3-Mercaptopyruvate Sulfurtransferase of *Leishmania* Contains an Unusual C-terminal Extension and Is Involved in Thioredoxin and Antioxidant Metabolism*

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Cytosolic 3-mercaptopyruvate sulfurtransferases (EC 2.8.1.12) of *Leishmania major* and *Leishmania mexicana* have been cloned, expressed as active enzymes in *Escherichia coli*, and characterized. The leishmanial single-copy genes predict a sulfurtransferase that is structurally peculiar in possessing a C-terminal domain of some 70 amino acids. Homologous genes of *Trypanosoma cruzi* and *Trypanosoma brucei* encode enzymes with a similar C-terminal domain, suggesting that this feature, not known in any other sulfurtransferase, is a characteristic of trypanosomatid parasites. Short truncations of the C-terminal domain resulted in misfolded inactive proteins, demonstrating that the domain plays some key role in facilitating correct folding of the enzymes. The leishmanial recombinant enzymes exhibited high activity toward 3-mercaptopyruvate and catalyzed the transfer of sulfane sulfur to cyanide to form thiocyanate. They also used thiosulfate as a substrate and reduced thioredoxin as the accepting nucleophile, the latter being oxidized. The enzymes were expressed in all life cycle stages, and the expression level was increased under peroxide or hypo-sulfur stress. The results are consistent with the enzymes having an involvement in the synthesis of sulfur amino acids *per se* or iron-sulfur centers of proteins and the parasite’s management of oxidative stress.

Sulfurtransferases (EC 2.8.1.1–5) are widely distributed enzymes of prokaryotes and eukaryotes (1, 2). The enzymes catalyze the transfer of sulfane sulfur from a donor molecule, such as thiosulfate or 3-mercaptopyruvate, to a nucleophilic acceptor, such as cyanide or mercaptoethanol. However, the natural sulfane donors and acceptors and the physiological functions of most sulfurtransferases remain uncertain.

The rhodanese family sulfurtransferases are thought to occur in the majority of organisms (1), with the mammalian enzymes being the most extensively studied (3, 4). The first elucidated role of mitochondrial bovine liver rhodanese was the detoxication of cyanide to form thiocyanate, which is harmless and excreted by the kidney. This role could be important, especially in the epithelial cells lining the gut (5), but is thought not to account for the wide distribution of these sulfurtransferases in different cell types (2). Another putative function of at least some sulfurtransferases is the provision of sulfane sulfur required for the formation of the iron-sulfur centers of proteins, notably respiratory proteins (6–9).

Sulfurtransferases may also play a part in the management of the cytotoxicity of reactive oxygen species in aerobic tissues (4). Bovine rhodanese has a 1000-fold higher affinity for the reduced form of thioredoxin than for cyanide and so may function in peroxide detoxification (4, 10). A reaction analogous to that of sulfane-loaded sulfurtransferase with thioredoxin is also thought to be a critical step in the synthesis of thiouridine (7, 11, 12), and the formation of thiocarboxylate during thiamine biosynthesis by the multidomain protein ThI of *Escherichia coli* (7, 12). Sulfurtransferases have also been implicated in the synthesis of biotin (13) and molybdopterin (14). Moreover, a role for sulfurtransferases in assimilatory sulfate reduction by transferring a molecule of sulfide to O-acetyl-l-serine in the synthesis of cysteine has been postulated (15).

Most sulfurtransferases have an N-terminal “structural” domain and a C-terminal domain containing the active site (1, 16, 17). The vertebrate rhodanases have been extensively studied in attempts to understand the part played by the N-terminal structural domain in the correct folding and stability of the enzymes. Current evidence suggests, however, that correct protein folding also requires the assistance of a chaperone molecule (18, 19).

