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Two pathways for cysteine biosynthesis in *Leishmania major*

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Abbreviations: RTS, reverse transulfuration; CS, cysteine synthase; SAT, serine acetyltransferase; CBS, cystathionine β -synthase; CGL, cystathionine γ -lyase; MST, mercaptopyruvate sulfurtransferase; 3-MP, 3-mercaptopyrivate; OAS, O-acetylserine; Lmj, *Leishmania major*; Tc, *Trypanosoma cruzi*; Hs, *Homo sapiens*; Sc, *Saccharomyces cerevisiae*; At, *Arabidopsis thaliana*; Tv, *Trichomonas vaginalis*; PLP, pyridoxal 5-phosphate; SS, serine sulfhydrase.

Synopsis

Genome mining and biochemical analyses have shown that *L. major* possesses two pathways for cysteine synthesis - the *de novo* biosynthesis pathway comprising serine acetyltransferase (SAT) and cysteine synthase (CS) and the reverse transsulfuration (RTS) pathway comprising cystathionine β -synthase (CBS) and cystathionine gamma-lyase (CGL). The *L. major* CS (LmjCS) is similar to the type A CSs of bacteria and catalyses the synthesis of cysteine using O-acetyserine and sulfide with K_m s of 17.5 and 0.13 mM, respectively. LmjCS can use sulfide provided by the action of mercaptopyruvate sulfurtransferase (MST) on 3-mercaptopyruvate (3-MP). LmjCS forms a bi-enzyme complex with *Leishmania* SAT (and *Arabidopsis* SAT), with residues K222, H226 and K227 of LmjCS being involved in the complex formation. LmjCBS catalyses the synthesis of cystathionine from homocysteine, but, unlike CBS of mammals, also has high cysteine synthase activity (but with the K_m for sulfide being 10.7 mM). In contrast, LmjCS does not have CBS activity. CS was up-regulated when promastigotes are grown in medium with limited availability of sulfur amino acids. Exogenous methionine stimulated growth under these conditions and also the levels of intracellular cysteine, glutathione and trypanothione, whereas cysteine had no effect on growth or the intracellular cysteine levels; correlating with the low rate of transport of cysteine into the cell. These data suggest that cysteine is generated endogenously by promastigotes of *Leishmania*. The absence of CS from mammals and the clear differences between CBS of mammals and *Leishmania* suggest that each of the parasite enzymes could be a viable drug target.

Introduction

Leishmania is a protozoan parasite that is the causative agent of a spectrum of diseases collectively known as the leishmaniases, there being some 60000 new cases annually [1]. Some aspects of the metabolism of *Leishmania* have been investigated in detail [2] notably that involving the low molecular weight thiol trypanothione, a conjugate of glutathione (GSH) and spermidine, which appears to play a pivotal role in maintaining intracellular redox homeostasis and providing defence against oxidative stress [3]. The synthesis of glutathione and so trypanothione depends on the availability of cysteine. This sulfur-containing amino acid is an essential growth factor for the related trypanosomatid *Trypanosoma brucei* [4] whereas *T. cruzi* can generate it from homocysteine [5]. In contrast, it is synthesised *de novo* in the microaerophilic parasitic protozoa *Entamoeba*, *Giardia* and *Trichomonas* - in which cysteine itself is the key antioxidant and redox buffer [6,7]. This study was undertaken to establish how *Leishmania* obtains cysteine and to characterise the mechanisms involved.

There are two routes known for the generation of cysteine, the *de novo* biosynthesis pathway, otherwise called the sulphydrylase pathway [7], and the reverse transsulfuration pathway (RTS) that converts homocysteine to cysteine in two steps - catalysed by cystathionine β -synthase (CBS, EC 4.2.1.22) and cystathionine γ -lyase (CGL, EC 4.4.1.1) (RTS; see Figure 1) [8]. Mammals contain only the latter pathway, but the situation is more complex in other groups. The *de novo* biosynthetic pathway for cysteine also comprises two steps, catalysed by serine acetyltransferase (SAT, EC 2.3.1.30) and cysteine synthase (CS, EC 4.2.99.8). In plants, distinct isoforms of SAT and CS exist in the mitochondria, plastids, and cytosol [9], while

bacteria contain only one isoform of SAT and CS although there are two types of the latter - designated CS type A (CS-A) and CS type B (CS-B) [10]. SAT, CS and CBS activities have been identified in *T. cruzi*, but only the genes for SAT and CBS have been described [5], whereas *E. histolytica* contains multiple genes for SAT and CS [6]. *T. vaginalis* also has multiple type B CS genes but lacks the SAT gene; instead it uses phosphoserine as the precursor for synthesis of cysteine [11].

We had identified genes apparently encoding SAT and CS in *Leishmania* [12], but the genome also contains genes encoding all four enzymes of the transsulfuration pathway (both forward and reverse). Thus the *in silico* evidence suggested a functional redundancy in *Leishmania* for the generation of cysteine. The aim of this study was to determine whether or not the predicted enzymes function as postulated and to characterise those that do. Moreover, we wished to determine the interplay between two routes whereby cysteine may be synthesised, to investigate how they may be regulated to control cysteine homeostasis, and whether uptake of exogenous cysteine is also important. One notable way in which biosynthesis of cysteine is controlled in some organisms is through complex formation between SAT and CS, thus the potential for such complex formation in *Leishmania* has been studied. As mammals lack the *de novo* biosynthesis of cysteine, this pathway, if operational and important in *Leishmania*, could be exploitable as a drug target.

Experimental

Strains and cultivation of *L. major* - Promastigotes of *L. major*

(MHOM/IL/80/Friedlin) were used throughout this study. They were grown at 27°C in either Modified Eagle's Medium designated HOMEM (Gibco, UK) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FCS) for routine maintenance [12] or, for the experiments investigating the use of exogenous organic sulphur sources, in Sulphur Depleted Medium (designated SDM) comprising Minimum Essential Medium (MEM) (ICN-biomedical) without an exogenous sulfur source (none of cysteine, cystine, methionine and serine) and supplemented with 20% (v/v) dialysed heat inactivated fetal calf serum (dHiFCS) (Sigma-Aldrich, UK), 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 (0.035 mg.L⁻¹) and folic acid (0.02 mg.L⁻¹) or in RPMI 1640 without cystine and methionine (Sigma) and supplemented with MgCl₂ (0.1 g.L⁻¹), HEPES pH 7.2 (40 mM), bioppterin (5 µg.ml⁻¹), hemin (1% v/v) and 20% (v/v) dHiFCS. The sulfur source required for growth of *L. major* promastigotes was investigated by adding methionine (300 µM), cysteine (300 µM), homocysteine (300 µM), serine (280 µM), O-acetylserine (250 µM) or sodium sulfide (80 µM) with and without serine (300 µM). The medium without an exogenous sulfur source acted as the control in these experiments. Cell densities were estimated using an improved Neubauer hemocytometer.

Identification and cloning of SAT, CS, and CBS of *L. major* - The genome database for *L. major* (<http://www.ebi.ac.uk/Tools/blast2/parasites.html>) was searched using the TBLASTN algorithm with protein sequences involved in cysteine *de novo* biosynthesis and RTS pathways from bacteria, yeast, plants and parasitic protozoa as queries to identify putative *L. major* proteins involved in these pathways. Expectation values (*E*-value) of the TBLASTN output, amino acid sequence alignments, and

identification of putative key domains known to be key components for enzymatic activities were all studied to determine if there was significant orthology. The *Leishmania* proteins identified were assigned as orthologues potentially involved in cysteine biosynthesis and have been annotated in www.geneDB.org with the systematic names serine acetyltransferase (SAT, LmjF43.2850), cysteine synthase (CS, LmjF36.3590), cystathionine γ -lyase (CGL, LmjF35.3230); cystathionine β -synthase (CBS; LmjF17.0250). Enzymes potentially comprising a forward transsulfuration pathway were also identified and annotated as CBL (LmjF32.2640) and CGS (LmjF14.0460).

