THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 3, Issue of January 17, pp. 1480–1486, 2003

Printed in U.S.A.

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

Received for publication, September 13, 2002, and in revised form, October 25, 2002
Published, JBC Papers in Press, November 4, 2002, DOI 10.1074/jbc.M209395200

Roderick A. M. Williams‡§, Sharon M. Kelly¶, Jeremy C. Mottram‖, and Graham H. Coombs‡ ‡‡**

From the Divisions of ‡Infection and Immunity and ¶Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ and the ‖Wellcome Centre for Molecular Parasitology, University of Glasgow, Anderson College, Glasgow G11 6NU, Scotland, United Kingdom

Cytosolic 3-mercaptopyruvate sulfurtransferases (EC 2.8.1.2) 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 detoxification of cyanide to form thiocyanate, which is harmless and excreted by the kidney. This role could be important, especially in the epithelial cells lining the gut (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 ThiI 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 rhodaneses 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,

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ313201 and AJ313202.

§ Supported by a Commonwealth scholarship.

** Medical Research Council (United Kingdom) Senior Research Fellow.

‡‡ To whom correspondence should be addressed: Div. of Infection and Immunity, University of Glasgow, Joseph Black Bldg., Glasgow G12 8QQ, Scotland, UK. Tel.: 44-141-330-4777; Fax: 44-141-330-3516; E-mail: g.coombs@bio.gla.ac.uk.

we describe the cloning, expression, and characterization of a sulfurtransferase 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/Friedlin) 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 from 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 79 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 μM E-64, 2 mM 1,10-phenanthroline, 4 μM 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.

Identification, PCR Amplification, and Cloning of 3-Mercaptopyruvate Sulfurtransferase (MST)[1] 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 TELT buffer (50 mM Tris-HCl, pH 8.0, 62.5 mM EDTA, 2.5 M 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 NT22 (5′-ACGCGCACCTTCGATGTGGCC-3′) and NT41 (5′-ATTTCATCCACAAGGTAGTGATGC-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 LMEXSLI (5′-TAACGCTATATAAGTATCAGTTTC-3′) and LMEXSLII (5′-AGTATCAGTTTCTGTACTTTATTG-3′) were used for 5′-RACE. The perfect match primers NT21 (5′-CACCGCCCGGCATCCGCTACC-3′) and NT54 (5′-ATCGACTGGTGCATGGCGAAC-3′), based on consensus sequences of the genes detailed above, and oligo(T) primers with an adaptor attached, UAP (5′-CUACUACUACUAGGCCACGCGTGGACTAGTAC-3′) and AUAP (5′-GGCCACGCGTGGACTAGTAC-3′), were used to amplify the 3′-ends of the genes with Expand High Fidelity *Pwo*/*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, LmajMSTΔ300–370, LmajMSTΔ320–370, LmajMSTΔ338–370, and LmajMSTΔ360–370, encoding truncated proteins lacking 70, 50, 32, and 10 amino acids of the C terminus (rLmajMSTΔ300–370, rLmajMSTΔ320–370, rLmajMSTΔ338–370, and rLmajMSTΔ360–370, respectively), were amplified from cDNA by PCR with the Expand High Fidelity system. For this, the 5′-perfect match primer NT42 (5′-CGCTGACATATGTCTGCTCCTGCTGCGCCGAA-3′) was used for all constructs, 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′-TTCCTCGAGTGGAGGGGCGGAAGAGGCCGCTGT–3′) for LmajMST, NT56 (5′-TTCCTCGAGCGGCAGCGGCGTCACCGC-3′) for LmexMST, NTMST1 (5′-TTCCTCGAGGCTGCGCATTATGG-3′) for LmajMSTΔ300–370, NTMST2 (5′-TTCCTCGAGCTTCGGGTTGTCGCCGA-3′) for LmajMSTΔ320–370, NTMST3 (5′-TTCTCGAGCTCCGCATCGGGTCTCTCG-3′) for LmajMSTΔ338–370, and NTMST4 (5′-TTCCTCGAGGACGCGGCCGCTCTTGA-3′) for LmajMSTΔ360–370. An *Nde*I restriction site (underlined) was added to the 5′-end of NT42, and an *Xho*I restriction site (underlined) was added to NT55, NT56, NTMST1, NTMST2, NTMST3, and NTMST4 to facilitate cloning and purification. The PCR products were cloned into pGEM-T to generate plasmids designated as pLmajMST, pLmexMST, pLmajMSTΔ300–370, pLmajMSTΔ320–370, pLmajMSTΔ338–370, and pLmajMSTΔ360–370, respectively, and sequenced.

