaltypBCS
AtcyCS
StcCS
ZmCS
OsCS
AtchCS
StchCS
SoCS
SomitCS
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ShoACS
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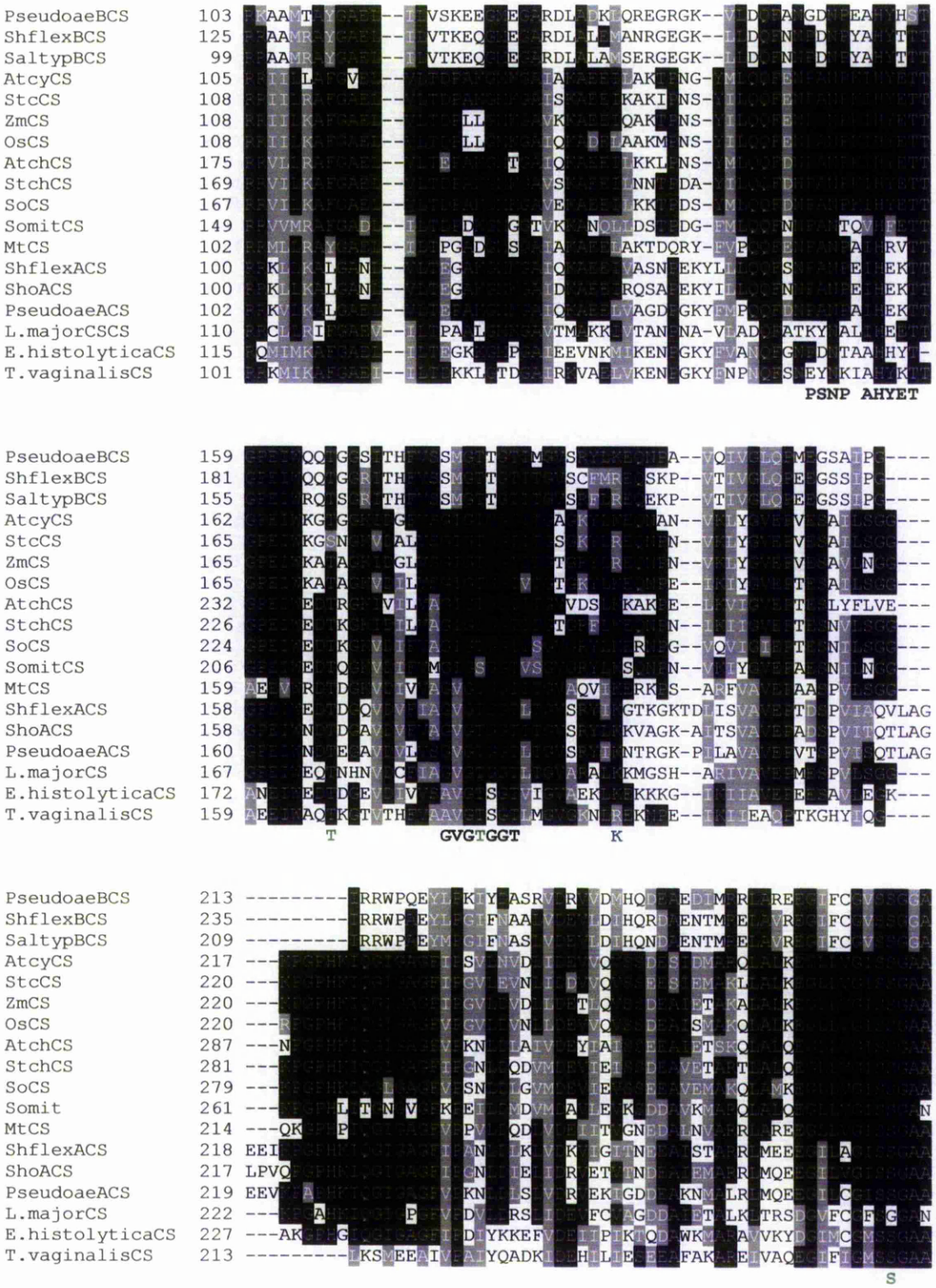
L.majorCS
E.histolyticaCS
T.vaginalisCS
####
K
GNT


PseudoaeBCS 103
ShflexBCS 125
SaltypBCS 99
AtcyCS 105
StcCS 108
ZmCS 108
OsCS 108
AtchCS 175
StchCS 169
SoCS 167
SomitCS 149
MtCS 102
ShflexACS 100
ShoACS 100
PseudoaeACS 102
L.majorCSCS 110
E.histolyticaCS 115
T.vaginalisCS 101
PSNP AHYET
PseudoaeBCS 159
ShflexBCS 181
SaltypBCS 155
AtcyCS 162
StcCS 165
ZmCS 165
OsCS 165
AtchCS 232
StchCS 226
SoCS 224
SomitCS 206
MtCS 159
ShflexACS 158
ShoACS 158
PseudoaeACS 160
L.majorCS 167
E.histolyticaCS 172
T.vaginalisCS 159
T
GVGTGGT
K
PseudoaeBCS 213
ShflexBCS 235
SaltypBCS 209
AtcyCS 217
StcCS 220
ZmCS 220
OsCS 220
AtchCS 287
StchCS 281
SoCS 279
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E.histolyticaCS 227
T.vaginalisCS 213
S

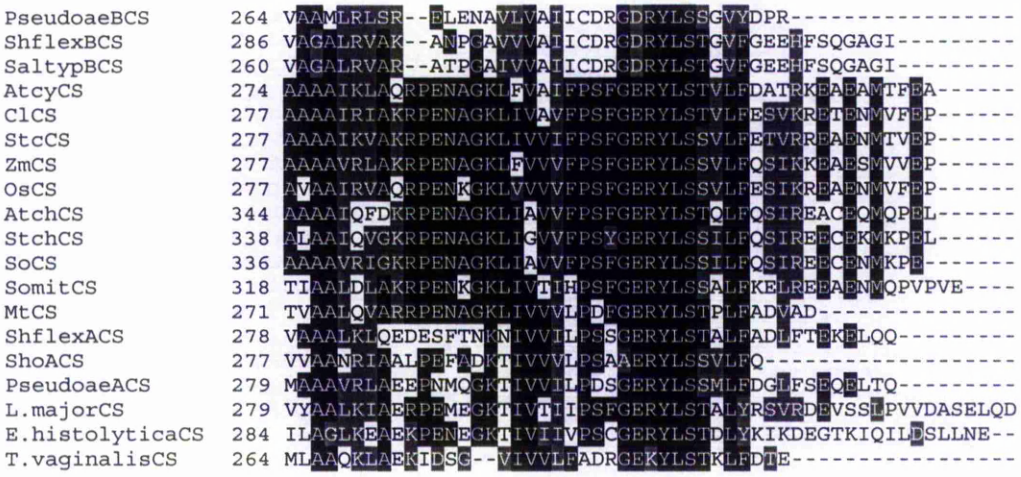


Figure 5.13. Multiple alignment of the deduced amino acid sequences of CS from *L. major* and other representative organisms. Sequences are as follows: *Pseudomonas aeruginosa*, PseudoeBCS (GenBank accession no. NC 002516), *Shigella flexneri*, ShflexBCS (GenBank accession no. NP 708275 GI:24113765), *Salmonella typhimurium*, SaltypCS (GenBank accession no. NC 003197.1), *Arabidopsis thaliana* cytosolic isoform, AtcyCS (GenBank accession no. AAK76499.1 GI:15027937), *Solanum tuberosum* plastid-targeting isoform, StcCS (GenBank accession no. AAC25636.1 GI:3290022), *Zea mays*, ZmCS (GenBank accession no. CAA59798.1 GI:758353), *Oryza sativa*, OsCS (GenBank accession no. AAK71541.1 GI:14626273), *Arabidopsis thaliana* chloroplast targeting isoform, AtchCS (GenBank accession no. S48695 GI:1084347), *Spinacia oleracea*, SoCS (GenBank accession no. P32260 GI:12644209), *Spinacia oleracea* mitochondria targeting isoform, Somit (GenBank accession no A55450 GI:107191), *Shigella flexneri*, ShflexACS (GenBank accession no. NP 708269), *Shewanella oneidensis*, ShoACS (GenBank accession no. NP 718475 GI:24374432), *Pseudomonas aeruginosa*, PseudoeACS (GenBank accession no. AE004699.1), *Leishmania major*, L.majorCS (this study), *Entamoeba histolytica*, E.histolyticsCS (GenBank accession no. BAA21916.1 GI:2346964), *Trichomonas vaginalis*, T.vaginalisCS (Goodall, 2001). Black shades represent identical amino acids, grey shades indicate conserved amino acid substitutions. Consensus sequence for the putative pyridoxal phosphate-binding motif (P*XX*SVKDR) is marked (#). The residues known to be important for the catalytic activity of spinach CS-A (blue and green in figure) by site-directed mutagenesis (Saito *et al.*, 1993) are highlighted in bold at the bottom of the alignment.

Phylogenetic reconstruction of the conserved regions of CS homologues from various organisms with the NJ method shows that they all fall within a single clade. However, CSs from plants were grouped together with the organellar (mitochondria and plastid –light blue) CSs clearly distinguishable from the cytosolic forms (blue in Figure 5.10). The bacterial proteins were clearly distinguishable into the cytosolic

type A (dark red) and plastidic type B (red) isoforms. The A-isoforms of the enzyme were closely related to the plant enzymes, which in turn were also closely related to LmajCS. The bacterial CS type B proteins on the other hand, were closely related to the *E. histolytica* CS protein. CS from *T. vaginalis* sequence though close to the type B enzyme and *E. histolytica* CS was the most divergent CS. The relationship of the trichomanda CS to the type B protein and not LmajCS is an interesting observation, as the type B bacterial enzymes, are expressed under anaerobic conditions in these organisms.

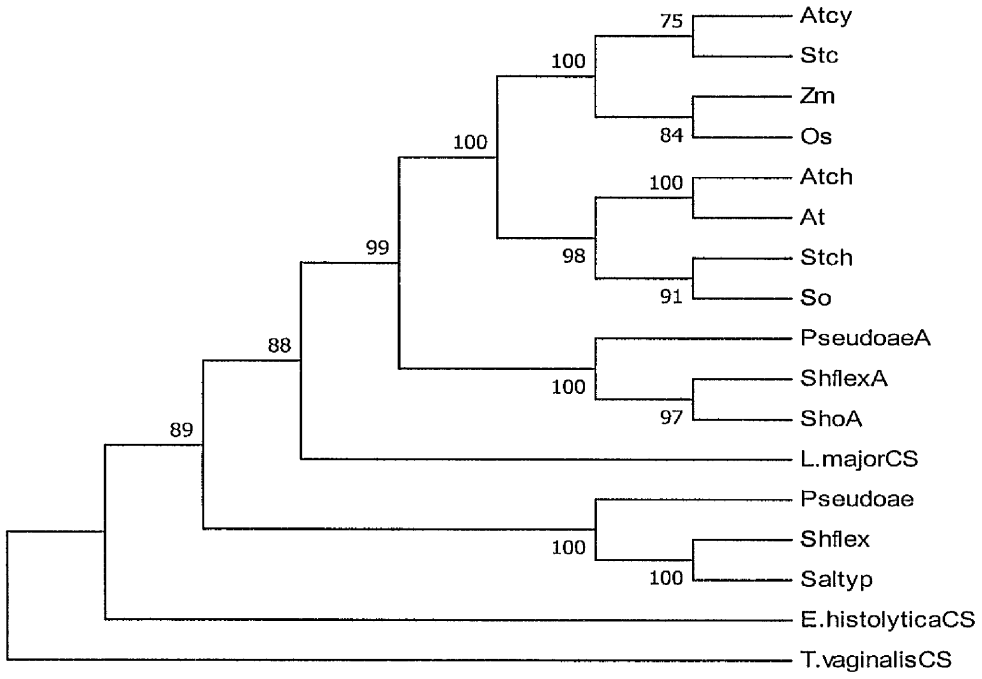


Figure 5.14 Phylogenetic analysis of conserved regions of LmajCS and CS from other organisms. Regions showing high homology (residues 11-328, LmajCS numbering) in the CS pile-up were used for phylogenetic analysis. Accession numbers of the SAT amino acid residues used are detailed in Figure 5.13.

5.12. *Genomic Organisation of the LmajCS*

To assess the copy number of the leishmanial *CS*, genomic DNA was digested with four restriction enzymes and analysed by southern blotting using the *LmajCS* cDNA as probe

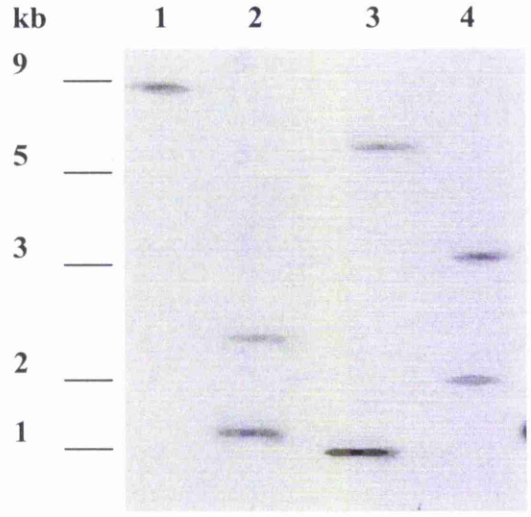


Figure 5.15. Genomic organisation of *LmajCS*. 5 µg of genomic DNA from *L. major* was digested with a variety of restriction endonucleases, seperated by agarose electrophoresis, transfered onto a nylon membrane, and then hybridized with [^{32}P] dATP-labelled LmajCS probe corresponding to the entire ORF. The restriction endonucleases used were *EcoR*I (lane 1), *Pst*I (lane 2), *Sal*I (lane 3), *Bsa*I (lane 4), The sizes of the DNA standards are indicated in kilobase pairs.

(Figure 5.15). Enzymes used that do not cut the gene itself, *Eco*RI, resulted in a single major hybridising DNA fragment, while *Bsa*1, *Sal*I and *Pst*I which cuts the ORF once, resulted in two bands each (Figure 5.15). The hybridisation patterns obtained as well as, the information on genomic environment apparent from the data in the genome database of *L. major,* indicate that the *LmajCS* gene is probably single copy.

5.14 Expression profile and biochemical characterization of recombinant LmajCS

Expression of LmajCS in *E. coli* strain BL21(DE3) under control of a T7 promoter, at growth and induction conditions of 37°C and 1 mM IPTG, resulted in the formation of insoluble inclusion bodies (Figure 5.16a). A culture conditions of 15°C and 0.5 mM IPTG, significantly influenced the degree of solubility of LmajCBS (Figure 5.16b). Recombinant LmajCS (rLmajCS) expressed in *E. coli* was purified using affinity chromatography as described for LmajMST.

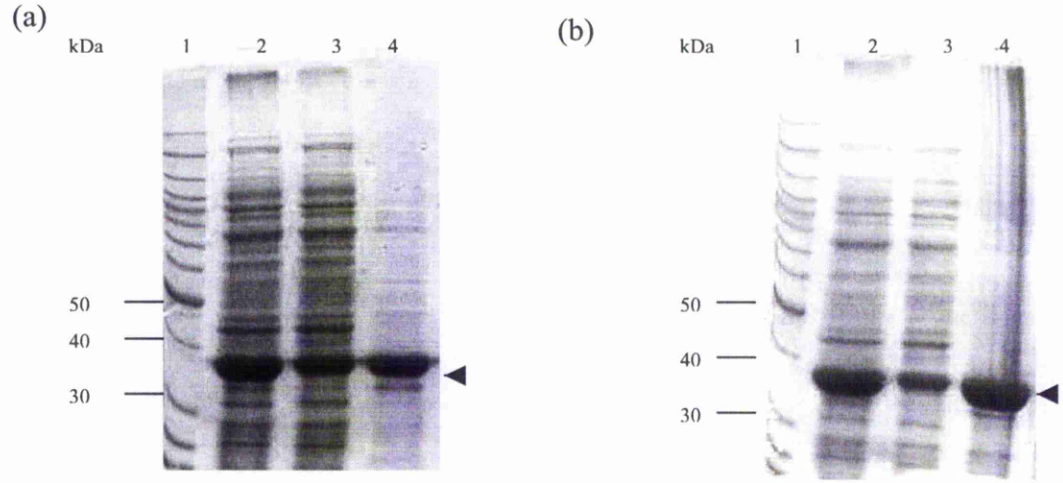


Figure 5.16. SDS-PAGE analysis showing the solubility of LmajCS protein at different growth temperatures. (a) SDS-PAGE analysis of LmajCS expressed at 37°C. *E. coli* expressing LmajCS was lysed in 250 mM sodium phosphate buffer (pH 8.0), containing 0.3 M NaCl (buffer A) and centrifuged at 13,000 x g for 30 min. Lanes 1, molecular mass standards, Lanes 2 and 3, supernatant and pellet factions of crude lysates of *E. coli* expressing LmajCS (arrowed). Lane 4, Purified rLmajCS eluted from the nickel column with Buffer A with 500 mM imidazole (arrowed). Estimated recombinant protein size: 35 kDa. (b) SDS-PAGE analysis of LmajCS expressed at 15°C. *E. coli* expressing LmajCS was lysed in 250 mM sodium phosphate buffer (pH 8.0), containing 0.3 M NaCl (buffer A) and centrifuged at 13,000 x g for 30 min. Lane order (a) as above.

The storage conditions for LmajCS were optimised and standardised to ensure that the recombinant protein was in optimal conditions, during characterisation. The storage conditions investigated reflected methods described previously for a *Trichmonas* protein (TvCS) (Goodall, 2001). The stability of the enzyme was determined by monitoring CS activity over a period of time when stored at 4°C. Preparation of the enzyme for storage involved an overnight dialysis of enzyme eluted from the column in 20 mM Tris-HCl, pH 7.9, and 25 μM PLP at 4°C to remove the excess salts (0.25 M imidazole and 0.3 M NaCl) contained in the elution buffer. The dialysed enzyme was stored at 4°C either in the dialysis buffer itself or the buffer with 50% (v/v) glycerol added. It was shown that the enzyme was stable for 4 weeks in both storage conditions. Subsequently, it was routinely stored in 20 mM Tris-HCl, pH 7.9, and 25 μM PLP at 4°C

CS activity was measured by assessing cysteine production using a stopped assay (Section 2.10). To optimise the CS assay, saturating concentrations of substrates were used and activity measured as a function of time and protein concentration. The reaction progressed linearly under these assay conditions for 2 min (Figure 5.17).

(a)

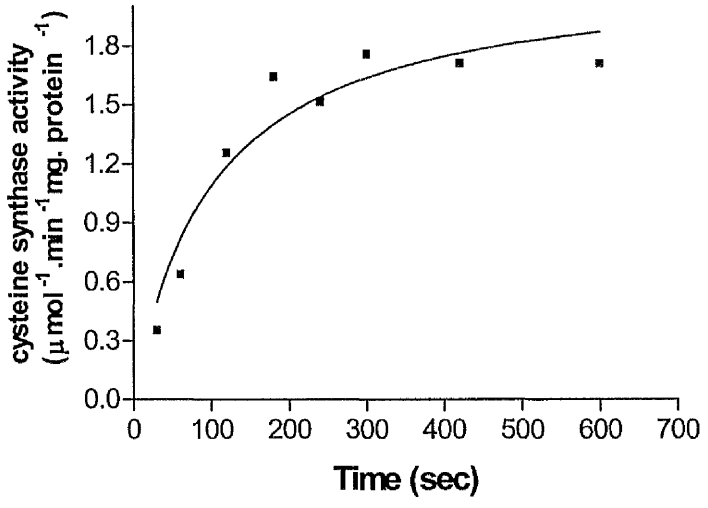


Figure 5.17. Cysteine production. The rate of cysteine production was linear over a 2 minutes incubation time at 37°C. Assay mix contained 10 mM *O*-acetyl serine, 1 mM Na_2S, 25 μM PLP and 0.5 μg of enzyme in a total volume of 100 μl.

A sigmoid instead of a hyperbolic curve was obtained while doubling the amount of protein (at ranges between 1 $\mu g.\mu l^{-1}$-15 $\mu g.\mu l^{-1}$) in the assay mix suggests a reversible reaction hence a lag a low protein concentrations (Figure 5.18). The rate of activity approximately doubled (and was linear) between 3 $\mu g.\mu l^{-1}$ and 7.5 $\mu g.\mu l^{-1}$ (Figure 5.13b).

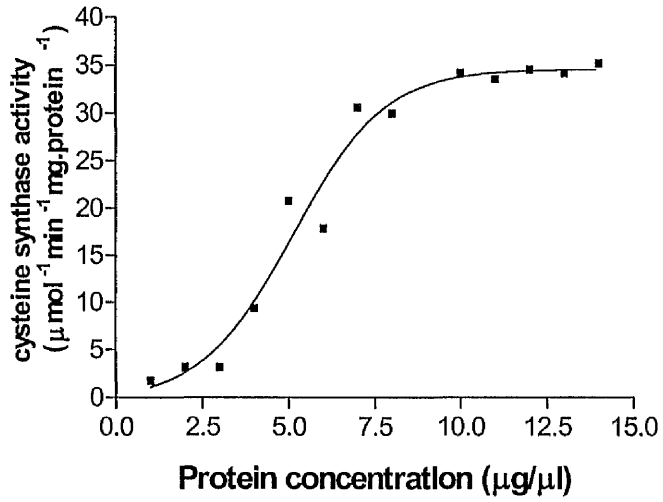
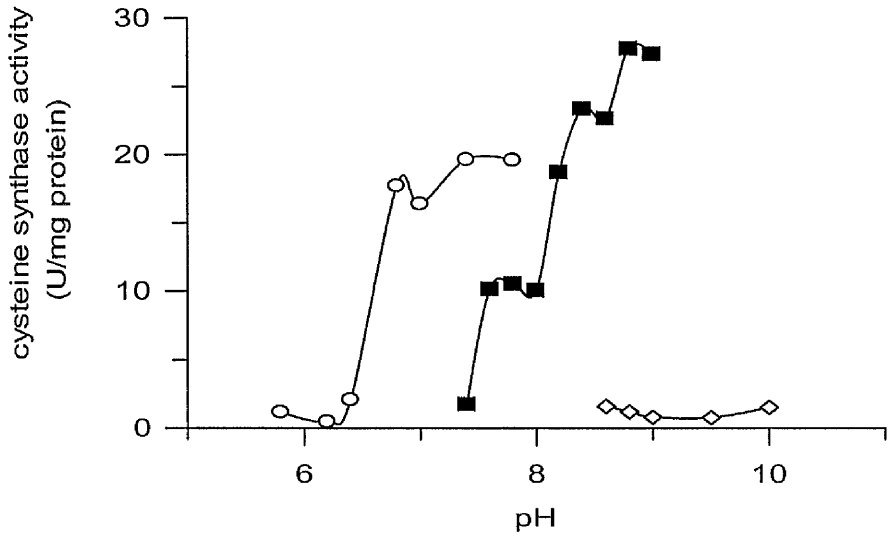


Figure 5.18. The effect of LmajCS concentration on cysteine formation. Increasing LmajCS concentration in the cysteine synthase ass incubated for 2 minutes at 37°C produced a sigmoid curve, but was directly proportional to cysteine produced expressed as μmoles/mi of protein between 3 μg/μl and 7.5μg/μl.

The buffer used greatly influenced the activity of rLmajCS. With phosphate, the enzyme was most active in the pH range 6.8-7.8 (Figure 5.19a) while with Tris-HCl it was most active between pH 8.0-8.9. Since the highest activity was recorded for Tris-HCl, it was selected as the buffer of choice at pH 8 for the *in vitro* assay. LmajCS also catalyses a reverse reaction (known as cysteine desulfurase), which produces sulfide from cysteine and β-mercaptoethanol. The pH optimum for this reaction was similar to that of the forward reaction (8.4-8.8) except that there was no detectable activity below pH 8.2 (Figure 5.19b). Cysteine synthase activity data were

subsequently derived from a 2 min assay in Tris-HCl pH 8.0 to minimize the effect of the 'apparent' reverse reaction which was also optimal at the same pH range (between pH 8.4 and 8.9) as the forward reaction.