Genomic DNA (gDNA) of *L. major* was isolated as described by [13]. The open reading frames (ORFs) of the genes putatively involved in the *L. major* cysteine synthesis pathways were amplified by PCR using the Expand High Fidelity PCR system (Roche Molecular Biochemicals) with gene-specific primers modified with appropriate restriction sites (to facilitate cloning into their respective expression vectors) detailed in Table S1. All PCR assays were carried out in a GeneAmp 9600 PCR system (PerkinElmer Life Sciences) for 30 cycles of denaturation (94°C, 15 s), annealing (65°C, 15 s) and extension (72°C, 2 min). Each ORF was verified by nucleotide sequencing (MBSU, University of Glasgow, UK) and cloned into the pET expression vectors (Invitrogen) pre-digested with appropriate restriction enzymes to produce the plasmids detailed in Table S1. The plasmids were used to transform BL21(DE3) for recombinant protein expression to generate recombinant fusion proteins containing a 6x His-tag, which facilitates purification using affinity chromatography.

Protein expression and purification - *L. major* SAT, CS and CBS were expressed from a clone of BL21(DE3) transformed with their respective plasmids (Table S1). Overnight cultures in Luria Bertani (LB) medium were used to inoculate fresh LB supplemented with 100 $\mu\text{g ml}^{-1}$ ampicillin or 25 $\mu\text{g ml}^{-1}$ kanamycin (Table S1) and grown at 37°C until an OD₆₀₀ of 0.6 was reached. Expression of recombinant protein was induced with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) overnight at 15°C. Cells were harvested and resuspended in 5 ml of buffer A (50 mM sodium phosphate, 0.3 M NaCl, pH 8.0 and 25 μM pyridoxal phosphate (PLP)) containing 5 mM imidazole and disintegrated by sonication. Soluble fractions were recovered by centrifugation at 16,000 $\times$ g for 30 min at 4°C. Proteins were affinity purified by applying the soluble fraction to a 13-ml nickel-nitrilotriacetic column (bioCAD 700E workstation) pre-equilibrated with buffer A. The column was washed with 60 ml and 30 ml of buffer A containing 20 mM and 60 mM imidazole, respectively. The His-tagged fusion proteins were then eluted with 500 mM imidazole in buffer A. The SAT-CS bi-enzyme complex was purified in a similar manner from BL21(DE3) co-expressing the N-terminal 6x His tagged SAT and the non-tagged *L. major* CS from separate plasmids (Table S1). The eluants of CS and CBS were dialysed against 50 mM Tris-HCl, pH 7.9, 25 μM PLP overnight at 4°C whereas the eluants containing SAT and the SAT-CS bi-enzyme complex were dialysed against 50 mM Tris-HCl, pH 7.9 at 4°C overnight. All proteins were stored at 4°C and their protein concentrations determined with a Bio-Rad protein assay kit (Bradford) using BSA as a standard. SAT and CS of *Arabidopsis thaliana* were expressed and purified as described previously [14] using the same expression constructs (gifts from Prof R. Hell, Germany) and used as positive controls in this study. Site directed mutagenesis of *L. major* CS and *Arabidopsis* CS was performed with the primer and plasmid combinations specified in

Table S1 using the QuickChange™ site-directed mutagenesis kit (Stratagene) according to manufacturer's instructions.

Enzymic activities of SAT, CS, and CBS - Cysteine synthase activity was determined at 37°C in a 500 µl reaction containing 200 mM potassium phosphate, 1 mg ml⁻¹ BSA, 1 mM EDTA, 0.2 mM PLP, 30 mM OAS, 3 mM Na₂S and 15 ng CS or 0.1 µg CBS as appropriate. The enzyme and substrate were pre-incubated for 5 min at 37°C before starting the reaction by addition of sodium sulfide. Samples were taken after 5, 10 and 15 min at 37°C and the cysteine concentration was quantified using the method of [15]. The first step of this procedure, in which the sample is diluted 5-fold in 0.4 M nitrous acid, effectively stops the reaction. Serine sulphydrylase (SS) activity of CS, in which serine combines with sulphide to form cysteine, was determined in the same way as described for the CS assay above, except that 50 mM serine was used instead of OAS. To determine whether sulphide produced by the action of mercaptopyruvate sulfurtransferase (MST) on β-mercaptopyruvate (MP) could be utilized by CS for cysteine biosynthesis, assays were run for 5 min with varying concentrations of MP (ranging from 0-10 mM) and excess β-mercaptoethanol (5 mM) and 0.5 µg MST to generate sulfide that could subsequently condense with OAS. 30 mM OAS and 0.5 µg CS were added to the reaction after the preincubation and incubated for a further 5 min; the amount of cysteine produced was then quantified as described above.

Cystathionine production from homocysteine and serine (CBS activity) was determined in a 100 µl reaction containing 50 mM Tris-HCl, pH 7.3, 10 mM serine, 10 mM homocysteine, 0.25 mM PLP and 0.1 µg enzyme. All components were equilibrated to 37°C and the reaction initiated with enzyme. After 2-5 min incubation at 37°C, the reaction was stopped with 50 µl 20% (w/v) TCA and incubated on ice for 10 min. The mixture was centrifuged for 5 min at 16,000 x g and the supernatant used for cystathionine analysis as described by [5]. The amount of cystathionine formed was determined by adding 1 ml of ninhydrin reagent (10 g.L⁻¹ in glacial phosphoric acid) to 130 µl assay supernatant, boiling (5 min), cooling on ice (2 min) and incubating for 20 min at room temperature for colour development. The absorbance was measured at 455 nm. A standard curve was prepared with cystathionine (0-500 nmol) dissolved in ninhydrin reagent and treated as described above to quantify the amount of cystathionine formed. Cystathionine production from homocysteine and cysteine (homocysteine sulphydrylase activity) was determined in a 100 µl reaction containing 50 mM Tris-HCl, pH 7.3, 0.3 mM homocysteine, 5 mM cysteine, 0.25 mM PLP and 0.3 µg enzyme. Again, all components were equilibrated at 37°C and the reaction initiated with enzyme. The amount of cystathionine formed was quantified as described above.

Sulfide production in the cysteine desulphydration reaction, in which cysteine combines with β-mercaptoethanol to form S-2-hydroxyethylcysteine and hydrogen sulfide [16], was monitored by trapping the sulfide with lead acetate [12]. The 1 ml reaction mixture contained 50 mM Tris-HCl, pH 8.0, 10 mM cysteine, 20 mM β-mercaptoethanol, 0.1 mM PLP, 0.33 mM lead acetate and 300 ng CBS or CS (to start the reaction). The hydrogen sulfide released during incubation at 37°C was determined by monitoring the production of lead sulphide through its absorbance at

360 nm. Homocysteine desulfurase activity was measured similarly except that 5 mM of homocysteine was used instead of cysteine.

SAT catalyzes the formation of O-acetylserine and CoA from serine and acetyl-CoA. A 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) assay was used to quantify CoA production as described by [17]. In this, DTNB reacts with the sulfhydryl compound CoA to form TNB⁻, which has an extinction coefficient at 412 nm of 13600. The assay was carried out at 37°C in 100 mM Tris-HCl, pH 8.0, with 0.1 mM acetyl CoA, 1 mM serine, 0.4 mg ml⁻¹ DTNB and 5-50 µg enzyme. The reaction was initiated by the addition of serine and the absorbance at 412 nm was monitored continuously at 37°C for 5-10 min.

Kinetic parameters were calculated using Grafit 5 (Erithacus Software Ltd) software.

Western blot analysis and SDS-PAGE - Rabbit polyclonal α -CS and α -CBS antisera were raised against recombinant proteins of each of SAT, CS and CBS of *L. major* by the Scottish Antibody Production Unit (Carluke, UK), using standard protocols. Parasite lysates were produced by resuspension of parasite pellets in lysis buffer (0.25 M sucrose, 0.25% (v/v) Triton X-100, 10 mM EDTA, 10 µM E-64, 2 mM 1,10-phenanthroline, 4 µM pepstatin A, and 1 mM phenylmethylsulfonyl fluoride). Lysates were centrifuged at 13,000 x g for 30 min at 4°C, and an aliquot of the resulting supernatant (8 µg protein) were subjected to Western blot analysis as described previously [12]. The polyclonal immune rabbit antisera against SAT, CS, CBS, MST and thiol-dependent reductase 1 (TDR1) were diluted 1:2000 (v/v), 1:5000(v/v); 1:2000(v/v); 1:2000(v/v) and 1:5000(v/v), respectively, in Tris-buffered saline containing 1% (w/v) low fat dried milk and 0.1% (v/v) Tween 20. Monoclonal immune mouse serum recognising the His-tag of the proteins was used at 1:5000 (v/v) in the same buffer. Bound antibody was detected using horseradish peroxidase-coupled secondary α -mouse (1:5000) and α -rabbit (1:5000) antibodies (Scottish Antibody Production Unit) and ECL Western blotting detection reagents (Pierce Ltd) according to the manufacturer's recommendations. The antisera against MST and TDR1, used as loading controls, have been described previously [12,18]. ImageJ (<http://rsb.info.nih.gov/ij/index.html>) was used to quantify the Western blot signals in order to make comparisons.

