The Crystal Structure of Leishmania major 3-Mercaptopropionate Sulfurtransferase

A THREE-DOMAIN ARCHITECTURE WITH A SERINE PROTEASE-LIKE TRIAD AT THE ACTIVE SITE*

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Leishmania major 3-mercaptopropionate sulfurtransferase is a crescent-shaped molecule comprising three domains. The N-terminal and central domains are similar to the thiosulfate sulfurtransferase rhodanese and create the active site containing a persulfurated catalytic cysteine (Cys-253) and an inhibitory sulfite coordinated by Arg-74 and Arg-185. A serine protease-like triad, comprising Asp-61, His-75, and Ser-255, is near Cys-253 and represents a conserved feature that distinguishes 3-mercaptopropionate sulfurtransferases from thiosulfate sulfurtransferases. During catalysis, Ser-255 may polarize the carbonyl group of 3-mercaptopropionate to assist thiolic attack, whereas Arg-74 and Arg-185 bind the carboxylate group. The enzyme hydrolyzes benzyol-Arg-p-nitroanilide, an activity that is sensitive to the presence of the serine protease inhibitor N\(^{-}\)p-tosyl-L-lysine chloromethyl ketone, which also lowers 3-mercaptopropionate sulfurtransferase activity, presumably by interference with the contribution of Ser-255. The L. major 3-mercaptopropionate sulfurtransferase is unusual with an 80-amino acid C-terminal domain, bearing remarkable structural similarity to the FK506-binding protein class of peptidylprolyl cis/trans-isomerase. This domain may be involved in mediating protein folding and sulfurtransferase-protein interactions.

Sulfurtransferases (EC 2.8.1.1–5) catalyze the transfer of sulfane sulfur from a donor molecule to a thiolic acceptor. These enzymes are widely distributed in plants, animals, and bacteria (1-3) and have been implicated in a wide range of biological processes. For example, sulfurtransferases may be involved in the formation and maintenance of iron-sulfur clusters in protein (4, 5), detoxification of cyanide (6, 7), degradation of cysteine (8), biosynthesis of the molybdenopterin cofactor of xanthine oxidase (9), selenium metabolism (2, 10), and thiamine and 4-thiouridine biosynthesis (11, 12). The expression of specific sulfurtransferases is up-regulated under conditions of peroxide or hypo-sulfur stress, osmotic shock, and phage infection (13), suggesting that such enzyme activity is protective of the cell and/or involved in repair processes. Nevertheless, despite intensive study, the biological functions and identification of the physiological substrates of sulfurtransferases remain uncertain.

The archetypal sulfurtransferase is rhodanese, a thiosulfate: cyanide sulfurtransferase (TST) able to catalyze the transfer of the thiosulfate sulfur to cyanide in vitro. The related 3-mercaptoalkanoyl sulfurtransferase (3-mercaptoalkanoyl:cyanide sulfurtransferase [MST]), first discovered in rat liver (14), catalyzes reactions similar to those catalyzed by rhodanese, but uses 3-mercaptoalkanoyl in preference to thiosulfate as the donor in the two-step reaction,

\[
\text{HSCH}_2\text{COO}^- + E \leftrightarrow \text{CH}_2\text{COO}^- + ES
\]

** Step 1

\[
ES + \text{CN}^- \leftrightarrow E + \text{SCN}^-\]

** Step 2

where E represents the free enzyme and ES the enzyme-sulfur adduct.

Crystal structures of rhodanases have been elucidated and analyzed in detail (15-20). The enzyme consists of two domains that, despite a low level of sequence identity, are structurally homologous. Each domain, often referred to as a rhodanese domain, is constructed from a five-stranded \(\beta\)-sheet core surrounded by five \(\alpha\)-helical sections. The active site, with a catalytic cysteine, is situated in a cleft formed at the interface of the domains, although it is mainly constructed from residues associated with the C-terminal domain. For that reason, this domain is often termed the active domain, whereas the N-terminal domain is described as inactive.