*Leishmania* parasites are widespread and important parasites of humans and dogs. The diseases they cause are most prevalent in the tropics and subtropics, although leishmaniasis is also endemic in Southern Europe and has been reported in the United States (20). There is a pressing need to improve ways of treating the diseases; and in particular, there is a requirement for better chemotherapy (21). *Leishmania* is an excellent organism in which to investigate the possible roles of sulfurtransferases in antioxidant and sulfur amino acid metabolism. The parasites exist intracellularly in macrophages while within their mammalian host and are thought to be particularly well adapted to survive against oxidative stress arising from the immune mechanisms of the host. *Leishmania* is unusual in possessing trypanothione, a conjugate of glutathione and spermidine, as a major cellular thiol and apparently uses this and associated enzymes as a prime means of protection against oxidative damage (22, 23). However, the source of cysteine, essential for trypanothione, is unknown. In this study,
we describe the cloning, expression, and characterization of a sulfotransferase of the protozoan parasites Leishmania major and Leishmania mexicana and provide data on its unusual structure and possible roles.

**EXPERIMENTAL PROCEDURES**

Cultivation of L. major and L. mexicana—Promastigotes of L. major (MHOM/JL/80/Friedlieb) and L. mexicana (MNYC/BZ/62/M379) were normally grown in modified Eagle’s medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum at 27 °C (24). Metacyclic promastigotes of L. major were purified for a stationary phase population of cells using the agglutination assay described by Sacks et al. (25). Amastigotes of L. mexicana were purified from infected BALB/c mice or grown axenically as described (26). The bloodstream form of Trypanosoma brucei strain 427 was grown in rats and isolated by DEAE ion-exchange chromatography as described (27). The procyclic form of T. brucei strain 427 was grown at 27 °C in semi-defined medium containing 10% (v/v) fetal bovine serum and 3.5 mg/ml hemin (28). Parasites were harvested and used immediately or pelleted and stored at −20 °C. Parasite lysates were produced by resuspension of parasite pellets in lysis buffer comprising 0.25 M sucrose, 0.25% (v/v) Triton X-100, 10 mM EDTA, and a mixture of protease inhibitors (10 μg E-64, 2 mM phenylmethylsulfonyl fluoride, 4 μg pepstatin A, and 1 mM phenylmethylsulfonyl fluoride). Lysates were centrifuged at 13,000 × g for 5 min at 4 °C, and the resulting supernatant (designated the soluble fraction) was retained for analysis. Protein concentrations were determined according to the Bradford procedure (Bio-Rad) using bovine serum albumin as the protein standard.

**PCR Amplification, Cloning of 3-Mercaptopyruvate Sulfotransferase (MST)** of L. major (LmajMST) and L. mexicana (LmexMST)—Total RNAs of L. major and L. mexicana promastigotes were isolated using TRIzol (Invitrogen) and reverse-transcribed with Superscript II reverse transcriptase (Invitrogen) into single-stranded cDNA as described by the manufacturer. Genomic DNAs of L. major and L. mexicana were isolated using TEL buffer (50 mM Tris-HCl, pH 8.0, 6.25 mM EDTA, 2.5 mM LiCl, 49% (v/v) Triton X-100) as described (29). The 5′- and 3′-ends of the genes from both L. major and L. mexicana were amplified using 5′- and 3′-RACE kits (Invitrogen). The degenerate reverse gene-specific primers NT2 (5′-ACGCCGACCTTC-GATGTGCGC-3′) and NT4 (5′-ATTCTACATACACGATGCTAGTGC-3′), based on conserved motif sequences from the alignment of the putative rhodanese of L. major (GenBank™/EBI accession number AF163772) and bacterial homologs in the NCBI Database, and the spliced leader primers LMEXLS1 (5′-TAACCGTATTAAGTAGTACGT-TTC-3′) and LMEXLS2 (5′-AGTAGATCAGTTCTGATTCATTGTG-3′) were used for 5′-RACE. The perfect match primers NT21 (5′-ACGCCGACCTTC-GATGTGCGC-3′) and NT22 (5′-AGATCAGTTCTGATTCATTGTG-3′), based on consensus sequences of the genes detailed above, and oligo(T) primers with an adaptor attached, UAP (5′-CUACUACUAUCAGGACCCGTCGTAGTAC-3′) and AUAP (5′-GCAGCAG CTGAGCTAGTAC-3′), were used to amplify the 3′-ends of the genes with Expand High Fidelity Felo/Taq polymerase. Both the 5′- and 3′-ends of the genes were cloned into the pGEM-T vector (Promega) and sequenced.