Cysteine transport assay - Transport of [³⁵S]-L-cysteine (American radiochemicals) into *L. major* promastigotes was performed using the oil stop technique as described previously [19]. Briefly, promastigotes were harvested and washed with CBSS pH 7.4 (33 mM HEPES, 98 mM NaCl, 4.6 mM KCl, 0.3 mM CaCl₂, 0.07 mM MgSO₄, 5.8 mM NaH₂PO₄, 0.3 mM MgCl₂ and 14 mM D-glucose) and resuspended in CBSS at 2 x 10⁸ cells ml⁻¹. Cells (100 µl) were then incubated with 25 or 50 nM [³⁵S]-L-cysteine together with non-radioactive cysteine in a microcentrifuge tube containing 200 µl oil (7:1 [v/v] dibutyl-phthalate/mineral oil; $d = 1.018$ g.ml⁻¹) for a period at 25°C, as indicated in Results. Incubations were terminated by centrifugation of cells through the oil at 16,000 x g for 1 min. This separates the radiolabelled cells from extracellular radioactivity. The tubes were frozen in liquid nitrogen and the tips containing the cell pellets cut off, whereupon the contents were solubilised in 2% (w/v) SDS for 30 min. Three ml of scintillation fluid (Optiphase HiSafe III; Perkin-Elmer) were added and left overnight at room temperature. Radioactivity in the cell pellet was then determined using a 1450 MicroBeta Trilax liquid scintillation counter.

The zero uptake background was determined with cells prepared as described above, but with all operations at 0°C and with immediate termination by centrifugation. The assays were carried out using triplicates and transport values were determined by subtraction of the zero uptake background values. Kinetic parameters were calculated using Grafit 5 (Erithacus Software Ltd) software.

Analysis of intracellular thiols levels using HPLC - *L. major* promastigotes were harvested from late log phase cultures using 2500 x g for 10 min and resuspended at 2.5×10^7 in 50 µl of 40 mM N-[2-hydroxyethyl]-piperazine-N'[3-propanesulphonic acid], 4 mM diethylenetriamine pentaacetic acid, pH 8.0, containing 0.7 mM tris(2-carboxyethyl)phosphine and incubated at room temperature for 45 min. Monobromobimane was added to give a final concentration of 1 mM and the mixture was heated for 3 min at 70°C. Extracts were deproteinised by addition of an equal volume of 4 M methanesulphonic acid, pH 1.6, and incubated on ice for 30 min. Proteins were removed by centrifugation at 16,000 x g for 7 min at 4°C and the resultant supernatants were analysed by HPLC (using a UltiMate HPLC system [Dionex] and a GEMINI C18 column [Phenomenex]). The mobile phase consisted of two solvents: solvent A, 0.25% acetic acid; solvent B, 100% acetonitrile. Metabolites were separated at a flow rate of 0.55 ml min⁻¹ by application of the following gradient (% of solvent B): 0 min, 0%; 10 min, 0%; 40 min, 8%; 100 min, 15%; 110 min, 50%; 111 min, 0%; 121 min, 0%. Thiols were detected using a fluorescence detector (excitation, 365 nm; emission, 480 nm). Glutathione, cysteine and trypanothione were identified by comparison of their retention times with those of their respective standards. A standard curve for quantitation was achieved by integration of peak areas of known quantities of thiols, and experimental peak areas were compared with this curve to determine nmol of thiol (10⁸ cells)⁻¹. Total thiols were calculated as the sum of cysteine, glutathione and 2 x trypanothione concentrations. Differences were analyzed using parametric Student's 2-tailed *t* tests. The data presented are from three independent experiments.

Results

1. Identification of genes encoding enzymes of cysteine biosynthesis in *L. major*

We identified *Leishmania* genes likely to encode enzymes involved in cysteine biosynthesis by searching the *Leishmania* genome databases (<http://www.ebi.ac.uk/Tools/blast2/parasites.html>) using protein sequences from plants, bacteria, yeast and parasitic protozoa orthologues as the queries. The *L. major* sequences identified were then back-searched against the SWISSPROT database to confirm gene identity. These analyses suggest that *L. major*, *L. infantum* and *L. braziliensis* all have enzymes comprising two cysteine biosynthetic routes, the *de novo* biosynthesis and the RTS pathways (Table 1, Figure 1). The *L. major* genome contains single copy genes, validated by Southern blot analysis (data not shown), for CBS (*LmjCBS*; LmjF17.0250) and CGL (*LmjCGL*; LmjF35.3230) of the RTS pathway, and CS (*LmjCS*; LmjF36.3590) and SAT (*LmjSAT*; LmjF34.2850) of the *de novo* biosynthesis pathway. Each of the four predicted enzymes has the key residues and motifs reported to be required for activity, as detailed below. In addition, *L. major* encodes cystathionine γ-synthase (*LmjCGS*; LmjF14.0460) and cystathionine β-lyase (*LmjCBL*; LmjF32.2640) forming the forward transsulfuration pathway (TS), implying that the parasites also can convert cysteine into methionine. A similar search of the *T. cruzi* genome (www.geneDB.org), a related parasite, also identified

orthologues of both biosynthetic pathways, although there appear multiple copies of all the genes (Table 1). The *T. brucei* genome (www.geneDB.org), on the other hand, contains single copies of genes encoding enzymes of the RTS pathway but lacks genes encoding the proteins of the cysteine *de novo* biosynthetic and TS pathways. *LmjSAT* encodes a protein of 411 amino acids (45.5 kDa) with significant identities to the multiple SATs of other protozoa - *T. cruzi* (38.4-39.1%) and *E. histolytica* (21.7-30.4%) (Figure S1A). The hexapeptide structural domain required for acyltransferases activity (residues 274-380; *L. major* numbering) and the β -cluster (residues 277-364) that interacts with CS to form the bi-enzyme complex (Figure S1A) [20,21] are also conserved. The residues likely to be involved in acetyl CoA binding comprise Cys³⁷⁶, Pro³⁸³, Gln³⁹¹ and Ser³⁹³, respectively, in contrast to *Arabidopsis* where they are Ser²⁶³, Pro²⁷⁰, Gly²⁷⁷ and Pro²⁷⁹ [22,23].

LmjCS encodes a protein of 342 amino acids (35.5 kDa) with high sequence identities with CSs of other protozoa - 72% for *T. cruzi*, 42% for *E. histolytica* and 34-38% for *T. vaginalis* (Figure S1B). *LmjCS* has the four lysine residues (Lys⁴⁰, Lys⁵², Lys⁶⁷ and Lys¹⁹⁹) required for binding the pyridoxal phosphate (PLP) cofactor and thought to be essential for sulfhydrylase activity [24]. All CS enzymes have a similar structure and catalytic mechanism but show differences in substrate specificity determined in part by the structure of the β 8- β 9 loop that lines the entry to the active site [10]. The short loop found in the type A isoforms of bacterial and plant CSs [25] severely restricts access to the active site and these enzymes have a strict specificity for OAS and sulfide. The type B CS isoforms of bacteria [25], thermophiles [26] and *Trichomonas* [11] have an extended loop containing a conserved charged residue that allows the use of larger, negatively charged molecules such as *O*-phosphoserine and thiosulfate as substrates. Sequence comparison shows that *LmjCS* has the short β 8- β 9 loop similar to bacterial type A CS isoforms and thus we predicted that it has a substrate preference for sulfide and OAS. *LmjCS* also contains the residues (K²²², H²²⁶ and K²²⁷) implicated for plant [27] and bacterial type A [28] CSs in binding to SAT which, together with the presence of the β -cluster residues in *LmjSAT*, suggested that the two are likely to interact to form a *LmjSAT*-*LmjCS* bi-enzyme complex; the equivalent in other organisms plays roles in activating SAT and/or regulating CS activity [27,29]. These residues are absent from type B CS isoforms that do not form a complex with SAT.