(a)



(b)

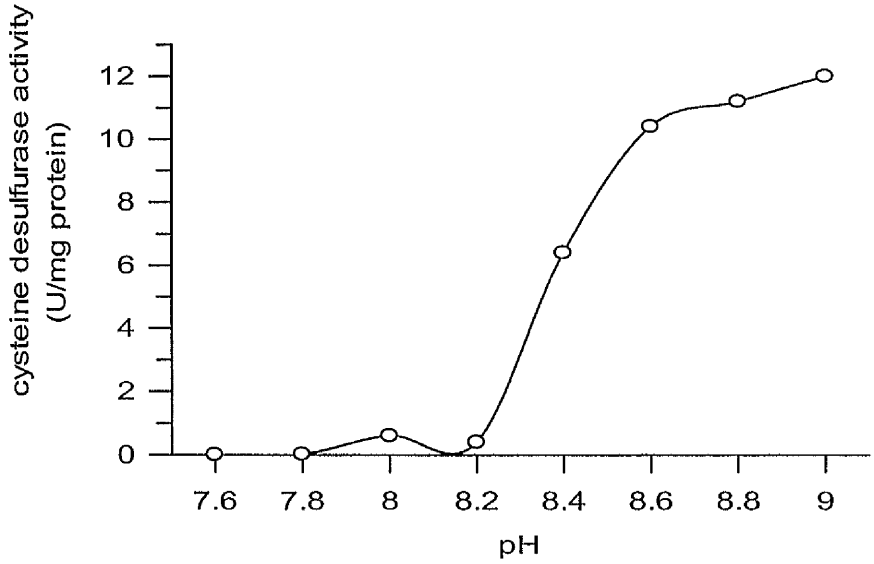
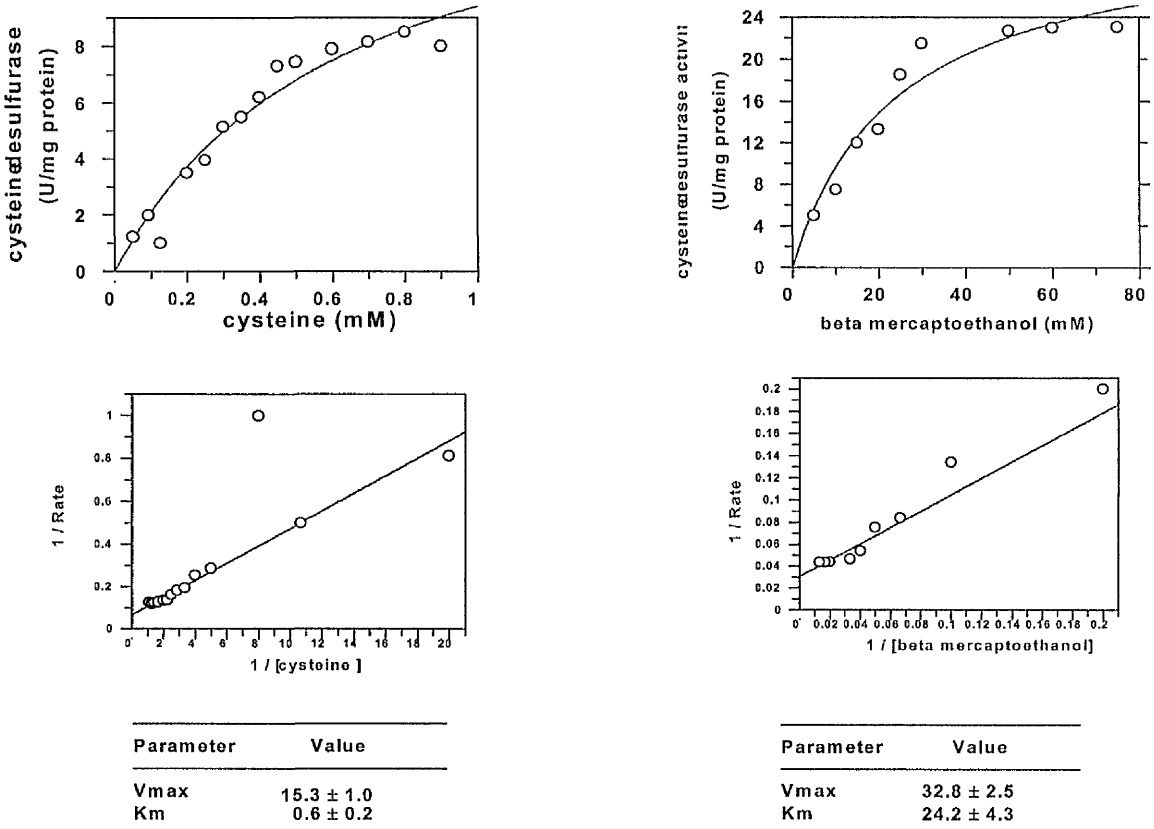


Figure 5.19a. pH optimum of LmajCS catalysing cysteine synthesis. The complete system contained in 1 ml: *O*-acetylserine 10 mM), hydrogen sulfide (1 mM) and buffers (200 mM; sodium phosphate (open circle), Tris-HCl (closed squares) and CHAPS (open diamond)). Data points are mean values of three assays given as μmoles/min/mg of protein. **(b)** pH optimum of CS catalysing cysteine desulfurase activity. The complete system contained in 1 ml: cysteine (5 mM), β-mercaptoethanol (5 mM) and Tris-HCl buffer (200 mM). The production of lead sulfide was monitored spectrometrically at 360 nm. Data points are mean values of three assays given as μmoles/min/mg of protein.

Purified recombinant LmajCS (rLmajCS) had a CS activity of 33.5 ± 0.2 μmol.min^{-1} mg.protein^{-1} (n=3, mean ± SD). rLmajCS also catalysed cysteine desulfurase activity at 9.8 ± 3.5 μmol.min^{-1}mg.protein^{-1} (n=3 mean ± SD) with a K_m for L-cysteine of 0.62 ± 0.15 mM (n=3) and a K_m of 24.2 ± 6.4 for β-mercaptoethanol (Figure 5.20). The enzyme cannot use homocysteine as substrate (homocysteine desulfurase) nor was the production of sulfide from cysteine without without β-mercaptoethanol (the activity known as activated serine sulfurhydrase) detected. CS activity was found in extracts of wild type parasites (0.07 μmol.min^{-1}.mg.protein^{-1}).



Parameter	Value
Vmax	15.3 ± 1.0
Km	0.6 ± 0.2

Parameter	Value
Vmax	32.8 ± 2.5
Km	24.2 ± 4.3

Figure 5.20. Kinetics analysis of LmajCS cysteine desulfurase activities. (a) Cysteine saturation curve and Michaelis Menten plot. Kinetics parameters (V_{max} and K_m) calculated by Grafit4 (Erithacus Software Limited) are also given. Data points are mean values of three independent expressions. The complete system is detailed in Figure 5.19b, but cysteine concentration varied from 0.05 – 0.9 mM. (b) β-mercaptoethanol saturation curve and Michaelis Menten plot. Kinetics parameters (V_{max} and K_m) calculated by Grafit4 (Erithacus Software Limited) are also given. Data points are mean values of three independent protein preparations. β-mercaptoethanol concentration varied from 5-70 mM. The complete system is detailed in Figure 5.19b.

5.14 Expression of LmajCS in different developmental forms of the parasite

The expression of the leishmanial LmajCS was also analysed using polyclonal antibodies raised against rLmajCS to determine the extent to which the enzyme is stage-specific. It was found that each developmental stage in the leishmanial life cycle showed cysteine synthase expression (Figure 5.21). Since the L. major amastigote pellet used in the Western blot analysis was not pure (exclusively free from host cell contamination), comparision of the expression levels among the life cycle stages cannot be made at this point. The rabbit antiserum was strongly reactive to purified rLmajCS and but recognized proteins at 35 kDa and 75 kDa in soluble extracts of *L. major*' amastigotes, log phase, stationary phase and metacyclic promastigotes (Figure 5.21). The 75 kDa band, was much larger than the SAT protein but was the approximately the sum of the molecular masses of rLmajSAT and rLmajCS. SAT/CS complexes have been reported in the literature, but are known to dissociate with a high concentration of *O*-acetyl-*L*-serine (OAS) (Mino *et al.*, 1999). The 75 kDa band did not dissociate with 1 mM OAS but the tightness of the SAT/CS complex differs from one isoenzyme to another (Mino *et al.*, 1999).

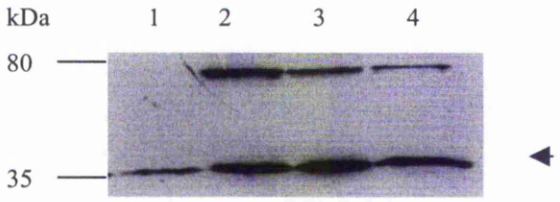


Figure 5.21. Western blot analysis of expression of leishmanial CS during the life cycle. Eighty micrograms of soluble cell extract was subjected to Western blot analysis using the rabbit α-rCS antiserum. Expression during the life cycle, *L. major* amastigote harvested from lesions of BALB/c mice (lane 1), *L. major* mid-log phase promastigotes (lane 2), *L. major* stationary phase promastigotes (lane 3), *L. major* metacyclic promastigotes (lane 4). Estimated molecular weight of LmajCS is arrowed. RLmajCS is expressed in all life cycle stages of the parasite.

5.15 LmajCS is not up-regulated in response to oxidative stress.

To find out whether the gene is involved in the mechanism by which the parasite detoxify oxygen reactive species within it host, *L. major* promastigotes was cultured in medium supplemented with varying concentrations of H_2O_2 (0.1-10 mM) to mimic oxidative stress produced by the host immune responses. The effect of the H_2O_2 on the expression of CS in *L. major* over 24 h incubation at 27°C was examined. The IC_{50} was estimated to be 0.26 mM (Figure 5.22).

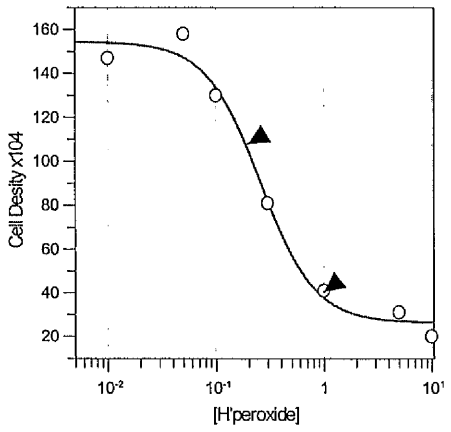


Figure 5.22. IC_{50} determination of *L. major* promastigotes exposed overnight to hydrogen peroxide. Promastigotes were exposed to varying concentrations of hydrogen peroxide ranging from 10 mM to 0.01 mM. The IC_{50} was estimated to be 0.26 mM. The sample of cells harvested and analysed by western blot analysis in (b) are arrowed. Initial cell density was 1.6 x 10^6. Growth at the end of the 24 h incubation in H2O2 was assessed by direct counting using the improved Neubauer hemocytometer under the microscope.

Western blot analysis of the lysate of selected cultures (0.0 mM, 0.5 mM, 1 mM) representative of the wide spectrum of H_2O_2 concentration used, showed no difference on the expression levels of the CS gene under the range of concentrations used (Figure 5.23).

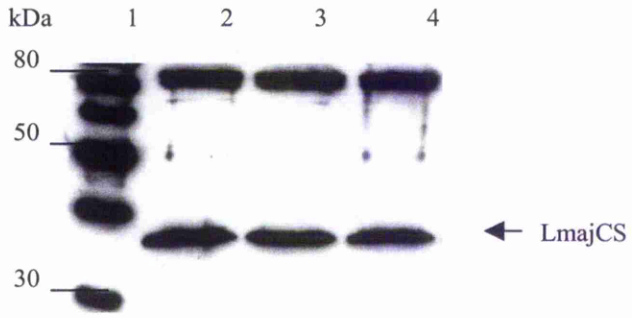


Figure 5.23. Expression in leishmanial CS in promastigotes subjected to oxidative stress. Western blot analysis of expression of leishmanial CS in cells subjected to oxidative stress. Eighty micrograms of soluble cell extracts from parasites exposed overnight to varying concentration of hydrogen peroxide were subjected to western blot analysis using the rabbit α-rCS antiserum. Molecular mass standards (lane 1), hydrogen peroxide at 0.0 mM (lane 2), hydrogen peroxide at 500 μM (lane 3), hydrogen peroxide at 1000 μM (lane 4). Arrowed is the LmajCS monomer.

5.16 Resistance of L. major *over-expressing the SAT and CS genes to oxidative stress*

The absence of regulation of LmajCS expression under oxidative stress could be due to either the LmajCS gene playing no vital role in the detoxification of reactive oxygen species or the LmajCS is not a rate-limiting enzyme and is expressed at high levels in the cysteine biosynthetic pathway. Hence there was no need for its regulation under to oxidative stress. To test these hypotheses, the genes involved in the *de novo* synthesis of cysteine, LmajSAT and LmajCS, were over-expressed in *L. major* promastigotes and the transgenic parasites characterised. CS activity of *Leishmania* overproducing LmajCS gene showed 5-fold increase in CS activity (0.4 $\mu mol.min^{-1}.mg\ protein^{-1}$) relative to the control, pGL607 (0.06 $\mu mol.min^{-1}.mg\ protein^{-1}$) over-expressing the antibiotic marker alone. Western analysis of the cell extracts of promastigotes over-expressing CS and SAT also showed an increase (of the 35 kDa

(Figure 5.24 a) and 45 kDa (Figure 5.24b) bands) in protein expression above that of the control transfected with the empty vector.

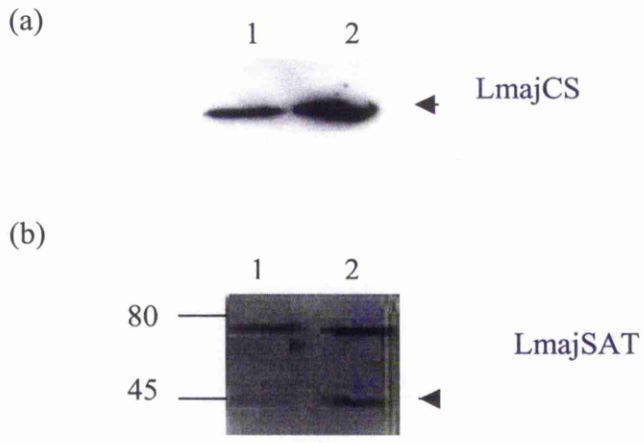


Figure 5.24a. Western blot analysis of *L. major* promastigotes expressing of *LmajCS* gene from an episome. 80 μg of soluble extracts from parasite were subjected to western blot analysis using the rabbit α-rCS antiserum. *L. major* promastigote with empty vector expressing the blasticidin antibiotic marker (lane 1), *L. major* promastigotes over-expressing the LmajCS gene (lane 2).

Figure 5.17b. Western blot analysis of *L. major* promastogotes expressing of *LmajSAT* gene from an episome. 80 μg of soluble extracts from parasite were subjected to western blot analysis using the rabbit α-rSAT antiserum. *L. major* promastigote with empty vector expressing the blasticidin antibiotic marker (lane 1), *L. major* promastigotes over-expressing the LmajSAT gene (lane 2).

To evaluate whether SAT or CS individually affect *L. major*'s resistant to oxidative stress, transgenic parasites confirmed to be over-expressing the SAT and CS genes at a higher level relative to the control, were to exposed the same concentrations of hydrogen peroxide or t-butyl hydroperoxide or cumene hydroperoxide detailed in Chapter 4. The cell densities of both over-expressing cell lines and their control cell line over-expressing the antibiotic resistant gene were estimated initially on addition

of the stressors. By the end of the 6 days incubation period, (wild type cells reached a late log phase density of 5 x 10^7 cell.ml^{-1} corresponding to an absorbance of 0.95 ± 0.34 at 405 nm with p-nitrophenyl phosphatase (acid phosphatase assay). The SAT and CS overproducing *Leishmania* (pGL607SAT and pGL607CS) were resistant relative to the control cells to the concentration of the oxidants used. At 1 and 6 days after the addition of stressors, the relative cell density of SAT overproducing cell line estimated by the acid phosphatase assay were 0.31 ± 0.01 and 0.57 ± 0.03 for hydrogen peroxide, 0.33 ± 0.02 and 0.51 ± 0.07 for t-butylperoxide, and 0.33 ± 0.01 and 0.57 ± 0.09 for cumene hydroperoxide (Figure 5.25a). The CS overproducing cell lines on the other hand recorded an absorbance of 0.30 ± 0.01 and 0.56 ± 0.02 for hydrogen peroxide, 0.33 ± 0.01 and 0.54 ± 0.02 for t-butylperoxide, and 0.31 ± 0.01 and 0.55 ± 0.01 for cumene hydroperoxide for the same time points (Figure 5.25b). The cell density for the control cells, under the same conditions and time points were 0.29 ± 0.06 and 0.31 ± 0.02 for hydrogen peroxide, 0.32 ± 0.01 and 0.35 ± 0.03 for t-butylperoxide, and 0.33 ± 0.02 and 0.37 ± 0.01 for cumene hydroperoxide (Figure 5.25c).

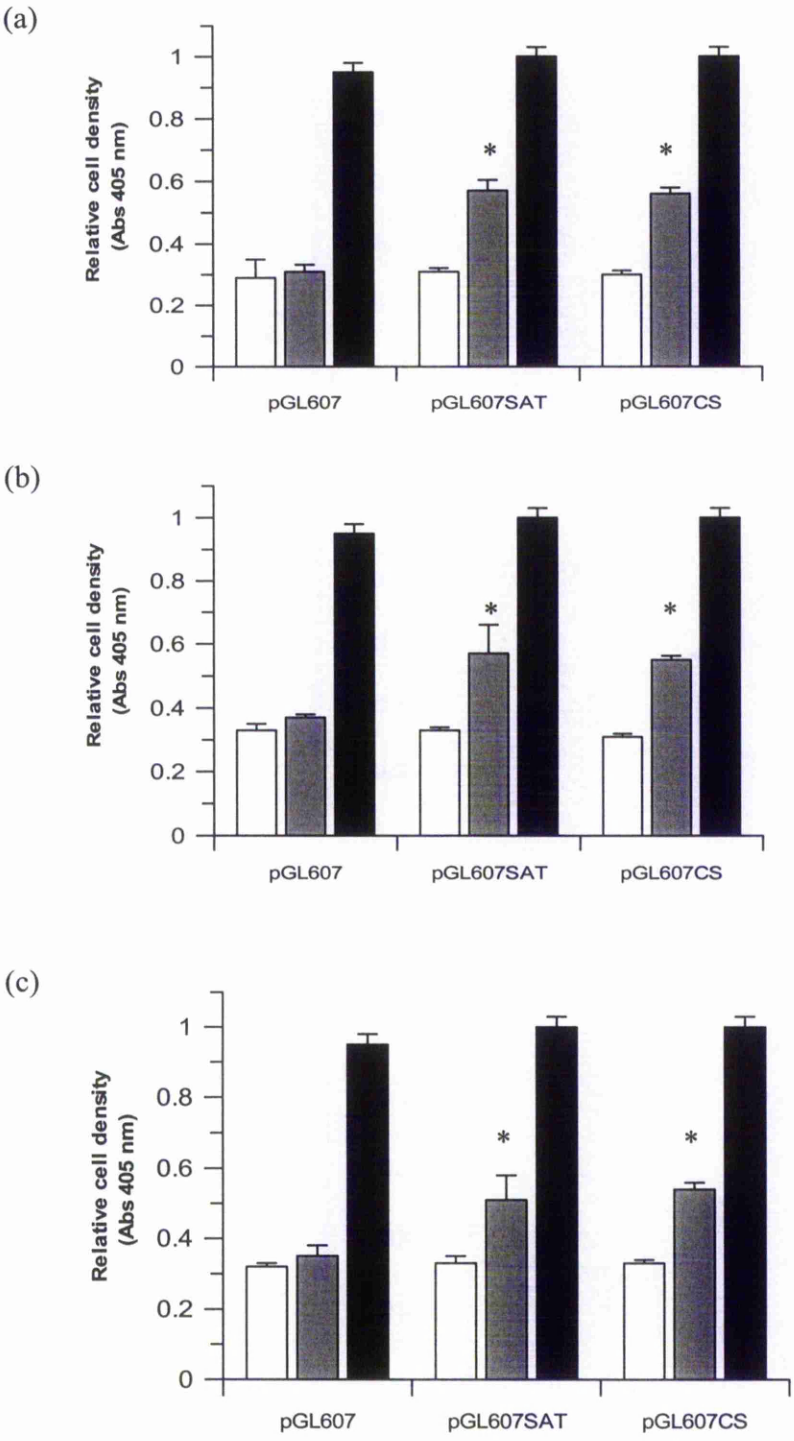


Figure 5.25 Relative cell density of *L. major* promastigotes subjected to oxidative stress. *L. major* promastigotes transformed with pGL607, pGL607SAT or pGL607CS and incubated at 27°C for 6 days in the presence (grey) or absence (black) of (a) 0.5 mM H_2O_2 or (b) 10 μM cumene hydroperoxide or (c) 10 μM t-butylperoxide. The relative cell relative density of viable cells was estimated by the acid phosphatase assay at day 6. Open bars represents the relative cell densities prior to the addition of the oxidant. Differences considered statistically significant by Student's t test ($p<0.05$) between the pGL607 and pGL607CS transformants or between pGL607 and pGL607SAT transformants are marked with asterisks.

The cells exposed to H_2O_2 were further analysed at the activity and protein expression levels. CS activity in the lysate of the pGL607CS cells exposed to H_2O_2 increased (to 0.35 μmol.min.mg protein^{-1}) compared with the control (0.06 μmol.min.mg protein^{-1}). Western analyses of the same extracts (Figure 5.26) also show an increase above the control levels of protein exposed to H_2O_2.

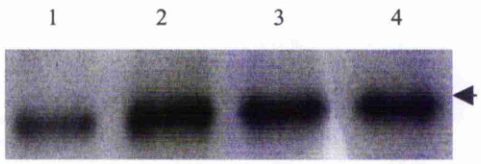


Figure 5.26 Western blot analysis of expression of leishmanial CS in cells cells over-expressing the LmajSAT, LmajCS and *L. major* cystathionine β-synthase (LmajCBS) proteins subjected to oxidative stress from hydrogen peroxide at 500 μM. Eighty micrograms of soluble cell extract was subjected to western blot analysis using the rabbit α-rCS antiserum. Control, pGL607 (lane 1), pGL707SAT (lane 2), pGL607CS (lane 3), and pGL607CBS (lane 4). Arrowed is the LmajCS monomer.

5.17 LmajCS is not up-regulated in response to heavy metals

Recently, strains of *Leishmania* resistant to the trivalent antimonial, pentostam have been isolated from clinical cases. The mechanism by with the parasite develop resistance against heavy metals is unknown. In plants, CS is regulated by stress produced by heavy metals. Heavy metals, e.g. cadmium, have been shown to induces the biosynthesis of phytochelatin peptides from GSH and cysteine. Phytochelatins have not been reported in trypanosomatids. The heavy metals are believed to have similar mode of action. To investigate whether the cysteine biosynthetic pathway play a role in heavy metal detoxification, promastigotes were grown in either cadmium chloride (2.75 μg.ml^{-1}), or copper sulfate (0.18 μg.ml^{-1}) or potassium

antimony tartrate (36.1 μg.ml^{-1}), or sodium arsenite (0.34 μg.ml^{-1}) overnight at 27°C. The concentration used were those known to inhibit growth by 50% during 24 h incubation (data not shown). Western analysis of lyaste (Figure 5.27) showed no significant difference in the expression levels of the CS gene when promastigotes were exposed to stress produced by heavy metals, relative to the control cells grown in normal medium.

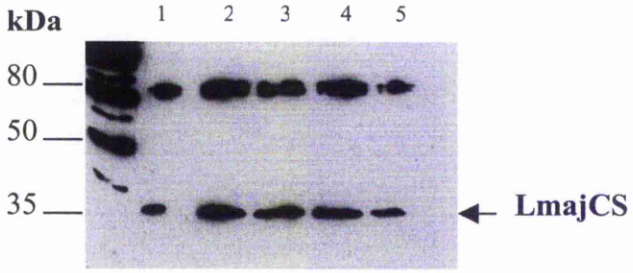


Figure 5.27. Western blot analysis of expression of leishmanial CS in cells subjected to heavy metals. 80 μg of soluble cell extracts exposed overnight to concentration of heavy metals known to kill 50% of promastigotes, and were subjected to western blot analysis using the rabbit α-rCS antiserum. Molecular mass standards, (left hand lane); control cells grown in the absence of stress, (lane 1); cadmium chloride at 2.75 μg.ml^{-1}, (lane 2); sodium antimony tartrate at 36.1 μg.ml^{-1}, (lane 3); copper sulfate at 0.18 μg.ml^{-1}, (lane 4); sodium arsenite at 0.34 μg.ml^{-1}, (lane 5). The estimated molecular weight of LmajCS is arrowed.

5.20 Sulfur requirement in L. major

L. major promastigotes are dependent on an exogenous source of cysteine for growth and survival, but the presence of a functional *de novo* biosynthetic pathway for cysteine biosynthesis makes the sulfur requirement of *L. major* a bit puzzling. Thus, the dilemma between leishmanial sulfur requirements and ability to synthesize cysteine *de novo* was investigated in two ways. Firstly, to find out the expression levels of CS when *L. major* are grown in medium devoid of an exogenous sulfur

source and, secondly to determine what sulfur source was vital for the parasites growth and survival.

L. major promastigotes grown in a medium devoid of exogenous sulfur source (MEM lacking cysteine, and methionine and supplemented by dialysed FCS) showed an apparent increase in CS expression levels relative to the control grown in normal medium (Figure 5.28). Whether this is a genuine increase is debatable as no activity assay was carried out to supplement the data from western analysis. This effect, if valid, might suggest that the CS gene is involved in cysteine synthesis, and its ability to synthesise cysteine is hampered if the substrates or intermediates involved are limiting.

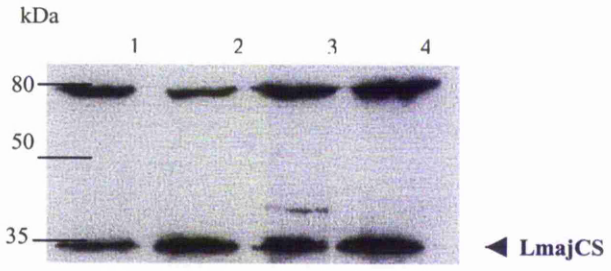


Figure 5.28. Western blot analysis of expression of leishmanial CS in cells over-expressing the SAT, CS and CBS proteins of promastigotes subjected to hypo-sulfur stress. 80 μg of soluble cell extract was subjected to western blot analysis using the rabbit α-rCS antiserum. Control cells in the absence of stress (lane 1), SAT overexpressing cell line (lane 2), CS overexpressing cell line (lane 3), CBS overexpressing cell line (lane 4). The estimated molecular weight of LmajCS is arrowed.

To investigate whether the substrate were limiting within the parasite and needed to be salvages from the medium, as well as the sulfur requirement of *L. major* promastigotes, the MEM medium was supplemented with either methionine (0.2

mg.L^{-1}) or cysteine (0.2 mg.L^{-1}) or homocysteine (0.2 mg.L^{-1}) or serine (0.2 mg.L^{-1}) or *O*-acetylserine (0.2 mg.L^{-1}) or hydrogen sulfide (0.15 mg.L^{-1}). Survival of *L. major* promastigotes was hardly affected without a sulfur source (sulfur deprived) but there were no appreciable growth. A similar scenario was observed in cells grown in the same medium supplemented with either 0.2 mg.L^{-1} cystathionine for the same duration. Promastigotes in medium supplemented with 0.15 ml.L^{-1} H_2S failed to thrive during the 6 days period (Figure 5.29). This slight increase in growth of promastigotes cultures in sulfur-deleted medium was not sustainable. They failed to thrive when attempts were made to sub-passage them which might suggest that the parasites 'sulfur-pool' was depleted in the absence of a sulfur source in the first passage to aid survival.