Plasmids were digested with *Nde*I and *Xho*I and ligated into *Nde*I/*Xho*I-digested pET21a$^+$ vector (Invitrogen) to produce the respective pET constructs (*e.g.* pETLmajMST). The plasmids were used to transform *E. coli* BL21 Codon Plus (DE3)RP cells (Stratagene). Expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside for 5 h at 15 °C. Cells were pelleted and resuspended in 5 ml of buffer A (50 mM sodium phosphate and 3 M NaCl, pH 8.0) with 5 mM imidazole and disintegrated 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-nitrilotriacetic acid column (bioCAD® 700E work station) pre-equilibrated with buffer A. The column was washed with 60 ml of buffer A containing 5 mM imidazole and then with 300 ml of buffer A containing 60 mM imidazole. The His-tagged recombinant proteins were eluted with 250 mM imidazole in buffer A. The eluant was dialyzed with excess 20 mM Tris-HCl, pH 7.9, and 1 mM $Na_2S_2O_3$ at 4 °C overnight and stored at −20 °C. Approximately 25 mg of soluble recombinant proteins (LmajMST and LmexMST) were obtained from 1-liter cultures of *E. coli*.

Refolding of Denatured Enzyme—Denaturation and reactivation analyses of rLmajMSTΔ338–370, rLmajMSTΔ360–370, and rLmajMST 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 $Na_2S_2O_3$ and 6 M urea for 120 min at 20 °C. Attempts to reactivate the proteins involved one of two procedures: (*a*) 25 μl of denatured protein at 1.0 mg/ml were diluted in 75 μl of refolding buffer containing 0.2 M 2-mercaptoethanol, 50 mM $Na_2S_2O_3$, 10 mM KCl, and 10 mM $MgCl_2$; 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 sulfurtransferase 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 Hybond™-N$^+$ membrane (31) by capillary transfer. Probes were prepared from 1110-bp *Nde*I/*Xho*I ORF fragments from pETLmajMST and pETLmexMST using a Prime-It kit (Stratagene) and purified on Microspin S-200 HR columns (Amersham Biosciences). Filters were hybridized overnight with [α-^{32}P]dATP-labeled LmajMST 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—Sulfurtransferase activity was determined for recombinant sulfurtransferases 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-mercaptopyruvate 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 M Tris-HCl, pH 7.3, 10 mM KCN, and enzyme extract. The reaction was initiated by the addition of thiosulfate or 3-mercaptopyruvate (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 ($Fe(NO_3)_3$, 50 g/liter; and 65% HNO_3, 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 2-mercaptoethanol and 3-mercaptopyruvate or thiosulfate. This was detected using lead acetate and measuring the production of lead sulfide. The reaction mixture (1 ml) contained 0.1 M Tris-HCl, pH 7.3, 0.2 mM lead acetate, 5 mM mercaptoethanol, and enzyme extract. The reaction was started by the addition of 5 mM sodium thiosulfate or 3-mercaptopyruvate. The reaction at 37 °C was monitored continuously by detecting the formation of lead sulfide spectrophotometrically at 360 nm. The molar extinction coefficient was taken as 5205 cm^{-1} M^{-1} (32).

The oxidation of reduced thioredoxin by recombinant sulfurtransferase 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 μg *Trichomonas vaginalis* thiore-

[1] The abbreviations used are: MST, 3-mercaptopyruvate sulfurtransferase; LmajMST, *L. major* MST; rLmajMST, recombinant LmajMST; LmexMST, *L. mexicana* MST; RACE, rapid amplification of cDNA ends; ORF, open reading frame; bis-ANS, 1,1′-bis(4-anilino)naphthalene-5,5′disulfonic acid; contig, group of overlapping clones.

doxin reductase, and 5 μg *T. vaginalis* thioredoxin[2] in 0.1 M 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-mercaptopyruvate or thiosulfate. 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 $\times$ 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 2.5×10^5 ml^{-1} 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) dialyzed heat-inactivated fetal bovine serum. Crude lysates of the parasites were obtained as described above for further analyses.