CBS is also a PLP-dependent enzyme, evolutionarily related to CS and in most cases with some CS activity [14]. *LmjCBS* encodes a protein of 359 amino acids (39.2 kDa) which has three (Lys⁴², Lys⁵³ and Lys²⁰²) of the four lysine residues reported to be required for activity of other CSs (Figure S1C) [30]. The fourth position in *LmjCBS* is, instead, Glu⁶⁶. *LmjCBS* has relatively high amino acid sequence identity with *LmjCS* (31%) and quite high identities with the region comprising the catalytic domain (residues 1-359; [5]) of CBSs from *L. tarentolae* (83%), *T. cruzi* (56-66%), *H. sapiens* (49%) and *S. cerevisiae* (42%). *LmjCBS*, however, differs from HsCBS in lacking the haem-binding motif and regulatory domain at the N- and C-termini, respectively [31]. The haem-binding motif acts as a redox sensor [32], whereas the regulatory domain controls the tetrameric state of the protein and so CBS activity [32]. Interestingly, the *Saccharomyces* protein (ScCBS) also lacks the N-terminal haem-binding motif [33] but does possess the C-terminal regulatory domain [8,34] whereas TcCBS lacks the C-terminal regulatory domain but has an N-terminal extension (Figure S1C) – although this does not bind haem [5]. *LmjCBS*, TcCBS and ScCBS all also lack the oxidoreductase (CysXXCys) motif of HsCBS [35].

LmjCGL encodes a protein of 552 amino acids (60.6 kDa) and has the PLP-binding moiety (between positions 197-202) and the reactive lysine (Lys¹⁹⁹; *L. major* numbering) required for activity (Figure S1D) [36]. *LmjCGL*'s identities with other CGLs are *Bacillus subtilis* (38%), *S. cerevisiae* (35 %), *H. sapiens* (34%), *T. brucei* (72%) and *T. cruzi* (80%).

2. CS of *L. major* catalyzes the synthesis of cysteine from O-acetylserine

Soluble recombinant *LmjCS* (r*LmjCS*) was produced in large quantities (~25 mg.L⁻¹) using the pET21a+ expression plasmid in BL21(DE3) *E. coli* and purified to apparent homogeneity for analysis (Figure S2). r*LmjCS* activity was optimal at pH 8.0 and 37°C and was stable for several weeks without any appreciable loss of activity when stored at 4°C (data not shown). The apparent K_{cat} s of r*LmjCS* using O-acetylserine (OAS) and sodium sulfide were 2047 sec⁻¹ and 2669 sec⁻¹ for OAS and sulphide, respectively (Table 2). The K_m for sulfide was low (0.13 mM). No activity was detected between OAS and methanethiol or sodium thiosulfate (the detection limit being 0.6 μ mol min⁻¹ mg protein⁻¹). r*LmjCS* was unreactive towards O-acetylhomoserine (50 mM), O-succinylhomoserine (50 mM), O-phosphoserine (100 mM) or serine (50 mM) and sodium sulfide (the detection limit being 0.2 μ mol min⁻¹ mg protein⁻¹). r*LmjCS* had no detectable CBS activity that forms cystathionine from serine and homocysteine (the detection limit being 0.4 μ mol min⁻¹ mg protein⁻¹). r*LmjCS* also has cysteine desulfurase activity hydrolysing cysteine to yield sulfide, but only in the presence of β -mercaptoethanol, with a K_{cat} s of 88.2 sec⁻¹ and 99.0 sec⁻¹ for cysteine and β -mercaptoethanol, respectively (Table 2). It has been proposed that this desulfurase activity has a reaction mechanism similar to the sulfhydrylase reaction [16]. In this, cysteine binds to the enzyme and sulfide by β -elimination to form the α -aminoacrylate intermediate, β -mercaptoethanol then acts as the nucleophile in the second half-reaction to produce S-2-hydroethylcysteine. No desulfurase activity was detected with homocysteine (50 mM) and β -mercaptoethanol or with cysteine or homocysteine and DTT (the detection limit being 0.2 μ mol min⁻¹ mg protein⁻¹). r*LmjCS* was relatively insensitive to propargylglycine (PAG) and hydroxylamine, inhibitors of various PLP-dependent enzymes, with 1 mM PAG and hydroxylamine inhibiting r*LmjCS* sulfhydrylase activity only by 1% and 3%, respectively. On the other hand, 1 mM phenylhydrazine-HCl inhibited CS sulfhydrylase activity of r*LmjCS* by 36 $\pm$ 0.2 %.

The source of the sulfide for the CS activity in *Leishmania* is unclear. Prokaryotes, plants and enteric protozoa assimilate inorganic sulphur into organic sulfur *via* the sulfate reduction pathway [6]. Our genome search suggests that this pathway is absent from *L. major*. Sulfide produced by the action of mercaptopyruvate sulfurtransferase (MST) on 3-mercaptopyruvate in a linked assay with r*LmjCS* gave a specific activity for CS of 1114 $\pm$ 208 μ mol min⁻¹ mg protein⁻¹, with the amount of cysteine produced roughly proportional to the amount of 3-mercaptopyruvate added up to 3 mM (Figure 2).

3. *LmjCBS* can synthesise both cystathionine and cysteine

Recombinant *LmjCBS* (r*LmjCBS*) was produced at high quantities (~32 mg.L⁻¹) in *E. coli* and the enzyme purified to apparent homogeneity (Figure S2) showed multiple enzymatic activities. It was optimally active at pH 8.0 and 37°C for all of its activities and was stable as purified for several weeks at 4°C without any appreciable loss of

activity (data not shown). The CBS activity of rLmjCBS, utilising homocysteine and serine to generate cystathionine (Figure 3, reaction I), had K_{cat} s of 97.7 sec^{-1} and 51.9 sec^{-1} for homocysteine and serine, respectively (Table 2). rLmjCBS was also capable of the β -replacement reaction, 'homocysteine sulphydrylase' (Figure 3, reaction II), in which cysteine and homocysteine form cystathionine and hydrogen sulfide. The homocysteine sulphydrylase reaction was only apparent at concentrations of cysteine $>0.1 \text{ mM}$, whereas homocysteine at concentrations greater than 0.3 mM were inhibitory (Figure S3). Consequently, the kinetic parameters of LmjCBS for this reaction was carried out at homocysteine concentrations (up to 0.3 mM) that were not inhibitory and obeyed Michealis Menton kinetics (Figure S3). The resultant apparent K_{cat} s were 11.1 sec^{-1} and 26.2 sec^{-1} for homocysteine and cysteine, respectively (Table 2). rLmjCBS also showed remarkably high CS activity with OAS and sodium sulfide (Figure 3, reaction III), the apparent K_{cat} s being 2269 sec^{-1} and 1516 sec^{-1} , respectively. However, the K_m for sulphide was relatively high (10.7 mM). rLmjCBS also had serine sulphydrylase activity (cysteine being formed from serine and sodium sulfide; Figure 3, reaction IV) with K_{cat} s for serine and sodium sulfide being 14.2 sec^{-1} and 14.1 sec^{-1} , respectively (Table 2). LmjCBS was also capable of the 'activated serine sulphydrase' activity, otherwise known as cysteine desulfurase (which generates S-hydroxyethylcysteine and hydrogen sulfide from cysteine and β -mercaptoethanol; Figure 3, reaction V), with K_{cat} s of 38.3 sec^{-1} and 20.7 sec^{-1} for cysteine and β -mercaptoethanol, respectively (Table 2). In the absence of β -mercaptoethanol there was a small activity, about 10% of that in the presence of β -mercaptoethanol; this is called reverse serine sulphydrase and forms serine and hydrogen sulfide from cysteine.