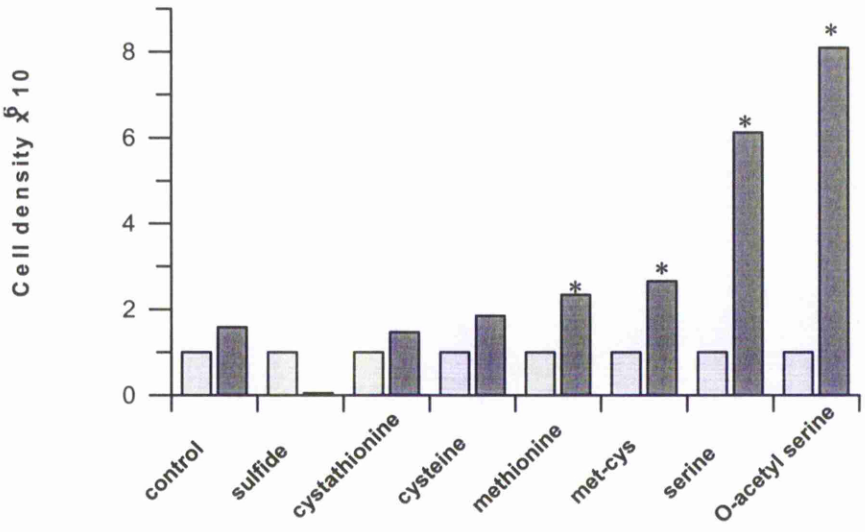


Figure 5.29 Cell density of *L. major* promastigote in variable growth conditions. *L. major* promastigotes were incubated at 27°C for 5 days either in MEM medium lacking serine and an exogenous sulfur source (control) or supplemented with sulfide (0.15 mg.L^{-1}), cystathionine (0.2 mg.L^{-1}), cysteine (0.2 mg.L^{-1}), methionine (0.2 mg.L^{-1}), serine (0.2 mg.L^{-1}) and *O*-acetyserine (0.2 mg.L^{-1}). The cell density of cells was estimated initially (white) and at day 5 (grey). Differences considered statistically significant by Student's t test ($p<0.05$) between initial and final cell densities are marked with asterisks.

These results show that *L. major* like the *T. brucei* bloodstream forms require cysteine for growth, but it further suggest that parasite can synthesise cysteine *in vivo* to maintain it growth if the substrates and intermediates in the pathway are not limiting. The demand of *L. major* promastigotes for serine suggests a link between the *de novo* synthetic pathway of cysteine and the serine biosynthetic pathway. This link is bound to posses some form of regulation as serine within organisms is known be limiting (Brunold, 1993) so a massive increase in growth when it becomes saturated in the growth medium is not suprising.

5.21 Discussion

In this chapter, I have demonstrated the existence of a pathway for the *de novo* synthesis of cysteine in *L. major* and established the importance of cysteine in the parasite's growth and resistance to oxidative stress. This pathway has been reported in bacteria (Kredich *et al.*, 1969) and plants (Blaszczyk *et al.*, 1999) and more recently in the protozoa *E. histolytica*, *E. dispar* and *T. cruzi* (Nozaki *et al.*, 1998, 1999, 2001). A putative ORF of SAT in the *Plasmodium yoeli* database might suggest that this apicomplexan parasite, like the amoebae and trypanosomatids has the *de novo* cysteine biosynthetic pathway. However, the recently published results of the *Plasmodium falciparum* sequencing project suggest this species lacks the pathway (Gardner *et al.*, 2002).

Whilst the presence of putative *LmajSAT* and *LmajCS* suggest the existence of *de novo* synthesis of cysteine within the parasite, the amplification and expression of LmajSAT resulted in a soluble but apparently inactive recombinant protein. Activity was recovered from the first few drops eluted from the Ni^{2+} affinity column during purification, but the total eluant at the end of the purification exercise was inactive.

There is the possibility that first few drops of LmajSAT from the column coupled with and was activated by the *E. coli* CS during preparation for purification. The entire purification process might have diluted and dissociated the complex and hence inactivated the enzyme. This is rather speculative and needs to be verified. Nevertheless, factors potentially responsible for only an apparent inactive protein being produced could be: (a) inactivation of LmajSAT by imidazole in the elution buffer, (b) Instability of LmajSAT, (c) cold inactivation SAT at 0°C (Mino *et al.*, 2001), (d) difficulty in optimising the assay conditions and (e) uncoupled LmajSAT is by itself inactive. The bi-enzyme complex (SAT with CS) is also known to activate SAT (Kredich *et al.*, 1969), stabilise it and reduce the rate of cold inactivation rate of SAT (Mino *et al.*, 2001). A question one needs to ask at this point is, does the LmajSAT/LmajCS form a bi-enzyme complex *in vivo*? I am tempted to speculate that the presence of the SAT/CS heteromization domain at the C-terminal end of LmajSAT and the presence of a second band of approximately 75 kDa (the approximate sum of the molecular masses of the CS and SAT proteins) observed during western analyses of both proteins are suggestive that such an interaction exists within the parasite. However, the trypanosomal SAT (TcSAT) produced as recombinant GST-fusion protein (Nozaki *et al.*, 2001) was active, albeit at low level (Nozaki *et al.*, 2001). The enzyme was inactive after thrombin digestion of the GST protein, suggesting that some protein-protein interaction was vital for activity of this trypanosomatid SAT.

Interaction of SAT with CS is not a new phenomenon and has been reported extensively in the literature for plants (Bogdanova and Hell, 1997; Mino *et al.*, 2000; 2001). The SAT/CS system is an important model for the study of protein-protein

interactions. This is now plausible for the *L. major' de novo* cysteine biosynthetic pathway since the two genes in the pathway have been identified.

The LmajCS was present and expressed in each life cycle stage of the parasite. Although these enzymes exist in multiple isoforms in plants (Murillo and Leustek, 1995; Gutierrez-Marcos *et al.*, 1996; Setya *et al.*, 1996), southern blot analysis have shown that the SAT and CS genes exist as single copy genes in *L. major*. A gene deletion study of LmajSAT and LmajCS is in progress. Clones of single replacement SAT/HYG and CS/HYG transgenic strains, resistant to hygromycin (500 $\mu g\ ml^{-1}$), are yet to be characterized for integration of the cassette into the LmajSAT and LmajCS loci. Information from the characterization of these lines might throw some light on the *in vivo* function of the cysteine biosynthetic pathway, although deletion of both alleles is likely to be more informative.

A reverse cysteine desulfurase activity, which produces sulfide from cysteine and β-mercaptoethanol was detected using LmajCS. The functional significance of this reverse reaction in *L. major* is unknown, but it is interesting to note that with the bacterium, *Methanosarcina thermophila,* cysteine and sulfide in the growth medium fully repressed the expression of CS activity whereas cysteine as the sole sulfur source in the absence of sulfide produced a 3-fold elevation of CS expression and a corresponding elevated level of sulfide (Borup and Ferry, 2000). This was rather surprising if one assumed that CS functions only in the synthesis of cysteine. These results suggest another role for CS other than cysteine biosynthesis. One possible alternative role is that CS can mobilize sulfur from cysteine for other biosynthetic

activities. CS has been shown to be involved in Fe-S cluster formation (Flint *et al.*, 1996). Further research is needed to find out if this is an *in vivo* phenomenon of *L. major*.

Studies on cysteine uptake and requirements are limited in protozoa. *Giardia spp.* (Lugan and Nash, 1994) cannot synthesize cysteine *de novo* even though cysteine is essential for the parasites' growth. It is puzzling and sounds a bit paradoxical to find out in the literature that *E. histolytica* (Nozaki *et al.*, 1998) and *T. vaginalis* (Goodall, 2001) can synthesize cysteine *de novo* but require cysteine in its growth medium for survival. High levels of intracellular cysteine have been reported in these anaerobic parasites. With *T. brucei*, growth of the bloodstream forms was dependent on cysteine (Duzenko *et al.*, 1992). *L. major* promastigotes in medium devoid of an exogenous source of cysteine and supplemented with dialysed serum (1000 kDa cut-off) also failed to thrive (this study). Dialysis would have removed various components of normal serum, including purines, heam, and pterines. However, addition of purines and amino acid supplements ably compensated for the low molecular weight molecules lost in dialysis and produced a different growth pattern. The medium was able to maintain growth for only one passage, suggesting that the parasite' 'sulfur store' (i.e. cysteine or components involved in cysteine synthesis) was being used up. Replenishment of the medium with serine, *O*-acetylserine, methionine, cysteine and a combination of cysteine and methionine stimulated growth. Cysteine stimulation of leishmanial growth was not as significant as that reported for *T. brucei* (Duzenko *et al.*, 1992). This was not surprising because cysteine in growth medium is prone to auto-oxidation by traces of copper. Auto-oxidation can, however, be minimized by either continuous replenishment or the addition of bathocuproine

sulfonate to the medium (Duzenko *et al.*, 1992). Methionine alone or in combination with cysteine did significantly increase the growth of *L. major*. *L. major* promastigotes in this study failed to thrive in the concentration of H_2S used. The higher eukaryotic plants, unlike *L. major,* can use H_2S is as sulfur source in limited sulfate (SO_4^{2-}) supply (De Kok *et al.*, 1997; Stuiver and De Kok, 1997; De Kok *et al.*, 1998; 2000).

Serine is produced from 3-phosphoglycerate (an intermediate in the glucose metabolism) and its incorporation with sulfide to synthesise cysteine links the *de novo* synthesis of cysteine with both carbohydrate and nitrogen metabolism (Brunold, 1993). The regulatory interactions between nitrate and sulfate assimilation have already been described (Kopriva *et al.*, 2002), but the mechanism is not yet clear. Several studies have shown that expression of different isoforms of CS is regulated differently under both sulfur and nitrogen starvation (Takahashi and Saito, 1996; Warrilow and Hawkesford, 1998). In plants (Takahashi *et al.*, 1997) and bacteria (Kredich, 1993), OAS acts as a transcriptional regulator of the sulfate assimilation genes.

Serine, OAS and sulfide availability are limiting and strongly controlled in the cysteine biosynthetic pathway (Rennenberg, 1983; Neuenschwander *et al.*, 1991). Cysteine at low and high concentrations inhibited SAT (Brunold and Suter, 1982; Noji *et al.*, 1998) and sulfate uptake and reduction (Herschbach and Rennenberg, 2001) respectively, but an excess of either can circumvent the internal control system (Neuenschwander *et al.*, 1991; Buwalda *et al.*, 1993; Saito *et al.*, 1994). These findings agree with my findings with OAS in a sulfur-depleted medium containing *L.*

major promastigotes. The addition of OAS to *L. major*'s growth medium (devoid of an exogenous sulfur source) removed the limitation imposed by low concentration of OAS, hence a rapid synthesis of cysteine and subsequently increase in cell density of the parasite. More work needs to be done to pinpoint the exact role(s) of these compounds and understand the sulfur requirements of *L. major*. These results so far can benefit from kinetic analysis of the uptake of these 'amino acid sulfur sources' that stimulate growth, as well as further feeding experiments and growth studies on transgenic cell lines over-expressing the genes involved in the *de novo* synthesis of cysteine.

Cysteine is used by anaerobic organisms to maintain a low redox potential and detoxify reactive oxygen species (Fahey *et al.*, 1984), but in aerobic organism, it is known to be involved in the synthesis of secondary metabolites involved in a number of detoxification reactions e.g. glutathione, metallothionines, phytochelatins and tryparedoxin. Hence, it is known to be involved in the resistance of organisms to oxidative stress, and in metal detoxification. In recent years, resistance to antimonials used as first line drugs in the treatment of *Leishmania* infections has been increasingly reported. The mode of action of these drugs is unknown and the mechanisms by which drug resistance comes about is speculative with research data at the moment being limited. Knowledge of the mechanisms involved in drug resistance will help in the development of new drugs. SbIII, the toxic trivalent derivative (SbV) is known to bind to thiol groups on proteins, to deplete cellular glutathione and to generate oxidative stress (Tandon *et al.*, 2002). In plants, the removal of heavy metals such as arsenic is by phytochelatins, enzymatically synthesized from the small tripeptide glutathione (*γ-L*-glutamyl-*L*-cysteinglycine) in response to toxic levels of the heavy

metals (Noctor *et al.*, 1998; Xiang and Oliver, 1998; Wemmie *et al.*, 1994; Vido *et al.*, 2001). Glutathione in turn is synthesized by a two step ATP-dependent reaction catalysed by gamma-glutamylcysteine synthetase and glutathione synthetase. These reactions are regulated by feedback control of the gamma-glutamylcysteine synthetase by GSH as well as cysteine availability as exogenous addition of cysteine has been shown to increase GSH content (Farago *et al.*, 1994). Recently, yeast resistance to cadmium exposure was shown to be regulated by the presence of methionine in the medium and proteins involved in regulating sulfur assimilation, thus demonstrating a link between sulfur metabolism and glutathione production (Stephen *et al.*, 1997; Dormer *et al.*, 2000). A proteomic approach recently adopted by Vido *et al.*, (2001) showed that the levels of at least 54 proteins (including proteins with well characterised roles in countering oxidative stress) were elevated in response to cadmium exposure in keeping with the hypothesis that heavy metal exposure results in oxidative stress. However, they also noted that some of the largest increases were of those proteins involved in the biosynthesis of sulfur-containing amino acids; methionine, cysteine and the tripeptide glutathione (Vido *et al.*, 2001). However, little work has been carried out on the regulation of cysteine biosynthesis under heavy metal stress. In this study, wild type promastigotes exposed to heavy metals (copper sulfate (0.5mM), cadmium chloride (0.3 mM), arsenate (0.2 mM), and potassium antimony tartrate (0.4 mM)), and oxidative stress (hydrogen peroxide 0.5 mM) showed no difference in the expression levels of CS relative to the control cells. A plausible explanation might be the CS protein is abundantly expressed in the parasite as reported for the plants CS (Nakamura *et al.*, 1987; Ruffet *et al.*, 1994.) and is non-regulatory. If this is true for the *L. major* CS protein, then the unaltered expression of LmajCS under heavy metal stress is not surprising.

To investigate whether a link does exist, transgenic *L. major* overexpressing the SAT and CS genes individually were prepared and exposed to the oxidants, hydrogen peroxide, cumene hydroperoxide and tert-butyl hydroperoxide. Both cell lines were relatively resistant and showed appreciable growth relative to wild type promastigotes in the presence of these oxidants. This provides evidence that enzymes involved in the *de novo* synthesis of cysteine play a role in *L. major*'s defence against oxygen reactive species. Further, it might suggest that CS in *L. major* was perhaps non-regulatory or abundantly expressed. A similar treatment of the transgenic cell lines over-expressing SAT and CS with heavy metals might be informative. These over-expression studies have proved to be useful in defining the *in vivo* function of both CS and SAT. However this information need to be substantiated by gene deletion studies.

Chapter 6

The trans-sulfuration pathways in L. major *and characterization of cystathionine β-synthase.*

6.0 Introduction

The trans-sulfuration pathways constitute part of the metabolic inter-conversion of the sulfur-containing amino acids cysteine and methionine (Figure 6.1). The forward pathway (trans-sulfuration), transforms cysteine into homocysteine via the intermediate *L*-cystathionine by the sequential action of the enzymes cystathionine γ-synthase (CGS) and cystathionine β-lyase (CBL). This pathway has been identified in bacteria (Clausen *et al.*, 1997; 1998), fungi (Sienko and Paszewski, 1999; Dobric *et al.*, 2000) and plants (Maimann *et al.*, 2000; Breitinger *et al.*, 2001) (Figure 6.1). Conversely, the reverse trans-sulfuration is catalysed by the enzymes, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CGL). This pathway occurring in mammals (Kraus *et al.*, 1978; Nagasawa *et al.*, 1984; Kery *et al.*, 1994) and fungi (Flavin, 1971; Ono *et al.*, 1994) although the actinomycetes is a notable exception to this rule (Ono *et al.*, 1994) (Figure 6.1).

These enzymes belong to the ubiquitous pyridoxal-5'-phosphate-dependent (PLP) family which catalyses reactions involving amino acids as substrates. Generally, the amine group of the PLP conjugates with its amino acid substrates causing the bonds around the Cα, Cβ or Cγ positions to break, hence their classification into α, β and γ enzymes (Alexander *et al.*, 1994). Further, this classification into distinct families also depends on their folds (Alexander *et al.*, 1994) and are believed to be the product of multiple evolutionary events (Salzmann *et al.*, 2000). The consequence is a

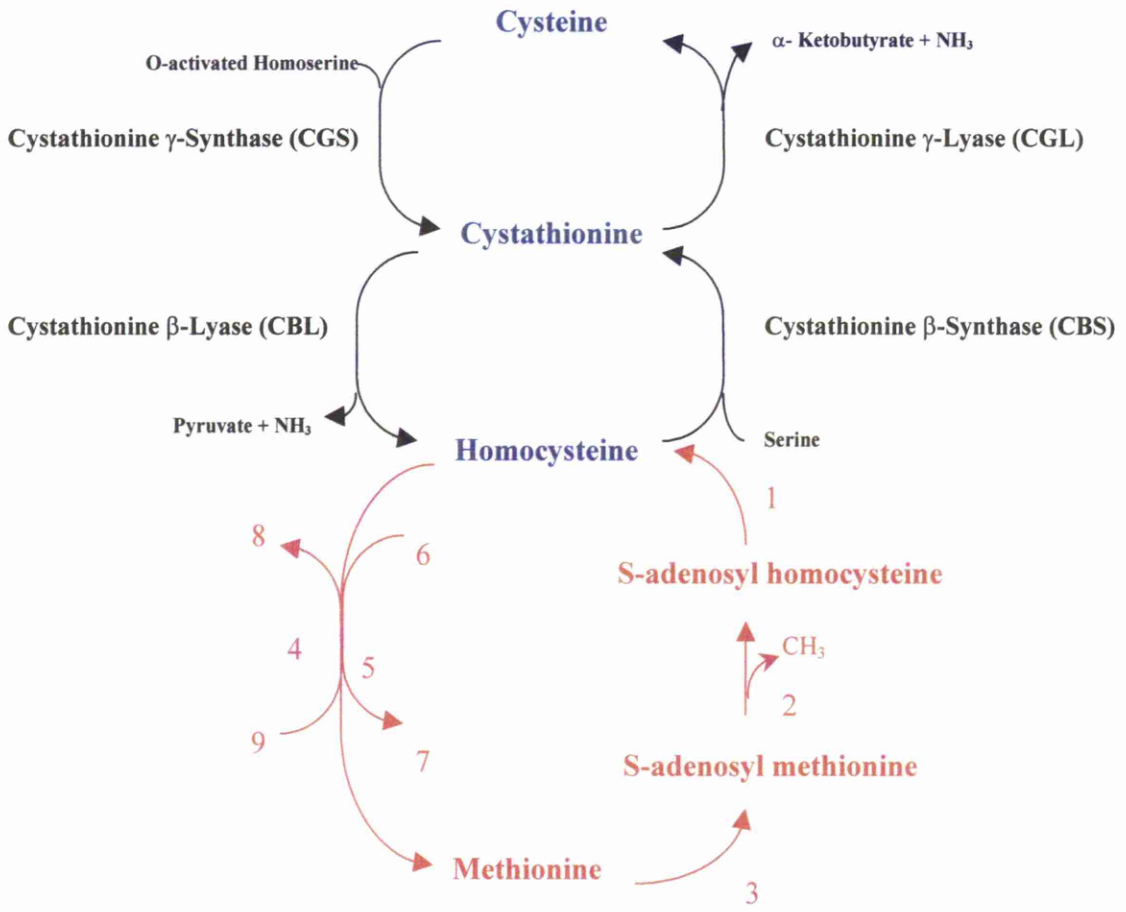


Figure 6.1 Overview of the trans-sulfuration and methionine cycle reactions. Enzymes catalysing key steps are indicated. The trans-sulfuration pathway is shown in black. The left panel represents reactions of the forward transsulfuration pathway occurring in bacteria, plants and fungi; the right panel shows the reverse transsulfuration pathway found in fungi and mammals. The methionine cycle and intermediates involved in individual reactions shown in red. Enzymes of the cycle are: 1, S-adenosyl homocysteine hydrolase; 2, methyltransferase; 3, methionine adenosyltransferase; 4, 5-methyltetra hydrofolate homocysteine methyltransferase. The metabolites are 6, betaine homocysteine methyltransferase; 6, dimethyl glycine; 7, betaine; 8, tetrahydrofolate; 9, 5-methyltetrahydrofolate

versatile enzyme capable of catalysing transamination, decarboxylation, racemisation, replacement and elimination reactions dependent on the enzyme's active site architecture. CBL, CGS and CGL show extensive sequence homology and belong to PLPγ family (Alexander *et al.*, 1994) while CBS is unrelated and belongs to the PLPβ family.

Cystathionine *β*-synthase has been purified from major phyla, the nematodes (Papadopoulus *et al.*, 1996), helminthes (Walker *et al.*, 1991b), fungi (Jhee *et al.*, 2000), mammals (Kery *et al.*, 1994, Taoka *et al.*, 1999) and more recently from protozoa (Nozaki *et al.*, 2001). They are all dependent on PLP for activity. The mammalian CBS enzyme is further dependent on S-adenosylmethionine (AdoMet) and heme (Kery *et al.*, 1994, Taoka *et al.*, 1999) for activity. Results are conflicting as to whether the fungi enzymes are dependent on AdoMet (Ono *et al.*, 1994; Jhee *et al.*, 2000).

In mammals, CBS is believed to be the rate-limiting step of the reverse transsulfuration pathway (Roper and Kraus, 1992). Further, it plays a direct role in detoxification of homocysteine in cells, and an indirect role in the detoxification of reactive oxygen species. Under oxidising conditions, CBS activity is enhanced leading to a subsequent increase in the glutathione production (Taoka *et al.*, 1998).

The genome sequencing projects of *Leishmania major* is currently approximately 55% complete, but have so far produced HTGS and ORFs with deduced similarity to enzymes involved in the metabolic pathway for sulfur-amino acid metabolism. In this

chapter, HTGS and ORFs will be used to construct a predictive metabolic map, which provides information that gives a framework for understanding cysteine and methionine biosynthesis in the parasite. The characterisation of CBS and its possible involvement in oxidative stress are also presented.

6.1 Material and Methods

6.2. PCR amplification of LmajCBS

The ORF of the gene encoding *LmajCBS* was amplified from the gDNA by PCR with the Expand High Fidelity system using a thermal cycle of 94°C for 2 min; 30 cycles of 94°C for, 65°C for 30s, 72°C for 3 min and a final step of 72°C for 7 min. For this, the 5'-perfect match primer CBS1 (5'CGCTGACATATGTCTGCTCCTGCTGCGCCGAA3') and a 3' perfect match primer CBS3 (5'TTGCGGCCGCCGCTGAGTGCCGCTCTCGTACT3') containing the *Nde*I and *Not*I restriction sites (underlined) to facilitate cloning were used. The PCR products were cloned into pGEM-T (Qiagen) to generate plasmids designated as *pGEMTLmajCBS*. Plasmids were propagated in *E. coli* DH5α cells and plasmid DNA was prepared by the Qiagen method and sequenced on both strands (MWG Biotech. or University of Glasgow Sequencing Service). Plasmids *pGEMTLmajCBS* was digested with *Nde*I and *Not*I and the restriction endonuclease fragment of 1.1 kb, encoding the CBS sequences amplified, were ligated into *NdeI/Not*I-digested pET21a^{+} vector (Invitrogen) to produce *pETLmajCBS*.

6.2.1 Expression and Purification of CBS

LmajCBS was expressed and purified using the same procedures described for LmajMST (Chapter 3) except that (a) expression was induced by 0.5 mM IPTG for 5

hours at 15°C. (b) cells were pelleted and resuspended in 5 ml of sonication buffer A with 25 μM PLP and (c) the eluant was dialysed with excess 500 mM Tris-HCl, pH 8.0, with 25 μM PLP at 4°C overnight to remove salt and imidazole and then stored at 4°C until analysed. Approximately 34 mg soluble, recombinant protein (rLmajCBS) was obtained from a 1 litre culture of induced *E. coli.*

6.2.2 *Production of LmajCBS over-expressing and knock-out plasmids*

The ORF of the *LmajCBS* gene was amplified by PCR (denaturation at 94°C for 2 min, 30 cycles of 94°C for 15 sec, 64°C for 30 sec 72°C for 2 min and a final extension of 7 min at 72°C) using oligonucleotide primers CBSr1 (5'GATATCCATATGGCATCCGCAAACCCCCG3') and CBSr2 (5'GATATCCTACTGAGTGCCGCTCTCGTACTTGGCCAGCT3') containing the *EcoRV* restrictions site (underlined) to facilitate subcloning into the re-expression vector, pGL607. The amplified LmajCBS gene was subcloned into the pGEM-T vector (Promega) to give *pGEMTrLmajCBS* and subsequently into the *Sma*I-cut pGL607 re-expression vector to generate the plasmid *pGL607CBS* which was subsequently used to transfect *L. major* promastigotes as detailed in Chapter 2. The re-ligated *Sma*I-cut pGL607 without any insert acted as a control and was designated pGL607.

