Methionine adenosyltransferase (MAT, EC 2.5.1.6)-mediated synthesis of S-adenosylmethionine (AdoMet) is a two-step process consisting of the formation of AdoMet and the subsequent cleavage of the tripolyphosphate (PPPi) molecule, a reaction induced, in turn, by AdoMet. The fact that the two activities, AdoMet synthesis and triphosphate hydrolysis, can be measured separately is particularly useful when the site-directed mutagenesis approach is used to determine the functional role of the amino acid residues involved in each. The present report describes the cloning and subsequent functional refolding, using a bacterial expression system, of the MAT gene (GenBank accession number AF179714) from Leishmania donovani, the etiological agent of visceral leishmaniasis. The absolute need to include a sulfhydryl-protection reagent in the refolding buffer for this protein, in conjunction with the rapid inactivation of the functionally refolded protein by N-ethylmaleimide, suggests the presence of crucial cysteine residues in the primary structure of the MAT protein. The seven cysteines in L. donovani MAT were mutated to their isosteric amino acid, serine. The C22S, C44S, C92S and C305S mutants showed a drastic loss of AdoMet synthesis activity compared to the wild type, and the C33S and C47S mutants retained a mere 12% of wild-type MAT activity. C106S mutant activity and kinetics remained unchanged with respect to the wild-type. Cysteine substitutions also modified PPPi cleavage and AdoMet induction. The C22S, C44S and C305S mutants lacked in triphosphatase activity altogether, whereas C33S, C47S and C92S retained low but detectable activity. The behavior of the C92S mutant was notable: its inability to synthesize AdoMet combined with its retention of triphosphatase activity appear to be indicative of the specific involvement of the respective residue in the first step of the MAT reaction.

**Keywords:** methionine adenosyltransferase; S-adenosylmethionine; polyamines; site-directed mutagenesis; cysteine; Leishmania donovani; trypanosomatids.
Long-term exposure of African trypanosomes to the irreversible ornithine decarboxylase inhibitor α-difluoromethylornithine (DFMO) leads to massive intracellular build-up of AdoMet and a potential state of hypermethylation, causing cellular death in the parasite [14]. The information on MAT in trypanosomatids is scant, however. Yarlett et al. [15] described two isozymes with different kinetic constants isolated from Trypanosoma brucei extracts, which, unlike the host enzyme, are only poorly inhibited by AdoMet. A recent report describes the molecular cloning and characterization of a recombinant MAT-II enzyme from Leishmania infantum (similar to mammalian MAT II). The protein contains seven cysteine residues which function in AdoMet synthesis and triphosphatase activities in the recombinant enzyme.

Materials and methods

Reagents, cells and libraries

DNA modification and restriction enzymes were from Boehringer Mannheim. Thermus aquaticus (Taq) polymerase was from Promega and Pyrococcus furiosus (Pfu) polymerase was from Stratagene (La Jolla, CA, USA). Leishmania donovani promastigotes (insect flagellated form) and L82 (Ethiopian) genomic library EMBL-3 were a kindly gift by J.C. Meade (University of Mississippi, USA). Heterologous expression in bacteria was performed in E. coli XL-1Blue strain. All other chemical and reagents were of the highest quality available.

Amplification of MAT2-encoding fragment

To generate a DNA probe, the polymerase chain reaction (PCR) was employed using degenerated oligonucleotides in both, forward and reverse orientations, corresponding to the phylogenetically conserved regions EGHPDK and PGGIVF, respectively. The reaction mixture contained 50 mM KCl, 10 mM Tris/HCl, pH 9.0, 1% (v/v) Triton X-100, 2.5 mM MgCl, 200 pmol of each oligonucleotide primer and 2.5 units of Taq polymerase in a final volume of 50 μL. The amplified product (639 bp) was originated using the sense primer 5′-GAG GCC CAC/T CCC/G GAC/T AAG-3′ and the antisense primer 5′-GGG GCC GGC G/ AAT G/CAC GAA-3′ corresponding to the above expected amino acid sequences, was subcloned into pGEM-T (Promega) and sequenced.