4. LmjCS enhances the activity of LmjSAT *in vitro*

LmjSAT was generated as recombinant enzyme (rLmjSAT) in soluble form using a *E. coli* system, but expression was low ($\sim 0.5 \text{ mg.L}^{-1}$). Using standard assay conditions described for other SATs, we failed to detect SAT activity towards serine and acetyl CoA with the purified rLmjSAT (the detection limit being $0.2 \mu\text{mol min}^{-1} \text{ mg protein}^{-1}$) whereas recombinant SAT of *Arabidopsis*, used as a positive control, showed activity of $2.8 \mu\text{mol min}^{-1} \text{ mg protein}^{-1}$. This finding for rLmjSAT was surprising and contrasted to that reported for *T. cruzi* SAT (TcSAT) [5], although LmjSAT has only 17% identity to TcSAT. We hypothesised that LmjSAT needs to be activated by LmjCS. When LmjSAT, tagged at the N-terminus with a 6x His-tag, was co-expressed with LmjCS (without a His-tag) in *E. coli* and the resultant LmjSAT then affinity-purified (see below) and assayed, SAT activity was detected (specific activity of $90 \text{ nmol min}^{-1} \text{ mg protein}^{-1}$; a rate 4-fold higher than background under the experimental conditions used) at a rate comparable to that reported for TcSAT [5]. No kinetic analysis was possible due to low availability of rLmjSAT. These data suggested that LmjCS stabilises and/or enhances LmjSAT activity, perhaps through formation of a complex.

5. LmjSAT interacts with LmjCS to form a complex *in vitro*

The formation of a SAT-CS complex has been described previously for plant proteins [14,37]. We investigated whether the *Leishmania* proteins do likewise by His-tagging rLmjSAT at its N-terminus (to give His-LmjSAT) and co-expressing in *E. coli* with LmjCS (devoid of any tag). Purification was then conducted using affinity chromatography targeting the His-tag. The expectation was that if LmjCS interacts with His-LmjSAT, the complex should be retained on the column; elution could then be achieved with either OAS (10 mM), which would remove CS from the complex, or

imidazole (500 mM), which would remove the whole complex by interfering with the His-binding. This procedure has been validated for plant enzymes [14,29,37]. SDS-PAGE analysis of the eluates in our experiments revealed that rLmjCS and rLmjSAT were indeed retained on the column and eluted by 500 mM imidazole (Figure 4A; Figure 4Bi, lane 1) similarly to a positive control involving the *Arabidopsis* SAT-LmjCS complex (Figure 4Bii, lane 1). Initial elution of the column with wash buffer (50 mM Tris HCl, pH 8.8, 300 mM NaCl, 80 mM imidazole) containing 10 mM OAS resulted in just rLmjCS being recovered in the eluate (Figure 4Bi, lane 3). rLmjSAT was subsequently eluted by washing the column with elution buffer containing 500 mM imidazole (Figure 4Bi, lane 4). rLmjSAT expressed alone in *E. coli* and purified under similar conditions is shown in Figure 4Bi, lane 2.

With *A. thaliana* CS (designated AtOASTL or AtCS), the positively charged residues K²¹⁷, H²²¹ and K²²² located in loops β 8- β 9 govern its interaction with SAT, and removal of the residues through site-directed mutagenesis disrupts complex formation [27]. Alignment of LmjCS with the AtOASTL revealed that the residues are conserved in LmjCS (K²²², H²²⁶ and K²²⁷) (Figure 4Ci). Thus we generated two mutants (designated LmjCS(K222A) and LmjCS(H226A/K227A) and analysed them for interactions with LmjSAT. As predicted, LmjCS(K222A) and LmjCS(H226A/K227A) failed to interact with the native LmjSAT (Figure 4Cii, lanes 2 and 3) while the native LmjCS bound well (Figure 4Cii, lane 1). Further, our interaction assay also showed that rLmjSAT can interact with CS from *Arabidopsis* (AtOASTL) to form a rLmjSAT/AtOASTL complex (Figure 4Cii, lane 4). As expected, a AtOASTL mutant designated AtOASTL(K217A) showed no interaction with rLmjSAT (Figure 4Cii, lane 5). The CS of *T. vaginalis* (TvCS) also did not interact with rLmjSAT (Figure 4Cii, lane 6), as expected as it is a type B CS and lacks the key residues (Figure 4Ci and [11]). We also showed that SAT from *A. thaliana* interacts with rLmjCS in a similar manner, while LmjCS(K222A) and LmjCS(H226A/K227A) showed much less binding to the *Arabidopsis* SAT (Figure 4Bii).

6. Methionine and not cysteine is essential for the growth of *L. major* promastigotes

It has been reported that cysteine is an essential growth factor for *T. brucei* [4]. We now know that this protozoon encodes genes for only the RTS pathway for cysteine generation, which may be inadequate to satisfy its cysteine requirement. It seemed likely, however, that the existence of the two cysteine biosynthetic pathways in *Leishmania* would make it insensitive to the absence of an exogenous source of cysteine. This hypothesis was tested using *L. major* wild type promastigotes cultured in a semi-defined medium depleted of serine, cysteine and methionine (designated sulphur-depleted medium: SDM) and supplemented with 20% (v/v) dialysed foetal calf serum (dFCS). Cultures were initiated with promastigotes at 2.5×10^5 cells ml⁻¹ and growth monitored over 5 days. Promastigotes in the control experiments were maintained in normal medium supplemented with 10% (v/v) normal FCS. As expected, promastigotes maintained in normal medium grew well but growth was retarded in promastigotes maintained in SDM although they remained motile and viable (Figure 5). The addition of exogenous cysteine or methionine at 300 μ M had no significant effect on the growth of *L. major* promastigotes (Figure 5A), nor did addition of thiosulfate. Exogenous serine (at 300 μ M), however, resulted in enhanced growth (Figure 5A). These data are consistent with the absence of genes encoding proteins involved in the *de novo* synthesis of serine in this protozoon [6] and thus with

exogenous serine being essential [38]. Thus serine was added to SDM (this medium being designated SSDM) and a reanalysis of the importance of the other additions was carried out (Figure 5B). Of all the substances added, only methionine restored promastigote growth to that of the control cells maintained in normal medium (Figure 5B). Addition of cysteine failed to significantly stimulate growth of promastigotes maintained in SSDM ($P>0.5$). Higher concentrations of cysteine ($> 300 \mu\text{M}$) retarded promastigote growth; indeed death ensued within 24 h at concentrations of 10 mM (data not shown). In total, these data suggest that *L. major* differs from *T. brucei* in that methionine and not cysteine is markedly beneficial for growth.

Next, we investigated the effect of the exogenous sources of organic sulfur on the thiol levels in *L. major* promastigotes cultured in RPMI (lacking cysteine and methionine) supplemented with cysteine or methionine by using HPLC (Figure 5C). Growth of *Leishmania* promastigotes in RPMI and SSDM was equivalent under all conditions tested (data not shown). Promastigotes cultured in normal medium were used as the controls (Figure 5C, lane 4). Compared with the control promastigotes, the total thiol levels were reduced 3.8-fold ($P<0.005$) in promastigotes cultured in RPMI and to 59% ($P<0.05$) in RPMI supplemented with cysteine. In contrast, the total thiol levels in promastigotes cultured in RPMI supplemented with methionine were slightly higher (1.4-fold, $P<0.05$) than those in the control promastigotes in normal medium (Figure 5Ci). The decreased thiol levels in *L. major* promastigotes cultured in RPMI or RPMI supplemented with cysteine correlated well with the reduced growth observed (Figure 5B). Analysis of the levels of cysteine, glutathione and trypanothione revealed detailed differences (Figure 5Cii). The thiol concentrations in control cells are similar to those previously reported [39]. Compared with promastigotes grown in normal medium, cells grown in RPMI showed reduced levels of all thiols. Cells grown in RPMI with methionine had similar levels of cysteine and glutathione to the control but trypanothione levels were increased 2.4-fold ($P<0.005$). Cells grown in RPMI supplemented with cysteine showed increased levels of glutathione (2.3-fold, $P<0.05$) and trypanothione (2.8-fold, $P<0.05$) compared with promastigotes cultured in just RPMI, which apparently accounted for the increase in total thiol content (2.2-fold higher, $P<0.05$). The levels of glutathione and trypanothione in RPMI supplemented with cysteine were relatively similar to the levels in the control cells grown in normal medium. Importantly, however, addition of exogenous cysteine did not result in an increase in the intracellular level of cysteine, which was only 12 % of the level seen in the control cells.