Analysis of LmajMST and Its Truncated Derivatives Using CD Spectroscopy and Binding of Bis-ANS—CD spectral measurements of rLmajMSTΔ338–370, rLmajMSTΔ360–370, and rLmajMST were performed on a Jasco J-600 spectropolarimeter at 20 °C. All samples were in 20 mM sodium phosphate buffer, pH 7.4. Protein concentrations for far-UV and near-UV CD measurements were 0.26 and 1.1 mg/ml, respectively, and samples were scanned in 0.05-cm and 0.5-mm path length cells, respectively. CD data were calculated in terms of mean molar residue ellipticity (θ) using mean residue weights of 109 for each enzyme (34, 35). The availability of hydrophobic binding sites on each of the proteins was also assessed using bis-ANS. Protein samples (50 μg/ml) were mixed with bis-ANS (to 30 μM) in 20 mM phosphate buffer, pH 7.8, containing 50 mM $Na_2S_2O_3$, and fluorescence was monitored (excitation at 395 nm and emission at 500 nm) at 25 °C using an LS55 luminescence spectrometer (PerkinElmer Life Sciences).

RESULTS

Cloning Genes Encoding Sulfurtransferases in L. major and L. mexicana—Searches of *L. major* HTGS sequence in the GenBank™/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™/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 thiosulfate sulfurtransferases, which are ~23% identical within the main domains (excluding the C-terminal extensions of the *Leishmania* sequences). Sequence analysis of the sulfurtransferases from vertebrates, plants, yeast, bacteria, and protozoa (Fig. 1) showed that the leishmanial proteins are more similar to other MSTs (with an active-site motif of CG(S/T)GVTA) than to eukaryotic thiosulfate sulfurtransferases and rhodaneses (with an active-site motif of CRKGVTA). Interestingly, prokaryotic genes thought to encode thiosulfate sulfurtransferases lack the CRKGVTA motif, although, in most cases, the substrate specificity has not been fully analyzed. As the catalytic active-site CGSGVTA motif of the leishmanial genes (*underlined* in Fig. 1) is considered to be predictive of an MST rather than a rhodanese or thiosulfate sulfurtransferase (49), the genes were designated LmajMST and LmexMST.

Intriguingly, both of the leishmanial sulfurtransferases contain an additional C-terminal domain compared with all other