Cloning of L. donovani MAT-II (MAT2 gene)

To isolate the full-length clone, a leishmanial L82 (Ethiopian) genomic library was screened as follows. 50 000 bacteriophages were blotted onto nylon membranes and further treated at 42 °C for 4 h in 5 × Denhardt’s reagent, 5 × NaCl/Cit (75 mM sodium citrate, 750 mM NaCl, pH 7.0), 50 mM sodium phosphate pH 6.5, 100 μg/mL single-stranded calf thymus DNA and 50% formamide [17]. Membranes were hybridized at 42 °C in the same solution containing 10−6–10−7 cpm of the random-priming 32P-labeled 639 bp PCR fragment. By stringent washings and autoradiography exposure, one positive recombinant colony was obtained. After three rounds of screening of a L. donovani L82 (Ethiopian) EMBL-3 genomic library, only one bacteriophage was isolated with the use of colony plaque hybridization [17]. The isolated bacteriophage was digested with restriction endonucleases, electrophoresed on 0.8% (w/v) agarose gels and transferred onto nylon membranes by the method of Southern [18]. The Southern blot was probed to the labeled 639 bp PCR fragment under the conditions described above. A 6.0 kb Smal fragment that hybridized to the probe was ligated to pGEM-3Zf(+) and transformed into XL-1Blue E. coli. Large-scale plasmid preparations of pGEM-3Z containing the 6.0 kb Smal fragment were prepared using Qiagen columns following the manufacturer’s instructions. A restriction map of the 6.0 kb Smal fragment was generated using a variety of restriction endonucleases, and the labeled 639 bp PCR product as probe. A new fragment of 2.0 kb obtained by digestion with Ava I was isolated, subcloned into pGEM-3Zf(+) and sequenced on both strands, using synthetic oligonucleotide primers by the Sanger method [19]. Analyses of nucleotide and amino acid sequences were performed on BLAST algorithm from the National Centre of Biotechnology Information database.

Site-directed mutagenesis

The full-length MAT2 gene was amplified by PCR using L. donovani genomic DNA as template. The sense and antisense primers were: 5′-CGG TAT CTA TCG TCT CCC TTC TCT TC-3′ and 5′-GGG GTA CCC CTT ACT CGA CCA TCT TCT TGTC GCA C-3′ containing the nucleotides 1–24 and 1179–1156 of L. donovani radiographic coding sequence, respectively. The 1.2 kb fragment containing the MAT2 gene was subcloned into BamHI/KpnI sites of pBluescript M13(+) SK (Stratagene, La Jolla, CA, USA). This plasmid, called pSK-MAT2 was the template for mutagenesis experiments using the Quick-Change site-directed mutagenesis kit following the manufacturer’s instructions.

The oligonucleotides employed for site-directed mutagenesis were: 5′-CATCCAGACAAAGCTTGGCGATCAGTATGCCGAC-3′ (C22S), 5′-GGCTGTGCTT GACCGCCGAC TCTGCGGGGCGACC GCGGCCCAG-3′ (C33S), 5′-GTTTCTCGA AAGGTGTTCCGAGGATCGTGCTCC GCGGAA-3′ (C45S), 5′-GTTCGCTGCGTTACGCGTCCGGC GGGCATG-3′ (C47S), 5′-CTG GACTACGAGTCCGC AATGTCGTTGGCGGCTGC-3′ (C92S), 5′-CAATCGCGCGGAGATCACCACCAGCCGCAAC-3′ (C106S), 5′-GGCCATCCGCGGCAGAGAGCT CGTCCGGC-3′ (C305S), respectively. The substituted bases are in italic, sense to the coding strand. The PCR reaction contained 20 ng of plasmid pSK-MAT2 as template, 250 ng each of the mutagenic oligonucleotide, 100 μM dNTPs, 5 μL of 10 × Pfu-buffer and 2.5 units of
Pfu-polymerase in a total volume of 50 µL. Reactions were carried out in a Mastercycler gradient thermocycler (Eppendorf) and consisted of a 5-min cycle at 94 °C, followed by 12 cycles at 94 °C for 30 s, 55 °C for 1 min and 68 °C for 12 min and ending with 10 min at 68 °C. PCR-products were incubated in presence of DpnI in order to digest the parental DNA template. The purified fragments were used to transform XL1-Blue E. coli, and four to six clones were sequenced to assure that site-directed mutation had been introduced accurately. Typically, the efficiency of mutagenesis was around 80%. Selected mutants were sequenced to confirm the lack of undesirable additional mutations, and then subcloned into the BamHI/KpnI sites of the bacterial expression vector pQE30 (QIA-express System, Qiagen).