Promastigotes maintained in SSDM had increased levels of LmjCS, as judged by Western blot analysis (Figure 5Di, lane 1). This increase was validated by densitometric analysis using ImageJ software (Figure 5Dii). LmjCS and LmjCBS in promastigotes growing in SSDM with cysteine (Figure 5C, lane 3) or methionine (Figure 5C, lane 4) was relatively similar to the level in wild type cells in normal nutrient-rich medium (Figure 5C, lane 2). These data suggest that enzymes from cysteine biosynthetic pathways are present and presumably functional in promastigotes, and that the level of CS can be adjusted in attempts to accommodate variations in the availability of exogenous amino acids.

7. Transport of [^{35}S]-L-cysteine into *L. major* promastigotes

Having established that an exogenous source of cysteine does not stimulate growth of *L. major* promastigotes and has little effect on the intracellular cysteine levels,

whereas exogenous methionine does, we examined the ability of promastigotes to utilize an exogenous source of cysteine. *Leishmania* is known to take up exogenous methionine [40]. A transport assay with [35 S]-L-cysteine showed that *L. major* promastigotes can take up cysteine and that the transport was linear for at least 5 min (Figure 6A, insert) but declined markedly by 25 min (Figure 6A). [35 S]-L-cysteine transport measured as a function of different concentrations of extracellular cysteine showed a typical Michaelis-Menton hyperbolic curve and displayed an apparent K_m of $229 \pm 39 \mu\text{M}$ ($n = 3$) and a $V_{\max}$ of $16.9 \pm 1.3 \text{ pmol min}^{-1} (10^7 \text{ cells})^{-1}$ (Figure 6B). The transport of cysteine was investigated using a range of non-radioactive cysteine, which showed that as expected cysteine inhibited [35 S]-L-cysteine transport into promastigotes (Figure S4). These results show that cysteine can be transported into *L. major* promastigotes, at only at a low rate.

Discussion

This study has shown that *L. major* possesses enzymes of each of two pathways for cysteine synthesis - the *de novo* biosynthesis pathway comprising serine acetyltransferase (SAT) and cysteine synthase (CS) and the reverse transsulfuration (RTS) pathway comprising cystathionine β -synthase (CBS) and cystathionine γ -lyase (CGL). Biochemical analysis of the recombinant CS has shown that the enzyme has high activity with O-acetylserine (OAS) and sulfide as substrates (Table 2) but is unable to utilise thiosulfate and phosphoserine. This substrate preference suggest that the *Leishmania* enzyme is similar to type A CSs of bacteria and has a $\beta\delta$ - $\beta\theta$ surface loop that restricts access to the active site pocket. The protein structure predicted from the gene sequence is in agreement with this (Figure S1B). The type B CSs of bacteria [25] and the CSs of *A. pernix* [26] and *T. vaginalis* [11] all have a different structure in this region, centred around a conserved positively charged residue that confers greater flexibility and allows the use of larger, negatively charged substrates (Figure S1B).

It has been shown that type A CSs of bacteria and *Arabidopsis* CS form bi-enzyme complexes with SAT, that are dissociated by OAS in the absence of sulfide [14,29,41]. The C-terminal residues of SAT bind to sequence motifs in the $\beta\delta$ - $\beta\theta$ surface loop and the substrate binding-loop in the active-site cleft; this completely fills the active site of CS and results in inhibition of its activity [27,29,42]. The substrate binding loop is conserved in all CS enzymes whereas the SAT-binding motif in the $\beta\delta$ - $\beta\theta$ loop is not found in the type B CSs of bacteria, which do not form a complex with SAT. Consistent with this, the CS enzymes of organisms that do not contain genes for SAT, notably *A. pernix* [42] and *T. vaginalis* [11], also lack this binding motif in the $\beta\delta$ - $\beta\theta$ loop. The gene sequences of the CSs of *Leishmania* show that the proteins do contain this SAT-binding motif and the consequent prediction that the enzyme will form a complex with SAT was confirmed in this study (Figure 4). Mutagenesis of LmjCS also indicated that residues equivalent to those of AtOASTL are involved in the binding to SAT (Figure 4).

The function of the CS-SAT bi-enzyme complex in *Leishmania* remains to be proven, but based on the findings for bacteria and plants [14,17,37,44] it is likely to have a regulatory role. The two enzymes show opposite responses to being part of the complex: SAT is inactivated or less active when released from the complex, whereas CS is completely inactive when in complex with SAT but highly active as a free

enzyme [14,29,45]. The data for *Leishmania* SAT is consistent with this, as SAT activity could only be detected when co-expressed with CS, but how this plays a part in cysteine homeostasis requires additional study.

The source of sulfide for cysteine synthesis in *L. major* is unclear. Plants, bacteria, and *Entamoeba* assimilate inorganic sulphur *via* the sulfide reduction pathway, but genes encoding enzymes of this pathway appear to be absent from the *L. major* genome. We postulated that the sulfide required by CS may be provided by the action of mercaptopyruvate sulfurtransferase (MST) on 3-mercaptopyruvate. This enzyme has previously been implicated in sulfide production and antioxidant defence [12] and we have now shown that LmjCS can indeed use sulfide emanating from the activity of MST to form cysteine at a rate similar to that when sulfide itself is provided (Figure 2). Such activity *in vitro*, however, does not mean that this is the *in vivo* source; this suggestion needs to be validated by genetic studies. An alternative is that sulfide could potentially be provided exogenously for the parasite in the parasitophorous vacuole, and/or the insect gut, although evidence that this is so is currently lacking.

This study has also established that *L. major* CBS is clearly different from mammalian CBSs; it lacks the N-terminal haem-binding motif and the C-terminal regulatory domain present in mammalian CBSs, moreover it is capable of using a wide variety of substrates (Table 2). LmjCBS does, unsurprisingly, possess CBS activity - to produce cystathionine (Figure 3; Table 1), but can also form cystathionine and sulfide with homocysteine and cysteine *via* β -replacement reaction, and was capable of producing sulfide *via* the cysteine desulfurase reaction by hydrolysing cysteine (Figure 3; Table 1). LmjCBS, however, can also form cysteine from OAS and sulfide. The K_{cat}/K_m of LmjCBS towards OAS is ~ 7 -fold higher than that of LmjCS, however its K_{cat}/K_m for sulfide is some 140-fold lower (Table 2). This suggests that CS and CBS are adapted to different physiological conditions, in which the relative concentrations of the two substrates are changed. Unfortunately, there is little or no information on the concentration of the two substrates in the various developmental stages of the parasite. However, there is some evidence that amastigotes have increased capacities for β -oxidation, the TCA cycle, mitochondrial electron transport and oxidative phosphorylation [2,46]. This could result in increased availability of acetyl CoA for OAS synthesis and a decreased availability of sulfide due to increased utilisation for the synthesis of iron sulfur clusters and lipoic acid. Under these circumstances, one would predict that CS rather than CBS would be functional in *de novo* cysteine biosynthesis. However, these speculative suggestions need to be rigorously tested experimentally.

Other CBSs also have CS activity. Human CBS, previously thought to lack CS activity [47], has recently been reported to have such activity but with a very low catalytic efficiency of K_{cat}/K_m $135 \text{ s}^{-1} \text{ M}^{-1}$ [48]. The difference between HsCBS and CBS from lower eukaryotes (which have significant CS activity, detailed below) is thought to be due to the active site pocket of HsCBS being constricted by the N-terminal haem-binding and C-terminal regulatory domains (Figure S1C) - such that it cannot accommodate the large acetyl group of OAS at its serine-binding site; whereas CBSs lacking one or other of domains can [48]. The CBSs from *T. cruzi* (TcCBS) and *S. cerevisiae* (ScCBS) have significantly greater CS activities than HsCBS, with the K_{cat} s for OAS being 12.5 sec^{-1} and 19.2 sec^{-1} , respectively, and K_m s of 4.9 mM and 1.3 mM for OAS and 4.1 mM and 16.6 mM for sodium sulfide, respectively [5,8]. These K_{cat} values are similar to those of the CBS activities of the same enzymes [43]. This is a very different situation from the *L. major* CBS, which has a CS activity

23-fold higher than its CBS activity (Table 2). We suggest that the explanation for the much higher CS activity of LmjCBS is that it lacks both the N- and C-terminal extensions that in the human enzyme constrict entry to the active site cleft, whereas both ScCBS and TcCBS have one of these extensions (Figure S1C). Interestingly, only three of the eight *T. cruzi* CBS genes have an N-terminal extension (www.geneDB.org), and the isoform with the longest extension is the one characterised [5]. The other *T. cruzi* CBS isoforms encoded in the genome (www.geneDB.org) may have more similar biochemical properties to the *L. major* CBS.