Single-targeted gene-replacement constructs were obtained by PCR amplification (denaturation at 94°C for 2 min, 30 cycles of 94°C for 15 sec, 64°C for 30 sec 72°C for 2 min and a final extension of 7 min at 72°C) of the 5'-untranslated region (5'UTR) of *LmajCBS* using primers KOCBS1 (5'AAGCTTCTCGCTCGGTCTCTCAAGTGGGGCAACGCT3') and KOCBS2

(5'GTCGACTGTGGAGGAGAGAGGAGGGGGTGGGGC3') and of the 3'-UTR of *LmajCBS* using KOCBS3 (5'CCCGGGTAGAGAGGCAGCGGCAGCAAGAGA3') and KOCBS4 (5'AGATCTTCTGTTTTCAGGGCGCAATGAGAAAAGAAGGGGT3'). The nucleotides used in primer design targeting the UTRs were obtained from the on-going *L. major* sequencing project. The primers (KOCBS1, KOCBS2, KCBSO3 and KOCBS4) were modified with the *HindIII*, *SalI*, *SmaI* and *Bgl*II restriction sites, respectively. They DNA fragments were subcloned into the pGEMT vector to give pGEMTαCBS-5F and pGEMTαCBS-3F and sequenced. The *SmaI/Bgl*II LmajCBS 5'-UTR PCR DNA fragment, the *HindIII/SalI* LmajCBS 3'-UTR PCR DNA fragment and the *Hind*III/*Bgl*II DNA fragment containing the hygromycin phosphatase gene were ligated consecutively into pGL345 to generate the knock-out plasmid pGL345αCBS. *L. major* promastigotes were transfected with this construct as described in Chapter 2. Positive clones were analysed by southern blotting using the [α-^{32}P] dATP labeled 5'-UTR as probe.

6.3 *Results*

6.4 Identification and cloning of the cystathionine β-synthase gene of Leishmania major *(LmajCBS)*

The cystathionine β-synthase gene (CBS) (EC 4.2.1.22), also commonly referred to as serine hydrolyase, of *L. major* was identified by BLAST search of the *L. major* database (http://www2.ebi.ac.uk/blast2/parasite.html) using the amino acid sequence of TcCBS from *T. cruzi* (Nozaki *et al.*, 2001) as the query sequence. The approximately 1.1 kb ORF of *LmajCBS* (EMBL/Genebank Accession No. AC AC019154_4), was amplified with gene-specific primers CBS1 and CBS3 as forward

primer and reverse primers after 30 cycles of PCR (Figure 6.2a). Sequencing and blastx analysis showed that the nucleotides sequence encoded a protein possessing strong homology to CBS from various organisms. LmajCBS was expressed and purified as soluble proteins from *E. coli* (Figure 6.2b). The predicted amino acid sequences of LmajCBS of *L. major* comprised 359 residues (Figure 6.3) with a calculated molecular mass of 39.2 kDa (Figure 6.2b).