MAT-II overexpression and refolding

MAT was overexpressed as described previously [20]. Briefly, the 1.2 kb MAT2 gene was subcloned into pQE30 as described above, and transformed into XL1-Blue E. coli competent cells. Overnight cultures prepared from single colonies were used to inoculate 100 mL of LB medium plus ampicillin (100 µg/mL). Cells were grown to D600 ≈ 0.5 and isopropyl-β-D-galactoside (IPTG) was added to a final concentration of 0.1 mM. After induction, growth was continued for 3 h. Cells were harvested by centrifugation, washed with saline solution, and stored at −70 °C until use.

Cell pellets were disrupted by sonication at 4 °C with a U50-control (Kika Labortechnich) sonifier [10 pulses of 30 s at 30-s intervals, at 50 W in 10 mL of 50 mM Tris/Cl, pH 8.0, containing 0.5 mM NaCl, 0.1% (v/v) 2-mercaptoethanol, and protease inhibitors (2 µg/mL aprotinin, 1 µg/mL pepstatin A, 0.5 µg/mL leupeptin, 2.5 µg/mL antipain, 0.1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride)].

Soluble and insoluble fractions (including inclusion bodies), were separated by centrifugation at 10 000 g for 10 min. Pellets from the insoluble fraction, were washed four times with 0.1 M Tris/HCl, pH 7.0, 10 mM MgSO4, 5% (v/v) Triton X-100 and 4 mM urea. A final wash with the same buffer without Triton X-100 and urea was performed. The inclusion bodies were solubilized with 50 mM Tris/HCl pH 8.0 (refolding buffer) containing 8 M urea and 10 mM MgSO4, during 24 h at 10 °C. Protein refolding was performed by a fourfold dilution refolding buffer up to a concentration of 2 M urea. The resulting suspension was then dialysed three times at 4 °C against refolding buffer containing the additives to be assayed, in order to remove the urea.

Refolding process from denatured MAT-enriched inclusion bodies was followed by measuring MAT-activity in withdrawn aliquots at different times, and by monitoring MAT intrinsic fluorescence quenching [21]. Fluorescence measurements were performed at 30 °C using excitation and emission wavelengths of 290 and 350 nm, respectively. Samples were maintained in the cuvettes for 12 min for the fluorescence signal to reach a constant value. All fluorescence data were corrected when necessary for dilution and for fluorescence background of the refolding buffer used.

MAT assay

MAT activity was assayed as described previously [22]. The assay contained in 250 µL total volume, 5 mM L-methionine, 1 mM ATP (containing [2,8-3H]adenosine 5′-triphosphate, 46 Ci mmol⁻¹, Amersham), in 100 mM Tris/HCl, pH 8.0, 240 mM KCl, 12 mM MgCl₂, and 10 mM dithiothreitol. The reaction was stopped with 4 mL ice-cold water. Reaction mixtures were loaded onto AG 50 W-X4 cation exchange columns, washed twice with 10 mL water and eluted with 4 mL of 3 M NH₄OH. Samples, previously neutralized with 1 mL of glacial acetic acid, were measured in a scintillation counter using 10 mL of Optiphase-Hisafe 3 (Wallac) cocktail for aqueous mixtures. One unit of MAT activity is defined as the amount of enzyme that catalyses the formation of 1 µmol AdoMet per hour and per milligram of protein. Protein was determined using the Bradford method [23]. Each data point was measured by triplicate and presented as the mean. Kinetic parameters for L-methionine an ATP were assessed under steady-state conditions. Kinetic constants for L-amino acid were determined in presence of 0.05–5 mM ATP and 0.025–2.5 mM L-methionine, whereas nucleoside constants were ascertained in presence of 0.05–1 mM L-methionine and 0.05–5 mM ATP.

