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Short communication

Biochemical characterization of serine acetyltransferase and cysteine desulfhydrase from *Leishmania major*

Daniela Marciano [a], Marianela Santana [a], Brian Suárez Mantilla [b], Ariel Mariano Silber [b], Cristina Marino-Buslje [a,b], Cristina Nowicki [a,*]

[a] Instituto de Química y Fisicoquímica Biológica IQUIFIB-CONICET, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, C1113AAD, Buenos Aires, Argentina
[b] Departamento de Parasitologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, Av. Lineu Prestes 1374, São Paulo, Brazil

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ABSTRACT

Cysteine metabolism exhibits atypical features in *Leishmania* parasites. The nucleotide sequence annotated as ***LmjF32.2640*** encodes a cysteine desulfhydrase, which specifically catalyzes the breakdown of cysteine into pyruvate, NH_3 and H_2S. Like in other pathogens, this capacity might be associated with regulatory mechanisms to control the intracellular level of cysteine, a highly toxic albeit essential amino acid, in addition to generate pyruvate for energy production. Besides, our results provide the first insight into the biochemical properties of *Leishmania major* serine acetyltransferase (SAT), which is likely involved in the two routes for *de novo* synthesis of cysteine in this pathogen. When compared with other members of SAT family, the N-terminal region of *L. major* homologue is uniquely extended, and seems to be essential for proper protein folding. Furthermore, unlike plant and bacterial enzymes, the carboxy-terminal-C_{10} sequence stretch of *L. major* SAT appears not to be implicated in forming a tight bi-enzyme complex with cysteine synthase.

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Leishmania parasites are the causative agents of a spectrum of neglected diseases. The current clinical treatments are far from being satisfactory since the available drugs are highly toxic and of low efficacy [1]. These pathogens exhibit unusual metabolic features; therefore, the understanding of their biochemical peculiarities is of great interest for public health. In *Leishmania major*, unlike in mammals, serine acetyltransferase (SAT) and cysteine synthase (CS) are involved in the *de novo* synthesis of cysteine. Moreover *L. major* cystathionine β synthase (CBS), an enzyme with unexpectedly broad substrate specificity, is predicted to participate in this biosynthetic route alternatively to CS. *L. major* CBS appears to be capable of condensing O-acetyl-serine (OAS) or L-serine with sulfide to generate cysteine. Also, this enzyme is expected to utilize L-serine and L-homocysteine for cystathionine production [2]. Hence in *L. major*, SAT is expected to produce the OAS needed by CS and CBS to generate cysteine. SATs and CSs are typically functional in plants and bacteria, the activities of these enzymes are strictly regulated by two complementary mechanisms (i) the strong feedback inhibition of SATs by cysteine; and (ii) the cysteine synthase complex (CSC) formation, which as a result of specific protein–protein interactions leads to a notable SAT activation and CS inactivation [3]. On the other hand, in eukaryotes and prokaryotes cystathionine is a pivotal intermediate for both transsulfuration pathways. Therefore in mammals, CBS specifically catalyzes the condensation of L-serine and L-homocysteine to form cystathionine, the latter is cleaved into cysteine by action of cystathionine γ lyase (reverse transsulfuration pathway). By contrast, in plants and bacteria, cystathionine is an essential intermediate for methionine biosynthesis (forward transsulfuration pathway). In these organisms, cystathionine β lyases (CBLs) cleave cystathionine into pyruvate, NH_3 and L-homocysteine, which is subsequently methylated by methionine synthase. Both transsulfuration pathways were predicted to be operative in *Leishmania* parasites (http://eupathdb.org/eupathdb/).