The functional significance of the multiple reactions carried out by *L. major* CBS is unknown but the kinetic parameters of this protein do not rule out that these reactions potentially occur within the cell and may confer selective advantage and flexibility to parasite within its hosts. The possibility that CBS functionally acts *in vivo* as a CS cannot yet be ruled out. Indeed, the finding that the *T. cruzi* CBS gene can overcome a growth defect of both *S. cerevisiae* (WB63yCBSΔ lines- deficient in CBS) and *Escherichia coli* (NK3 lines- a double mutant deficient the bacterial type A (cysK) and type B (cysM) CSs) suggests that this trypanosomatid CBS can functionally act as a CBS and a CS *in vivo* [5]. Thus *L. major* CBS has both CS and CBS activities, and this clearly distinguishes it from LmjCS in that the latter has no detectable CBS activity.

Western analyses showed that CS and CBS are both present in promastigotes of *L. major* (Figure 5), data confirmed for other *Leishmania* species by the global proteomic analyses reported to date - with there being some evidence of protein levels changing between different developmental stages [46]. In an attempt to unravel which pathways operate in promastigotes, we carried out the experiments monitoring parasite growth when different sulfur amino acids are available exogenously (Figure 5). These experiments showed that the parasite does not benefit significantly from an exogenous source of cysteine, in contrast to report for *T. brucei* [4]. Interestingly, however, methionine promoted growth (Figure 5) which correlates with a previous report that it is transported into promastigotes and augments the amino acid pool; importantly it is used predominantly for cystathionine biosynthesis [49]. Our data on intracellular thiol levels confirm this in that addition of exogenous methionine results in thiol levels including cysteine relatively similar to those in normal promastigotes grown in full medium, presumably from conversion of methionine *via* the transsulfuration pathway, whereas addition of exogenous cysteine had little effect on intracellular cysteine levels, although glutathione and trypanothione levels were relatively similar to those in cells grown in normal medium (Figure 5C_i). These data suggested that, unlike methionine, cysteine may not be transported at a sufficient rate to maintain the intracellular pool of cysteine at the level required for normal growth.

Our transport data (Figure 6) confirm this, for although transport occurred its rate was some 200-fold lower than cysteine transport into *T. brucei* [50] and the K_m for cysteine is some 10-fold higher than that for transport of methionine into *Leishmania* [40]. The low V_{max} for transport of cysteine coupled with the K_m of $>200 \mu M$, when the concentration of cysteine in the medium is about $70 \mu M$, is likely to mean that this is not a mechanism used by the parasite for obtaining cysteine. The importance of the cysteine *de novo* biosynthetic pathway was reiterated by the observation that promastigotes in medium lacking an exogenous organic source of sulfur had an up-regulated level of CS (Figure 5D), presumably in an attempt to counter the lack of intracellular cysteine. Thus our results show that *Leishmania* differs considerably not

only from *T. brucei*, which relies upon exogenous cysteine [4,50], but also *T. cruzi*, which transports cysteine *via* a highly specific transporter with a relatively low K_m (49 μ M) to maintain its intracellular cysteine pool and iron-sulfur formation [51]. Our growth data (Figure 5A) also confirmed that serine was an essential amino acid for *Leishmania* that is salvaged by promastigotes and used for growth [38]. This is consistent with the absence from its genome of genes involved in serine biosynthesis [52]. Together these data are consistent with both the CS and CBS pathways operating in promastigotes and that methionine can be converted to cysteine, thus suggesting that CBS certainly functions in cystathionine synthesis *in vivo*.

A key question is why *Leishmania* has two pathways for generating cysteine when many cells cope very well with just a single source. One likely explanation is the availability of exogenous nutrients differs considerably between the parasite's environments in its sandfly and mammalian hosts and that the potential for two functional synthetic routes in the parasite reflects this. For instance, maybe *de novo* synthesis *via* CS occurs in one stage and synthesis from methionine occurs in another. The generation of genetic mutants lacking key genes could be an informative approach to answer these questions. A second possibility is that the two routes occur in different sub-cellular compartments within *Leishmania*. However, neither CS nor SAT contains clear targeting signals for location to organelles and preliminary evidence suggests both are cytosolic. It would be interesting to investigate whether intentionally targeting them to an organelle would have any impact upon cysteine homeostasis in the parasite.

As CS is absent from humans and *L. major* CBS is divergent from the mammalian homologue, both proteins potentially could represent good drug targets. The discovery that the ten C-terminal residues of SAT are inhibitory to CS [53] could form a framework for which specific inhibitors of CS can be designed, whereas the ability of *L. major* CBS to use more bulky substrates than can the human enzyme suggests a way to specifically target the parasite CBS.

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Figure legends

Figure 1. Cysteine biosynthesis pathways in *L. major*. Enzymes present in *L. major* and the reactions they catalysed identified by *in silico* analysis using BLAST algorithmn are given with their geneDB systematic name. Key:- CS, cysteine synthase; SAT, serine acetyltransferase; CBS, cystathionine β -synthase; CGL, cystathionine γ -lyase; CBL, cystathionine β -lyase; CGS, cystathionine γ -synthase.

Figure 2. Activity of enzymes potentially involved in the *de novo* biosynthesis of cysteine. Relationship between the cysteine synthase activity and the sulfide produced from the action of mercaptopyruvate sulfurtransferase on different concentrations of 3-mercaptopyruvate. The reaction mixture containing 3-mercaptopyruvate, β -mercaptoethanol (5 mM) and 0.5 μ g rLmjMST was incubated for 5 min prior to addition of O-acetylserine (30 mM) and 0.5 μ g LmjCS and further incubation for 5 min. The amount of cysteine produced was quantified as described by [15]

Figure 3. A general scheme of reactions catalysed by LmjCBS. Substrates used by the enzyme to synthesize cystathionine and cysteine as well as degrade cysteine are given. Key to reactions: I, cystathionine β -synthase; II, homocysteine desulfurase; III, cysteine synthase; IV, serine sulfydryase; V, cysteine desulfurase.

Figure 4. The LmjSAT-LmjCS complex from *L. major*. (A) His-LmjSAT co-purified with untagged LmjCS and analysed by SDS-PAGE analysis including staining with Coomassie blue. Lanes 1 shows the proteins eluted with 1 M imidazole under denaturing conditions; they are consistent with there being a LmajSAT-LmajCS complex. (Bi) Western blot analysis using α -His and α -LmjCS on eluants from affinity chromatography of LmjSAT in complex with LmjCS and eluted with 500 mM imidazole (lane 1) or first treated with wash buffer containing 10 mM OAS (lane 3) and subsequently with 500 mM imidazole (lane 4). Lane 2 shows the eluant from lysates of *E. coli* expressing rLmjSAT alone. (Bii) SDS-PAGE analysis including staining with Coomassie blue of eluants from affinity chromatography of AtSAT in complex with LmjCS (lane 1), LmjCS(K222A) (lane 2) and LmjCS (H226A/K227A) (lane 3). Molecular masses (kDa) are shown on the left. (Ci). Sequence alignment of LmjCS (this study), AtOASTL [9] and TvCS [11] showing positively charged residues involved in SAT binding marked with asterisks; they were subsequently replaced with an alanine by site-directed mutagenesis to generate the mutants LmjCS(K222A) and LmjCS(H226A/K227A). (Cii) Western blot analysis using α -LmjSAT and α -LmjCS on eluants from affinity chromatography of LmjSAT (shown in top panel) in complex with various CS proteins (bottom panel) and eluted with 1M imidazole. α -LmjCS recognises AtOASTL and TvCS. Key: LmjCS (lane 1), LmjCS(K222A) (lane 2), LmjCS(H226A/K227A) (lane 3), AtOASTL (lane 4),

AtOASTL(K217A) (lane 5) and TvCS (lane 6). Molecular masses (kDa) are shown on the left.