Tripolyphosphatase activity

The final reaction volume was 400 µL containing 100 µL of the enzymatic solution and 50 mM Tris/HCl, pH 7.8, 100 mM KCl, 7 mM MgCl₂, 1 mM dithiothreitol and 20–200 µM range of PPP. The reaction was carried out for 30 min at 30 °C and stopped by the addition of 1 mL of the stop solution [0.5% ammonium molibdate (w/v), 2% H₂SO₄ (v/v), 0.5% SDS (w/v) and 10 μL 10% (w/v) ascorbate]. After 5 min, absorption at 750 nm was measured.

Results and discussion

A probe for screening the L. donovani EMBL-3 genomic library was developed on the grounds of a partial amino acid sequences from the organisms appearing in Fig. 1 and

![Fig. 1. Scheme of the alignment of cysteine residues of MATs from different origins based on the previously reported [16]. GenBank™ accession numbers are as follows: L. donovani (AF179714); P. falciparum (AF097923); Saccharomyces cerevisiae (M23368); E. coli (K02129) and rat liver (S06114).](image-url)
PCR amplification (see ‘Materials and methods’). Translation of the 639 bp PCR fragment in all six possible reading frames revealed that several stretches of the peptide sequence predicted from one of the six, conserved high homology with the amino acid sequences of others MAT proteins submitted to GenBankTM database. The PCR product was used to screen the DNA library for this parasite to isolate the full-length clone. The isolated bacteriophage was used for subsequent analysis; a 6.0 kb Smal-digested fragment was hybridized to the PCR fragment and digested with Awl to obtain a 2.0 kb fragment, the product ultimately sequenced. The nucleotide sequence of the L. donovani MAT2 clone (GenBank™ accession number AF179714) was identical to the sequence found for L. infantum [16], with a single 1179 bp long ORF encoding 394 amino acid residues and a calculated molecular mass of 42 000 Da (data not shown). The MAT2-encoding sequence conserved a high G/C codon bias. L. donovani MAT2 contains all the motifs that bind to ATP, metals and the active site: the nonapeptide GGGAfSGKD, at position 269–277, corresponds to a P-loop that forms a part of the ATP binding site [24]. The y-phosphate moiety is hydrolyzed from triphosphate at conserved Arg255. The Asp19 and Asp282 residues have been described to bind Mg2+ [25] and Glu45 is involved in K+ binding [26]. L. donovani also conserved the hexapeptide GAGDQG at position 118–123, associated with the active site of the enzyme. The genomic organization of the MAT2 gene in the L. donovani genome was ascertained by digestion of the entire genomic DNA from L. donovani L82 (Ethiopian) cells with different endonucleases, after which the fragments were blotted and probed with the labeled 639 bp PCR fragment. The number of bands obtained after cleavage with the restriction enzymes is indicative of the presence of two copies of the MAT2 gene in the L. donovani genome, a result that concurs with findings described previously for L. infantum [16].

L. donovani MAT contains seven cysteine residues (Cys22, 33, 44, 47, 92, 106 and 305) per enzyme subunit. The location of the cysteine residues, based on the alignment recently reported for L. infantum MAT [16], is shown in Fig. 1. When compared to the mammalian enzyme, the cysteine residues in L. donovani MAT-II at positions 22 (which corresponds to Cys35 in rat liver MAT), 44 (Cys57 in rat liver), and 92 (Cys105 in rat liver) are found to remain invariant in most of the sequences aligned. The cysteines at positions 33 and 47 are found to be conserved in the Plasmodium falciparum MAT sequence [27]. Cys305 aligns with the cysteine at position 295 in the E. coli enzyme. In addition to these Cys residues, L. donovani contains a specific cysteine at position 106.

E. coli strain XL-1Blue cells transformed with pQE30-MAT, were induced with 0.1 mM IPTG. Aliquots were harvested at different times (30 min, 1 h, 2 h, 3 h), lysed and spun at 13 000 r.p.m. in a microfuge for 15 min. Proteins from the supernatants and pellets were analyzed by SDS/PAGE under reducing conditions. In the absence of IPTG, MAT expression was nil. With IPTG induction, however, a protein with an estimated molecular mass of 48 kDa was found to accumulate. The recombinant protein formed primarily in inclusion bodies. Successive washes of inclusion bodies with 4 M urea and 5% (v/v) Triton X-100 removed most of the contaminating proteins, producing a homogeneous MAT-II preparation, as shown by SDS/PAGE gels (Fig. 2, lane 2). His-tag affinity chromatography (Fig. 2, lane 3) showed that no further purification was obtained with this step. A single band with an estimated molecular weight of 48 kDa (Fig. 2, lane 4) was observed when Western analysis was conducted using a polyclonal MAT antibody [16] and whole L. donovani extract.