Due to the known biological relevance of sulfur-containing amino acids in protozoan parasites [4] we addressed the functional characterization of the putatives *L. major* SAT (**LmjF34.2850**) and CBL (**LmjF32.2640**) by cloning and heterologous expression of the recombinant enzymes. Unlike predicted, ***LmjF32.2640*** did not encode a CBL, but rather an enzyme that specifically catalyzed the decomposition of cysteine into pyruvate, H_2S, and NH_3. Hence, hereafter this coding sequence will be referred as cysteine desulfhydrase (CD). Notably, *L. major* CD displayed the highest sequence relatedness (about 50% identity, E: $4e^{-137}$) with numerous bacterial homologues yet experimentally uncharacterized. However, when *L. major* CD was compared with functionally characterized enzymes, a significant identity was observed with *Escherichia coli* CBL (37% identity). Also, a quite high sequence relat-

* Corresponding author. Tel.: +54 11 4964 8291; fax: +54 11 4962 5457.
E-mail address: cnowicki@criba.edu.ar (C. Nowicki).

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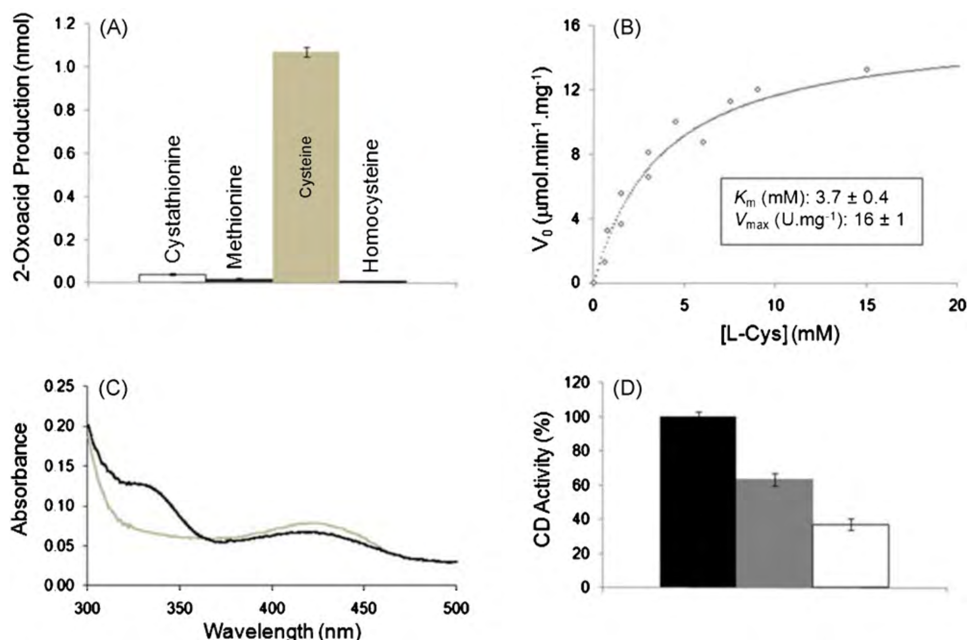

Fig. 1. Functional characterization of *L. major* CD. The nucleotide sequence corresponding to ***LmjF32.2640*** (http://eupathdb.org/eupathdb/) was amplified by PCR using genomic DNA as template in addition to specific forward (5′**GCTAGC**ATGCCCAATCAGTCATTTG3′, NheI site in bold) and reverse primers (5′**AAGCTT**GCAGCCCATCGACTCC3′, HindIII site in bold). The amplified DNA fragment was cloned into pET24a$^+$ (Novagen), the 6xHis-tagged *L. major* enzyme was expressed in *E. coli* BL21(DE3)plysS cells with 0.1 mM of isopropyl-D-thiogalactopyranoside (IPTG) at 28 °C for 4 h. The recombinant protein was purified by affinity chromatography following standard procedures. (A) Analysis of the substrate specificity. The capability of the recombinant *L. major* enzyme to catalyze the breakdown of sulfur-containing metabolic intermediates such as L-homocysteine, L-cysteine, L-methionine and LL-cystathionine was estimated by measuring the formation of 2-oxoacids with 2,4-dinitrophenylhydrazine. (B) Kinetic characterization. The production of SH_2 was determined spectrophotometrically by following the formation of SPb (apparent molar turbidity coefficient of colloidal PbS at 360 nm, ε_M 1.31×10^4 M^{-1} cm^{-1}) with lead acetate [6]. The initial rate (V_0) of H_2S production was measured as a function of the substrate concentration (L-cysteine), the experimental data were fitted to a hyperbola and the apparent kinetic parameters calculated by applying the Gauss–Newton algorithm [7]. The obtained values represent the mean of four determinations ± SD and are indicated in the inset of the figure. (C) Absorption spectra. The absorption spectra of *L. major* CD were recorded at 25 °C, in 100 mM Tris–HCl buffer, pH 8.0, which contained the recombinant *L. major* CD (0.4 mg ml^{-1}) in addition to 15 mM of 2-mercaptoethanol. The spectrum of *L. major* enzyme in absence of L-aminoethoxyvinylglycine (AVG) is indicated in grey line and that obtained in presence of 1 mM of this inhibitor in black line, respectively. (D) Inhibitory effect of AVG. The capability of *L. major* CD to catalyze the breakdown of cysteine was assayed in presence of 0 (■), 0.15 μM (■) and 1000 μM (□) of AVG. The activity was measured upon 2 min pre-incubation of the leishmanial enzyme with AVG as previously described [8]. The values are the means of four independent measurements and the error bars represent the SD.