Rhodanese-related enzymes are composed of either two rhodanese domains or a single, catalytically active rhodanese domain (1, 21). The rhodanese-like domain has been observed in association with other protein domains, e.g. in mitogen-activated protein kinase phosphatases (22, 23). The similarity be-

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* The abbreviations used are: TST, thiosulfate:cyanide sulfurtransferase; MST, 3-mercaptopropionate:cyanide sulfurtransferase; LmMST, L. major 3-mercaptopropionate:cyanide sulfurtransferase; pNA, p-nitroanilide; SeMet, selenomethionine; BisTris propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane; Bz-, benzyo-; TLCK, N\(^{-}\)p-tosyl-L-lysine chloromethyl ketone; TPCK, tosylphenylalanyl chloromethyl ketone; r.m.s.d., root mean square deviation; FKBP, FK506-binding protein; MIP, macrophage infectivity potentiator protein; PPase, peptidylprolyl cis/trans-isomerase.

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between rhodanese and this phosphatase extends from the overall fold to spatial conservation of the active-site cysteine utilized by both enzymes. This suggests that the rhodanese fold is optimized for activation of a catalytic cysteine.

Although there is no structure yet available for any MSts, several observations suggest that they are evolutionarily and structurally related to TSTs (4, 6). The two types of enzyme catalyze the sulfurtransferase reaction via the formation of a persulfide sulfur covalently bound to the thiol of a catalytic cysteine and display significant levels of sequence similarity, and some are immunologically cross-reactive. However, the different preferred in vivo substrates suggest that the enzymes have different in vivo substrates and physiological roles.

Despite the presence of a conserved catalytic cysteine, suggestive of a similar mechanism, the amino acid composition and location of charged residues in the active site of TSTs and MSts are distinct (21). In TSTs, two large and basic residues within the hexapeptide motif Cys-Arg-Lys-Gly-Val-Thr follow the catalytic cysteine. In MSts, the Arg-Lys pair is replaced by a Gly-Ser or Gly-Thr combination. Mutation of these particular residues to those observed in the other family of enzymes results in partial conversion to that activity, i.e., MSt becomes more rhodanese-like and vice versa (24, 25). Studies on the sulfurtransferase SseA, an Escherichia coli protein involved in serine sensitivity, have reinforced the observation that the sequence following the active-site cysteine can distinguish sulfurtransferases as TSTs or MSts (26), but the structural consequences of such non-conservative amino acid differences to the active site of MSts were unclear.

Williams et al. (27) recently identified and characterized a cytosolic MSt from the parasitic trypanosomatid Leishmania major (LmMSt). Expression of this enzyme is up-regulated in L. major promastigotes during conditions of oxidative stress, suggesting an involvement in detoxification of peroxides; and, in common with E. coli and mammalian MSts (28–30), LmMSt is able to utilize thioredoxin as the thiophilic acceptor. It was also reported that LmMSt can fold independently (27), in contrast to many other sulfurtransferases, which require molecular chaperones to assist such a process (31). It was hypothesized that the unusual 80-amino acid C-terminal extension in LmMSt may play a part in the folding process, particularly as short truncations of this region resulted in misfolded protein (27).

The availability of a stable and active recombinant enzyme allowed us to initiate a crystallographic study to delineate structure-activity relationships in an MSt with the aim of characterizing the active site, investigating the roles of the two residues immediately following the catalytic cysteine, determining the structure of the C-terminal extension, and providing an MSt model for detailed comparisons with TSTs. A number of assays using peptidyl-p-nitroanilide (pNA) substrates were carried out seeking to identify additional enzyme activities.

**Materials and Methods**

**Sample Preparation and Crystallization—**Recombinant LmMSt was expressed and purified according to Williams et al. (27). Initial crystallization trials used the sparse matrix approach (32), applying the hang-dropping vapor diffusion technique with reagents from Hampton Research. Crystals were grown overnight under Crystal Screen I condition 46 (18% (w/v) polyethylene glycol 8000, 100 mM sodium cacodylate (pH 6.5), and 200 mM calcium acetate) at 20 °C. Optimization of these conditions resulted in crystals with approximate dimensions of 0.3 × 0.3 × 0.2 mm³ from a drop consisting of 1 μl of protein (0.5 mg/ml) and 1 μl of reservoir (14% (w/v) polyethylene glycol 8000, 50 mM sodium cacodylate (pH 6.5), and 160 mM calcium acetate). Crystals were cryoprotected with 20% glycerol and maintained at −170 °C for transportation to beamline ID29 at the European Synchrotron Radiation Facility (Grenoble, France), where data were measured.