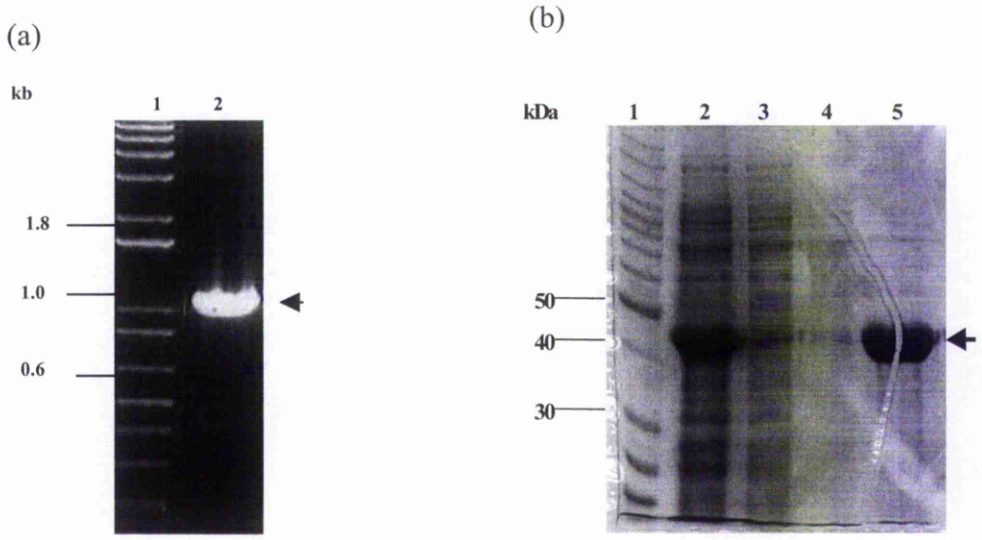


Figure 6.2 (a) Ethidium bromide stained 0.7% agarose gel showing the PCR product of the LmajCBS of *Leishmania major*. Lane 1, DNA standards; Lane 2, *LmajCBS* (arrowed). (b) Purification of rLmajCBS under native conditions. The soluble protein in the supernatant fraction was purified on a nickel chelator column. The His-tagged protein was eluted by addition of Buffer A with 250 mM imidazole. SDS-PAGE analysis of the following samples: Lane 1, molecular mass standards; lanes 2 and 3, supernatant and pellet fractions of a crude extract, respectively, of the recombinant *E. coli* cells; lane 4 wash with Buffer A with 60 mM imidazole; lane 5 purified rLmajCBS eluted from the nicked chelator column with Buffer A with 500 mM imidazole. Estimated recombinant protein (arrowed) size: 39.1 kDa.

```
          M   A   S   A   N   P   R   N   Q   I   L   A   N   A   L   E   A   V   G   N
    1 ATG GCA TCC GCA AAC CCC CGC AAC CAA ATC CTG GCC AAC GCC CTC GAG GCG GTC GGC AAC
      TAC CGT AGG CGT TTG GGG GCG TTG GTT TAG GAC CGG TTG CGG GAG CTC CGC CAG CCG TTG
          T   P   C   I   R   L   N   R   V   P   Q   K   H   G   I   R   C   E   V   V
   61 ACC CCT TGC ATC CGC TTG AAC CGC GTC CCA CAG AAG CAT GGC ATT CGG TGT GAG GTC GTG
      TGG GGA ACG TAG GCG AAC TTG GCG CAG GGT GTC TTC GTA CCG TAA GCC ACA CTC CAG CAC
          A   K   C   E   F   F   N   P   G   G   S   V   K   D   R   I   G   K   Q   M
  121 GCC AAG TGC GAA TTC TTC AAC CCT GGC GGC AGC GTG AAG GAT CGC ATC GGC AAG CAG ATG
      CGG TTC ACG CTT AAG AAG TTG GGA CCG CCG TCG CAC TTC CTA GCG TAG CCG TTC GTC TAC
          V   L   D   A   E   K   N   G   T   L   K   P   G   S   V   I   V   E   A   T
  181 GTA CTC GAC GCC GAA AAG AAC GGG ACG CTC AAG CCG GGA TCC GTG ATT GTG GAG GCT ACA
      CAT GAG CTG CGG CTT TTC TTG CCC TGC GAG TTC GGC CCT AGG CAC TAA CAC CTC CGA TGT
          S   G   N   T   G   I   G   L   S   M   A   A   A   I   R   G   Y   H   M   V
  241 AGT GGC AAC ACT GGC ATC GGT CTC TCC ATG GCG GCG GCG ATC CGC GGC TAC CAC ATG GTG
      TCA CCG TTG TGA CCG TAG CCA GAG AGG TAC CGC CGC CGC TAG GCG CCG ATG GTG TAC CAC
          I   T   M   P   K   K   M   S   H   E   K   E   T   T   L   Q   S   L   G   A
  301 ATC ACG ATG CCG AAG AAG ATG TCG CAC GAG AAG GAG ACG ACG CTA CAG TCC CTT GGC GCC
      TAG TGC TAC GGC TTC TTC TAC AGC GTG CTC TTC CTC TGC TGC GAT GTC AGG GAA CCG CGG
          E   V   I   R   T   E   T   S   L   P   Y   D   H   P   D   S   L   I   G   V
  361 GAG GTG ATC CGC ACG GAG ACG TCT CTT CCG TAC GAC CAC CCG GAC AGC CTC ATC GGG GTC
      CTC CAC TAG GCG TGC CTC TGC AGA GAA GGC ATG CTG GTG GGC CTG TCG GAG TAG CCC CAG
          A   R   R   L   R   D   E   K   G   Y   V   L   L   D   Q   Y   T   N   P   S
  421 GCT CGC CGC CTG CGC GAT GAG AAG GGC TAT GTG CTG CTG GAC CAG TAC ACG AAC CCC AGC
      CGA GCG GCG GAC GCG CTA CTC TTC CCG ATA CAC GAC GAC CTG GTC ATG TGC TTG GGG TCG
          N   P   G   A   H   Y   E   F   T   G   Q   E   I   Y   D   Q   C   G   S   K
  481 AAC CCG GGT GCG CAC TAC GAG TTT ACT GGC CAG GAG ATC TAC GAC CAG TGT GGC AGT AAG
      TTG GGC CCA CGC GTG ATG CTC AAA TGA CCG GTC CTC TAG ATG CTG GTC ACA CCG TCA TTC
          V   D   M   V   V   I   C   A   G   T   G   G   T   I   T   G   V   A   K   K
  541 GTC GAC ATG GTA GTC ATC TGC GCC GGA ACC GGT GGC ACG ATC ACT GGA GTG GCC AAG AAG
      CAG CTG TAC CAT CAG TAG ACG CGG CCT TGG CCA CCG TGC TAG TGA CCT CAC CGG TTC TTC
          L   K   E   L   I   P   G   V   I   V   V   G   V   D   P   V   G   S   I   I
  601 CTG AAG GAA CTC ATC CCC GGA GTC ATC GTT GTC GGT GTA GAC CCG GTC GGC AGC ATC ATC
      GAC TTC CTT GAG TAG GGG CCT CAG TAG CAA CAG CCA CAT CTG GGC CAG CCG TCG TAG TAG
          A   D   P   E   H   P   G   E   P   V   M   Y   H   V   E   G   I   G   Y   D
  661 GCC GAT CCC GAA CAC CCC GGC GAG CCT GTC ATG TAC CAC GTA GAG GGA ATC GGC TAT GAC
      CGG CTA GGG CTT GTG GGG CCG CTC GGA CAG TAC ATG GTG CAT CTC CCT TAG CCG ATA CTG
          F   V   P   D   V   C   E   R   K   Y   V   D   R   W   V   K   T   R   D   Q
  721 TTT GTG CCA GAT GTG TGC GAG CGT AAG TAC GTC GAT CGC TGG GTC AAG ACG CGC GAT CAG
      AAA CAC GGT CTA CAC ACG CTC GCA TTC ATG CAG CTA GCG ACC CAG TTC TGC GCG CTA GTC
          E   S   F   D   L   A   L   E   L   H   R   E   E   G   L   L   V   G   G   S
  781 GAG AGT TTC GAC CTC GCC CTC GAG CTG CAC CGC GAG GAG GGT CTG CTG GTC GGC GGT AGC
      CTC TCA AAG CTG GAG CGG GAG CTC GAC GTG GCG CTC CTC CCA GAC GAC CAG CCG CCA TCG
          S   G   S   A   M   A   G   V   L   E   A   A   K   D   L   R   P   D   Q   R
  841 AGC GGC TCC GCG ATG GCC GGC GTG TTG GAG GCA GCC AAG GAC CTT CGC CCG GAT CAG CGC
      TCG CCG AGG CGC TAC CGG CCG CAC AAC CTC CGT CGG TTC CTG GAA GCG GGC CTA GTC GCG
          C   V   V   L   M   A   D   G   I   R   N   Y   M   G   K   F   A   D   V   N
  901 TGC GTC GTG CTC ATG GCG GAC GGC ATC CGC AAC TAC ATG GGG AAG TTT GCC GAT GTC AAC
      ACG CAG CAC GAG TAC CGC CTG CCG TAG GCG TTG ATG TAC CCC TTC AAA CGG CTA CAG TTG
          W   M   I   E   H   G   F   R   Q   G   E   V   T   R   P   T   Y   D   T   L
  961 TGG ATG ATC GAG CAC GGC TTC CGG CAG GGT GAG GTG ACC CGC CCC ACC TAC GAC ACG CTG
      ACC TAC TAG CTC GTG CCG AAG GCC GTC CCA CTC CAC TGG GCG GGG TGG ATG CTG TGC GAC
          K   K   E   L   E   E   V   K   A   K   L   A   K   Y   E   S   G   T   Q
 1021 AAG AAG GAG CTG GAG GAG GTC AAG GCG AAG CTG GCC AAG TAC GAG AGC GGC ACT CAG
      TTC TTC CTC GAC CTC CTC CAG TTC CGC TTC GAC CGG TTC ATG CTC TCG CCG TGA GTC
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Figure 6.3 Nucleotide sequence of *L. major* CBS gDNA and deduced amino acid sequence. Nucleotides are numbered in the 5' to 3' direction, beginning with the first residue at the start codon and ending at the codon preceeding the stop codon.

6.5 The domain architecture of Cystathionine beta-synthase

The human CBS consists of a heme domain (residues 1-50), 'catalytic core' with a pyridoxal 5' phosphate (PGGSVKD) motif, and a regulatory domain composed of two 'CBS domains' (Figure 6.4). CBS from yeasts lacked the heme motif (Jhee *et al.*,

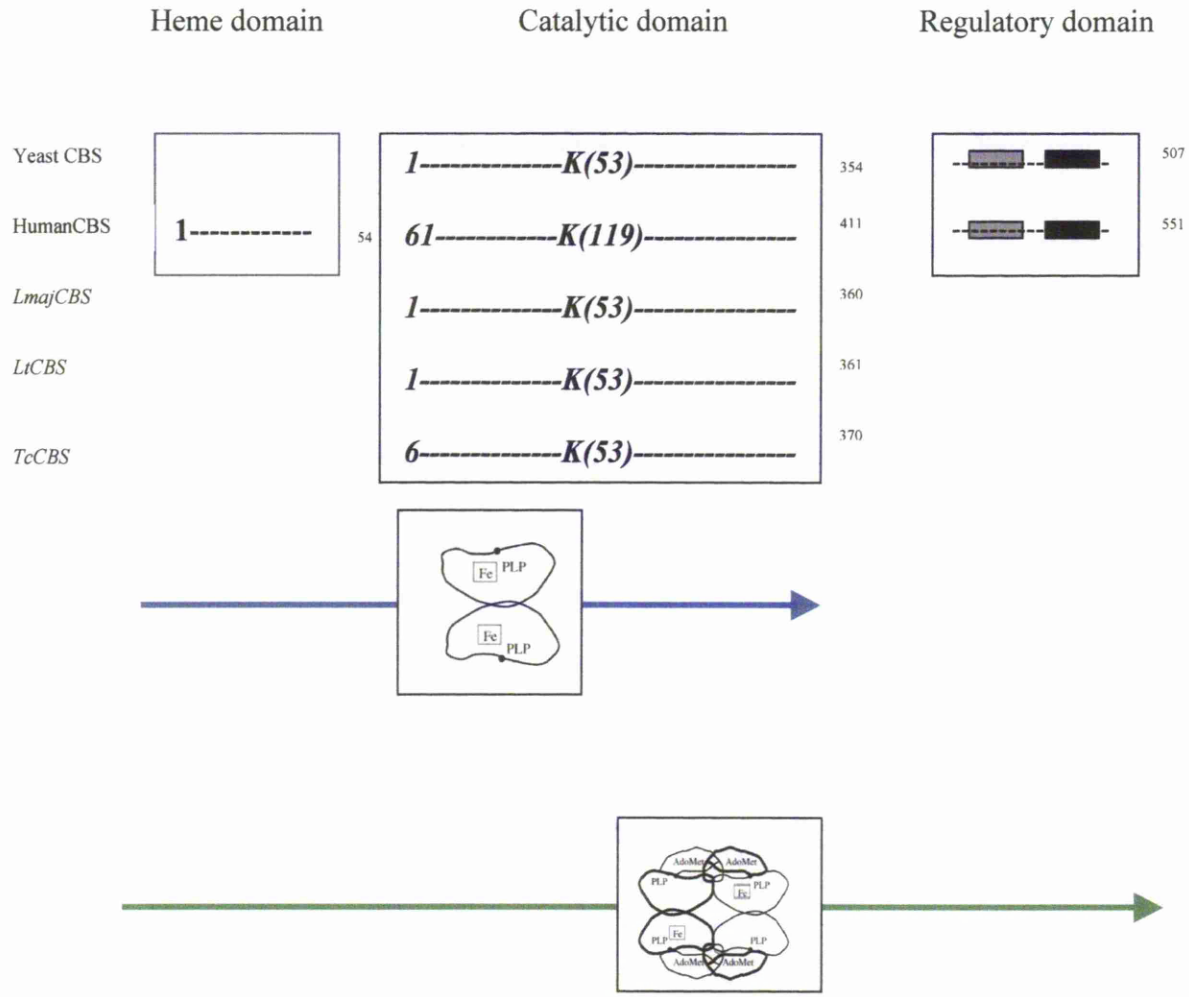


Figure 6.4. (a) A scheme of the domain architecture of CBS. The scheme shows a comparison of CBS from other organisms with the three domains of the CBS from mammals (Kery et al., 1994). The residue numbers for the lysine residues (K) that bind PLP for yeast CBS (Jhee *et al.*, 2000), human CBS (Kery *et al.*, 1994), TcCBS (Nozaki *et al.*, 2001), LtCBS (GenBank accession no. AAF71541.1 GI:8037933) and LmajCBS (this study) are indicated. The regulatory domain of human and yeast CBS contain CBS1 (Bateman, 1997) and CBS2 (Shan *et al.*, 2001). The green and blue arrows represent the mammalian CBS protein with all domain and lacking the CBS regulatory domain respectively. The oligomeric structure of full length mammalian CBS (green) has of four PLP and AdoMet and heme binding sites while the truncated CBS (blue) is a dimmer with only two PLP and heme binding sites.

2000; Maclean *et al.*, 2000, Nozaki *et al.*, 2001) but possess the CBS domains. Results are conflicting as to whether the CBS domain in yeast contain the AdoMet regulatory motif (Jhee *et al.*, 2000; Ono *et al.*, 1994).

The LmajCBS as well as CBS from other trypanosomatids, TcCBS (Nozaki *et al.*, 2001) and LtCBS (GenBank accession no. AAF71541.1 GI:8037933) lack the 90-120 amino acid regulatory CBS domains present in the yeast and mammals proteins, and the heme motif from the mammals, but possess the 'catalytic domain'. The lack of the CBS domain in LmajCBS makes it a relatively short protein (359 vs. 551 amino acid of the mammalian enzyme) which is comparable with CBSs from other trypanosomatids (Nozaki *et al.,* 2001) and cysteine synthase (CS) from a variety of organisms.

Native human CBS forms tetramers and higher multimers in solution, while truncated mutants or partially trypsin-digested CBS made up of either the catalytic core or the heme and catalytic domains are dimers (Figure 6.4) suggesting that the C-terminal domain is involved in the interaction of the subunits to form higher oligomeric structures (Oliveriusava *et al.*, 2002). Thus one can speculate that LmajCBS is a dimer. However, TcCBS, with 66% identity to LmajCBS, is a homotetramer (Nozaki *et al.*, 2001), implying that the C-terminal region in yeast and mammalian CBSs is not exclusively responsible for tetrameric formation as suggested previously (Taoka *et al.*, 1999).

6.5.1 Sequence alignment and phylogenetic analysis of CBS amino acid sequences

The deduced amino acid sequence of LmajCBS (Figure 6.5) has significant identities with CBS from *L. tarentolae* (83%), and *T. cruzi* (66%) (Table 6.1).

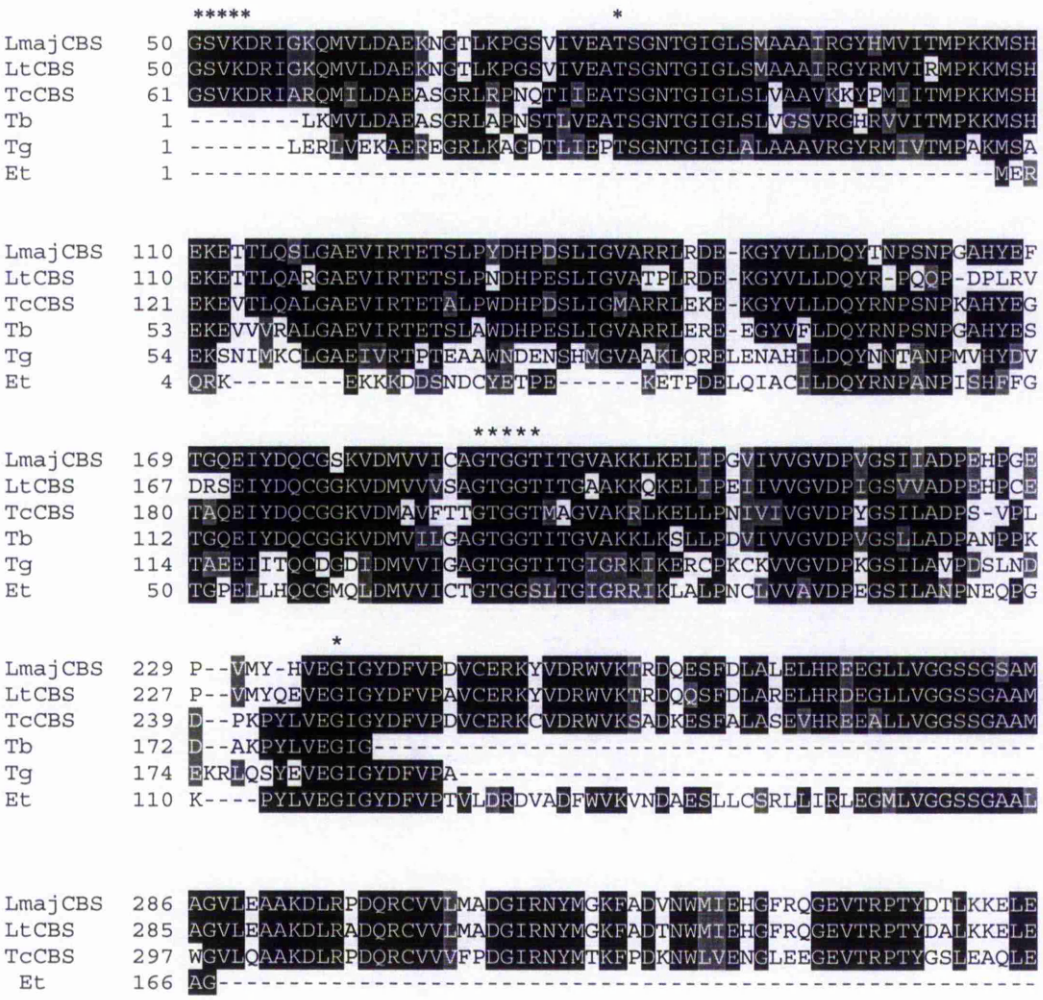


Figure 6.5. Multiple alignment of partial amino acid sequences of CBS from *L. major* and other protozoa. Sequences of CBSs are as follows; LmajCBS, *L. major* (this study), LtCBS, *Leishmania tarentolae* (GenBank accession no. AAF71541.1 GI:8037933); TcCBS, *T. cruzi* (Nozaki *et al.*, 2001), Tb, *T. brucei* (accession no. AQ648388), Et, *Eimeria tenella* (accession no AI 757666), Tg, *Toxoplasma gondii* (accession no.BM004334). These sequences were aligned using Clustal W program (Thompson *et al.*, 1994) and boxshade. Black shades indicate identical amino acids, grey shades indicate conservative amino acid substitutions. The consensus sequences for the putative pyridoxal phosphate-binding domain (PXXSVKDR) and other motifs vital for CBS activity are indicated with (*)

Further, blast searches of the databases maintained by genome sequencing services showed that the LmajCBS possesses strong identity to HTGS from the protozoa *Eimeria tenella* (47%), *Toxoplasma gondii* (52%) and *T. brucei* (52%) (Table 6.1).

Table 6.1 Sequence identities of selected PLP-dependent enzymes of the β family involved in sulfur amino acid metabolism to *L. major* cystathionine β-synthase

Phylum	Organism	CBS Identity (%)	CS Identity (%)
Protozoa	*Leishmania tarentolae*	83	-
	Trypanosoma cruzi	66	-
	Trypanosoma brucei	52	-
	Leishmania major	100	31
	Eimeria tenella	47	-
	Toxoplasma gondii	52	-
	Trichomonas vaginalis	-	28
	Entamoeba histolytica	-	30
Nematodes	*Caenorhabditis elegans*	51	-
Vertebrates	*Rattus norvegicus*	51	-
	Homo sapiens	49	-
	Oryctolagus cuniculus	49	-
Bacteria	*Escherchia coli*	-	32
	Pseudomonas aeruginosa	36	-
	Mycobacterium tuberculosis	41	-
Plants	*Arabidopsis thialiana*	-	34
	Spinach	-	34
Yeast	*Pichia pastoris*	45	-
	Saccharomyces cerevisiae	42	-

Accession numbers are detailed in Figure 6.6

This suggests that the trypanosomatids and apicomplexans are like yeast and mammals in having a functional reverse trans-sulfuration pathway (Figure 6.1). However, a metabolic map constructed from genes identified, by homologous similarities, from the recently 'finished' genome sequence of *Plasmodium falciparum* suggests that this apicomplexan lacks enzymes involved in sulfur amino acid synthesis (Gardner *et al.*, 2002). LmajCBS is more similar to CBSs from yeast (42-

45%) and other eukaryotes (49-51%) than the putative CBSs from bacteria (36-41%) (Table 6.1).

Sequence alignment of LmajCBS with amino acid sequences of CBSs from other organisms shows that LmajCBS has all the residues known to be vital for activity of the enzyme. The PLP coenzyme domain, by sequence homology encompassing residues 16-308 (http://www.ncbi.nih.nlm.gov/) has the phosphate moiety (PGGSVKD) between residues 48-54 with the reactive lysine at the 53rd position (Figure 6.6). In HsCBS, the phosphate motif interacts with the phosphate binding loop which in LmajCBS comprises of gly^{189}, thr^{190}, gly^{191}, gly^{192} and thr^{193} and the residues try^{156} and gly^{236} which determine the substrate specificity of the protein. LmajCBS does not contain the oxidoreductase (CXXC) motif conserved in the mammalian forms (HsCBS) (Meier *et al.*, 2001), suggesting that the enzyme might not be redox sensitive (Figure 6.6).

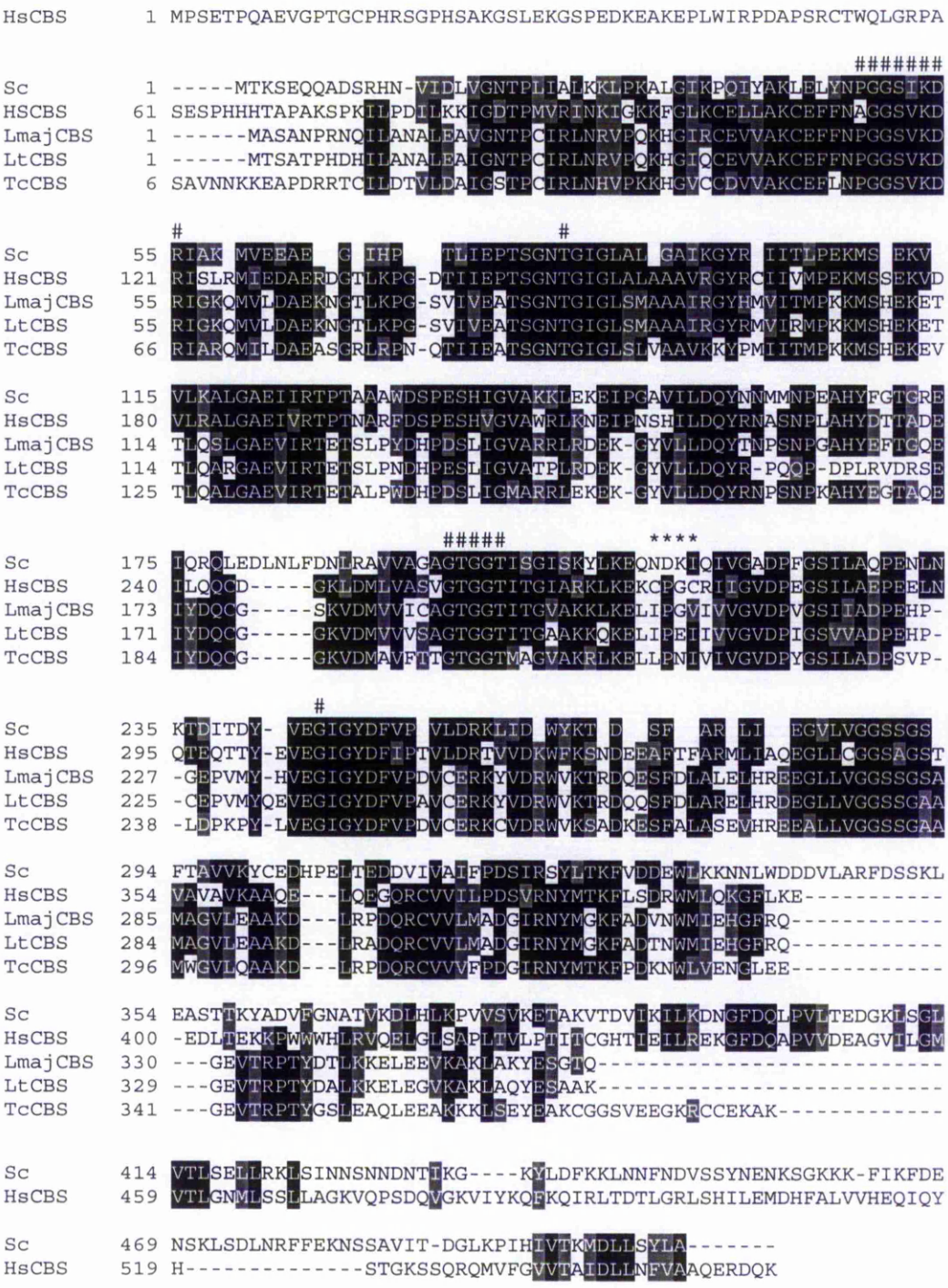
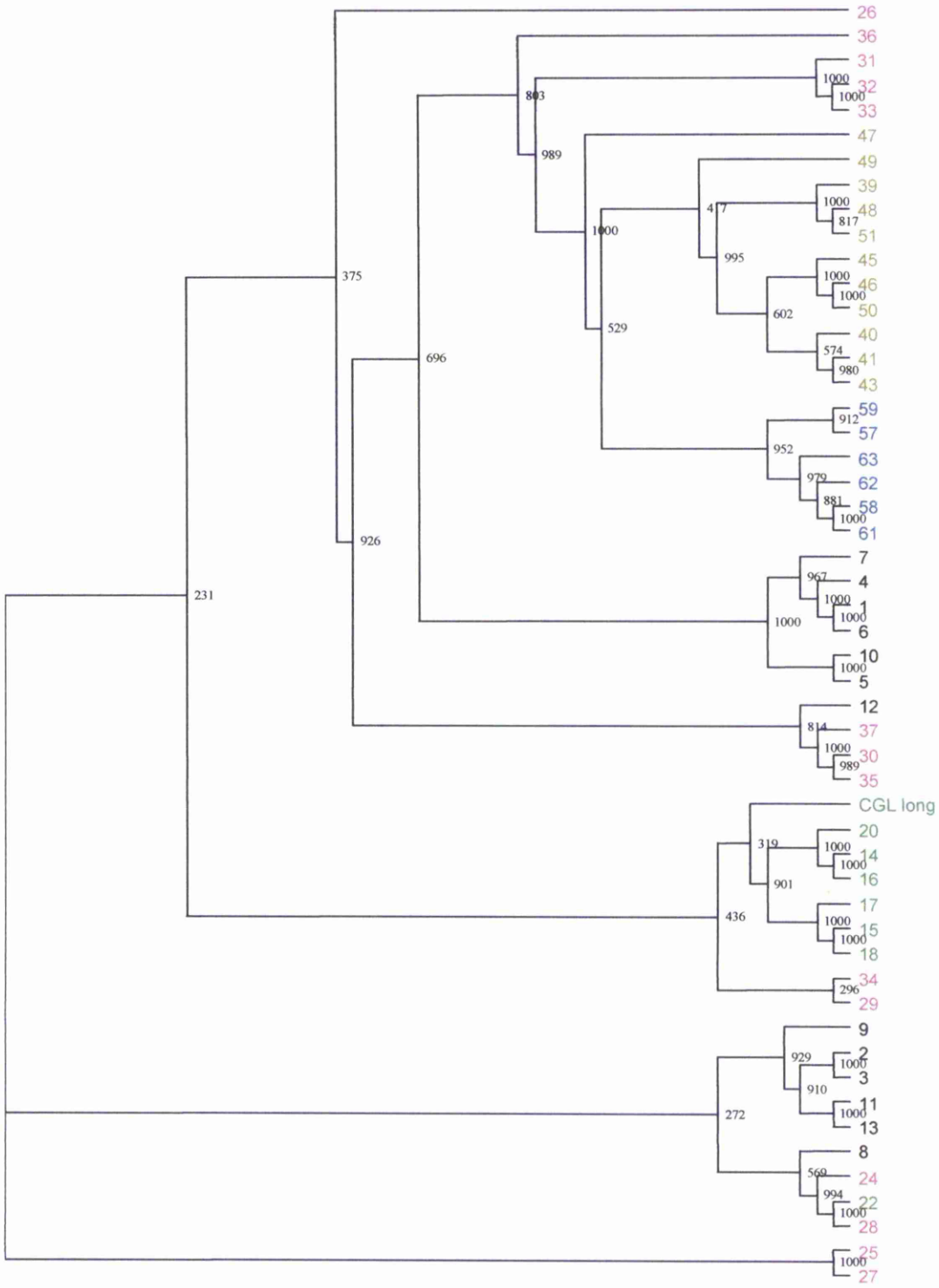


Figure 6.6 Multiple alignment of deduced amino acid sequences of CBS from *L. major* and other representative organisms. Sequences as follows HsCBS, *Homo sapiens*, ScCBS, *Saccharomyces cerevisiae* (Accession no. X72922), LmajCBS *L. major* (this study), LtCBS, *Leishmania tarentolae* (GenBank accession no. AAF71541.1 GI:8037933), TcCBS, *T. cruzi* (Nozaki *et al.*, 2001). These sequences were aligned using Clustal W program (Thompson *et al.*, 1994) and boxshade. Black shades indicate identical amino acids, grey shades indicate conserved amino acid substitutions. The consensus sequences for the putative pyridoxal phosphate-binding domain (PXXSVKDR) and other motifs vital for CBS activity are indicated with (#) while the oxidoreductase motif of HsCBS is highlighted with (*).

Phylogenetic analysis of CBS (Figure 6.7) produced clades representative of its domain architecture; mammalian heme and AdoMet-dependent (39, 48, 51), fungal heme-independent and AdoMet-dependent (41, 43), the trypanosomatids heme and AdoMet independent (45, 46, 50) proteins) of CBS proteins suggesting independent evolutionary events from a common ancestor. The *C. elegans* CBS (40) compared with other HsCBS has an unusually long N-terminal extension (NTE) of 140 amino acid sequences when compare with the mammalian CBS and it branches close to the yeast CBS. The long NTE might suggest the presence of a heme domain, but characterization of native CBS from nematodes has shown it to be heme-independent (Papodopoulus *et al*, 1996). Thus the NTE must have some other function. Two putative bacterial CBS from *M. tuberculosis* (47) and *P.aeruginosa* (49) were distinct and occurred as independent groups in the tree.


26
36
31
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881
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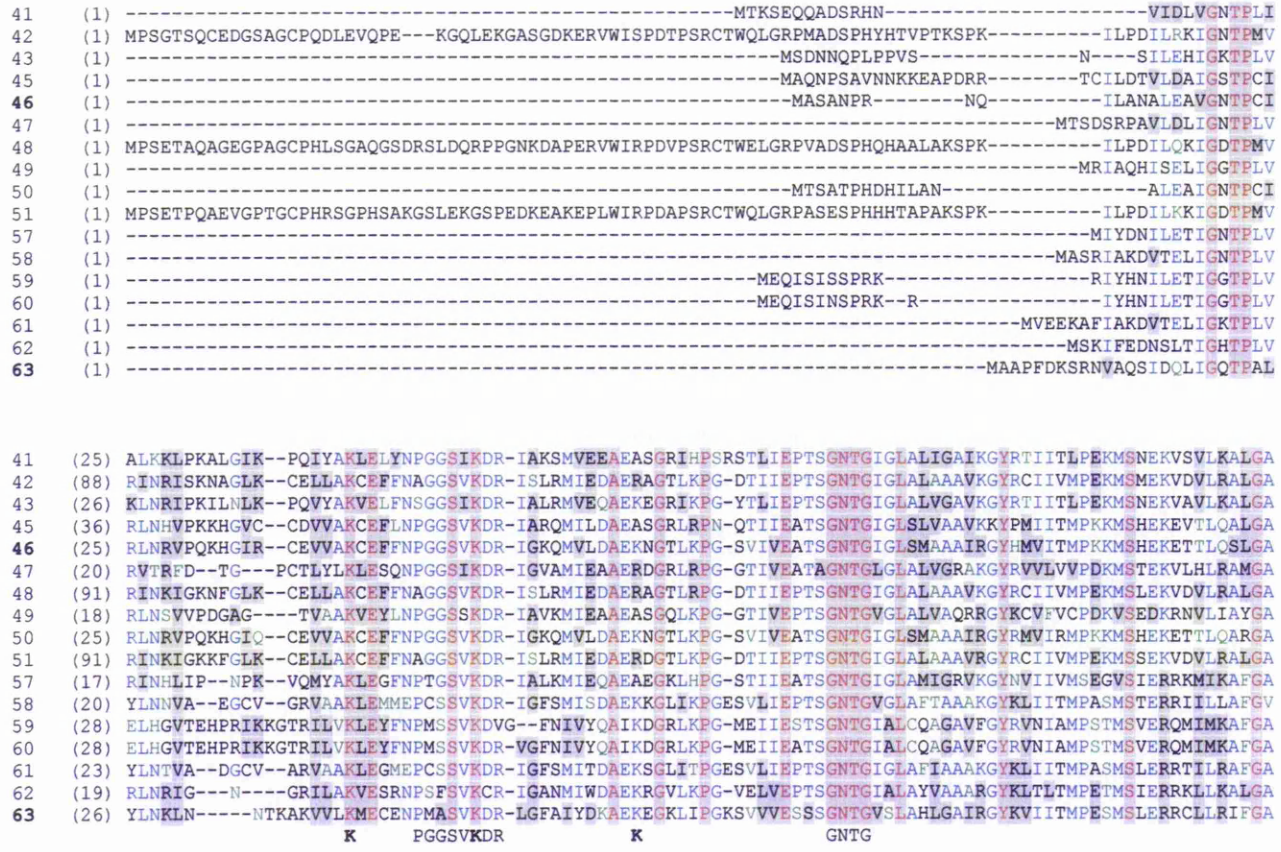
1000
61
375
926
7
967
4
1000
1
1000
6
231
1000
10
1000
5
12
814
37
1000
30
989
35
CGL long
20
319
1000
14
1000
16
901
17
436
1000
15
1000
18
34
296
29
9
929
2
1000
3
910
11
272
1000
13
8
569
24
994
22
1000
28
25
1000
27

Figure 6.7 Phylogenetic analysis of PLP-dependent enzyme sequences involved in the trans-sulfuration pathways. The phylogenetic tree was plotted using treeview (Page, 1996). CBL (black) were from: 1. METC_ECOLI-*E. coli* (accession no. GI 1207031 P06721), 2. *Arabidopsis thaliana* (accession no.AAA99176 GI:704397), 3. *Solanium tuberosum* (accession no. AAF74980 GI:8439539), 4. MET_HAEIN-*H. influenzae* (accession no. P44527), 5. METC_BORAV *Bordetella avium* (accession no.Q07703), 6. *Salmonella typhimurium* (accession no.ND462076.1 GI:16766461), 7. METC_YEAST *Saccharomyces cerevisiae* (accession no. P43623), 8.*Bacillus subtilis* (yjcJ) (accession no. Z99110), 9. *L. major* (this study), 10. *Bordetella avium* (Accession no. AAA22978 GI:304266), 11. *Neurospora crassa* (accession no. AF401237), 12. *Thermoplasma volcanism* (accession no. NP111526 GI:13541838), 13. *Aspergillus nidulans* (accession no.U28383). CGL (green) were from:14. CYS3_YEAST *Saccharomyces cerevisiae* (accession no. P31373), 15. CGL_HU_MAN-*Homo sapiens* (accession no.P46807), 16. *Saccharomyces cerevisiae* (accession no. P31376), 17. *C. elegans* (accession no. P55216), 18. CGL_RAT-*Rattus norvegicus* (accession no. P18757), 19. *E. herbicola* (accession no. S52983), 20. *Neurospora crassa* (accession no. AAK94040, GI:14024541), 22. *Bacillus subtilis* (Accession no.U93874), 23. *L. major* (This study), CGS (pink) were from: 24. METB_HELPY-*H. pylori* (accession no. P56069), 25. METB_MYCLE-*Mycobacterium leprae* (accession no. P46807), 26. METB_ECOLI *E. coli* (accession no. AAB0371 GI:305042), 27. METB_HAEIN-*H. influenzae* (Accession no. P44502), 28. *Bacillus subtillus* (accession no. NP 39063 GI:16079779), 29. *Mycobacterium tuberculosis* (accession no. AL021897), 30. METB_ARTH-*Arabidopsis thaliana* (accession no. BAA24699 GI:2852454), 31. MET7_YEAST- *Saccharomyces cerevisiae* (accession no. P47164), 32. MET7_NEUCR *N. crassa* (accession no. P38675), 33. *S. pombe* (accession no. AL031349), 34. METB_HERAV *H. auranticicus* (accession no. P24601), 35. *M. crystallinum* (accession no. AF069317), 36. YARZ_YEAST (Accession no. P47164), 37. Zea mays (Accession no.). CBS (yellow) were from: 39. *Rattus norvegicus* (Accession no. NP036654 GI:6978611), 40. *Caenorhabditis elegance* (accession no. NP 509686 GI:17570589), 41. *Saccharomyces cercevisiae* (accession no. BAA03953, GI:416161), 43. *Pichia pastoris* (accession no. BAB78511, GI:17298074), 45. *Trypanosoma cruzi* (accession no. AAK16403, GI:13241626), 46. *Leishmania major* (this study), 47. *Pseudomonas aeruginosa* (accession no. AAG03788 GI:9946253), 48. *Oryctolagus cuniculus* (accession no. AAF71541, GI8037933), 49. *Mycobacterium tuberculosis* (accession no.), 50. *Leishmania tarentolae* (accession no. AAF71541, GI:8037933), 51. *Homo sapiens* (accession no. AAH10242, GI:16307396) CS (blue) were from: 57. *Trichomonas vaginalis* (Goodall, 2001), 58. *Arabidopsis thaliana* (accession no.AAM91285 GI:22136414), 59. *Entamoeba histolytica* I (accession no. BAA21916, GI:234694), 60. *Entamoeba histolytica* II (accession no. BAA22144, GI:2366771), 61. Spinach (accession no. S35094, GI:421796), 62. *E. coli* (accession no.AAC75467, GI:1788754), 63. *Leishmania major* (this study).