edness (close to 30% identity) was revealed with other members comprised within the subgroup of the Cys/Met-metabolism related enzymes (Supplementary material, Fig. 1SA and SB). The phylogenetic reconstruction of 29 protein sequences belonging to Iγ PLP-dependent enzymes performed by using the neighbor-joining, maximum parsimony and maximum likelihood methods, consistently showed that the *L. major* CD (**LmjF32.2640**) was grouped jointly with the other leishmanial orthologues (**LinJ32_V3.2780**, **LbrM32V2.2880** and **LmxM31.2640**). Remarkably, this subgroup was included within the same clade that bacterial CBLs such as the enzymes from *E. coli* and *Salmonella enterica*. Moreover, these analyses revealed that *L. major* also exhibited an homologue not functionally characterized yet, but annotated as CBL-like protein (**LmjF14.0460**). Notably, this putative enzyme was clustered within the same clade as plant CBLs (Fig. 1SB).

The recombinant *L. major* CD was expressed with a 6xHis-Tag attached to its C-terminus and purified to protein homogeneity by affinity chromatography. According to the predicted nucleotide sequence, a single protein band with a relative molecular mass of 45 kDa was evidenced in SDS-PAGE (Supplementary Fig. S2). On the other hand, in gel filtration chromatography under native conditions, the recombinant *L. major* CD exhibited an elution volume which perfectly matched the value expected for a tetrameric protein (not shown). This result fitted in well with the homotetrameric organization reported for the majority of members of the subfamily of Cys/Met-metabolism PLP-dependent enzymes. The substrate specificity of **LmjF32.2640** was explored by monitoring the production of 2-oxoacids upon incubation with L-cysteine, L-homocysteine, L-methionine and LL-cystathionine. Unexpectedly, the recombinant *L. major* enzyme was completely unable to catalyze the breakdown of cystathionine into pyruvate, NH_3 and L-homocysteine. However, this enzyme specifically catalyzed the decomposition of cysteine into H_2S, NH_3 and pyruvate (Fig. 1A). At saturating concentration of L-cysteine, the recombinant enzyme exhibited an apparent K_m of 3.7 ± 0.4 mM and a V_{max} of 16.0 ± 1 U mg^{-1} (Fig. 1B). Like most of the members of the Iγ-subfamily PLP-dependent enzymes, the recombinant *L. major* CD displayed the canonical absorption spectrum at neutral pH range with a maximum in the region of 420 nm (Fig. 1C). In the presence of 1 mM L-aminoethoxyvinylglycine (AVG), the spectrum of *L. major* CD exhibited a decrease at 420 nm with a concomitant increase at 341 nm. Moreover like plant and bacterial CBLs, *L. major* CD decomposed cysteine less efficiently in the presence of AVG, in fact its catalytic competence was lowered in parallel to the increase of the inhibitor concentrations (Fig. 2D).