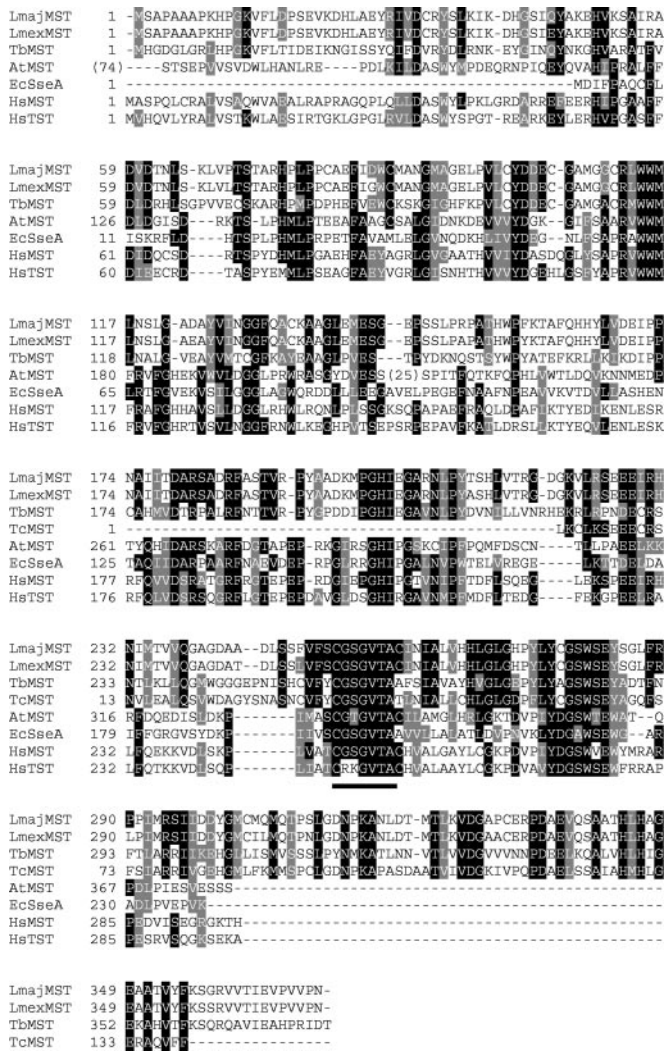

FIG. 1. **Alignment of deduced amino acid sequences of sulfurtransferases from *Leishmania* and representative organisms.** The sequences are as follows: LmajMST and LmexMST (this study); *T. brucei* (*Tb*) MST (GenBank™/EBI accession number AC091553); *T. cruzi* (*Tc*) MST (accession number AI667879); *Arabidopsis thaliana* (*At*) MST (accession number BAA85148; gi:6009981); *Escherichia coli* (*Ec*) SseA protein (accession number P31142); *Homo sapiens* (*Hs*) MST (accession number P25325); and *H. sapiens* thiosulfate sulfurtransferase (*TST*) (accession number NM003312.2; gi:17402865). These sequences were aligned using AlignX (VectorNTI). *Black shading* indicates identical amino acids; *gray shading* indicates conserved amino acids. The active-site sequence is *underlined*. The unique N-terminal and hinge domains of *A. thaliana* MST have been omitted; the number of amino acids that have been removed are indicated in *parentheses*. The *T. cruzi* sequence is incomplete and comprises only the C-terminal segment of the protein.

[2] G. H. Coombs, G. D. Westrop, P. Suchan, G. Puzova, T. Hirt, T. M. Embley, J. C. Mottram, and S. Muller, submitted for publication.

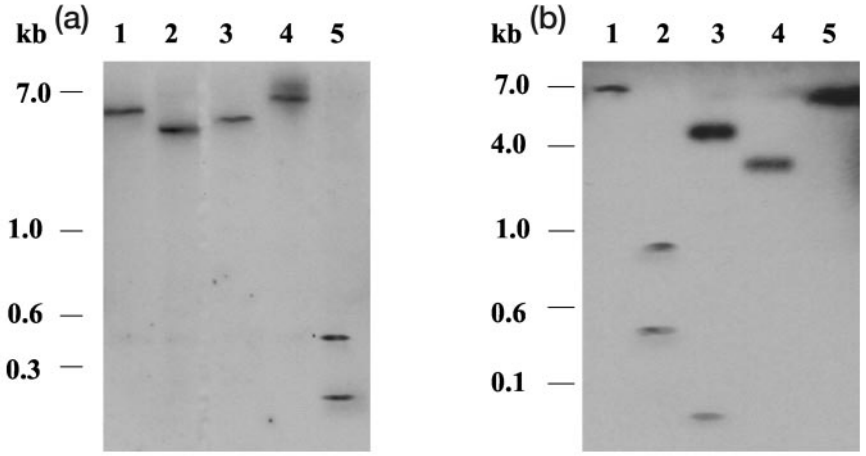

FIG. 2. **Genomic organization of leishmanial MSTs.** 5 μg of genomic DNAs from *L. major* (*a*) and *L. mexicana* (b) were digested with a variety of restriction endonucleases; separated by agarose gel electrophoresis; transferred to a nylon membrane; and hybridized with [^{32}P]dATP-labeled LmajMST and LmexMST cDNA probes corresponding to the entire ORFs, respectively. The restriction endonucleases used were *Pst*I (*lane 1*), *Bsa*I (*lane 2*), *Sal*I (*lane 3*), and *Eco*RI (*lane 4*), and *Sau3*A1 (*lane 5*) for *L. major* (*a*) and *Pst*I (*lane 1*), *Sau*3A1 (*lane 2*), *Bsa*I (*lane 3*), *Cla*I (*lane 4*), and *Sal*I (*lane 5*) for *L. mexicana* (*b*). The sizes of DNA standards are indicated in kilobase pairs.

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 cDNAs as probes (Fig. 2). With *L. major*, the enzymes used that did not cut the gene itself (*Pst*I, *Sal*I, *Bsa*I, and *Cla*I) resulted in a single major hybridizing DNA fragment, whereas *Sau*3A1, which cuts the ORF, resulted in multiple fragments (Fig. 2*a*). 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 dipeptidyl peptidase III (EC 3.4.14.4). The Southern data for the LmexMST gene (Fig. 2*b*) similarly show that the enzymes *Pst*I, *Sal*I, and *Cla*I did not cut the gene itself and resulted in a single major hybridizing DNA fragment, whereas *Sau*3A1 and *Bsa*I, both of which cut the ORF, resulted in multiple fragments. These data suggest that the gene of *L. mexicana* is also single copy.