6.5.2 Comparison between cystathionine β-synthase and cysteine synthase

It has been speculated that CBS and CS are evolutionary related (Alexander *et al.*, 1994) and there are reports of CBS proteins showing vestigial CS activity (Papodopoulus *et al*, 1996; Nozaki *et al.*, 2001). Despite, the higher identity of LmajCBS with other CBS amino acid sequences (36-88%), it also showed significant identity with CS amino acid sequences 31%, 32%, 34%, 28%, and 31% identity with LmajCS, *E coli*, plant, *T. vaginalis* and *E. histolytica* CSs, respectively (Table 6.1).

Multiple alignments showed that similarity between their sequences was primarily confined to the PLP-binding domain (Figure 6.8). The blocks of identity between the two enzymes were confined to the motifs known to be vital for activity of CS activity from *Salmonella* and Spinach (Vaara, 1992) (Figure 6.8). They include the PLP moiety (PXXSXKDR) between positions 48-55 (LmajCBS numbering) and three lysines at positions 42, 53, and 202, (but not the lysine at position 67) in the LmajCBS (Figure 6.8). The N- and the C- terminal non-catalytic regions showed virtually no similarity (Figure 6.8). Despite the high identity and similar characteristic between the two groups of β family PLP- dependent enzymes, phylogenetic analysis suggest that they evolved independently from a common ancestor (Figure 6.7).



41 (122) EIIRTPTAAAWDSPESHIGVAKKLEKEIPGAVILDQYNNMMNPEAHYFGTGREIQRQLEDLNLFDNLRAVVAGAGTGGTISGISKYLKEQNDKIQIVGAD
42 (184) EIVRTPTNARFDSPESHVGVAWRLKNEIPNSHILDQYRNASNPLAHYDDTAEEILQQCDG-----KVDMLVASAGTGGTITGIARKLKEKCPGCKIIGVD
43 (122) EIIRTPTEAAWDSPESHIGVARRLEKEIPNAVILDQYGNVNNPDAHFYTTGAEIWDQTNG-----KVTHGVAGAGTGGTITGIAKFLKSKNPNIQIIGAD
45 (132) EVIRTETALPWDHPDSLIGMARRLEKEKGY-VLLDQYRNPSNPKAHYEGTAQEIYDQCGG-----KVDMAVFTTGTGGTMAGVAKRLKELLPNIVIVGVD
46 (121) EVIRTETSLPYDHPDSLIGVARRLRDEKGY-VLLDQYTNPSNPGAHYEFTGQEIYDQCGS-----KVDMVVICAGTGGTITGVAKKLKELIPGVIVVGVD
47 (113) EVHITRSDVGKGHPEYYQDVAARLAQDIPGAFFADQFNNPANPLAHECGTGPELWAQTGHD-----LDAIVVGVGSSGTLTGLTRFFQKVQPELEMVLAD
48 (187) EIVRTPTNARFDSPESHVGVAWRLKQEIPNSHILDQYRNASNPLAHYDTTAEEILQQCDG-----KLDMLVASAGTGGTITGIARKLKEKCPGCQIIGVD
49 (112) EVVVCPTAVPPHDPASYYSVSDRLVRDIDGAWKPDQYANPEGPASHYVTTGPEIWADTEG-----KVTHFVAGIGTGGTITGAGRYLKEVSGGRVRIVGA
50 (121) EVIRTETSLPNDHPESLIGVATPLRDEKGY-VLLDQYRPQQPDPLRVDR--SEIYDQCGG-----KVDMVVVSAGTGGTITGAAKKQKELIPEIIVVGVD
51 (187) EIVRTPTNARFDSPESHVGVAWRLKNEIPNSHILDQYRNASNPLAHYDTTADEILQQCDG-----KLDMLVASVGTGGTITGIARKLKEKCPGCRIIGVD
57 (111) EIILTDKKLGTDGAIR--KVAELVKENPGKYFNPNQFSNEYNKIAHYKTTAEEIWAQTKG-----TVTHFVAAVGTSGTLMGVGKNLREKNPEIK-----
58 (115) ELVLTDPAKGMKGAIA---KAEEILAKTPNGYMLQQFENPANPKIHYETTGPEIWKGTGG-----KIDGFVSGIGTGGTITGAGKYLKEQNANVKLYG--
59 (125) ELILTEGKKGMPGAIE--EVNKMIKENPGKYFVANQFGNPDNTAAHHYT-ANEIWEDTDG-----EVDIVVSAVGTSGTVIGVAEKLKEKKKGIKIIAVE
60 (126) ELILTEGKKGMPGAIE--EVNKMIKENPGKYFVANQFGNPDNTAAHHYT-ANEIWEDTDG-----EVDIVVSAVGTSGTVIGVAEKLKEKKKGIKIIAVE
61 (118) ELILTDPAKGMKGAVQ---KAEEIRDKTPN-YILQQFENPANPKVHYETTGPEIWKGTGG-----KIDIFVSGIGTGGTITGAGKYLKEQNPDVKLIG--
62 (110) NLVLTEGAKGMKGAIQ--KAEEIVASNPEKYLLLQQFSNPANPEIHEKTTGPEIWEDTDG-----QVDVFIAGVGTGGTLTGVSRYIKGTKGKTDLISVA
63 (120) EVILTPAALGMKGAVT---MAKKIVTANPNAVLADQFATKYNALIHEETTGPEIWEQTNH-----NVDCFIAGVGTGGTLTGVARALKKMGSHARIVAVE
PSNP AHYET GVGTGGT K
41 (222) PFGSILAQPENLNKTDITD---YKVEGIGYDFVPQVLDRKLIDVWYKTDDKPSFKYARQLISNEGVLVGGSSGSTFTAVVKYCEDHPELTEDDVIVAIFP
42 (279) PEGSILAEPEELNQTEQTA---YEVEGIGYDFIPTVLDRAVVDRWFKSNDDDSFAFARMLISQEGLLCGGSSGSAMAVAVKAAQEL-K--EGQRCVVILP
43 (217) PHGSILALPESLNTSN-EQ---YKVEGIGYDFIPEVLDREIVDTWYKTDDKDFFKLARQLISEEGILVGGSSGSALSAAVKIINDA-KLDESAVVVVCP
45 (226) PYG--SILADPSVPLDPKP---YLVEGIGYDFVPDVCERKCVDRWVKSADKESFALASEVHREEALLVGGSSGAAMWGVLQAAKDL-R--PDQRCVVVFP
46 (215) PVG--SIIADPEHPGEPVM---YHVEGIGYDFVPDVCERKYVDRWVKTRDQESFDLALELHREEGLLVGGSSGSAMAGVLEAAKDL-R--PDQRCVVLMA
47 (208) PEG--SIMAEYSRSGTLGTPGSWAVEGIGEDFVPAIADLSSVRHAYSISDEESFAMARELLRVEGIPGGSSTGTLLAAALRFCREQ---KEPKRVVSFVC
48 (282) PEGSILAEPEELNQTEVTA---YEVEGIGYDFIPTVLDRTVVDRWFKSTDKEAFAFARMLIAQEGLLCGGSAGSAVAVAVKAAQEL-Q--EGQRCVVILP
49 (207) DP--------EGSVYSGGAGRPYLVEGVGEDFWPAAYDPSVPDEIIAVSDSDSFDMTRRLAREEAMLVGGSCGMAVVAALKVAEEAG---PDALIVVLLP
50 (213) PIG-SVVADPEHPCEPVMY---QEVEGIGYDFVPAVCERKYVDRWVKTRDQQSFDLARELHRDEGLLVGGSSGAAMAGVLEAAKDL-R--ADQRCVVLMA
51 (282) PEGSILAEPEELNQTEQTT---YEVEGIGYDFIPTVLDRTVVDKWFKSNDEEAFTFARMLIAQEGLLCGGSAGSTVAVAVKAAQEL-Q--EGQRCVVILP
57 (199) ----------IIEAQPTKGHYIQGLKSMEEAIVPAIYQADKIDEHILIESEEAFAKAREIVAQEGIFIGMSSGAAMLAAQKLAEKI----DSGVIVVLFA
58 (205) ------VEPVESAILSGGKPGPHKIQGIGAGFIPSVLNVDLIDEVVQVSSDESIDMARQLALKEGLLVGISSGAAAAAAIKLAQRP--ENAGKLFVAIFP
59 (217) PE--------ESAVLEGKAKGPHGIQGIGAGFIPDIYKKEFVDEIIPIKTQDAWKMARAVVKYDGIMCGMSSGAAILAGLKEAEKP--ENEGKTIVIIVP
60 (218) PE--------ESAVLEGKAKGPHGIQGIGAGFIPDIYKKEFVDEIIPIKTQDAWKMARAVVKYDGIMCGMSSGAAILAGLKEAEKP--ENEGKTIVIIVP
61 (207) ------LEPVESAVLSGGKPGPHKIQGLGAGFIPGVLDVNIIDEVVQISSEESIEMAKLLALKEGLLVGISSGAAAAAAIKVAKRP--ENAGKLIVAVFP
62 (203) VEPTDSPVIAQALAGEEIKPGPHKIQGIGAGFIPANLDLKLVDKVIGITNEEAISTARRLMEEEGILAGISSGAAVAAALKLQEDES--FTNKNIVVILP
63 (212) PM--------ESPVLSGGKPGAHKIQGIGPGFVPDVLDRSLIDEVFCVAGDDAIETALKLTRSDGVFCGFSGGANVYAALKIAERP--EMEGKTIVTIIP

Figure 6.8. Multiple alignment of deduced amino acid sequences of LmajCBS with CBS and CS from other representative organisms CBS were from: 41. *Saccharomyces cerevisiae* (accession no. BAA03953, GI:416161), 43. *Pichia pastoris* (accession no. BAB78511, GI:17298074), 45. *Trypanosoma. cruzi* (accession no. AAK16403, GI:13241626), 46. *Leishmania major* (this study), 47. *Pseudomonas aeruginosa* (accession no. AAG03788 GI:9946253), 48. *Oryctolagus cuniculus* (accession no.AAF71541, GI8037933), 49. *Mycobacterium tuberculosis* (accession no.), 50. *Leishmania tarentolae* (accession no. AAF71541, GI:8037933), 51. *Homo sapiens* (accession no. AAH10242, GI:16307396) CS were from: 57. *Trichomonas vaginalis* (Goodall, 2001), 58. *Arabidopsis thaliana* (accession no.AAM91285 GI:22136414), 59. *Entamoeba histolytica* I (accession no. BAA21916, GI:234694), 60. *Entamoeba histolytica* II (accession no. BAA22144, GI:2366771), 61. Spinach (accession no. S35094, GI:421796), 62. *E. coli* (accession no.AAC75467, GI:1788754), 63. *Leishmania major* (this study). These sequences were aligned using AlignX program of VecorNTI (Informax). Red prints on grey shades indicate identical amino acids, blue and green prints indicate conservative amino acid substitutions. The putative pyridoxal phosphate-binding motif (PXXSVKDR) and three of four lysine residues shown to be important for the catalytic activity of spinach CS-A by site directed mutagenesis (Vaara, 1992) are marked in bold. Motifs known to be involved CS activity are also shown. The leishmanial proteins 46 and 63 are highlighted in bold.

6.6 Genomic Organisation of the LmajCBS

The copy number of the leishmanial CBS was assessed by digesting genomic DNA with five restriction enzymes and analysing it by Southern blotting using the CBS ORF as probe (Figure 6.9). The enzymes used that do not cut the *L. major* gene itself, *BamH*I, *Eco*RI, *Nde*I, *Pvu*I, *Sac*I *Xba*I, and *Apa*I, resulted in a single major hybridising DNA fragment, while *Bsa*1, *Sac*II, *Puv*II, and *Sal*I which cut the ORF more than once, resulted in multiple bands (Figure 6.9). The hybridisation patterns obtained indicate the LmajCBS gene is probably single copy. Information on genomic environment apparent from the data in the genome database for *L. major* show that the gene does not occur in a tandem array (Data not shown). This, taken together with the Southern data, confirm that LmajCBS is single copy, which is in constrast with the six genes reported for the trypanosomatid, *T. cruzi* (Nozaki *et al.*, 2001).

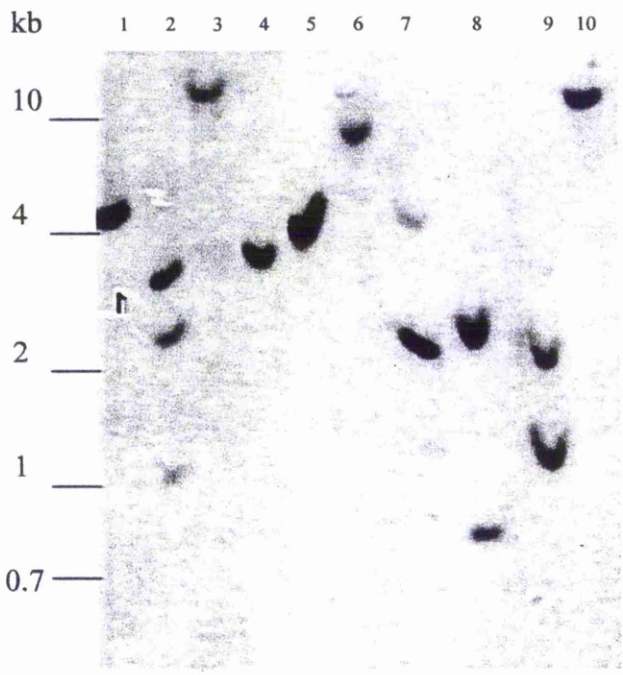


Figure 6.9 Genomic organization of leishmanial CBS. 5 µg of genomic DNA from *L. major* was digested with a variety of restriction endonucleases, separated by agarose gel electrophoresis, transferred onto a nylon membrane, and then hybridised with [^{32}P] dATP-labeled *LmajCBS* probe corresponding to the entire ORFs. The restriction endonucleases used were *BamH*I (lane 1), *Bsa*I (lane2), *Eco*RI (lane 3), *Nde*I (lane 4), *Pvu*I (lane5), *Sac*I (lane 6), *Sac*II (lane 7), *Sal*I (lane 8), *Pvu*II *and Nde*I (lane 9), and *Xba*I and *Apa*I (lane 10). The sizes of DNA standards are indicated in kilobase pairs.

6.7.1 Expression profile and biochemical characterization of recombinant CBS

Expression of LmajCBS in *E. coli* strain BL21(DE3) under control of a T7 promoter, at standard growth conditions of 37°C and 1 mM IPTG, resulted in the formation of insoluble inclusion bodies. However, conditions of 15°C and 0.5 mM IPTG significantly increased the degree of solubility of LmajCBS (Figure 6.2b). Limited characterization was carried out on purified recombinant LmajCBS (rLmajCBS). The enzyme was stable in 50 mM Tris, pH 8.0, containing 25 μM PLP for 4 weeks with and without glycerol at a protein concentration of 5 mg/ml judged by CBS activity. The 'CBS' assay was optimized to ensure saturating concentration of substrates for the incubation times and protein concentrations used. Optimum pH and temperature were not determined. Instead, as the pH and temperature optima reported in the literature ranged from 7.5-9 and 33-42°C respectively, the conditions were set at pH 8.0 and 37°C. The reaction progressed linearly under the standard assay conditions for about 10 min, while doubling the amount of protein in the assay mix approximately doubled the rate. Thus all activity data were subsequently derived in a 5 min assay in Tris-HCl, pH 8.0 at 37°C.

rLmajCBS showed multiple activities (Section 2.10). rLmajCBS showed CBS activity of 30.5 ± 0.2 $\mu mol.min^{-1}mg.protein^{-1}$. rLmajCBS also fromed cystathionine with cysteine and either homocysteine (14.0 ± 1.2 $\mu mol.min^{-1}mg.protein^{-1}$) or serine (12.4 ± 1.0 $\mu mol.min^{-1}mg.protein^{-1}$). rLmajCBS also show serine sulfurhydrase (SS), activity which forms cysteine from serine and sodium sulfide. The SS activity was 14.6 ± 0.9 $\mu mol.min^{-1}mg.protein^{-1}$. rLmajCBS showed a remarkably high CS activity (22.5 ± 1.7 $\mu mol.min^{-1}mg.protein^{-1}$). rLmajCBS also catalysed the activated sulfurhydrase reaction in which of hydrogen sulfide is generated from cysteine and β-

mercaptoethanol (9.4 ± 0.9 μmol.min^{-1}mg.protein $^{-1}$) with a K_m of 0.74 ± 0.5 mM for cysteine and a K_m of 4.4 ± 1.9 mM for β-mercaptoethanol. This hydrogen sulfide forming activity was relatively inhibited by 10mM cysteine.

6.7.2 Expression of CBS in L. major

CBS activity was found in extracts of wild type parasites (0.15 μmol.min^{-1}.mg.protein^{-1}). The transfected *Leishmania* line overexpressing the LmajCBS gene showed a 4-fold increase in CBS activity (0.6 μmol.min^{-1}.mg.protein^{-1}) relative to the control pGL607 (0.14 μmol.min^{-1}.mg.protein^{-1}) over-expressing just the antibiotic resistant marker. The expression of leishmanial CBS was also analysed using polyclonal antibodies raised against rLmajCBS. The rabbit antiserum was strongly reactive to purified rLmajCBS and also recognised a single 39 kDa protein in the soluble extract of wildtype promastigotes (Figure 6.10). Western analysis of the cell extracts of promastigotes over-expressing CBS showed an increase in protein expression above that of the control transfected with the empty vector (Figure 6.10).

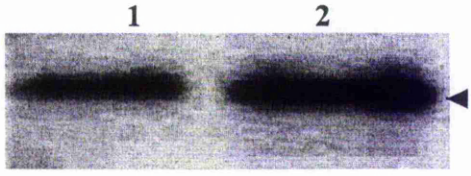


Figure 6.10. Western blot analysis of L. major promastigotes over expression of leishmanial CBS gene. *L. major* promastigotes with the vector expressing just the blasticiin resistant gene (lane 1); *L. major* promastigotes over-expressing the LmajCBS gene (lane 2).Just a single band was detected, at ~39 kDa (arrowed).

6.7.3 Over-expression of LmajCBS confers resistance of promastigotes to oxidative Stress

To help define the role of LmajCBS in oxidative stress, transgenic parasites confirmed to be over-expressing the LmajCBS gene at a higher level relative to the control, was exposed to the oxidants (hydrogen peroxide, t-butyl hydroperoxide and cumene hydroperoxide) at the concentrations detailed in Chapter 4. The cell densities of both the over-expressing cell line and their control over-expressing the antibiotic resistant gene were estimated initially on addition of the stressor and at the end of a 6 days incubation period. The LmajCBS over-expressing cell line pGL607CBS was resistant to the concentration of oxidants used relative to the control cells. At day 6 after the addition of the stressors, the relative cell dentity of CBS over-producing cell line estimated by the acid phosphatase assay was 0.65 ± 0.34; 0.52 ± 0.91; 0.53 ± 1.12 for hydrogen peroxide, t-butyl hydroperoxide and cumene hydroperoxide respectively. The cell density of the control cells under the same conditions and time points were 0.32 ± 0.33, 0.35 ± 0.56 and 0.32 ± 0.29 for hydrogen peroxide, t-butyl hydroperoxide and cumene hydroperoxide respectively. The starting cell density of 0.31 was used for both transgenic and control cells.

6.8 Identification of L. major *the PLP-γ family genes involved in sulfur amino acid metabolism*

Genes potentially involved in methionine and cysteine biosynthesis in *L. major* were identified from its genome sequence database using the strategy described in section 6.4. HTGS fragments with similarity to for the PLPγ genes, cystathionine γ-lyase (CGL) and cystathionine β-lyase (CBL) were analyzed by blastx. This showed that

Table 6.2 ORFs of genes identified by BLAST searches of the *L. major* genome potentially involved in sulfur amino acid metabolism

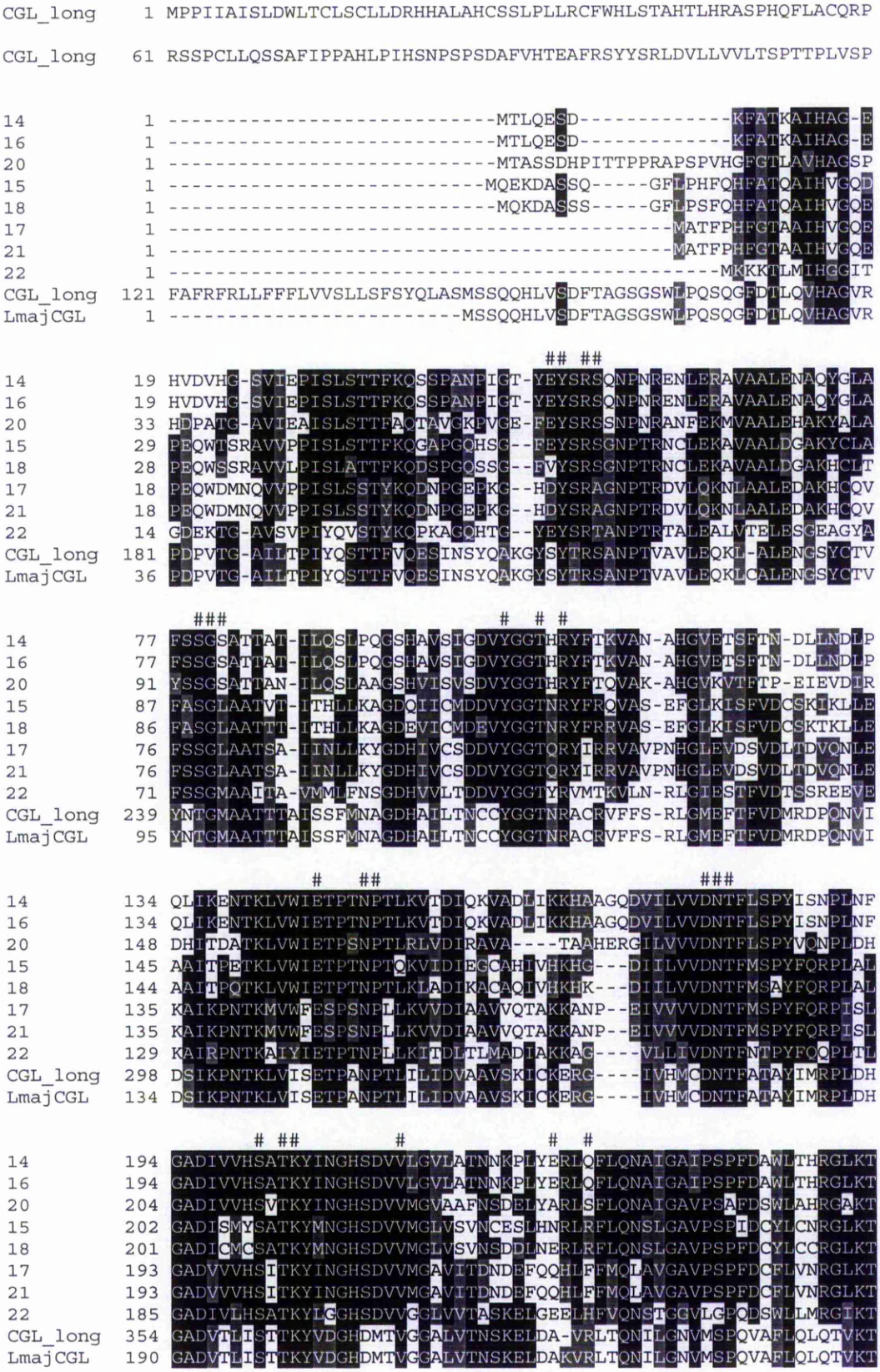
Family	Genes name	No. of nucleotides (kb)	Predicted amino acid residues	Est. molecular weight (kDa)	Chromosome no.	*Genebank Accession no*	Pathway
PLPγ	Cystathionine β-lyase	1518	506	55.4	32	AL359773	TS
	Cystathionine γ-synthase	-	-	-	-	-	
	Cystathionine γ-lyase	1656	552	60.6	16	AC019154	RTS
PLPβ	Cystathionine β-synthase	1120	359	39.2	16	AL499619	

RTS- reverse trans-sulfuration **TS-trans-sulfuration**

they encode proteins possessing strong identities to their respective PLPγ -dependent proteins from bacteria, plants and mammals (Table 6.2).

6.8.1 Cystathionine γ-lyase or γ-cystathionase (CGL: EC 4.4.1.1) is the second enzyme in the reverse trans-sulfuration pathway. It catalyses the cleavage of *L*-cystathionine to *L*-cysteine, α-ketobutyrate and ammonia. The putative LmajCGL gene, consisting of 1656 nucleotides encoding a protein of 552 amino acids with a predicted molecular mass of 60.6 kDa was identified on chromosome 16 (Table 6.2).

Sequence alignment of the deduced amino acid sequence of LmajCGL with known CGLs shows homology throughout its entire length to CGLs from other species. The sequence predicted an NTE for the *L. major* protein, but the presence of another methionine start downstream which aligns well with other CGL casts doubts on the putative start codon (Figure 6.11). Such uncertainty can, however, be resolved by 5'RACE PCR analysis. There is no structural information available for CGL but it shares a high homology and identity to CBL whose structure has been solved. Sequence alignment analysis of CBL and CGL collated with results from inhibition (Steegborn *et al.*, 1994) and x-ray diffraction studies (Clausen *et al.*, 1996) suggest that both enzymes the same overall fold and mode of action (Alexander *et al.*, 1994; Clausen *et al.*, 1996; 1998; Steeegborg, 1999). Based on the CBL structure from *E. coli*, LmajCGL has the PLP moiety and the reactive lysine (278^{th}) associated with activity conserved (Figure 6.11). The degree of identity between the amino acid sequences of LmajCGL and LmajCBL is 27%.



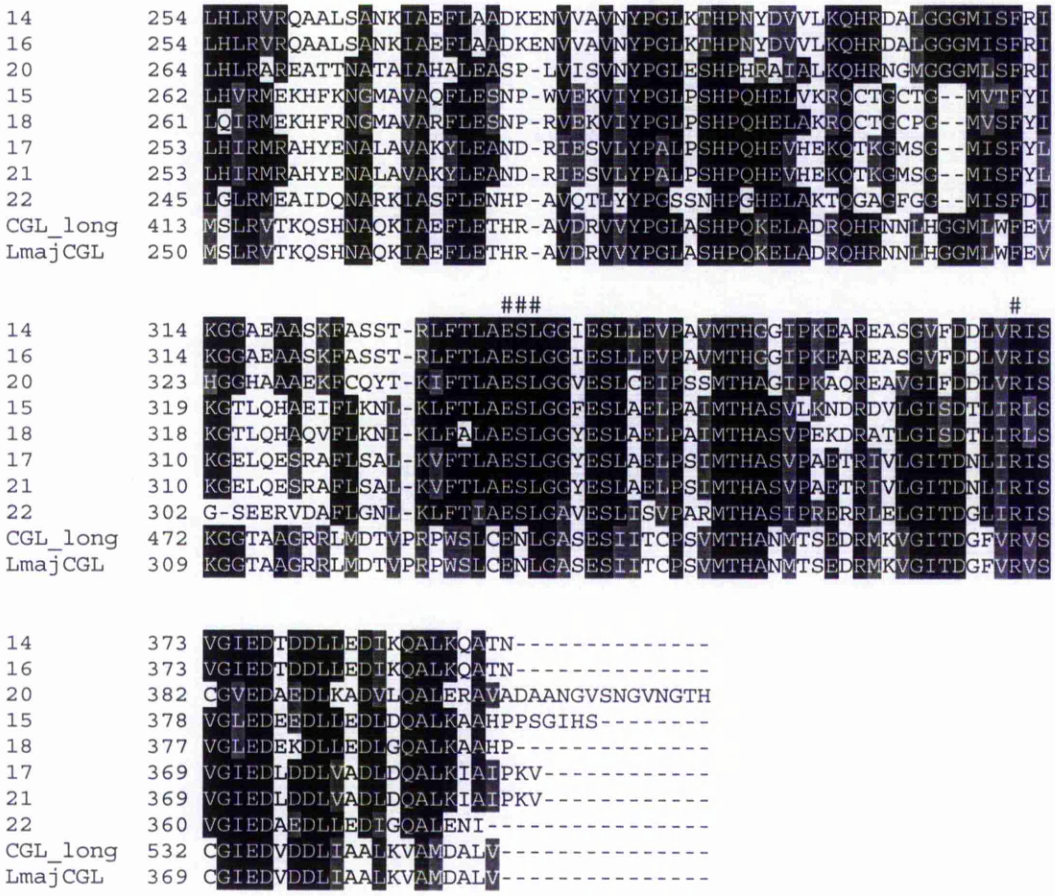


Figure 6.11. Multiple alignment of deduced amino acid sequences of CGL from *L. major* and from other representative organisms. Sequences as follows: CGL were from: 14. CYS3_YEAST *Saccharomyces cerevisiae* (accession no. P31373), 15. CGL_HU_MAN-*Homo sapiens* (accession no. P46807), 16. *Saccharomyces cerevisiae* (Accession no. P31376), 17. *Caenorhabditis elegans* (Accession no. P55216), 18. CGL_RAT-*Rattus norvegicus* (Accession no. P18757), 19. *Erwinia herbicola* (accession no. S52983), 20. *Neurospora crassa* (accession no. AAK94040, GI:14024541), 22.*Bacillus. subtilis* (accession no.U93874), LmajCGL_long. *L. major* (this study) LmajCGL. *L. major* (this study). LmajCGL_long and LmajCGL are the deduced amino acid sequences with and without the unusual N-terminal extension (NTE). These sequences were aligned using the ClustalW and boxshade programs. Black shades indicate identical amino acids, grey shades indicate conserved amino acid substitutions. The putative PLP binding motif is identified between position 197 and 201, with lysine 199 (#) as the site of co-factor binding via a Schiff base in LmajCGL. Motifs important for activity of CGL from *E. coli* are marked (Vaara, 1992).

The amino acid sequence predicted for LmajCGL without the NTE showed significant identity and conserved residues to γ- family of PLP-dependent enzymes (Alexander *et*