The protocol for functional folding of the MAT-protein enriched insoluble aggregates was based on the procedure described by López-Vara et al. [20]. Briefly, two successive washes with 4 M urea containing 5% (v/v) Triton X-100 yielded the protein overexpressed in the inclusion bodies in a very pure (over 99%) state. Removal of the excess urea added, protein dilution and equilibrium dialysis was requisite to proper MAT folding, which was monitored by both enzyme activity and fluorescence quenching.

The presence of seven cysteines in L. donovani MAT-II suggests that sulfhydryl-protection reagents may be required for optimum refolding. Conformational transitions were observed (Fig. 3A) via fluorescence quenching during the refolding of L. donovani MAT-II in the presence of 10 mM dithiothreitol. Such transitions, which provoked fast and large fluorescence quenching effects (indicating strong stimulation of MAT activity), took place during the first 2 h. MAT activity (Fig. 3B) shows a sharp rise after 2 h of dialysis with 10 mM dithiothreitol, to plateau thereafter. Notably, when the MAT molecule refolded in the absence of dithiothreitol, it only quenched about half of the...
fluorescence quenched in the presence of the thiol, and no activity at all was recovered. All MAT enzymes require a divalent cation and most have bindingsites for both the Mg$^{2+}$-ATP substrate and for free Mg$^{2+}$ [25]. Another experiment, similar to the one described, was conducted in which MAT activity was monitored during the refolding process in the presence and absence of MgSO$_4$ (Fig. 3C,D). The Mg$^{2+}$ cation does not affect the refolding of wild-type MAT, and the MAT activity was observed to be similar in both cases.

The synthetic reaction catalyzed by MAT occurs in two consecutive steps: AdoMet and PPP$_i$ are first synthesized from methionine and ATP and then PPP$_i$ is subsequently hydrolyzed to PP$_i$ and Pi to allow the products to be released from the active site of the enzyme. Recombinant MAT activity was linear in terms of both time (up to 90 min) and protein concentration (data not shown). The steady-state activity at saturation of both substrates, i.e. 5 mM ATP and 5 mM L-methionine, was 12 μmol·mg$^{-1}$·h$^{-1}$ ($k_{cat} = 0.32$ s$^{-1}$) (Table 1). The enzyme showed slight sigmoid behavior with both L-methionine and ATP. Hill plots and the software package Enzfitter, were used for kinetic parameters calculations. Co-operativity, estimated to be $n = 2.3$ (ATP = 0.5 mM) declined with rising ATP levels to nearly 1 (ATP = 5 mM). $S_{0.5}$-values for L-methionine, not significantly affected by ATP, were estimated to be 250 ± 25 μM. Conversely, when assessed as a function of ATP at different L-methionine levels, MAT activity was sigmoid ($n = 1.8$). The $S_{0.5}$-values for ATP were calculated to be 27 ± 5 μM and the curve retained its sigmoid shape as concentrations of the L-amino acid were increased. The tripolyphosphatase activity of L. donovani recombinant MAT-II was measured under the standard assay conditions described in ‘Material and methods’. Tripolyphosphatase activity was linear over time and for protein concentration, and no Pi was observed to be released in the absence of recombinant MAT. Sigmoid behavior was found under steady state conditions, with a $k_{cat}$ 0.04 s$^{-1}$ and an $S_{0.5}$-value of 40 μM (Table 1).