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 $Na_2S_2O_3$, and 4 mg/ml bovine serum albumin.

Recombinant LmajMST and LmexMST had activity toward both 3-mercaptopyruvate 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-mercaptopyruvate 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 were not used by rLmajMST. The role of thiols such as mercaptoethanol, homocysteine, cysteine, and glutathione was analyzed using the sulfurtransferase assay involving sulfide trapping with lead acetate. In this assay, the enzyme showed activity in the absence of added 2-mercaptoethanol, but the addition of 5 mM 2-mercaptoethanol increased the rate by 20-fold to 345 μmol/min/mg of protein. Homocysteine (10 mM) and cysteine (50 μM), but not glutathione, also each increased the production of sulfide from mercaptopyruvate, but only by ~10-fold.

TABLE I
Activities of recombinant LmajMST and LmexMST

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

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

Organism/substrate	Kinetics
L. major	
Sodium thiosulfate	K_m = 1.7 mM V_{max} = 10.9 μmol/min/mg k_{cat} = 2.73 × 10^3 min^{-1}
Mercaptopyruvate	K_m = 0.2 mM V_{max} = 345 μmol/min/mg k_{cat} = 1.73 × 10^5 min^{-1}

Expression and Localization of Leishmanial MSTs—Sulfurtransferase activity was found in cell extracts prepared from each developmental stage in the leishmanial life cycle (Table II). Polyclonal antibodies raised against rLmajMST recognized a single 40-kDa protein in soluble extracts of *L. mexicana* amastigotes and promastigotes and *L. major* log phase, stationary phase, and metacyclic promastigotes (Fig. 3*a*).

The localization of the LmajMST gene was investigated us-

[3] Available at www.sanger.ac.uk.

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.

Stage	Sulfurtransferase	Rhodanese
	μmol/min/mg protein	*μmol/min/mg protein*
L. mexicana		
Amastigotes	0.33 ± 0.08 (4)	0.09 ± 0.01 (2)
Stationary phase promastigotes	0.60 ± 0.11 (4)	0.05 ± 0.02 (2)
L. major		
Log phase promastigotes	0.71 ± 0.02 (3)	0.02 ± 0.01 (2)
Stationary phase promastigotes	0.67 ± 0.05 (3)	0.09 ± 0.05 (2)
Metacyclic promastigotes	0.61 ± 0.07 (3)	0.03 ± 0.01 (2)

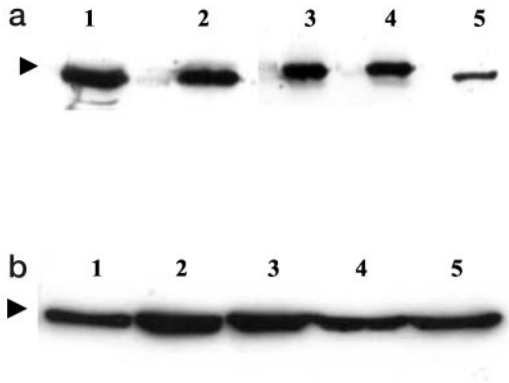

FIG. 3. **Western blot analysis of the expression of leishmanial MST during the life cycle and in cells subjected to hypo-sulfur and oxidative stress.** 80 μg of soluble cell extract were subjected to Western blot analysis using rabbit anti-recombinant MST antiserum. *a*, expression during the life cycle. *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.

ing immunofluorescence microscopy. Labeling was detected throughout the cytoplasm of the cell (data not shown), suggestive of a cytosolic location for LmajMST. The subcellular distribution of MST in promastigote fractions derived by differential centrifugation was also analyzed by Western blot analysis. This analysis similarly showed that the protein was recovered primarily in the cytosolic fraction (data not shown), whereas antibody raised against the lysosomal CPB cysteine protease of *L. mexicana* (36) detected protein in the small organelle fraction as expected. Enzyme activity analyses revealed similar results. These data together suggest that the leishmanial MST is a cytosolic enzyme. This is consistent with analysis using PSORT,[4] which indicated that the leishmanial sulfurtransferases lack any characteristic mitochondrial or other targeting sequence.