al., 1994) throughout its entire length. It has between 26-38%, 14-37%, 17-34% and 31-33% identities with CGL, CGS, CBL and methionine γ-lyase (MGL), respectively, (data not shown) from a variety of organisms (Table 6.3).

Table 6.3 Sequence identities of selected PLP-dependent enzymes of the γ family involved in sulfur amino acid metabolism to *L. major* cystathionine gamma-lyase. Number in parenthesis are arbituary numbers assigned to each protein analyzed in this chapter

Phylum	**Organism**	% Identity with LmajCGL		
		CGL	CBL	CGS
Bacteria	*Escherichia coli*	-	23 (1)	29 (26)
	Haemophilus influenzae	-	21 (4)	31 (27)
	Thermoplasma volcanium	-	22 (12)	-
	Erwinia. herbicola	34 (19)	-	-
	Helicobacter pylori			33 (24)
	Mycobacterium leprae			33 (25)
	Mycobacterium tuberculosis	-	-	36 (29)
	Halobacterium aranticicus	-	-	36 (34)
	Bordetella avium	-	25 (10)	-
	Salmonella typhimurium	-	22 (6)	-
	Bacillus subtilus	38 (22)	34 (8)	37 (28)
Fungi	*Saccharomyces cerevisiae*	35 (16)	17 (7)	20 (36)
	Neurospora crassa	35 (20)	25 (11)	14 (32)
	Aspergillus nidulans	-	26 (13)	-
	Saccharomyces cerevisiae	35 (14)	-	-
	Saccharomyces pombe	-	-	13 (33)
Mammals	*Homo sapiens*	34 (15)	-	-
	Rattus norvegicus	35 (18)	-	-
Nenatodes	*Caenorhabditis elegans*	34 (17)	-	-
Plants	*Arabidopsis thaliana*	-	31 (2)	31 (30)
	Solanum tuberosum	-	30 (3)	-
	Mesembryanthemum crystallinum	-	-	29 (35)
	Zea mays	-	-	31 (37)
Protozoa	*Leishmania major*	100 (9)	26 (23)	-

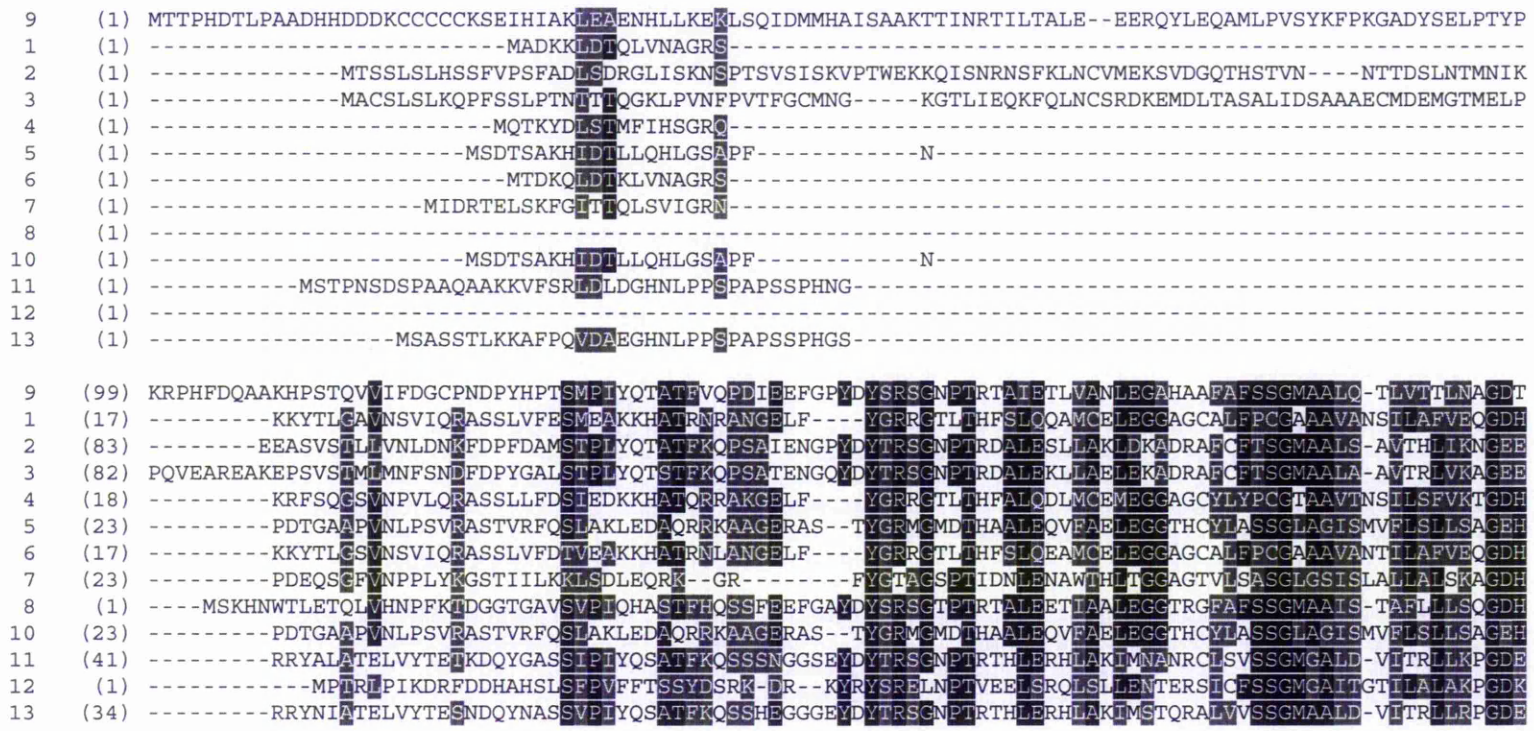
6.8.2 Cystathionine β-lyase or β-cystathionase (CBL: EC 4.4.1.8) catalyzes the cleavage of *L*-cystathionine to *L*-homocysteine, pyruvate and ammonia. *LmajCBL* is located on chromosome 32. The full length of LmajCBL has an ORF of 1518 nucleotides encoding a protein of 506 amino acids with a predicted molecular weight of 55.4 kDa (Table 6.2). CGL, CGS and CBL are involved in the cysteine-methionine biosynthesis pathways and belong to the same class of PLP-dependent enzymes that catalyses γ-replacement reactions of C_4 amino acid and β or γ cleavage of cysteine (Alexander *et al.*, 1994).

The deduced amino acid sequence of LmajCBL has significant identity to CBLs isolated and characterized from *A. thaliana* (36%, Breitinger *et al.*, 2001), *S. tuberosum* (39%, Maimann *et al.*, 2000) and *E. coli* (22%, Clausen *et al.*, 1996) protein (Table 6.4). It also showed relatively high identity to PLP-dependent enzymes that catalyse γ-replacement reactions (Table 6.4).

Table 6.4 Sequence identities of selected PLP-dependent enzymes of the γ family involved in sulfur amino acid metabolism to *L. major* cystathionine beta-lyase (legend as in Table 6.3. Number in parenthesis are arbituary numbers assigned to each protein analyzed in this chapter.

Phylum	*Organism*	% Identity with LmajCBL		
		CGL	CBL	CGS
Bacteria	*Helocobacter pylori*	-	-	38 (24)
Bacteria	*Halobacterium aranticicus*	-	-	34 (34)
Bacteria	*Escherichia coli*	-	22 (1)	33 (26)
Bacteria	*Haemophilus influenzae*	-	22 (4)	34 (27)
Bacteria	*Bordetella avium*	-	27 (10)	-
Bacteria	*Bacillus subtilus*	38 (22)	33 (8)	38 (28)
Bacteria	*Thermoplasma volcanium*	-	23 (12)	-
Bacteria	*Erwinia herbicola*	35 (19)	-	-
Bacteria	*Mycobacterium leprae*		-	34 (25)
Bacteria	*Mycobacterium*	-	-	34 (29)
Fungi	*Salonella typhimurium*	-	20 (6)	-
Fungi	*Saccharomyces cerevisiae*	32 (16)	22 (7)	20 (36)
Fungi	*Neurospora crassae*	34 (20)	37 (11)	13 (13)
Fungi	*Aspergillus nidulans*	-	36 (13)	-
Fungi	*Saccharomyces cerevisiae*	32 (14)	-	-
Fungi	*Saccharomyces pombe*	-	-	14 (33)
Mammal	*Homo sapiens*	34 (15)	-	-
Mammal	*Rattus norvegicus*	34 (18)	-	-
Nematode	*Caenorhabditis elegans*	35 (17)	-	-
Plants	*Mesembryanthemum*	-	-	25 (35)
Plants	*Arabidopsis thaliana*	-	36 (2)	25 (36)
Plants	*Solanum tuberosum*	-	39 (3)	-
Plants	*Zea mays*	-	-	20 (37)
Protozoa	*Leishmania major*	26 (23)	100 (9)	-

Great diversity between the sequences is evident in the N-terminal region of the CBL proteins (Figure 6.12). A PLP binding site found in PLP dependent enzymes was identified between 356–370 with lysine 364 as the site for co-factor binding via a schiff base (Alexander *et al.*, 1994).



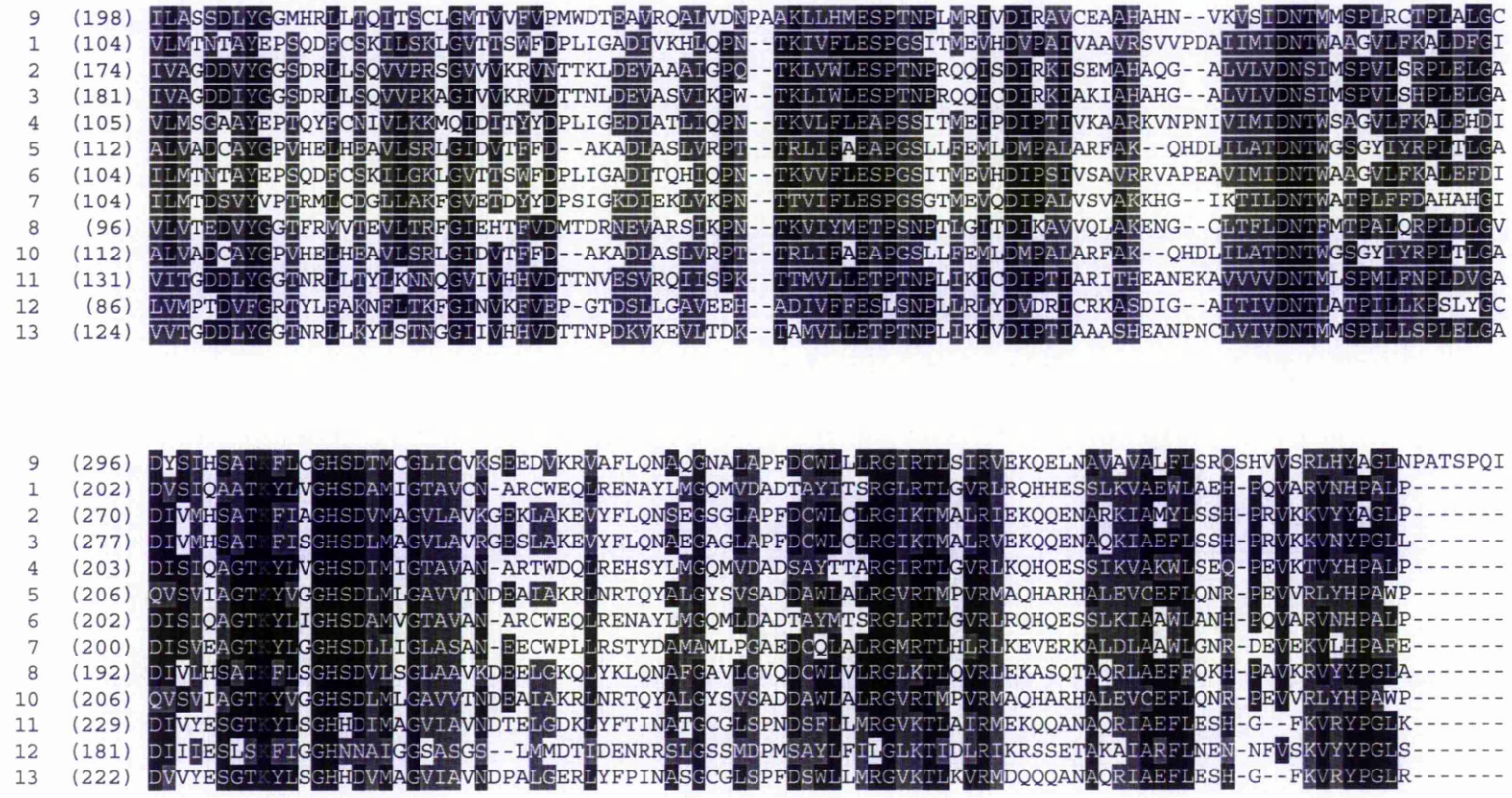


Figure 6.12. Multiple alignment of deduced amino acid sequences of CBL from *L. major* and from other representative organisms. Sequences as follows: 1. METC_ECOLI-*E. coli* (accession no. GI: 1207031 P06721), 2. *Arabidopsis thaliana* (accession no. AAA99176 GI: 704397), 3. *Solanium tuberesum* (accession no. AAF74980 GI: 8439539), 4. MET_HAEIN-*H. influenzae* (accession no. P44527), 5. METC_BORAV *Bordetella avium* (accession no. Q07703), 6. *Salomonella typhimurium* (accession no. ND 462076.1 GI: 16766461), 7. METC_YEAST *Saccharomyces cerevisiae* (accession no. P43623), 8.*Bacillus subtilis* (yjcJ) (accession no. Z99110), 9. L. *major* (this study), 10. *Bordetella avium* (10) (Accession no. AAA22978 GI:304266), 11. *Neurospora crassa* (accession no. AF401237), 12. *Thermoplasma volcanism* (accession no. NP111526 GI:13541838), 13. *Aspergillus nidulans* (accession no. U28383). These sequences were aligned using ClustalW and boxshade programs. shades indicate identical amino acids, grey shades indicate conserved amino acid substitutions. The putative PLP binding motif is identified between position 301 and 306, with lysine 304 (red) as the site of co-factor binding via a Schiff base in LmajCBL. Black

6.9.1 *Sequence alignment of PLPγ enzymes from* L. major *trans-sulfuration pathway*

Structural analysis of CBL from *E. coli* has highlighted the amino acids, (lys, asp and glu) crucial for activity of some PLPγ -dependent enzymes (Sienko and Paszewski, 1999). The alignment of CGS, CBL and CGL showed these residues to be conserved in the PLPγ- dependent enzymes involved in the trans-sulfuration pathway in *L. major* (Figure 6.13). The lys^{220} (Clausen *et al.*, 1996) and lys^{213} (Steegborn *et al.*, 1999) of EcCBL and HsCGL, respectively, which covalently bind to the PLP co-factor (Martel *et al.*, 1987) are conserved in LmajCBL and LmajCGL to lys^{203} and lys^{199} respectively (Figure 6.13). Two other residues involved in activity of EcCBL, asp^{205} and glu^{174}, were also conserved in the whole γ-family (Figure 6.13), and are asp^{280} and glu^{310} in LmajCBL and asp^{337} and glu^{251} in LmajCGL, respectively. Further, the PLPα-enzyme acid:pyruvate aminotransferase from *Pseudomonas putida* (Yonaha *et al.*, 1992; Clausen *et al.*, 1996) indicates that these residues corresponded to lys^{207} asp^{259} and glu^{226}. Methionine γ-lyase (MGL) from *T. vaginalis* (Mckie *et al.*, 1998) and *P. putida* (Inoue *et al.*, 1995; Hori *et al.*, 1996) have been shown to have these residues conserved as well (data not shown). These residues participate in PLP binding along with the lysine residue, thus, suggesting similar reaction mechamisms (Sleegborn *et al.*, 1999) and a possible evolutionary relationship of PLP-binding domain between the α- and γ- families (Alexander *et al.*, 1994; Clausen *et al.*, 1996). The α- and γ-family might be distantly related with one another, but they are clearly not homologous with the β-family (Figure 6.7).

6.9.2 Phylogenetic analysis of PLP γ enzymes

CBL, CGS and CGL catalyse γ-replacement reactions of C_4 amino acids and β- or γ-cleavage of cystathionine. Phylogenetic analysis of these proteins (Figure 6.7) showed that CBL form two classes. The LmajCBL together with the plants (*A. thaliania* (36%), *S. tuberosum* (39%), fungi (*Neurospora sp.*(36%), *A. nidulans* (36%) and the bacteria, *B. subtilus* (33%) CBS form one group while the bacterial proteins from *Thermoplasma volcanium* (23%), *B. avium* (21%), *E. coli* (22%), *H. influenzae* (22%) and a fungus, *S. cerevisiae* (22%) CBS formed another group. The bacterial CBL of *B. subtilus* had similar identity with CGL and CGS and were all clustered in the same group. The CGS protein formed several independent clades for plants (*Z. mays, A. thaliana, M. crystallinum*), bacteria *(E. coli, H. auranticius, M. tuberculosis, B. subtilus, H. influenzae, H. influenzae, M. leprae* and *H. pylori)* and yeast *(S. cerevisiae, N. crassa* and *S. pombe).* The bacteria CGS, (*M. tuberculosis and H. auranticius*) were in the same clade as the LmajCGL with an identity of 36%, suggesting that both bacterial proteins are closely related to cystathionine γ-lyase.

These results are suggestive that LmajCBL and LmajCGL are derived from a common ancestor, as proposed earlier for the corresponding bacterial MetB and MetC genes (Belfaiza *et al.*, 1986).

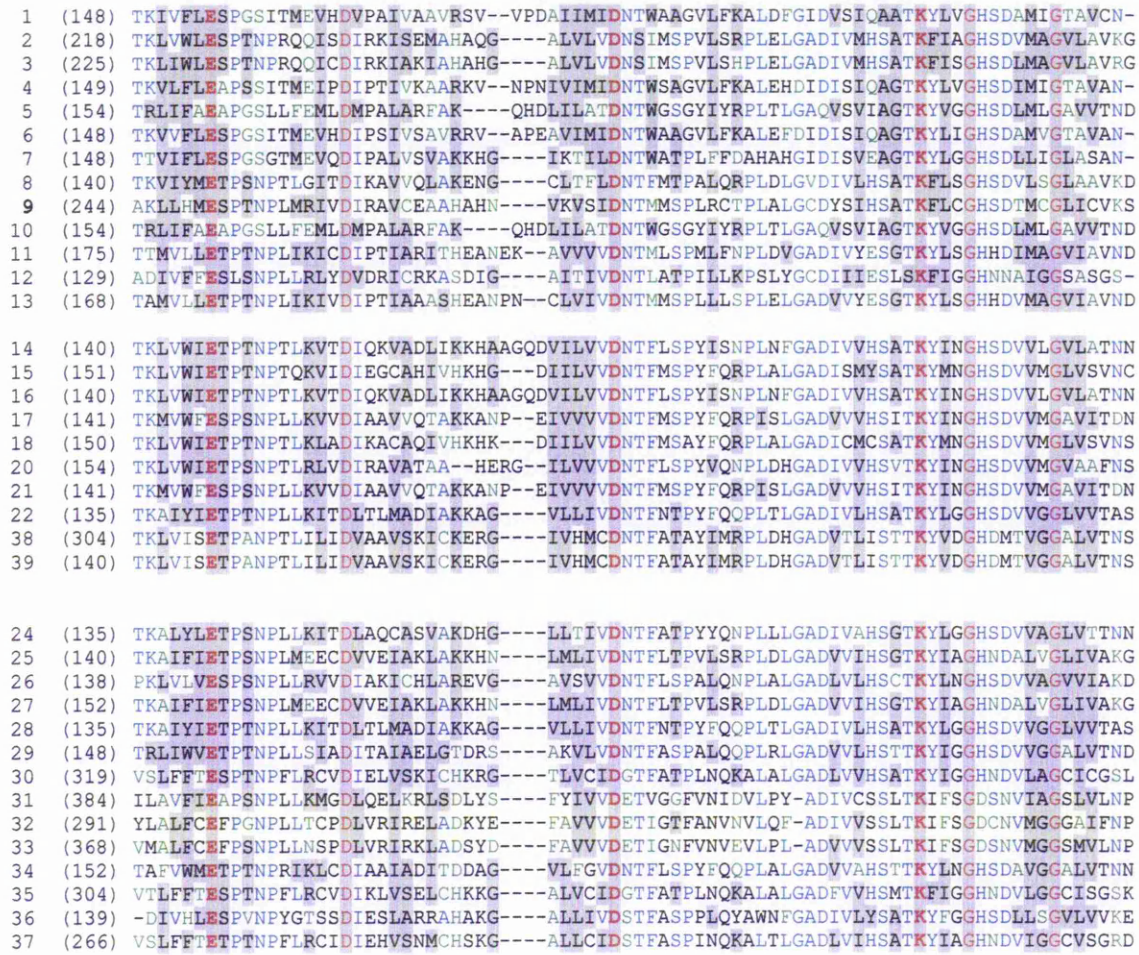


Figure 6.13. Alignment of the amino acid sequences of PLP-dependent enzymes. Amino acid residues of CGL, CGS and CBL from a variety of organisms aligned to show conserved residues (arrowed) that interact with PLP molecule. Amino acid numbering from first nucleotide of proteins aligned is given on the right of each protein. The organisms used and their accession no are given in Figure 6.6.

6.12 Discussion

In this chapter I have produced putative sequences of the PLPγ-dependent enzymes CGL and CBL and I have characterised the PLPβ-dependent enzyme CBS involved almost exclusively in the metabolism of amino acids. These putative amino acid sequences were identified in the genome database of *L. major* via extensive database mining with homologous sequences as query. These gene fragments were either overlapping GSS sequences made into a contig or a cosmid containing the gene's ORF within e.g. LmajCGL. The presence of these enzymes might suggest that *L. major* have a functional trans-sulfuration pathway involved in the interconversion of cysteine to methionine and vice versa.

The PLPβ enzyme LmajCBS have been identified cloned and expressed as a recombinant protein. Alignment of its deduced amino acid sequence with allied proteins from human and yeast shows it to be a shorter protein (369 vs. 552 vs. 506) and without the N-terminal heme (Meier *et al.*, 2001) and C-terminal CBS motifs (Kery *et al.*, 1998; Shan and Kruger, 1998; Jhee *et al.*, 2000; Maclean *et al.*, 2000).

The evolutionary non-conserved heme domain present in some CBSs plays a regulatory and/or structural role (Oliveriusava *et al.*, 2002) as well as enhancing the mammalian CBS affinity for PLP binding (Maclean *et al.*, 2000). CBS affinity for PLP diminishes without the heme domain (Maclean *et al.*, 2000; Nozaki *et al.*, 2001). The difference in protein structure probably relate to the fact that lower eukaryotes including yeast synthesize pyridoxal *de novo* (Chumnantana *et al.*, 2001) while humans salvage it from dietary sources (Nguyen *et al.*, 2001). It thus seems conceivable that the N-terminal domain heme domain represents an evolutionary

adaptation in mammals designed to augment retention of PLP by CBS. This is less vital in lower eukaryotes and so the heme domain is absent (Oliveriusava *et al.*, 2002). How trypanosomatids satisfy their pyridoxal requirement is unknown but extension of the argument above would suggest that they synthesize it.

The two copies of the CBS domain found in tandem in yeast and mammalian CBS enzymes (Bateman, 1997) are clearly absent from their trypanosomatid counterparts. The CBS domains are also linked to other non-homologous protein domains in two or four tandem copies e.g. inosine monophosphate dehydrogenase has two CBS domians un tandem associated with it. The general function is unknown, but its absence in *L. major* protein clearly suggest it does not play a role in CBS activity, whereas in mammals it plays regulatory (Finkelstein *et al.*, 1975) and autoinhibitory (Janosk *et al.*, 2001; Shan *et al.*, 2001) roles. It might function in similar fashion in other proteins carrying the domain and elucidation of its role in CBS could provide information on its function in a wide range of proteins. Recently, it has been shown to be required for tetramerization of CBS (Kery *et al.*, 1998; Oliveriusava *et al.*, 2002). Extensive deletion (Oliveriusava *et al.*, 2002) and trypsin digest (Kery *et al.*, 1998) analyses of HsCBS have produced a mutant protein that resembles the 'short CBS' catalytic domain of LmajCBS (Figure 6.4). A consequence of this is that dissociated from a tetramer to a dimer (Kery *et al.*, 1998). Further, CBS from the nematode, *P. rectivivus* (Papadopoulous *et al.*, 1996), and CS, which resemble the catalytic domain of LmajCBS in length, exist as dimers (Nozaki *et al.*, 2001), thus suggesting that LmajCBS might be a dimer as well. However, TcCBS, with 66% identity with LmajCBS, solely exists in the tetrameric form (Nozaki *et al.*, 2001), which might suggest that LmajCBS is a tetramer. Such findings cast doubts on the C-

terminal's domain's role in tetramerization (Taoka *et al.*, 1999; Nozaki *et al.*, 2001) but Oliveriusava *et al.*,(2002) extensive deletion studies of this domain recently provided further support for its role in the process. If LmajCBS also forms the higher oligomeric structure, then one can suggest it involves a different process. The 3D structure of the naturally occurring 'short forms' of CBS has not yet been solved to date. Consequently, in its absence, site-directed mutagenesis of specific residues and possibly internal deletion could be used to characterise which portion(s) are specifically associated with the organisation of CBS from trypanosomatids into higher molecular weight oligomers as reported by Nozaki *et al.*, (2001).

In general, CBS catalyses PLP-dependent reactions which result in the linkage of serine with sulfide from a sulfur source. Various sulfur sources have been reported (Nozaki *et al.*, 2001) thus explaining the multiple activities of CBS (Papodoulous *et al.*, 1996; Nozaki *et al.*, 2001). Sequence analysis of rLmajCBS have shown that it possesses all the motifs of other CBS known to be responsible for catalytic activity. As expected, rLmajCBS catalyses the PLP-dependent condensation of serine with homocysteine or cysteine to form cystathionine. It can also form of cysteine directly from serine or *O*-acetyl serine (OAS) and hydrogen sulfide (Braunstein *et al.*, 1971; Ono *et al.*, 1988; Kozich and Kraus, 1992). This reaction have also been reported in CBSs from humans (Kraus *et al.*, 1978), rats (Gomez-Bautista and Barrett., 1988), and the yeast CBS *S. cerevisiae* (Ono *et al.*, 1994).

rLmajCBS has the apparent ability to catalyse the replacement of the β-SH group of cysteine with β-mercaptoethanol to form a cysteine thioester and sulfide the activated serine sulfhydrase reaction. This reaction has previously been reported in *T. vaginalis*

(Thong and Coombs, 1987; 1985; Walker and Barrett, 1991a; 1992) and nematodes (Papodoulous *et al.*, 1996) but not in their mammalian host. But the mammalian form can produce hydrogen sulfide in the so-called cysteine desulfurase (C-DES) reaction. CBS activities which produce hydrogen sulfide have not been investigated in lower eukaryotes, but in humans it is believed that the hydrogen sulfide can act as a neuromodulator (Shan *et al.*, 2001). The levels of endogenous hydrogen sulfide in *L. major* cells is unknown, but if PLP-dependent enzymes like CBS can form sulfide from cysteine, then there is likely to be redundancy in sulfide production within the parasite which may reflect it importance in physiological reactions involving sulfur like the formation of iron sulfur clusters. Whether all these reactions shown to occur *in vitro* also happen *in vivo* is debateable. Their biological roles are unknown (Byrne *et al.*, 1988; Rolland *et al.*, 1993; Nozaki *et al.*, 2001) but CBS has been suggested to be involved in the detoxification of toxic thiols by direct conjugation with cysteine.

It was also not surprising to observe some vestigial CS activity with the rLmajCBS protein. This has been reported in *S. cerevisiae* (Ono *et al.*, 1994), *N. crassa* (Ono *et al.*, 1994) and TcCBS (Nozaki *et al.*, 2001). rLmajCBS shared a large degree of sequence identity to CS (28-34%) especially around the catalytic domain and three out of four reactive lysines which can bind the common elements (serine, pyridoxal phosphate and sulfide) were conserved. Further, the similarity of these two enzymatic reactions suggests that the condensation of serine with a sulfur compound to produce cysteine must have occurred early in evolution (Nozaki *et al.*, 2001). The notion that CBS might be a bifunctional protein involved in two cysteine synthesising pathways is not new. TcCBS and HsCBS have been shown to function as CBS in the CBS-deficient yeast strain WB63yCBSA (Kraus *et al.*, 1993; Nozaki *et al.*, 2001) and as

CS in *E. coli* (Nozaki *et al.*, 2001). This is shown by functional rescue or complementation in organisms lacking endogenous CBS and CS, respectively.