Native crystals of LmMSt diffracted to 2.7-Å resolution and display the tetragonal space group P4₁2₁2 with unit cell lengths of a = 100.6 Å and c = 67.3 Å. The asymmetric unit contains a single polypeptide with an approximate molecular mass of 42 kDa, a solvent content of ~49%, and V̅ = 2.4 Å³/Da.

The methionine auxotrophic strain of E. coli, B834(DE3), was heat shock-transformed with the plasmid carrying the gene for LmMSt (27) and selected on LB agar plates containing 100 μg/ml ampicillin. Bacteria were cultured in M9 minimal medium containing selenomethionine (SeMet). Expression of LmMSt was induced at mid-log phase with 0.6 mM isopropyl-β-D-thiogalactopyranoside, and cell growth continued overnight. Cells were harvested by centrifugation at 2500 × g, resuspended in binding buffer (20 mM Tris-HCl (pH 7.5), 500 mM NaCl, 5 mM imidazole, and 20% glycerol), and following the addition of lysozyme and proteinase K, the mixture was lysed using a French press. Insoluble debris was separated by centrifugation at 27,000 × g for 20 min at 4 °C, and the supernatant containing soluble LmMSt was passed through a 0.2-μm syringe filter and then applied to a Ni²⁺-resin column (HiTrap, Amersham Biosciences) pre-equilibrated with binding buffer. Following a wash with 20 mM BisTris propane and 10 mM imidazole (pH 7.5) using a BioCAD 700E (Applied Biosystems), the product was eluted with a linear imidazole gradient from 0 to 500 mM. Fractions were analyzed by SDS-PAGE, and those containing LmMSt were pooled and dialyzed overnight against 20 mM Tris-HCl (pH 8.0) and 50 mM NaCl. The sample was then subjected to anion-exchange chromatography on a 5-mL Sepharose column (Amersham Biosciences) and the resin was washed with 25 mM BisTris propane (pH 8.0), and LmMSt was found in the flow-through and wash fractions. These fractions were passed over a second Ni²⁺-resin column to concentrate the protein, and LmMSt was eluted with a linear imidazole gradient from 0 to 500 mM. Fractions containing LmMSt were again pooled and dialyzed overnight in 20 mM Tris-HCl (pH 8.0) and 50 mM NaCl. The dialyzed protein was concentrated (Centricon-10/Microcon-10, Millipore Corp.) to ~3.5 mg/ml for use in crystallization experiments. SDS-PAGE and matrix-assisted laser desorption ionization time-of-flight mass spectrometry were used to assess purity, and the latter technique was also used to confirm full incorporation of SeMet residues. Crystals of the SeMet MSt protein grew under the same conditions as, and are isomorphic with, the native crystals, but diffraction to lower resolution.

**Data Collection and Processing—**Data were measured at the European Synchrotron Radiation Facility (beamline ID14 E2H). A single SeMet derivative crystal was soaked in a solution of 70% reservoir and 30% glycerol for 10 s and flash-cooled in a stream of nitrogen gas at 170 °C. The wavelength used was 0.998 Å, and a highly redundant data set (180° × 1° oscillations; Data set 1) was measured on an ADSC Quantum3 detector, processed, and scaled using the HKL suite of programs (33). Processing statistics are presented in Table I.

**Single Wavelength Anomalous Dispersion Phasing, Structure Solution, Model Building, and Refinement—**Ten selenium positions were identified using the program SOLVE (34) and used for phases calculation using a figure of merit of 0.23 to 2.1 Å resolution (MLPHARE) (35). The first electron density maps were of poor quality, but density modification and histogram matching with the program DM (36) were spectacularly successful and increased the figure of merit to 0.76. The programs ARP/WARP (37) and MAID (38) were used to construct sections of the polypeptide backbone into this map. In addition, MOLREP (39) positioned a polyalanine model of bovine rhodanese (Protein Data Bank code 1ORB) (16) into the experimentally phased map. The results from all of these programs were combined to produce a polyalanine model of LmMSt onto which side chains were assigned using O (40). A subset of data (5%) was set aside for the calculation of Rmerge (41) and used as a guide for the refinement. The Hendrickson-Lattman coefficients derived from the DM calculations were included as phasing restraints in the early stages of refinement. Several rounds of refinement with CNS (crystallography and NMR system) (42) and REFMAC5 (43) interspersed with rounds of model building produced a model for LmMSt. The electron and difference density maps were noisy, especially on the selenium positions, and the highly redundant data set (180° × 1° oscillations; Data set 2) was used to complete the refinement (Data set 2) (see Table I). Further refinement with the addition of water molecules and ions completed the analysis. Residues 1–239 and 239–243 are disordered. The stereochemistry of the model was assessed with PROCHECK (see Table I for details) (44), and secondary structure was predicted using PROMOTIF (45).
and lead acetate to trap the H2S released (27). Inhibition of these ferase activity was measured using 3-mercaptopyruvate as substrate.