LmajMST Is Up-regulated in Response to Oxidative and Hypo-sulfur Stress—Exposure of *L. major* promastigotes to the oxidants hydrogen peroxide, cumene hydroperoxide, and *tert*-butyl hydroperoxide (at 300, 10, and 10 μM, respectively) led to the inhibition of growth by ~50% during 24 h of incubation. Differing effects upon the expression of MST were noted. The sulfurtransferase activity of parasites exposed to cumene hydroperoxide was increased (to 0.98 ± 0.01 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. 3*b*) 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. 3*b*), with the resultant sulfurtransferase activity being 0.85 ± 0.01 μmol/min/mg of protein.

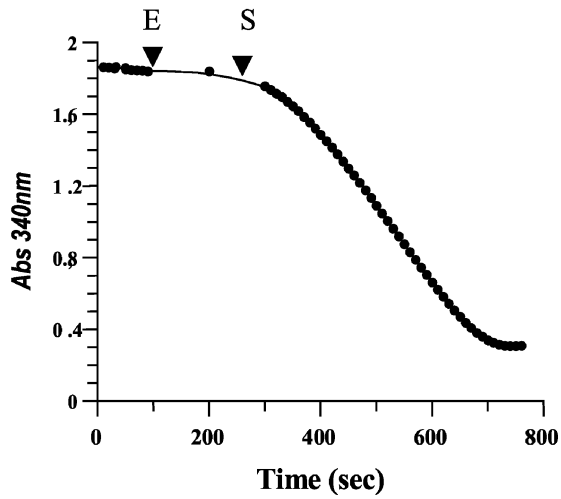

FIG. 4. **rLmajMST reacts with thioredoxin.** rLmajMST (enzyme (*E*)) and 3-mercaptopyruvate (substrate (*S*)) were added at the times indicated by the *arrows* to the reduced thioredoxin system, which was at equilibrium, and the subsequent NADPH oxidation was followed spectrophotometrically at 340 nm.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

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 386 μmol/min/mg of protein for 3-mercaptopyruvate). The apparent affinity for the reduced thioredoxin was high (K_m = 300 nM). Similar oxidation did not occur if any substrate was omitted or if 3-mercaptopyruvate was replaced by 10 μM cumene hydroperoxide.

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-

[4] Available at psort.nibb.ac.jp.

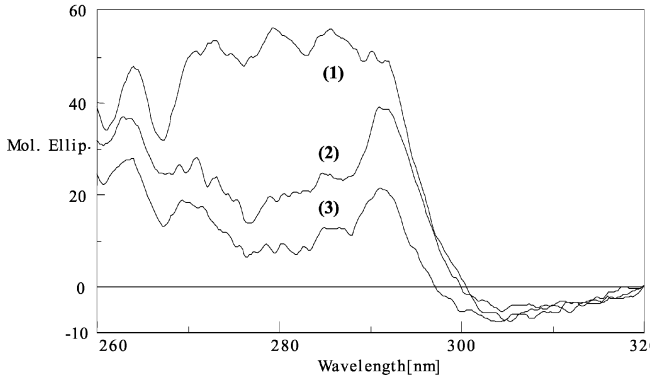

FIG. 5. **CD spectra of rLmajMST proteins.** The near-UV CD spectrum of full-length rLmajMST (*trace 1*) differed considerably from those of rLmajMSTΔ360–370 (*trace 2*) and rLmajMSTΔ338–370 (*trace 3*). *Mol. Ellip.*, molecular ellipticity in degrees/cm^2/dmol.

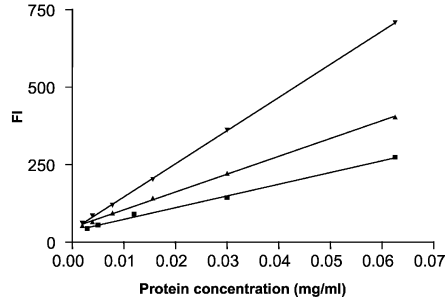

FIG. 6. **Bis-ANS fluorescence of rLmajMST proteins.** Full-length rLmajMST (■) bound less bis-ANS than did rLmajMSTΔ360–370 (▲) and rLmajMSTΔ338–370 (▼). The fluorescence intensity (*FI*) is given in arbitrary units.