PCR Amplification, Expression, and Purification of MSTs—The open reading frames (ORFs) of the genes encoding LmajMST and LmexMST and four constructs, LmajMSTΔ338–370, LmajMSTΔ320–370, LmajMSTΔ338–370, and LmajMSTΔ360–370, encoding truncated proteins lacking 20, 50, 32, and 10 amino acids of the C terminus (rLmajMSTΔ338–370, rLmajMSTΔ320–370, rLmajMSTΔ338–370, and rLmajMSTΔ360–370, respectively), were encoded from cDNA by PCR with the Expand High Fidelity system. For this, the 5′-perfect match primer NT24 (5′-CGCTGAGATATGTTGCTGCGC-GGAA-3′) was used for all constructions, together with the appropriate 3′-primer encoding the regions directly preceding the native stop codon and amino acids 300, 320, 338 and 360, respectively: NT55 (5′-TTCCTTGATATGAGGACTGCGC-GCCAT-3′) for LmajMSTΔ338–370, NT55 (5′-TTCCTTGATATGAGGACTGCGC-GCCAT-3′) for LmajMSTΔ320–370, NT55 (5′-TTCCTTGATATGAGGACTGCGC-GCCAT-3′) for LmajMSTΔ338–370, and NT55 (5′-TTCCTTGATATGAGGACTGCGC-GCCAT-3′) for LmajMSTΔ360–370, respectively. The PCR products were cloned into pGEM-T and transformed into the E. coli strain BL21 Codon Plus (DE3)Rf cells (Stratagene). Expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside for 5 h at 37 °C and the cells were pelleted and resuspended in 5 ml of buffer A (50 mM sodium phosphate and 3 mM NaCl, pH 8.0) with 5 mM imidazole and diluted by sonication, and the soluble fraction was recovered by centrifugation at 13,000 × g for 30 min at 4 °C. This was applied to a 13-mL nickel-nitriolotriacetic acid column (bioCAD® 700E wash station) pre-equilibrated with buffer A. The column was washed with 60 ml of buffer A containing 50 mM imidazole and then with 300 ml of buffer B containing 60 mM imidazole. The His-tagged recombinant proteins were eluted with 250 mM imidazole in buffer A. The eluant was dialyzed with excess 20 mM Tris-Cl, pH 7.9, and 1 mM Na2S2O3 at 4 °C overnight and stored at −20 °C. Approximately 25 mg of soluble recombinant proteins (LmA3T and LmexMST) were obtained from 1-liter cultures of E. coli.

**Refolding of Denatured Enzyme**—Denaturation and reactivation analyses of rLmA3TΔ338–370, rLmA3TΔ360–370, and rLmexMST with urea were based on protocols detailed by Bhattacharyya and Horowitz (30). Proteins were incubated at 1.0 mg/ml in 50 mM potassium phosphate, pH 7.8, containing 50 mM Na2S2O3 and 6 mM urea for 120 min at 20 °C. Attempts to reactivate the proteins involved one of two procedures: (a) 25 μl of denatured protein at 1.0 mg/ml were diluted in 75 μl of refolding buffer containing 0.2 mM 2-mercaptoethanol, 50 mM Na2S2O3, 10 mM KCl, and 10 mM MgCl2; or (b) an aliquot of the denatured sample was dialyzed at 4 °C against refolding buffer. Successful refolding and hence reactivation were monitored by assaying 10-μl samples using the standard sulfotransferase assay.