Figure 5. Cell density and thiol levels of *L. major* promastigotes in sulfur depleted media. (A) *L. major* promastigotes at 2.5×10^5 cells ml^{-1} were incubated at 27°C for 5 days in SDM medium (lacking an exogenous sulfur source) and supplemented with cysteine (300 μM), methionine (300 μM), thiosulfate (150 μM), or serine (300 μM). The cell densities were determined initially (represented by grey dashed lines) and at day 5 (black bars). Data are mean $\pm$ SE from 3 independent experiments. (B) Experiments were carried out as detailed in (A) but all treatments were supplemented with serine at 300 μM to give SSDM. Additional supplements were *O*-acetylserine (OAS, 250 μM) or 3-mercaptopyruvate (3-MP, 180 μM). Data are means $\pm$ SE from 2 independent experiments. (Ci) Total thiol content of *L. major* grown under different conditions: RPMI alone (1), RPMI supplemented with 300 μM methionine (2) or cysteine (3); HOMEM medium (4). Results are means $\pm$ S.D. from 3 extracts. (Cii) Intracellular levels of cysteine, trypanathione and glutathione of *L. major* grown under conditions as in Ci. Results are means $\pm$ S.D. from 3 extracts. (Di) Western blot analysis of lysates of promastigotes from (B). SSDM (lane 1), HOMEM nutrient-rich medium (lane 2), SSDM supplemented with cysteine (lane 3) and SSDM supplemented with methionine (lane 4). Molecular masses (kDa) are shown on the left. (ii) Densitometric analysis of signals from LmjCBS compared with TDR1 (top panel) and LmjCS compared with TDRI (bottom panel) using ImageJ software (<http://rsb.info.nih.gov/ij/index.html>).

Figure 6. Cysteine transport in *L. major* promastigotes. (A) Time dependence of cysteine incorporation using 50 nM [^{35}S]-L-cysteine and 25 μM non-radioactive cysteine with 2×10^7 promastigotes. Cysteine incorporation was linear for 5 min (insert). (B) Transport of cysteine during 5 min as a function of cysteine concentration. The experiment involved 2×10^7 promastigotes and various concentrations of cysteine, in all cases provided from a stock solution of 50 nM [^{35}S]-L-cysteine and 500 μM non-radioactive cysteine. The reciprocal plot is shown in the inset.

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Table 1. Genes identified in the genome of *L. major* orthologous to genes encoding enzymes of cysteine biosynthesis

Pathway	Gene name	GI number of query sequence ¹	Query organism	<i>L. major</i> systematic name in GeneDB*	Orthologues in other trypanosomatids			
					<i>L. infantum</i>	<i>L. braziliensis</i>	<i>T. cruzi</i>	<i>T. brucei</i>
De novo	Serine acetyltransferase	13241626	<i>T. cruzi</i>	LmjF34.2850	LinJ34.V2.2710	Absent	Tc00.1047053504013.40	Absent
	Cysteine synthase	2346964	<i>E. histolytica</i>	LmjF36.3590	LinJ36.2007.0420 V3	LbrM35.V2.3820	Tc00.1047053507165.50 Tc00.1047053507793.20	Absent
Reverse Transsulfuration (RTS)	Cystathionine β -synthase	70886353	<i>T. cruzi</i>	LmjF17.0250	LinJ17.2007.0420 V3	LbrM17.V2.0230	Tc00.1047053510381.10 Tc00.1047053506905.50 Tc00.1047053508175.60 Tc00.1047053508177.110 Tc00.1047053508177.120 Tc00.1047053508177.129 Tc00.1047053508241.140 Tc00.1047053509149.9 Tc00.1047053511691.10 Tc00.1047053511691.20	Tb11.02.5400
							Tc00.1047053510739.19	
							Tc00.1047053510661.250	
							Tc00.1047053510741.10	
	Cystathionine γ -lyase	399331	<i>S. cerevisiae</i>	LmjF35.3230	LinJ35.V3.3280	LbrM34.V2.October		Tb09.221.3330
Transsulfuration (TS)	Cystathionine β -lyase	16078253	<i>B. subtilis</i>	LmjF32.2640	LinJ32.V3.2789	LbrM32.V2.October	Absent	Absent
	Cystathionine γ -synthase			LmjF14.0460	LinJ14.V3.0470	LbrM14.V2.October	Absent	Absent

¹GI number is a unique series of digits assigned consecutively to each sequence record processed by NCBI (www.ncbi.nlm.nih.gov).

- www.geneDB.org

Table 2. Kinetic parameters of enzymes of cysteine synthesis

Enzyme	Activity	Substrates	K_m (mM)	K_{cat} (sec ⁻¹)	K_{cat}/K_m (M ⁻¹ sec ⁻¹)
<i>LmjCS</i>	O-acetylserine sulphydrylase ¹	O-acetylserine	17.5 ± 4.8	2047	1.2 × 10 ⁵
		Sulfide	0.13 ± 0.04	2669	2.0 × 10 ⁷
	Cysteine desulfurase ²	Cysteine	3.1 ± 0.7	88.2	2.9 × 10 ⁴
		β-mercaptoethanol	13.8 ± 0.6	99.0	7.2 × 10 ³
<i>LmjCBS</i>	O-acetylserine sulphydrylase ³	O-acetylserine	1.6 ± 0.4	2269	1.4 × 10 ⁶
		Sodium sulfide	10.7 ± 0.9	1516	1.4 × 10 ⁵
	Cysteine desulfurase ⁴	Cysteine	0.8 ± 0.5	38.3	4.8 × 10 ⁴
		β-mercaptoethanol	4.4 ± 1.9	20.7	4.7 × 10 ³
	Serine sulphydrylase ⁵	Serine	3.0 ± 1.3	14.2	4.7 × 10 ³
		Sodium sulfide	6.6 ± 4.2	14.1	2.1 × 10 ³
	Homocysteine sulphydrylase ⁶	Cysteine	7.1 ± 2.0	11.1	1.6 × 10 ³
		Homocysteine	0.54 ± 0.10	26.2	4.9 × 10 ⁴
	Cystathionine β-synthase ⁷	Homocysteine	6.9 ± 1.8	97.7	1.4 × 10 ⁴
		Serine	1.1 ± 0.8	51.9	4.7 × 10 ⁴

For kinetic analyses, ≥ 6 different substrate concentrations were used with at least two replicate assays.

¹ O-acetylserine sulphydrylase activity of *LmjCS*: the K_m for OAS was determined using 3 mM Na₂S with 1- 40 mM OAS and the K_m for Na₂S was determined using 30 mM OAS with 0.1-1 mM Na₂S

² Cysteine desulfurase activity of *LmjCS*: the K_m for cysteine was determined using 50 mM β-mercaptoethanol with 0.2- 20 mM cysteine and the K_m for β-mercaptoethanol was determined using 20 mM cysteine and 0.5-50 mM β-mercaptoethanol.

³ O-acetylserine sulphydrylase activity of *LmjCBS*: the K_m for OAS was determined using 3 mM Na₂S with 0.1-100 mM OAS and the K_m for Na₂S was determined using 100 mM OAS with 1-3 mM Na₂S

⁴ Cysteine desulfurase activity of *LmjCBS*: the K_m for cysteine was determined using 50 mM β-mercaptoethanol with 0.1-20 mM cysteine and the K_m for β-mercaptoethanol was determined using 20 mM cysteine and 1-50 mM β-mercaptoethanol.

⁵ Serine sulphydrylase activity of *LmjCBS*: the K_m for serine was determined using 50 mM Na₂S with 1-50 mM serine and the K_m for Na₂S was determined using 50 mM serine with 1-50 mM Na₂S

⁶ Homocysteine sulphydrylase activity of *LmjCBS*: the K_m for cysteine was determined using 0.3 mM homocysteine with 1-5 mM cysteine and the K_m for homocysteine was determined using 5 mM cysteine with 0.01-0.3 mM homocysteine.

⁷ Cystathionine β -synthase of LmjCBS: the K_m for serine was determined using 50 mM homocysteine with 1-50 mM serine and the K_m for homocysteine was determined using 50 mM serine with 1-50 mM homocysteine.

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Figure 1

Figure 2

Figure 3

Figure 4

C_i

ii

D i

ii

A

B