The crystals are tetragonal and display space group $P4_12_2_1$ and have improved diffraction to 2.1-

<table>
<thead>
<tr>
<th>TABLE I</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Structure of 3-Mercaptopyruvate Sulfurtransferase</strong></td>
</tr>
</tbody>
</table>

Numbers in parentheses correspond to the highest resolution shell, a bin of 2.17 to 2.10 Å. $R_{	ext{sym}} = \sum |I - \langle I \rangle| / \sum I$, where the summation is over all symmetry equivalent reflections. $R_{\text{atom}} = \sum |I(+) - I(-)| / 2\sum |I(+)| + |I(-)|/2$.

<table>
<thead>
<tr>
<th>Data set 1</th>
<th>Data set 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution range (Å)</td>
<td>20.2-10</td>
</tr>
<tr>
<td>No. reflections</td>
<td>348,345</td>
</tr>
<tr>
<td>No. unique reflections</td>
<td>24,450</td>
</tr>
<tr>
<td>Redundancy</td>
<td>14.2</td>
</tr>
<tr>
<td>Wilson B (Å$^2$)</td>
<td>35.8</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>99.9 (99.3)</td>
</tr>
<tr>
<td>$I/\sigma(I)$</td>
<td>42.1 (7.8)</td>
</tr>
<tr>
<td>$R_{	ext{free}}$ (%)</td>
<td>5.1 (28.2)</td>
</tr>
<tr>
<td>$R_{\text{all}}$ (%)</td>
<td>1.9</td>
</tr>
</tbody>
</table>

Protein residues/atoms | 360/2786 |
Water molecules/ions | 407/308 |
47/120/300 |
Waters/SO$_4$$^2-$/Ca$^{2+}$ | 54.4/56.9/65.0 |
5.0/9.0/1.7 |
1.0/0.0/0.0 |
3.0/0.0/0.0 |
Ramachandran analysis | 81.6 |
Favorable (%) | 16.4 |
Additionally favorable (%) | 2.0 |
Acceptable (%) | 0.0 |
zymes is 23%, and an overlay gives a root mean square deviation (r.m.s.d.) of 2.4 Å for 267 C-α pairs and a Z-score of 29.6. The Z-score is a measure of the statistical significance of the best alignment determined in DALI; and, typically, two dissimilar proteins have a Z-score of ≤2. MST matched against itself with a Z-score of 57. The DALI superposition was optimized using the graphics program O, and the r.m.s.d. was reduced to 1.7 Å for 224 C-α pairs. This is the superposition shown in Fig. 3a. Two other homologs of note were identified. First, E. coli GlpE (Protein Data Bank code 1GN0) (21), which shares a sequence identity of 22% with the central domain of LmMST, produces an r.m.s.d. of 2.5 Å over 101 C-α atoms and a Z-score of 11.0. This protein is encoded by a gene on the sn-glycerol 3-phosphate regulon (gpl) and is a single domain rhodanese-type sulfurtransferase. Second, the catalytic domain of human CDC25A phosphatase (Protein Data Bank code 1C25) (22), which shares a sequence identity of 22% with LmMST, produces an r.m.s.d. of 2.4 Å over 97 C-α pairs and a Z-score of 8.2. Despite only limited sequence conservation between these distinct enzyme families, the structural overlays indicate that secondary structure is well conserved, with large-scale structural differences restricted to surface loops.

Although only 12% identical in amino acid sequence, superposition of the N-terminal and central domains of LmMST gives an r.m.s.d. of 2.0 Å for 104 C-α pairs (Fig. 3b). This compares favorably with the superposition of the two domains

![Figure 2](image-url)
from bovine rhodanese, which gives an r.m.s.d. of 1.6 Å.