**Genomic Southern Blot Analysis**—Genomic DNA was extracted according to standard procedures (29). DNA (5 μg) was digested with the appropriate enzymes, fractionated by agarose gel electrophoresis, nicked, denatured, neutralized, and blotted onto a HybondTM-N+ membrane (31) by capillary transfer. Probes were prepared from 1110-bp NdeI/Xho ORF fragments from pETLmA3T and pETLmexMST using a Prime-It kit (Stratagene) and purified on Microspin S-200 HR columns. Filters were washed, exposed overnight with [32P]dATP-labeled LmA3T and LmexMST probes in Church-Gilbert hybridization solution. Filters were washed under high stringency and exposed to x-ray film (Konica Medical Film).

**Enzyme Activity Measurements**—Sulfotransferase activity was determined for recombinant sulfotransferases and soluble extracts of parasites by monitoring thiocyanate or sulfide formation as follows. The “rhodanese” assay was based on the formation of thiocyanate from the reaction between cyanide and either 3-mercaptoproprionate or thiosulfate and the detection of the thiocyanate by reacting it with an iron reagent to produce a red complex of FeSCN−, which absorbs at 460 nm. The optimized reaction mixture (1 ml) contained 0.1 mM Tris-Cl, pH 7.3, 10 mM Na2S2O3 and enzyme extract. The reaction was started by the addition of thiocyanate or 3-mercaptoproprionate (5 mM), and incubation was at 37 °C for 10 min. The reaction was stopped by the addition of 500 μl of formaldehyde, and 1.5 ml of iron reagent (Fe(NO3)3, 50 μl/liter; and 65% HNO3, 200 ml/liter) were added for FeSCN· complex formation. The assay was calibrated using NaSCN. The “sulfurtransferase” assay was based on the formation of hydrogen sulfide from the reaction between 3-mercaptoproprionate and 3-mercaptoproprionate or thiosulfate, which was detected using lead acetate and measuring the production of lead sulfide. The reaction mixture (1 ml) contained 0.1 mM Tris-Cl, pH 7.3, 0.2 mM lead acetate, 5 mM mercaptoproprionate, and enzyme extract. The reaction was started by the addition of 5 mM sodium thiosulfate or 3-mercaptoproprionate. The reaction at 37 °C was monitored continuously by detecting the formation of hydrogen sulfide spectrophotometrically at 380 nm. The molar extinction coefficient was taken as 5205 cm−1 M−1 (32).

The oxidation of reduced thioredoxin by recombinant sulfotransferase was measured by monitoring the reduction of oxidized thioredoxin by NADPH-specific thioredoxin reductase (4). A reaction mixture (1 ml) containing 20 μM NADPH, 2 μM Trichomonas vaginalis thiore-
doxin reductase, and 5 μg T. vaginalis thioredoxin in 0.1 x phosphate buffer, pH 7.0, was incubated at 37 °C for 5 min. A constant absorbance, as reported by Nandi et al. (4), was not achieved, as the thioredoxin reductase-thioredoxin system used has a low NADPH oxidase activity.2 0.1 μg of recombinant sulfurtransferase was added, and the reaction was started by the addition of 5 mM 3-mercaptopropionic acid or thiiosulfate. The reaction was incubated at 37 °C, and the consumption of NADPH was followed at 340 nm.

**Western Blot Analysis and SDS-PAGE—** Rabbit polyclonal anti-MST antibody was raised against rLmajMST by the Scottish Antibody Production Unit (Carluke, UK) using standard protocols. Parasite pellets were resuspended in an equal volume of lysis buffer, and supernatant (15,000 x g for 10 min) samples (10 μg of protein) were subjected to Western blot analysis as described previously (31) with polyclonal immune rabbit serum diluted 1:500 in Tris-buffered saline containing 1% (w/v) low fat dried milk and 0.1% Tween 20. Bound antibody was detected using horseradish peroxidase-coupled secondary antibodies (Scottish Antibody Production Unit) and ECL Western blotting detection reagents (Amersham Biosciences).