The capability of *Leishmania* parasites to catabolize cysteine producing H_2S has not been described so far. However, a similar capacity to decompose cysteine into pyruvate, H_2S and NH_3 was reported in pathogenic bacteria such as *Treponema denticola*, *Streptococcus anginosus*, *Mycobacterium tuberculosis*, among others. In *M. tuberculosis*, CD is expected to control the intracellular pool of this highly toxic albeit essential amino acid, as well as to provide pyruvate for energy production [5]. Interestingly, a large amount of evidence supports the current belief that endogenous gases such as H_2S can function as signaling molecules for physiological and pathological processes. The ability to produce H_2S is considered as a potential virulence factor of Gram-negative bacteria and is associated with the pathogenic processes these organisms cause

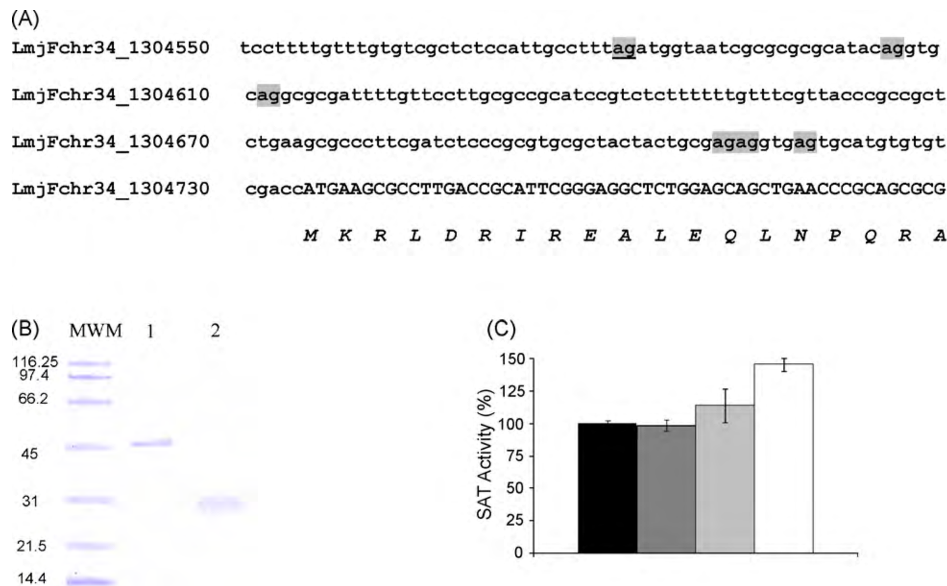

Fig. 2. Functional characterization of *L. major* SAT. (A) Identification of the splice leader site for *L. major* SAT. PCR reactions were performed using *L. major* cDNA as template, *Taq* DNA polymerase (Fermentas), a forward primer corresponding to part of the spliced leader sequence (5′CGCTATATAAGTATCAGTTTC3′), and a reverse primer (5′CAAGCGATGCCTTTAAAGCGTC3′) designed to anneal 456-pb downstream of the initiation ATG codon predicted for ***LmjF34.2850***. The figure depicts the nucleotide sequence comprised between the positions 1304550 and 1304790 of chromosome 34 of *L. major* Friedlin strain (http://eupathdb.org/eupathdb/). The splice leader acceptor site (AG) identified by RT-PCR 153-pb upstream from the ATG initiation codon predicted for the putative *SAT* (***LmjF34.2850***) is highlighted in grey and underlined. The 5′UTR is in lower case; possible *trans* splice acceptor sites (AG) are highlighted in grey; the nucleotide sequence downstream of the initiation ATG codon is in upper case, and the amino acid sequence corresponding to the first 19 residues is in italics. To assess the functionality of the putative *L. major* SAT (**LmjF34.2850**), three different constructs were made, which comprised the predicted full-length ORF and the two variants shortened in the 5′ends by the first 201 and 381 nucleotides, respectively. The nucleotide sequences were amplified by PCR using *L. major* genomic DNA as template in addition to forward and reverse primers specifically designed for each construct, the restriction enzymes sites (NdeI and XhoI) are indicated in bold: fw-Full_length_SAT: 5′**CATATG**AAGCGCCTTGACCGCATTC3′; fw-Δ201_SAT: 5′GC**CATATG**GAGTGGGTGATTGGC3; fw-Δ381_SAT: 5′AG**CATATG**GAGTCGAGAAGCACAAAAGG3′; and rev-Full_length_SAT: 5′**CTCGAG**TCAGATGCCAGAGCCATCC3′. The amplified DNA fragments were cloned into pET19a⁺ (Novagen). The recombinant proteins which exhibited their N-terminus extended by a 6xHis-Tag and a TEV cleavage site were expressed in *E. coli* Rosetta (DE3) with 0.1 mM IPTG, at 20 °C during 4 h. (B) SDS-PAGE analysis. The recombinant *L. major* SAT was purified by affinity chromatography and the protein homogeneity was assessed by SDS-PAGE and Coomassie brilliant blue staining. Lane MWM, molecular weight markers; the values in kDa are shown on the left side of the panel; lane 1, *L. major* SAT immediately heated in sample buffer after purification and lane 2, *L. major* SAT upon storage for short periods of time at room temperature or 4 °C. (C) Activation of *L. major* SAT by homologous CS. SAT activity was determined in 150 mM triethanolamine buffer, pH 7.4, which contained 0.1 mM acetyl-CoA, 20 mM L-serine and 0.2 mM 5,5′-dithio-bis(2-nitrobenzoic acid). The increase of absorbance at 412 nm ($\varepsilon_M^{2\text{-nitro-5-thiobenzoate}}$ $1.36 \times 10^4\,M^{-1}\,cm^{-1}$) was measured [13]. The extent of *L. major* activation by homologous CS was determined in presence of 0 (■), 0.9 (■), 4 (■) and 9 (□) molar excess of the recombinant homologous CS (calculated per monomer). The values are the means of four independent measurements, and the error bars represent the SD.