The feedback inhibition of MAT by AdoMet, was analyzed using the Dixon approach at L-methionine concentrations of 0.5–5.0 mM, resulting in a noncompetitive pattern with a $K_i$ value of 4 mM. By contrast, in a similar

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**Table 1. Kinetic characterization of refolded L. donovani MAT.** AdoMet synthesis and tripolyphosphatase activity were measured under steady-state conditions established in Material and methods. Hill plots were used to determine the kinetic constants of both activities. Results are the mean of four independent determinations ± SD.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>AdoMet synthesis</th>
<th>Tripolyphosphatase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific activity</td>
<td>12 μmol·mg$^{-1}$·h$^{-1}$</td>
<td>3.5 μmol·mg$^{-1}$·h$^{-1}$</td>
</tr>
<tr>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>0.32 s$^{-1}$</td>
<td>0.04 s$^{-1}$</td>
</tr>
<tr>
<td>$S_{0.5}$ (L-methionine)</td>
<td>250 ± 25 μM</td>
<td></td>
</tr>
<tr>
<td>$S_{0.5}$ (ATP)</td>
<td>27 ± 5 μM</td>
<td>40 ± 3 μM</td>
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analysis with ATP concentrations of 0.5–5.0 mM, a competitive inhibitory effect was found with a $K_i$ value for the nucleobase of 0.8 mM. Regardless to AdoMet synthesis, AdoMet was a nonessential activator of triphosphatase activity in the range of 5–100 μM.

The $k_{cat}$ values determined at saturating concentrations of triphosphatase, ATP and l-methionine showed that the rate of AdoMet synthesis is higher than the rate of triphosphatase cleavage. As the overall process should be dependent on the slowest reaction, triphosphatase activity may be thought to be responsible for the rate of AdoMet synthesis in leishmania. However, triphosphatase hydrolysis is activated several-fold when AdoMet occupies the active site, thus suggesting that the reaction forming AdoMet and PPP$_i$ is the step that determines the rate of the overall process, in which PPP$_i$ cleavage would be requisite to enzyme turnover [28].

The dependence of MAT activity on cysteine residues was assessed for both the AdoMet synthesis and triphosphatase activities in the presence of the sulphydryl reagent N-ethylmaleimide. The panels shown in Fig. 4 represent the time-course of AdoMet synthesis (Fig. 4A) and triphosphatase (Fig. 4B) inactivation when 1 mM N-ethylmaleimide was added to the incubation media. In both cases time-dependent inactivation was observed, which is indicative of irreversible binding to one/several of the sulphydryl moieties involved in the enzymatic process. The semi-inactivation times estimated for AdoMet synthesis and PPP$_i$ cleavage processes were estimated to be 3.9 and 11.8 min, respectively. However, the presence of 50 μM AdoMet in the incubation media caused bi-exponential decay in the presence of the sulphydryl reagent. The semi-inactivation time of the rapid process was calculated to be 1.6 min, whereas the slope of the slow semi-inactivation process was rather similar to the slope of the curve found when the medium did not contain AdoMet, with an estimated half-life of 10.5 min.

The role of the seven cysteine residues in *L. donovani* MAT was studied using the site-directed mutagenesis approach, in which seven single mutants were produced, each lacking one of the sulphydryl moieties. The amino acid chosen to replace Cys was serine, as it is regarded to be isosteric to cysteine and does not impact hydrophobicity [12]. All the mutants, named C22S, C33S, C44S, C47S, C92S, C106S and C305S, were expressed in *E. coli* and their products were refolded as described in [Material and methods]. MAT was measured for both AdoMet synthesis and triphosphatase activities in all the cysteine mutants for comparison to the wild-type protein (Fig. 5A–C).

Figure 5A shows the ability of the various *L. donovani* MAT cysteine mutants to synthesize AdoMet under the standard assay conditions. Site-directed mutations on the phylogenetically conserved cysteines Cys22, Cys44 and Cys92 yielded mutants completely lacking in any synthetic activity. The C305S mutant retained a scant 1% of the activity displayed by the wild type. The C33S and C47S mutants retained only 15% and 10%, respectively, of the $V_{max}$ under saturating conditions for both substrates, and no changes in affinity were found. Unlike the other mutants, C106S was not kinetically different from the wild-type protein. Structural analyses of mammalian MAT show that the cysteines positioned between Cys35 and Cys105 are located in the central domain of each subunit, in the interface between the two dimers comprising the tetrameric structure [29]. This domain contains five cysteine residues, two of them (Cys35 and Cys61) forming a disulfide bond which may be necessary for the tetrameric state of the enzyme. In addition, Cys69 is involved in the correct folding of the monomer, supporting the establishment of the disulfide bond [29,30]. Chromatographic and modeling studies show that Leishmania [16] and Plasmodium [27] MATs are dimers whose identical cysteine compositions in the central domain lack the homologous mammalian enzyme amino acids at positions 61 and 69 which are, in turn, involved in establishing the disulfide bond and proper folding to the tetrameric structure. Nevertheless, the substitution of two specific cysteines, Cys33 and Cys47, present in both species, originated a significant loss of enzymatic activity but no change in affinity.