**Effects of Oxidative and Hypo-sulfur Stress on the Expression of LmajMST—** Promastigotes were cultured for 24 h under standard conditions and from a starting density of 2.5 x 10^5/ml in either (a) the presence of concentrations of hydrogen peroxide, cumene hydroperoxide, and tert-butyl hydroperoxide that inhibited growth by ~50% (300, 10, and 10 μM, respectively) or in (b) RPMI 1640 medium lacking cysteine and methionine (Labtech) supplemented with 10% (v/v) polyconal immune rabbit serum diluted 1:500 in Tris-buffered saline containing 1% (w/v) low fat dried milk and 0.1% Tween 20. Bound antibody was detected using horseradish peroxidase-coupled secondary antibodies (Scottish Antibody Production Unit) and ECL Western blotting detection reagents (Amersham Biosciences).

**Results**

**Cloning Genes Encoding Sulfurtransferases in L. major and L. mexicana—** Searches of L. major HTGS sequence in the GenBank/TM/EBI Data Bank revealed a gene (gi:13122208) encoding a protein with similarity to sulfurtransferases. PCR cloning, coupled with 5’ and 3’-RACE, confirmed the identity of the gene, which was subsequently annotated as a sulfurtransferase (GenBank/TM/EBI accession number AJ313201). The AG dinucleotide spliced reader addition acceptor site was found to be 354 nucleotides 5’ of the ATG start codon, and the gene has a 1160-bp ORF and a 173-bp 3’-untranslated region. The same methodology was used to clone the L. mexicana homolog (accession number AJ313202), which has a 89-bp 5’-untranslated region, a 1160-bp ORF, and a 255-bp 3’-untranslated region. The predicted amino acid sequences of the sulfurtransferase of each Leishmania species comprise 370 residues, with a calculated molecular mass of 40.1 kDa.

**Features of Predicted Amino Acid Sequences of the Sulfurtransferases—** The predicted amino acid sequences of L. major and L. mexicana are 96.7% identical to each other and 47% identical to a putative T. brucei sulfurtransferase identified in the T. brucei genome data base. Of the other sulfurtransferases known, the most similar are the mammalian and plant mercaptopyruvate and thiiosulfate sulfurtransferases, which are ~23% identical within the main domains (excluding the C-terminal extensions of the Leishmania sequences).

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MSTs known. The domain, which comprises 70 amino acids compared with the human gene (GenBank™/EBI accession number P25325), has no sequence identity to any other known protein sequence. However, a similar C-terminal domain is also present in a homologous protein of T. brucei and also in the protein predicted from a contig compiled from genomic sequence survey (GSS) gene fragments identified in the Trypanosoma cruzi genome data base (Fig. 1). Thus, it appears that this domain of the protein is a feature characteristic of, and perhaps unique to, the MSTs of trypanosomatids.

Genomic Organization of the MSTs—To assess the copy number of the leishmanial MSTs, genomic DNA was digested with five restriction enzymes and analyzed by Southern blotting using the MST CDNs as probes (Fig. 2). With L. major, the enzymes used that did not cut the gene itself (PstI, SalI, BsaI, and ClaI) resulted in a single major hybridizing DNA fragment, whereas Sau3A1, which cuts the ORF, resulted in multiple fragments (Fig. 2a). The hybridization patterns obtained for L. major, together with analysis of the genome sequence in the vicinity of the gene, indicate that the LmajMST gene is single-copy. Analysis of the genome sequence data base\(^3\) showed that the MST of L. major is flanked at its 5′-end by NADP dehydrogenase (EC 1.6.99.3) and at its 3′-end by dipetidyl peptidase III (EC 3.4.14.4). The Southern data for the LmexMST gene (Fig. 2b) similarly show that the enzymes PstI, SalI, and ClaI did not cut the gene itself and resulted in a single major hybridizing DNA fragment, whereas Sau3A1 and BsaI, both of which cut the ORF, resulted in multiple fragments. These data suggest that the gene of L. mexicana is also single copy.