in mammals [9]. In addition, H_2S is also believed to be involved in plant–pathogen interactions [10]. It is tempting to speculate that in *Leishmania* parasites the production of H_2S might also be somehow related with a mechanism for establishing the infection in the mammalian host.

On the other hand, nucleotide sequences encoding putative SATs are highly conserved in *Leishmania* parasites such as *L. infantum*, *L. major*, *L. brasiliensis* and *L. mexicana* (>90% identity). In cell free-extracts of *L. mexicana* promastigotes, the specific activity of SAT was significantly higher (12.7 ± 0.3 nmol min^{-1} mg^{-1}) than the values determined for this enzyme in spinach stroma (0.53 nmol min^{-1} mg^{-1}) or in protein extracts from tobacco leaves (2.79 nmol min^{-1} mg^{-1}) [11,12]. Leishmanial putative SATs also revealed a significantly high sequence identity with counterparts from different sources such as bacteria (*Bacteroides vulgatus*, 53%; *Heamophilus influenzae* 35%; *E. coli*, 36%), protozoa (*Entamoeba histolytica* isozymes, around 42%; *Trypanosoma cruzi* 64%) and plants (*Nicotiana tabacum* 41%; *Arabidopsis thaliana*, 42%). Remarkably, the highest similarities corresponded to the sequence stretch which comprised approximately the last 230 residues of leishmanial SATs. Moreover, the N-terminal region of the leishmanial enzymes (the first 200 amino acids) was shown to be strictly conserved among these putative SATs as well as uniquely extended among the members of the serine acetyltransferase family (Supplementary Fig. S3). In line with these observations, RT-PCR experiments demonstrated that the AG recognized as the splice leader acceptor site was 153-pb upstream of the initiation ATG codon predicted in the genome databases (http://eupathdb.org/eupathdb/). These findings evidenced that the initial ATG indeed could correspond to the first methionine predicted for leishmanial SATs (Fig. 2A). In addition, five potential splice sites in the context of rich pyrimidine tracks were also identified upstream of the first ATG of the annotated *L. major* ORF (***LmjF34.2850***). The full-length *L. major* SAT (**LmjF34.2850**) in fact coded for a functional protein, the N-terminal 6xHis tagged form of the recombinant SAT rendered a soluble and active enzyme. As expected, a unique protein band with an apparent molecular mass of 45 kDa was evidenced in SDS-PAGE (Fig. 2B, lane 1). By contrast, the two truncated variants of *L. major* SAT in which the first 67 and 127 amino acids were deleted, systematically rendered insoluble proteins. These findings jointly with the RT-PCR experiments suggested that leishmanial SATs very likely represent the homologues with the largest subunits within the SAT family and that their extended N-terminal region might play an essential albeit still unknown role. The full-length *L. major* SAT yielded 1.3 mg of pure recombinant protein per liter of *E. coli* culture. However, the recombinant enzyme turned out to be outstandingly unstable at the protein level (Fig. 2B, lane 2), only when tested immediately upon purification *L. major* SAT exhibited a specific activity of 1.9 U mg^{-1}. The stability of the recombinant SAT was not improved in the presence of the homologous CS, and the recombinant *L. major* enzyme was only barely activated (40 ± 0.2%) in the presence of the *L. major* CS (Fig. 2C). Opposed to *L. major* SAT, values in the range of