The behavior observed for the cysteine mutants differed in terms of triphosphatase activity. Figure 5B shows the residual activity of single cysteine mutants assayed under standard saturation conditions, in the absence of AdoMet. The single cysteine mutants of *L. donovani* MAT, C22S, C44S and C305S, lacked triphosphatase activity. C33S maintained a mere one-tenth of the activity displayed by the wild type. However, the ability of C92S, C106S and C47S to cleave PPP$_i$ remained high, and in the case of C47S, even...
higher than the wild-type protein. The addition of 50 mM AdoMet (Fig. 5C) activated PPP\(_i\) hydrolysis more than 12-fold in the wild type and to a similar extent in the C106S mutant. Significant activation was also observed in the C47S and C33S mutants, although with different kinetic constants and sigmoid behavior. There was a notable lack of any AdoMet stimulation in the C92S mutant (Table 2). Whilst it showed significant triphosphatase activity in the absence of AdoMet, its activity was not enhanced in the presence of AdoMet. The Cys92 residue is thus involved in the stimulatory effect of PPP\(_i\) cleavage induced by AdoMet and may be the amino acid residue to be rapidly inactivated by N-ethylmaleimide (Fig. 4B). The residues homologous with leishmanial Cys92 in mammalian and E. coli MATs are amino acid residues Cys105 and Cys90, respectively [11,12]. Both are involved in the dimer/tetramer equilibrium of the enzyme, and E. coli Cys90 is also involved in the binding of ATP to the active site. Because C92S is unable to synthesize AdoMet but can cleave PPP\(_i\), the respective cysteine may plausibly be thought to be involved in the first step of the reaction (synthesis of AdoMet and PPP\(_i\)), with no role in PPP\(_i\) cleavage.

The results of these studies show that recombinant L. donovani MAT only folds properly in reducing environments. With the exception of Cys106, all the cysteine residues in the enzyme are needed for AdoMet condensation, PPP\(_i\) hydrolysis or AdoMet activation. The structural involvement of the cysteines at positions 22, 44 and 305 appears to be crucial to the overall process. By contrast, the mutants lacking cysteine residues at positions 33 and 47

![Fig. 5. Leishmania donovani recombinant MAT activity and effect of cysteine substitutions on AdoMet synthesis (A) and triphosphatase activity in absence of AdoMet (B) and presence of 50 μM AdoMet (C). Freshly refolded MAT (16 μg) and cysteine mutants, were assayed in presence of 1 mM ATP for AdoMet synthesis activity and under standard saturation conditions for triphosphatase activity. Each bar represents the average ± SD of triplicates.](image)

<table>
<thead>
<tr>
<th>Table 2. Kinetic parameters of triphosphatase activity of wild-type MAT and cysteine-mutants from L. donovani. Kinetics were performed under standard assay conditions using 16 μg of freshly folded recombinant protein. Activation kinetics were performed in the presence of 50 μM AdoMet.</th>
<th>Without AdoMet</th>
<th>With AdoMet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutant</td>
<td>(V_{\text{max}}) (μmol·h(^{-1})·mg(^{-1}))</td>
<td>(S_{0.5}) (μM)</td>
</tr>
<tr>
<td>Wild type</td>
<td>3.7</td>
<td>40</td>
</tr>
<tr>
<td>C33S</td>
<td>0.3</td>
<td>n.d.</td>
</tr>
<tr>
<td>C47S</td>
<td>5.3</td>
<td>63</td>
</tr>
<tr>
<td>C92S</td>
<td>1.9</td>
<td>52</td>
</tr>
<tr>
<td>C106S</td>
<td>3.5</td>
<td>38</td>
</tr>
</tbody>
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retained part of their AdoMet synthesis and triphospho-
phatase activities. As the C92S mutant, which completely
lacked the ability to synthesize AdoMet, retained triphos-
phatase activity but was not stimulated by exogenous
AdoMet, it may be concluded that the cysteine residue at
position 92 participated in the first of the two reactions that
comprise the process.