Biochemical Characterization of Recombinant MSTs—The LmajMST and LmexMST genes were cloned into pET21a\(^*\) for E. coli expression of soluble recombinant enzyme (LmajMST and LmexMST) with a C-terminal six-histidine tag (~25 mg/liter E. coli). The purified recombinant MST was highly pure as judged by SDS-PAGE analysis and stable for at least 12 months without loss of activity at -20 °C when stored in 20 mM Tris-HCl, pH 7.9, 1 mM Na₂SO₄, and 4 mg/ml bovine serum albumin.

Recombinant LmajMST and LmexMST had activity toward both 3-mercaptopropionate and thiosulfate in the assays for both rhodanese-like and sulfurtransferase-like activities (Table I). The enzyme was optimally active in the pH range 6.9–7.6 toward both substrates. However, rLmajMST was considerably more active toward 3-mercaptopropionate than thiosulfate, with a lower \(K_m\) and higher \(k_{cat}\). In contrast to the reported behavior of bovine liver rhodanese (9, 42), cysteine and homocysteine

\(^3\) Available at www.sanger.ac.uk.
Leishmanial MST Contains an Unusual C-terminal Extension

Table II

Sulfurtransferase activities in L. major and L. mexicana

Soluble extracts from the parasite life cycle stages were assayed for their sulfurtransferase activities using two methods: with 5 mM 3-mercaptopyruvate and 5 mM mercaptoethanol as substrates and monitoring the formation of hydrogen sulfide (sulfurtransferase) or with 5 mM 3-mercaptopyruvate and 10 mM cyanide as substrates and measuring the amount of ferrous thiocyanide complex formed (rhodanese). Activities are means ± S.D. from triplicate analyses and the number of experiments given in parentheses.

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<th>Rhodanese</th>
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<tr>
<td></td>
<td>μmol/min/mg protein</td>
<td>μmol/min/mg protein</td>
</tr>
<tr>
<td>L. mexicana</td>
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<tr>
<td>Amastigotes</td>
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<td>0.09 ± 0.01 (2)</td>
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<tr>
<td>L. major</td>
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<tr>
<td>Log phase promastigotes</td>
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<Fig. 3.> Western blot analysis of the expression of leishmanial MST during the life cycle and in cells subjected to hypo-sulfur and oxidative stress. 80 μg of soluble cell extract were subjected to Western blot analysis using rabbit anti-recombinant MST antiserum. a, expression during the life cycle. Lane 1, L. mexicana amastigotes; lane 2, L. mexicana stationary phase promastigotes; lane 3, L. major mid-log phase promastigotes; lane 4, L. major stationary phase promastigotes; lane 5, L. major metacyclic promastigotes. b, expression in L. major promastigotes subjected to hypo-sulfur and oxidative stress. Lane 1, control; lane 2, hypo-sulfur stress; lane 3, 10 μM cumene hydroperoxide; lane 4, 300 μM hydrogen peroxide; lane 5, 10 μM tert-butyl hydroperoxide.>

<Fig. 4.> rLmajMST reacts with thioredoxin. rLmajMST (enzyme (E)) and 3-mercaptopyruvate (substrate (S)) were added at the times indicated by the arrows to the reduced thioredoxin system, which was at equilibrium, and the subsequent NADPH oxidation was followed spectrophotometrically at 340 nm. Abs, absorbance.