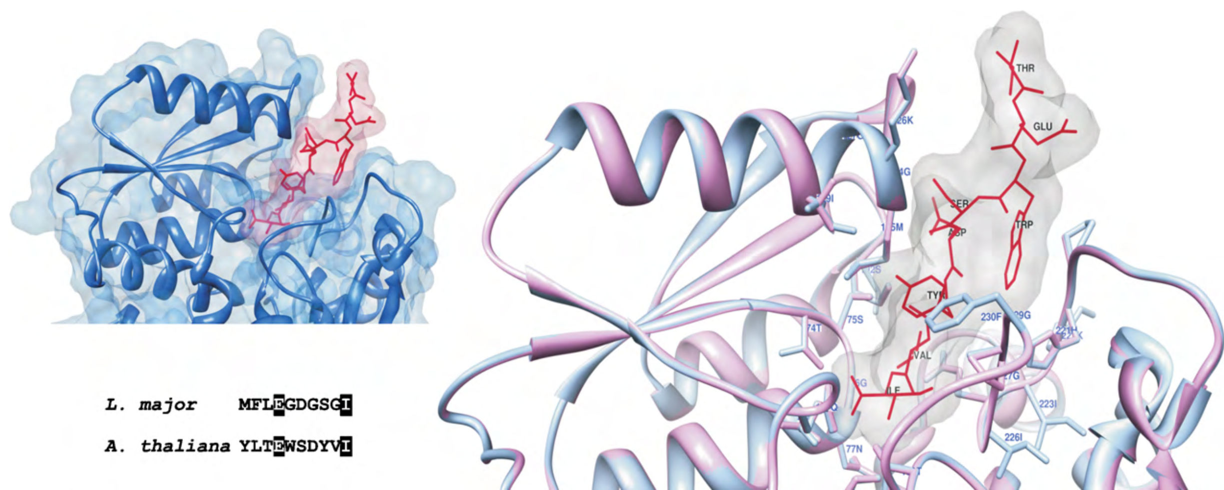

Fig. 3. Molecular model of the cavity of *L. major* CS which might potentially harbor the *L. major* SAT-C_{10} peptide. The sequence stretch comprising the residues 11–325 of *L. major* CS (**LmjF36.3590**) was modeled using the crystal structure of CS from *A. thaliana* in complex with the C_{10} peptide of the homologous SAT (PDB code, 2ISQ) as template [15] and the SwissModel server v 3.7 (http://swissmodel.expasy.org/) [18]. *L. major* model CS (pink) and template (light blue) are illustrated as ribbon representation on the right side of the panel, the residues that interact with *A. thaliana* SAT-C_{10} peptide are shown as sticks, and labels correspond to *A. thaliana* CS numbering. *A. thaliana* SAT-C_{10} peptide is indicated as red wire, and its surface is shown in grey. On the left side of the panel, the surface contacts between *A. thaliana* CS and the homologous SAT-C_{10} peptide are depicted. The figures were created using Chimera [19]. In the bottom of the figure, a comparison of the sequences corresponding to the last 10 amino acids of *L. major* and *A. thaliana* SATs are shown. Identical amino acids are indicated in white lettering on black background. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

2- and 7-fold activation have been reported for plant homologues [14,15].