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S-Adenosylmethionine: a control switch that regulates liver func-
3. Mato, J.M., Corrales, E.C., Martín-Duce, A., Ortiz, P., Pajares,
impaired trans-sulphuration pathway in liver disease: part I. Bio-
chemical implications. Drugs 40, 58–64.
transmethylation. Purification of the S-adenosylmethionine syn-
thetase from Baker’s yeast and its separation into two forms.
J. Biol. Chem. 252, 4506–4513.
ferase (S-adenosylmethionine synthetase) and S-adenosyl-
56, 251–282.
Characterization of a full-length cDNA encoding human liver S-
adensylmethionine synthetase: tissue-specific gene expression
8. De La Rosa, J., Ostrowski, J., Hryniewicz, M.M., Kredich, N.M.,
Chromosomal localization and catalytic properties of the
recombinant alpha subunit of human lymphocytes methionine
Cloning, expression and functional characterization of the beta
regulatory subunit of human methionine adenosyltransferase
the reactive sulfhydryl groups of S-adenosylmethionine syn-
tional roles of cysteine 90 and cysteine 240 in S-adenosylmethio-
Pajares, M.A. (1996) Site-directed mutagenesis of rat liver S-ad-
leishmanial effect of free and encapsulated sinifen against
Leishmania donovani infections in BALB/c mice. C. R. Acad. Sci.
III (308), 485–488.
polyamine metabolism on African trypanosomes. Acta Trop. 54,
225–236.
15. Yarlett, N., Garofalo, J., Goldberg, B., Cininelli, M.A., Ruggiero,
synthetase in bloodstream Trypanosoma brucei. Biochim. Biophys.
Acta 1181, 68–76.
16. Reguera, R.M., Balaña-Fouce, R., Pérez-Pertejo, Y., Fernández,
F.J., García Estrada, C., Cubría, J.C., Ordoñez, C. & Ordoñez, D.
(2002) Cloning, expression and characterization of methionine
adenosyltransferase in Leishmania infantum promastigotes. J. Biol.
Chem. 277, 3158–3167.
Laboratory, Cold Spring Harbor, New York.
DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98,
503–517.
with chain-terminating inhibitors. Proc. Natl Acad. Sci. USA 74,
5463–5467.
and characterization of rat liver methionine adenosyltransferase
from Escherichia coli inclusion bodies. Protein Expr. Purif. 19,
219–226.
Bautista, J.M. (1996) Unproductive folding of the human G6PD-
Expression of rat liver S-adenosylmethionine synthetase in
Escherichia coli results in two active oligomeric forms. Biochem.
J. 301, 557–561.
quantitation of microgram quantities of protein utilizing the
liver S-adenosylmethionine synthetase active site with 8-azido
site of S-adenosylmethionine synthetase. Roles of the active site
monovalent cation activation of S-adenosylmethionine synthetase,
using mutagenesis and uranyl inhibition. J. Biol. Chem. 270,
18277–18284.
27. Chiang, P.K., Chamberlin, M.E., Nicholson, D., Soubes, S., Su,
X.-Z., Subramanian, G., Lanar, D.E., Prigge, S.T., Scovill, J.P.,
Miller, L.H. & Chou, J.Y. (1999) Molecular characterization of
Plasmodium falciparum S-adenosylmethionine synthetase. Bio-
Energetics of S-adenosylmethionine synthetase catalysis. Bio-
chemistry 39, 4443–4454.
29. González, B., Pajares, M.A., Hermoso, J.A., Álvarez, L., Garrido,
B., Sufrin, J. & Sanz-Aparicio, J. (2000) The crystal structure of
tetrameric methionine adenosyltransferase from rat liver reveals
single disulfide bridge in rat methionine adenosyltransferase. Eur.
J. Biochem. 267, 132–137.