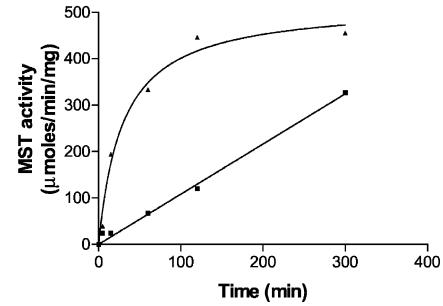

FIG. 7. **LmajMST refolds in the absence of a chaperone.** rLmajMST refolded to give active enzyme most efficiently when simply diluted into refolding buffer (▲), with 97% of the original activity being recovered by 2 h. Dialysis (■) also yielded active enzyme, but more slowly.

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 thiosulfate as the donor substrate. This substrate preference correlates well with the active-site residues being homologous to other eukaryotic MSTs rather than rhodaneses. 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-mercaptopyruvate 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 AAG10802) 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

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, sulfurtransferases 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 sulfurtransferase in *Leishmania* is that it functions to provide sulfide for the cysteine synthesis pathway. We have shown that LmajMST works in conjunction with both recombinant *L. major* cysteine synthase (GenBank™ accession number AL499624) and recombinant *L. major* cystathionine β-synthase (accession number AL499619) to synthesize cysteine *in vitro*. *T. cruzi* cystathionine β-synthase was also recently reported to possess cysteine synthase activity (45).

Eukaryotic MSTs can be cytosolic or mitochondrial (22, 42, 46). Both the immunolocalization and fractionation data suggest that the MST of *L. major* occurs within the cytosol. The only other report on a sulfurtransferase of a protozoon (33) suggest that an enzyme in *Euglena gracilis* is also distributed in the cytosol.

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

Acknowledgment—We thank N. C. Price for helpful advice with the CD analyses.