In an attempt to obtain additional evidence of the capability of leishmanial SATs to associate with homologous CSs by forming a plant-like complex, 1001 sequences of acetyltransferases retrieved by Blast search were aligned using COBALT. This analysis confirmed that beyond the last hexapeptide repeat, leishmanial SATs revealed insignificant sequence similarities with the other members of this family. Based on the high sequence relatedness between *L. major* and *A. thaliana* CSs, we decided to build a model of *L. major* counterpart, using as template the three-dimensional structure of *A. thaliana* CS in complex with the homologous SAT-C_{10} peptide [15]. Our results showed that the modeled *L. major* CS and the three-dimensional structure of *A. thaliana* CS displayed a rmsd of 0.2 Å. Consequently, the Ramachandran plot [16] showed that in the modeled CS 88.8% of the residues lay in the most favored regions, 11.2% were in the additionally allowed regions, and no residues were in the generously allowed or disallowed regions (not shown). The carbon backbones and the side chains of those amino acids from *A. thaliana* CS that interact with the homologous SAT-C_{10} peptide were perfectly superposed in the space with the equivalent residues of *L. major* CS. These results fitted in well with the reported interaction between *L. major* CS and *A. thaliana* SAT [2] as well as put in evidence the remarkable analogies between the binding pockets of both enzymes (Fig. 3). The sequence stretch which comprises the last ten amino acids of plant SATs is believed to be mostly responsible for the CSC formation [14,17], however the C-termini of leishmanial SATs do not exhibit significant similarities with the equivalent region of the *A. thaliana* counterpart. Notably, most of the amino acids of the C-termini of leishmanial homologues exhibit less bulky and less hydrophobic side chains, only the last Ile is strictly conserved (for surface complementation see left panel of Fig. 3). Hence, even though the leishmanial SAT-C_{10} peptide might enter within the cavity of CS, it seems unlikely that it could establish the same hydrogen bonds and van der Waals interactions as those that occur between the *A. thaliana* SAT-C_{10} peptide and CS. Since SAT and CS from *L. major* seem incapable of establishing tight protein–protein interactions, it is tempting to hypothesize that the regulatory mechanisms operative in these pathogens might not comprise the formation of the canonical plant bi-enzyme complex. In line with these findings, our phylogenetic analyses showed that SATs from *Leishmania* parasites seemed to be more closely related with orthologues from *E. histolytica* than with plant and bacterial counterparts, which are able to establish the bi-enzyme complex (Supplementary material Fig. 4S). It could be likely that in *Leishmania* sp., SAT and CS might have evolved from ancestral genes belonging to different organisms.

Our findings, jointly with those reported by Coombs and co-workers [2], indicate that cysteine metabolism exhibits atypical features in *Leishmania* parasites; two different *de novo* biosynthetic routes are likely to be functional in these pathogens, SAT being one of the enzymes involved in both processes. Due to this metabolic redundancy, these pathogens very likely need regulatory mechanisms to circumvent the toxicity of this amino acid. Hence, leishmanial CDs might play a pivotal role in those nutritional or metabolic conditions that raise the intracellular levels of cysteine. On the other hand, the incapability of **LmjF32.2640** to act as CBL brings into question the functionality of the forward transsulfuration pathway in *Leishmania* parasites. Currently, the putative enzymes predicted to be involved in the transsulfuration pathways are being functionally characterized in our laboratory. These studies would allow a better comprehension of the metabolic processes regarding the sulfur-containing amino acids in *Leishmania* parasites.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molbiopara.2010.06.004.

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