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LmajMST Oxidizes Reduced Thioredoxin—It has been reported that MSTs can both oxidize thioredoxin, a key intermediate in cellular redox reactions, and react with peroxides and that this may be a physiologically significant mechanism for combating oxidative challenges (4). Addition of rLmajMST and 3-mercaptopyruvate to a mixture of thioredoxin reductase, thioredoxin, and NADPH led to the rapid oxidation of NADPH (Fig. 4). Addition of thiosulfate rather than mercaptopyruvate resulted in activity, albeit at a lower level (37 μmol/min/mg of protein) compared with the control (Table II). In contrast, there were no significant changes in sulfurtransferase activity after exposure to tert-butyl hydroperoxide or hydrogen peroxide. Western analyses of the same cells extracts (Fig. 3b) also showed an increase above the wild-type levels of protein in the parasites exposed to cumene hydroperoxide stress. Promastigotes of L. major also responded to hypo-sulfur stress over 24 h by increased expression of MST (Fig. 3b), with the resultant sulfurtransferase activity being 0.85 ± 0.01 μmol/min/mg of protein.

The C-terminal Domain Is Required for LmajMST Activity—Four LmajMSTs were expressed with truncations in the C-terminal domain. Although the truncated proteins were expressed in E. coli in similar amounts compared with full-length rLmajMST, the removal of C-terminal peptides substantially changed the solubility of the protein. rLmajMSTΔ300–370, the protein lacking the entire C-terminal domain, and rLmajMSTΔ320–370 were expressed entirely in inclusion bodies irrespective of the induction and growth conditions used. Solubilization of the protein using 8 M urea and subsequent attempts to refold the enzyme failed to result in active enzyme. Shorter truncations of 24 or 10 amino acids yielded some soluble recombinant protein (~50 and 70%, respectively). However, these recombinant MSTs had greatly diminished enzyme activity (0.3 and 3 μmol/min/mg of protein, respectively) com-

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4 Available at psort.nibb.ac.jp.
pared with that of the full-length protein (343 μmol/min/mg of protein). CD analyses of the full-length and two soluble truncated proteins showed that all three had similar secondary structure contents (18% α-helix, 26% β-sheet, 23% turn, 33% other) when analyzed by the SELCON method (37). However, there was a marked difference in the near-UV spectra, especially in the region between 270 and 290 nm, between the wild-type protein and the truncated derivatives (Fig. 5). These results suggested that the consequence of the truncations was a less well folded protein with significant alterations to the environments of the tyrosine side chains. Bis-ANS fluorescence studies (Fig. 6) further supported the conclusions from the CD analyses in that the truncated proteins exhibited greater fluorescence, which is consistent with hydrophobic residues normally buried in the native enzyme being exposed in the truncated proteins.

Denatured LmajMST Refolds Unassisted—Mammalian rhodanese has been studied extensively as a model for protein folding (34, 38). However, refolding of rhodanese is relatively difficult because of the presence of multiple disulfide bonds and the ability of folding intermediates to form aggregates. Thus, rhodanese does not refold well unassisted, and either a chaperone or a detergent is required for success (38). LmajMST denatured using 6 M urea and then either dialyzed or simply diluted into refolding buffer regained its full enzyme activity without the need for assistance in the form of a chaperone or detergent (Fig. 7). In contrast, the truncated proteins similarly treated did not even regain the low level of activity that the enzymes possessed when they were initially purified from E. coli.

DISCUSSION

We have characterized at the molecular and biochemical levels an MST that is expressed throughout the life cycle of L. major and L. mexicana. The leishmanial single-copy genes predict an unusual sulfurtransferase that, in comparison with other known sulfurtransferases, has an additional C-terminal domain of some 70 amino acids. The discovery that this domain is also encoded in a gene of T. cruzi and T. brucei suggests that the feature is conserved among trypanosomatid parasites and so has some key function. Importantly, it distinguishes the parasites’ enzymes from their mammalian counterparts. A possible role of the unusual C-terminal domain was highlighted by the finding that the leishmanial MST can refold successfully without a chaperone, which is in contrast to the results reported for sulfurtransferases from other sources (21, 39–41). We hypothesized that the unusual C-terminal domain may play some part in this. The finding that the proteins with truncated C termini were misfolded and showed very little activity is consistent with this postulate.