REFERENCES

1. Bordo, D., and Bork P. (2002) *EMBO Rep.* **3,** 741–746
2. Nakamura, T., Yamaguchi, Y., and Sano, H. (2000) *Eur. J. Biochem.* **267,** 5621–5630
3. Luo, G. X., and Horowitz P. M. (1994) *J. Biol. Chem.* **269,** 8220–8225
4. Nandi, D. L., Horowitz, P. M., and Westley, J. (2000) *Int. J. Biochem. Cell Biol.* **32,** 465–473
5. Picton, R., Eggo, M. C., Merrill, G. A., Langman, M. J., and Singh, S. (2002) *Gut* **50,** 201–205
6. Marquet, A. (2001) *Curr. Opin. Chem. Biol.* **5,** 541–549
7. Lauhon, C. T., and Kambampati, R. (2000) *J. Biol. Chem.* **275,** 20096–20103
8. Duin, E. C., Lafferty, M. E., Crouse, B. R., Allen, R. M., Sanyal, I., Flint, D. H., and Johnson, M. K. (1997) *Biochemistry* **36,** 11811–11820
9. Ogata, K., and Volini, M. (1990) *J. Biol. Chem.* **265,** 8087–8093
10. Ray, W. K., Zeng, G., Potters, M. B., Mansuri, A. M., and Larson, T. J. (2000) *J. Bacteriol.* **182,** 2277–2284
11. Mueller, E. G., Palenchar, P. M., and Buck, C. J. (2001) *J. Biol. Chem.* **276,** 33588–33595
12. Palenchar, P. M., Buck, C. J., Cheng, H., Larson, T. J., and Mueller, E. G. (2000) *J. Biol. Chem.* **275,** 8283–8286
13. Tse Sum Bui, B., Escalettes, F., Florentin, D., and Marquet, A. (2000) *Eur. J. Biochem.* **267,** 2688–2694
14. Leimkuhler, S., and Rajagopalan, K. V. (2001) *J. Biol. Chem.* **276,** 22024–22031
15. Schmidt, A., and Jager, K. (1992) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **43,** 325–349
16. Bordo, D., Deriu, D., Colnaghi, R., Carpen, A., Pagani, S., and Bolognesi, M. (2000) *J. Mol. Biol.* **298,** 691–704
17. Trevino, R. J., Gliubich, F., Berni, R., Cianci, M., Chirgwin, J. M., Zanotti, G., and Horowitz, P. M. (1999) *J. Biol. Chem.* **274,** 13938–13947
18. Luo, G. X., and Horowitz, P. M. (1993) *J. Biol. Chem.* **268,** 10246–10251
19. Mendoza, J. A., Rogers, E., Lorimer, G. H., and Horowitz, P. M. (1991) *J. Biol. Chem.* **266,** 13044–13049
20. McHugh, C. P., Melby, P. C., and LaFon, S. G. (1996) *Am. J. Trop. Med. Hyg.* **55,** 547–555
21. Croft, S. L., and Yardley, V. (2002) *Curr. Pharm. Des.* **8,** 319–342
22. Miller, M. A., McGowan, S. E., Gantt, K. R., Champion, M., Novick, S. L., Andersen, K. A., Bacchi, C. J., Yarlett, N., Britigan, B. E., and Wilson, M. E. (2000) *J. Biol. Chem.* **275,** 33883–33889
23. Krauth-Siegel, R. L., and Coombs, G. H. (1999) *Parasitol. Today* **15,** 404–409
24. Brooks, D. R., Denise, H., Westrop, G. D., Coombs, G. H., and Mottram, J. C. (2001) *J. Biol. Chem.* **276,** 47061–47069
25. Sacks, D. L., Hieny, S., and Sher, A. (1985) *J. Immunol.* **135,** 564–569
26. Bates, P. A., Robertson, C. D., Tetley, L., and Coombs, G. H. (1992) *Parasitology* **105,** 193–202
27. Lanham, S. M., and Godfrey, D. G. (1970) *Exp. Parasitol.* **28,** 521–534
28. Brun, R., Jenni, L., Tanner, M., Schonenberger, M., and Schell, K. F. (1979) *Acta Trop.* **36,** 387–390
29. Medina-Acosta, E., and Cross, G. A. (1993) *Mol. Biochem. Parasitol.* **59,** 327–329
30. Bhattacharyya, A. M., and Horowitz, P. M. (2002) *Biochemistry* **41,** 422–429
31. Mottram, J. C., Kinnaird, J. H., Shiels, B. R., Tait, A., and Barry, J. D. (1993) *J. Biol. Chem.* **268,** 21044–21052
32. Thong, K. W., and Coombs, G. H. (1987) *Exp. Parasitol.* **62,** 143–151
33. Watanebe, F., Nakano, Y., and Kitaoka, S. (1985) *Agric. Biol. Chem.* **49,** 2203–2204
34. White, Z. W., Fisher, K. E., and Eisenstein, E. (1985) *J. Biol. Chem.* **270,** 20404–20409
35. Kelly, S. M., and Price, N. C. (1997) *Biochim. Biophys. Acta* **1338,** 161–185
36. Sanderson, S. J., Pollock, K. G. J., Hilley, J. D., Mendal, M., Halarie, P., Juliano, M. A., Juliano, L., Mottram, J. C., and Coombs, G. H. (2000) *Biochem. J.* **347,** 383–388
37. Sneerama, N., and Woody, R. W. (1993) *Anal. Biochem.* **209,** 32–44
38. Panda, M., and Horowitz, P. M. (2000) *J. Protein Chem.* **19,** 399–409
39. Ellis, R. J., and Hemmingsten, S. M. (1989) *Trends Biochem. Sci.* **14,** 339–342
40. Mendoza, J. A., Lorimer, G. H., and Horowitz, P. M. (1991) *J. Biol. Chem.* **266,** 16973–16976
41. Bhattacharyya, A. M., and Horowitz, P. M. (2001) *J. Biol. Chem.* **276,** 28739–28743
42. Nagahara, N., and Nishino, T. (1996) *J. Biol. Chem.* **271,** 27395–27401
43. Reckenfelderbaumer, N., Ludemann, H., Schmidt, H., Steverding, D., and Krauth-Siegel, R. L. (2000) *J. Biol. Chem.* **275,** 7547–7552
44. Laudenbach, D. E., Ehrhardt, D., Green, L., and Grossman, A. (1991) *J. Bacteriol.* **173,** 2751–2760
45. Nozaki, T., Shigeta, Y., Saito-Nakano, Y., Imada, M., and Kruger, W. D. (2001) *J. Biol. Chem.* **276,** 6516–6523
46. Papenbrock, J., and Schmidt, A. (2000) *Eur. J. Biochem.* **267,** 145–154