Results from database mining and blastx analysis of the gene content of *L. major* have shown that it has putative CGL and CBL genes involved in methionine and cysteine interconversion. Even though the genes of these PLPγ enzymes were not characterised, amino acid sequence analysis have shown that the *L. major* proteins had high identity to allied proteins and key residues vital for activity were conserved suggesting that these enzymes might have similar folds and catalytic mode of action. CGS was not identified and the absence of HTGS of CGS producing high hits may suggest that *L. major* lacked the gene or it is yet to be sequenced. The latter seems more likely. Blast results of non-redundant databases with HTGS from the *L. major* genome database used as query produced hits that had similarity to CGS, CBL and CGL. It is very difficult to predict the identity of short HTGS due to the high degree of similarity between these PLPγ enzymes (Mckie *et al.*, 1998). EcCGS and EcCBL share 37% similarity (29% identity) (Clausen *et al.*, 1998), similar quaternary structure (Litzenberger Holbrook *et al.*, 1990) and catalytic residues (Clausen *et al.*, 1998) but different chemical reactions. Dual roles of PLP proteins are not uncommon as their catalytic spectrum is not restricted to their physiological reactions (Steegborn *et al.*, 1999). CGS can catalyse β-replacement and α- or β-proton exchange reactions (Posen *et al.*, 1972; Guggenheim and Flavin, 1969), as well as CBL-like β-elimination reactions (Flavin, 1975). Similarly, reports have shown CGL and CBL carrying out the same function. CGL from yeast (Yamagata *et al.*, 1999) and rat liver (Uren *et al.*, 1978) show an appreciable CBL-like activity, i.e. they are able to cleave both C-γ-S and C-β-S bonds of L-cystathionine. Dobric *et al.*, (2000) have characterized a mixed

cystathionine γ/β lyase from *L. lactis*. This protein has the unique ability to carry out both αβ- elimination and αγ- elimination reactions on the cystathionine substrate. Hence it might be debatable as to how many proteins are actually involved in the trans-sulfuration pathway.

The structural information available for members of the PLPγ family has been limited until recently such that its evolutionary relationship to other PLP enzymes was unclear. Only one crystal structure of CBL from *E. coli* has been solved (Clausen *et al.*, 1996; 1998) and its reaction mechanism elucidated. The sequences of several PLPγ family genes are now available, and this has allowed sequence alignment, phylogenetic analyses and predictions of evolutionary relationship of *L. major* genes with PLPγ enzymes from other organism to be made. These analyses have shown these PLPγ enzymes to be closely related to themselves, as well as to the α-family but not to the β-family of proteins. This might suggest that the α- and β- family of PLP evolved independently from a single ancestor (Belfaiza *et al.*, 1986) hence the high number of clades observed.

The analysis so far is incomplete, however I believe that the data so far provides the framework in which in the longer term the trans-sulfuration pathway of *L. major* can be fully characterized. Homocysteine, occupies a key regulatory position between two competing pathways; remethylation of homocysteine to methionine (methionine salvage) and the biosynthesis of cysteine (Finkelstein, 2000). The relative flux between these two competing pathways depends on tissue content (Finkelstein and Martin, 1984; Selhub, 1999), the kinetic property of the constituent enzymes (Kutzbact *et al.*, 1967; 1984; Salzmann *et al.*, 2000), the concentration of substrates

and enzyme effectors (Finkelstein and Martin, 1984; Finkelstein *et al.*, 1975; Roper and Kraus, 1992), and the activity of other enzymes which may compete for the same substrates. The flux is roughly equal in mammals (45-82%, Goldberg *et al.*, 2000) and *T. brucei* (50%, Goldberg *et al.*, 2000).

The heme in HsCBS functions as oxygen carrier, electron carrier and catalyst. It binds to molecular oxygen, and triggers the oxygen-dependent increase in CBS activity. The spatial proximity between the heme binding and the oxidoreductase active site motifs (CXXC) in the HsCBS structure (Kabil *et al.*, 2001; Meier *et al.*, 2002) assist and aids the heme group in regulating CBS activity by redox changes thereby favouring transsulfuration over methionine cycle (Toaka *et al.*, 1998). This results in the detoxification of peroxides and corresponding increase glutathione production (Taoka *et al.*, 1998) from cysteine. *Leishmania* are unusual in possessing trypanothione, a conjugate of glutathione and spermidine, as a major cellular thiol and apparently using this and associated enzymes as a prime means of protection against oxidative damage (Krauth-Siegel and Coombs, 1999; Miller *et al.*, 2000). They are also likely to contain thioredoxin (Reckenfelderbaumer *et al.*, 2000), a key molecule in a number of redox reactions in cells but also central to the antioxidant peroxiredoxin cascade (Flohe *et al.*, 2002). Cysteine is the main constituent of these low molecular weight thiols, but the source of cysteine, essential for trypanothione production, is unknown.

L. major over-expressing the CBS gene was more resistant to oxidative stress induced by hydrogen peroxide, tert-butyl hydroperoxide and cumene hydroperoxide was markedly high under oxidative stress with cells over-expressing the CBS gene. This

resistance might exclude the influence of the methionine cycle, S-adenosylmethionine, polyamine and trypanothione syntheses as the likely route, but may add credence to the trans-sulfuration pathway and its association to the formation of compounds like cysteine, glutathione and their associated dithiols used to detoxify reactive oxygen species. In *T. brucei*, transsulfuration occur since the resulting AdoHcy formed is toxic when its accumulates in the cytosol (Hoffman *et al.*, 1980; German *et al.*, 1983). The rate of trans-sulfuration was 42-46% of cytosolic methionine levels and over 22 times the AdoMet pool. A study into the flux between these two pathways, and the over-expression of CBL and the double expression of both CBS and CBL might be significant as it might help us understand how the transsulfuration pathway is regulated in response to oxidative stress. Firm conclusions on the contribution of CBS in detoxifying reactive oxygen species can however be made when information on the cysteine and glutathione and dithiols levels with and without stress, and when gene deletion studies become available.

The LmajCBS gene exists as a single copy protein (TcCBS occurs as six copies in tandem) in the *L. major* genome. Gene deletion study is in progress. Clones of single-replacement CBS/HYG transgenic strains, resistance to hygromycin (500μg/ml) are yet to be characterised for integration of the cassette into the LmajCBS locus.

This work has shown that LmajCBS is clearly different for its mammalian host in domain architecture, regulation and some of the reaction it catalyses. Its ability to help the parasite cope with oxidation stress might suggest that the enzyme might be a potential drug target.

Chapter 7

Discussion

Overall, this study has investigated sulphur metabolism and its role in the *Leishmania major*'s protective mechanisms against oxidative stress. I have identified and characterised; (a) a sulfurtransferase of *L. major* and *L. mexicana;* (b) the pathways used by *L. major* to synthesise cysteine. *L. major,* like fungi (Morzycka *et al.*, 1979) and *T. cruzi* (Nozaki *et al.*, 2001), has the sulfydrylation and the reverse trans-sulfuration pathways (Figure 7.1). The enzymes cystathionine β-lyase and cystathionine β-synthase (organge) and cystathionine γ-synthase (dark red) are yet to be characterized and identified respectively.

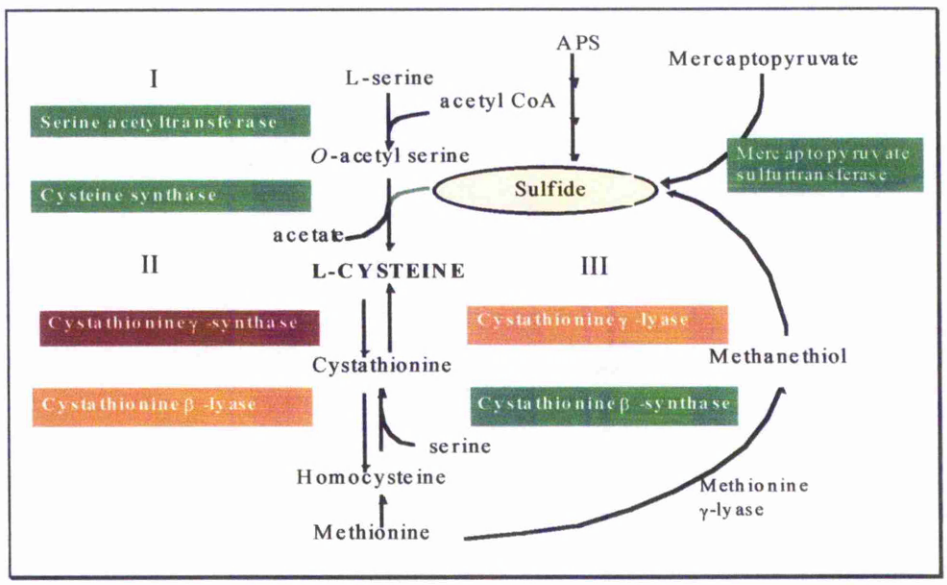


Figure 7.1 Overview of cysteine biosynthesis. The scheme shows the enzymes, serine acetyltransferase (yellow) and cysteine synthase (yellow) involved in the two-step *de novo* synthesis or sulfydrylation pathway (I) of cysteine from serine via the intermediate *O*-acetylserine (OAS). OAS condenses with sulfide from a variety of sources: the sulfate assimilatory pathway with (APS) being the initial substrate; methanethiol produced by the action of methionine γ- lyase on homocysteine; and the action of mercaptopyruvate sulfurtransferase on mercaptopyruvate. The trans-sulfuration pathway that inter-converts cysteine and methionine is also shown. Enzymes involved in this pathway are shaded dark red. The left panel (II) represents reaction of the forward trans-sulfuration pathway occurring in bacteria, plants and fungi; the right panel (III) shows the reverse trans-sulfuration pathway found in fungi and mammals

LmajMST like other MST/rhodanese proteins contains the structurally well-defined 'rhodanese' domains (N- and C-terminal) found as tandem repeats (Figure 7.2a), with an additional 70 amino acid C-terminal extension (LmajMSTCTE). Hence LmajMST is a 3-domain molecule whose structure can now be supported by results from X-ray diffraction analysis. Characterisation of LmajMSTCTE by mutagenesis and CD analysis has allowed us to conclude that it helps the protein attains its 3D conformation and full enzymatic property (Chapter 4).

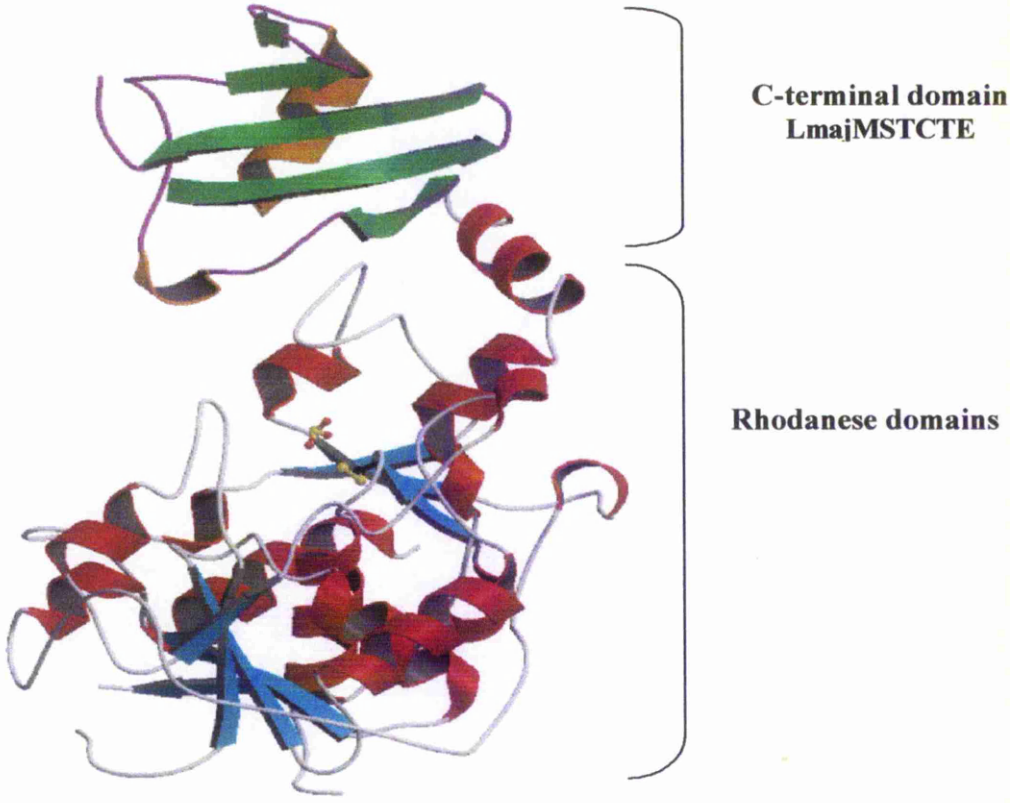


Figure 7.2a. The overall structure of LmajMST. Ribbon diagram of the secondary structure of the N- and C-termini (blue and red) forming the α/β topology 'rhodanese' fold and C-terminal extension (LmajMSTCTE, green and brown). The N- and C-domains are the same as those adapted for Rhobov and RhdA (Pleogman *et al.*, 1978; Bordo *et al.*, 2000). The active site Cys253 is represented in ball and stick. All drawings were prepared with Molscript (Kraulis, 1991) and Raster 3D (Merrit *et al.*, 1994.) Crystal structure was solved by Magnus Alphey, University of Dundee

Preliminary analysis of X-ray diffraction data has revealed that the interface between the LmajMSTCTE and the 'rhodanese' domains consist of strong hydrogen bonds (Q305-R289; E349-R191, Figure 7.2b). Truncations of LmajMSTCTE disrupted these bond, and resulted in the collapse of the 3D conformation of the mutant proteins, judged by a different CD UV spectrum and specific activity relative to the full length protein.

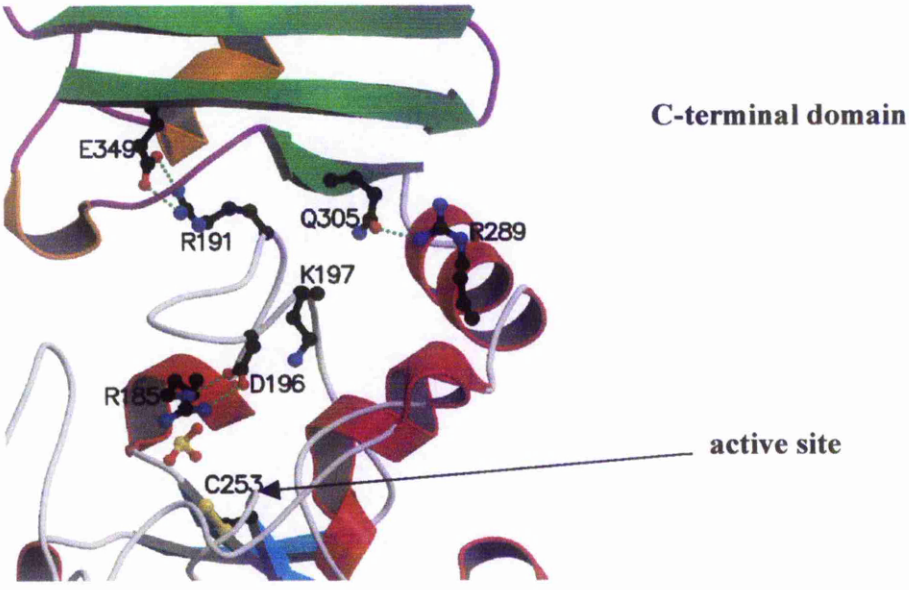


Figure 7.2b The interaction of LmajMSTCTE with the 'rhodanese' domain. Ribbon diagram of the interaction of hydrogen bonds between the interface of the 'rhodanese' domain (blue and red) and LmajMSTCTE (green and brown). The interacting residues Q305, R289, E349,and R191 are represented by ball and stick and the hydrogen bonds between them by green dotted lines. The active site Cys^{253} and other residues R185 D196 and K197 that forms the active site pocket are also represented with balls and sticks. All drawings were prepared with Molscript (Kraulis, 1991) and Raster 3D (*Merrit et al.*, 1994.). Crystal structure was solved by Magnus Alphey, University of Dundee

Sulfurtransferase/rhodanese catalyse the transfer of a sulfur atom from a suitable donor to a nucleophilc acceptor. Biochemical characterization of LmajMST has shown it to be a multifunctional sulfurtransferase (Figure 7.3) with a preference for mercaptopyruvate as a sulfur donor. This was not surprising because the CGSGVTA

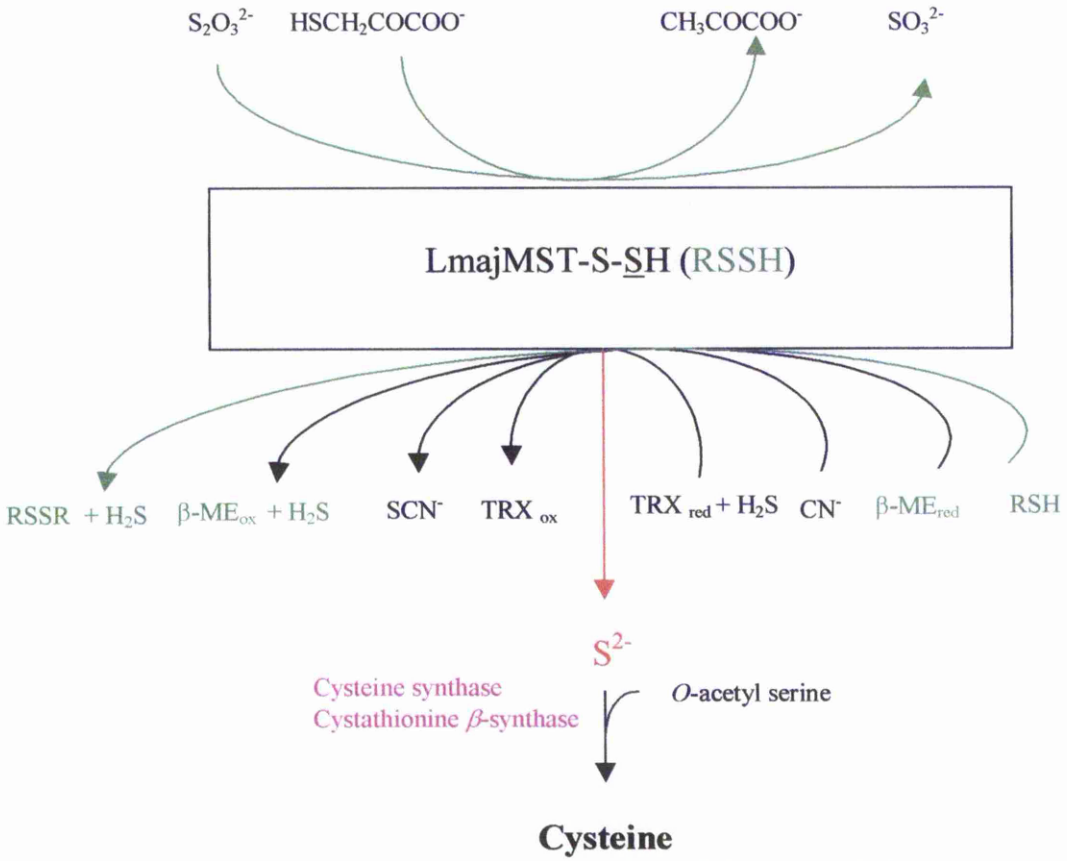


Figure 73. Schematic representation of the multifunctional role of MST. Reduced MST transfers sulfane sulfur from either thiosulfate or mercaptopyruvate, forms an enzyme-sulfane transition molecule which then transfers sulfane sulfur to: CN^- (black) to form SCN^- or $thioredoxin_{red}$ (black) with the formation of sulfide and $thioredoxin_{ox}$. The thiols (green), homocysteine, β-$mercaptoethanol_{red}$ and cysteine, homocysteine and glutathione (RSH) can react with enzyme intermediates, to form persulfide intermediate (RSSH), which then reacts with more thiols to form disulfides (RSSR) and H_2S. MST shows activity in the absence of an acceptor molecule (red). The sulfide produced works in conjunction with the recombinant *L. major* cysteine synthase (GenBank accesion number AL 499624) and cystathionine *β*-synthase (GenBank accession number AL 499619) to synthesize cysteine *in vitro*.

motif around the reactive cysteine residue -C^{253} (Figure 7.2) denotes a MST rather than a TST. LmajMST can utilise low molecular weight thiols such as cysteine, homocysteine, cyanide, β-mercaptoethanol, and reduced thioredoxin as acceptors substrates. Further, *in vitro,* it has been shown to interact with the cysteine biosynthetic pathway by providing sulfide, which condensed with OAS – a reaction catalysed by cysteine synthase. The over-expression of LmajMST in *L. major* promastigotes has lead not only to a 5-fold increase in sulfurtransferase activity (0.06 to 0.3 μmol/min/mg protein) but also to a 2-fold increase (3.3 ± 0.1 versus 6.5 ± 1.9 nmol/10^8 cells) in cysteine and ovothiol content (18.5 ± 0.9 versus 30.1 ± 3.1 nmol/10^8 cells) within the parasite (new results).

Ovothiol production involves the formation of mercaptohistidine via S-4(histidyl)-cysteine sulfoxide from cysteine, Fe^{2+}, histidine and PLP – a reaction catalysed by sulfoxide synthase and sulfoxide lyase (Figure 7.4, Vogt *et al.*, 2001). How MST fits into this pathway is hard to figure out based on current knowledge of the biosynthesis of the molecule. Vogt *et al.*, (2001) reported the formation of cysteine as a 'bi-product' of ovothiol production. Dithiothreitol (DTT) provides the sulfur for the formation of cysteine from S-4'(histidyl)-*L*-cysteine sulfoxide, suggestive that trypanosomatids possess a novel sulfurtranferase activity that can utilize DTT as S^{2-} sulfur donor. The increased cysteine observed in mutant parasites overexpressing the MST gene can either be attributed to this reaction or may suggest that MST can be a potential sulfide source for cysteine biosynthesis *in vivo*.

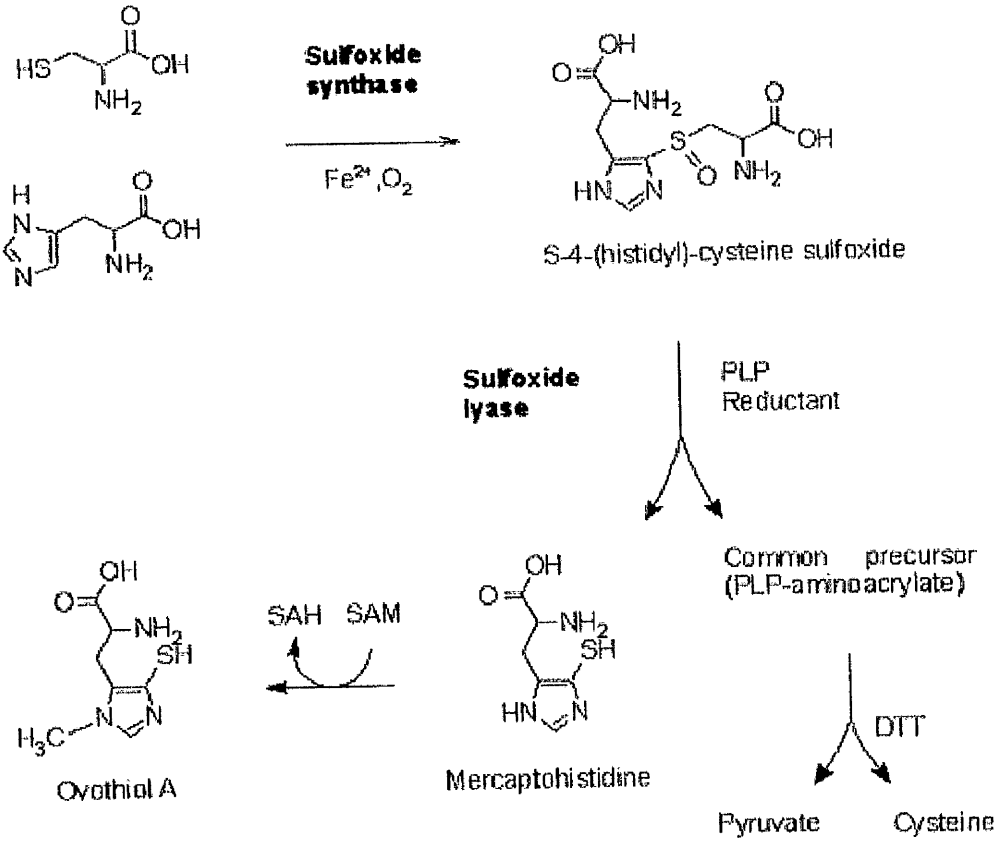


Figure 7.4. Ovothiol A biosynthetic pathway. DTT, dithiothreitol. Source: Vogt *et al.*, 2001

Ovothiols exist as reactive thiolate anions and they contribute to an enhanced reactivity with alkylating agents, reactive oxygen intermediates and other free radical species (Marjanovic *et al.*, 1995; Zoete *et al.*, 2000, Turner *et al.*, 1988) as well as providing protection against peroxinitrite-induced damage (Bailly *et al.*, 2000). Ovothiols have been implicated in the protection of trypanosomatids against oxidative stress (Ariyanayagam *et al.*, 2001). LmajMST in wild type promastigotes was up-regulated on exposure to 10 μM cumene hydroperoxide (Chapter 4), while promastigotes over-expressing the MST gene were resistant to the same oxidant.

Whether ovothiols play a role in the management of oxidative stress produced by cumene hydroperoxide is uncertain. What is clear from this study is that rLmajSAT, rLmajCS and rLmajCBS are potentially involved in the parasite's defence mechanisms against oxidative stress. The interaction of MST with thioredoxin (Trx) also implicates the protein in a possible role in detoxification of oxygen reactive (Nandi *et al.*, 2000).

Another opportunity provided by this study is the prossibility to analyse the interaction between Trx and LmajMST (at the μM range) as well as between LmajSAT and LmajCS. They can be useful models for studying protein-protein interactions in *Leishmania.*

The discovery of the cysteine biosynthetic pathways in *L. major*, though an interesting finding, raises the question as to why the parasite has retained this apparent redundant pathway for the synthesis of cysteine. The current viewpoint is based on the parasite's dependence on thioredoxin and tryparedoxin in the management of oxidative stress (Nozaki *et al.*, 2001). Many reports have emphasised the dependence of *T. brucei* bloodstream (Duzenko *et al.*, 1985), *T. vaginalis* (Gordon, 2001), *Giardia lamblia* (Gillin *et al.*, 1980; 1981) and *Entamoeba histolytica* (Nozaki *et al.*, 1998) on an exogenous sources of cysteine despite their capability to synthesise it. This study, however, shows that *L. major* can survive in the absence of an exogenous source cysteine, but growth was stimulated by cysteine, and methionine as well as substrates of the cysteine biosynthetic pathway that are limiting. When these limitations were overcame, the parasite cysteine biosynthetic pathway adequately satisfied its cysteine requirement in the absence of an exogenous source of cysteine or methionine. Thus

perhaps the biosynthetic pathway is sufficient to meet the parasites needs - at least at some stage during its life cycle.

Questions that need to be addressed about cysteine biosynthesis - trans-sulfuration and de novo within the parasite concern their localisation. Are they compartmentalised in organelles or are they cytosolic? If they are compartmentalised , then can one assume that compartmentalization, as in fungi (Ono *et al.*, 1994) is used as a tool to satisfy the demand for cysteine within the organism. Further, if these pathways are cytosolic, are they both functional at the same time? Do they both produce cysteine for the same reactions? Which pathway is essential for parasite's survival? Such information needs to be worked out and are quite invaluable in the screening for a novel drug targets. The *de novo* cysteine biosynthetic pathway is not known in vertebrates, a finding that could have important chemotherapeutic implications.

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