Analysis of the recombinant enzyme has clearly shown that the enzyme prefers 3-mercaptopyruvate to thiocysteine as the donor substrate. This substrate preference correlates well with the active-site residues being homologous to other eukaryotic MSTs rather than rhodanese. The \( K_m \) and \( V_{max} \) values obtained compare favorably with those reported for bacterial, plant, and vertebrate MSTs (1, 42). The very high activity toward 3-mercaptoppyruvate suggests that this could function as a natural substrate, although currently there is nothing known about the levels of this compound in Leishmania parasites or any roles that it may have.

The finding that the leishmanial sulfurtransferases can use thioredoxin as an acceptor with a \( K_m \) of 300 nM suggests that this could also be one natural substrate. It has been demonstrated previously that the thioredoxin from E. coli can serve as a sulfur acceptor substrate for the E. coli sulfurtransferase when thiosulfate is near its \( K_m \) (10), and it has also been shown that mammalian sulfurtransferases can utilize thioredoxin (4). At one time, thioredoxin was considered to be absent in Leishmania (see Ref. 25); however, a thioredoxin-like gene has been identified in L. major (GenBank™/EBI accession number AAC10802) and T. brucei (43). One possible function of the interaction between a sulfurtransferase and thioredoxin could be the involvement of the enzyme in reduction and detoxification of peroxides (4). The finding that the leishmanial enzyme cannot catalyze this reaction suggests that it must have some other role. The oxidation status of thioredoxin is thought to be crucial in regulating a number of cellular reactions, and so the oxidation by the leishmanial sulfurtransferase of reduced thioredoxin implicates this enzyme in similar processes.

The antioxidant machinery of trypanosomatids has been considered to rely almost exclusively on trypanothione and its associated enzymes (23). However, other enzyme systems may well also be involved. The up-regulation of LmajMST upon

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**Fig. 5.** CD spectra of rLmajMST proteins. The near-UV CD spectrum of full-length rLmajMST (trace 1) differed considerably from those of rLmajMSTΔ360–370 (trace 2) and rLmajMSTΔ338–370 (trace 3). Mol. Ellip., molecular ellipticity in degrees/cm²/dmol.

**Fig. 6.** Bis-ANS fluorescence of rLmajMST proteins. Full-length rLmajMST (■) bound less bis-ANS than did rLmajMSTΔ360–370 (▲) and rLmajMSTΔ338–370 (▼). The fluorescence intensity (FI) is given in arbitrary units.

**Fig. 7.** LmajMST refolds in the absence of a chaperone. rLmajMST refolded to give active enzyme most efficiently when simply diluted into refolding buffer (▲), with 97% of the original activity being recovered by 2 h. Dialysis (■) also yielded active enzyme, but more slowly.
exposure of *L. major* promastigotes to the oxidant cumene hydroperoxide shows that MST may be an important additional enzymatic mechanism for protection against oxidative stress.

The up-regulation of MST upon culturing *L. major* promastigotes without an exogenous source of sulfur suggests that the enzyme might also have a physiological role in sulfur amino acid metabolism. The expression of RhdA, a rhodanese-like protein of *Synechococcus* sp. strain PCC7942, is also induced by sulfur starvation (44). Furthermore, sulfotransferases have been implicated in the metabolism of sulfur as shown by the formation and regulation of iron-sulfur centers of proteins (9). Another possible role of the sulfotransferase in *L. major* is its expression in the mammalian form of the parasite (45). The results obtained provide compelling evidence that *Leishmania* contains a high level of activity of a structurally unusual sulfotransferase that has a strong activity toward 3-mercaptopypyruvate and thioredoxin and that plays some role in antioxidant and sulfur amino acid metabolism. The finding that the sulfotransferase in *Euglena gracilis* is also distributed within the cytosol. The only other report on a sulfotransferase of a protozoon (33) suggest that an enzyme in *Euglena gracilis* is also distributed in the cytosol.

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