The N-terminal domain of LmMST is cyan; the central domain is blue; and the C-terminal domain is red. The trace for rhodanese is magenta. The view is the same as in Fig. 2a. b, stereo view of the two rhodanese-like domains of LmMST superimposed. The N-terminal domain is colored cyan, and the central domain is blue. The active-site Cys-253 and the spatially equivalent N-terminal Asp-102 are included.

The function of the C-terminal domain is uncertain; therefore, clues were sought from an architectural comparison in DALI.

Comparison with FKBP revealed a sequence identity of 10%, and an overlay of 67 C-α pairs produced an r.m.s.d. of 2.3 Å and a Z-score of 6.0. In the case of MIP, the sequence identity is 12%, and the r.m.s.d. is 3.0 Å for 74 C-α pairs with a Z-score 5.7. FKBP is a peptidylprolyl cis-trans-isomerase (PPIase) class of immunophilin implicated in regulation of the mammalian immune response and basic cellular processes such as protein folding and trafficking (51). PPIases are often tightly associated with other proteins or are clearly distinguishable domains in larger polypeptides (52); and, in the case of LmMST, there is a distinct PPIase-like domain attached to a sulfurtransferase. MIP, a virulence factor implicated in host cell invasion, also displays PPIase activity and is found in bacteria such as Legionella pneumophila (50) and also in the trypanosomatid Trypanosoma cruzi (53).

The active site for the PPIase fold is a shallow pocket into which the inhibitor FK506 binds to form a complex that is a potent agonist of immunosuppression, exerting its effects through inhibition of the phosphatase activity of calcineurin (54). Superposition of the FKBP-FK506 complex onto the C-terminal domain of LmMST (Fig. 4) shows that the PPIase active site maps to a depression on the C-terminal domain of LmMST where the persulfurated Cys-331 is located. FK506 is included in Fig. 4 to highlight the position of Cys-331 within this potential ligand-binding pocket in the C-terminal domain of LmMST. In contrast to LmMST, the FKBP structure possesses extended loops and strands around the active-site pocket, including one formed by Thr-85 to Asn-94 and termed the “80s loop.” This loop acts as a flap to regulate access to the pocket (49, 54). Although lacking the extended loop structures, the altered position of the loop linking strand β15 with helix α11 (residues 330–335) of LmMST compared with that of FKBP places the side chains of Cys-331, Arg-333, Asp-335, and Arg-359 to interact with any potential ligands at this site (Fig. 5). Two salt bridges formed between Asp-335 and Arg-359 and between Glu-337 and Arg-359 are also present in the pocket, creating a distinct conformation that accommodates Cys-331 at one end (data not shown).

Assays for PPIase activity were carried out as described by Fischer et al. (55) using the FKBP- and cyclophilin-specific substrates succinyl-Ala-Leu-Pro-Phe-pNA and succinyl-Ala-Ala-Pro-Phe-pNA, respectively, but no activity could be detected (data not shown). However, the observation that truncated versions of LmMST do not express as soluble proteins suggests that the C-terminal domain makes an important contribution in stabilizing the overall fold (27). In this context, it is intriguing that the domain displays a fold common to a class of proteins implicated in regulating folding processes and in protein-protein associations and has a reactive cysteine placed in
the FKBP-like target-binding site. Further experiments will be required to determine how this domain contributes to the function of LmMST.

The MST Active Site—The active site is positioned in a cleft between the N-terminal and central domains (Fig. 2a) and is constructed from seven segments of the polypeptide. Five of these segments (residues 35–39, 72–75, 104–109, 193–196, and 253–258) are short loops between an α-helix and a β-strand; one is helix α6 (residues 180–185); and the longest stretch of polypeptide contributing directly to the active site (residues 209–221) encompasses β3, β10, and the loop leading into strand β11.

The floor of the active site is formed mainly by the loop between strand β12 and helix α8. Placed at the center of the loop is the side chain of the catalytic Cys-253, which is in the intermediate sulfur-substituted persulfide-containing state (Fig. 6). The conformation of the loop results in six amide groups from residues 254 to 259 placed to donate hydrogen bonds in toward the persulfide. On either side of the persulfide are the polar side chains of Ser-255 and Thr-258, the latter of which participates in a hydrogen bond with the persulfide. On either side of the persulfide groups from residues 254 to 259 placed to donate hydrogen bonds in toward the persulfide. On either side of the persulfide.

One side of the active-site cleft displays a basic patch formed by Arg-74, His-75, Arg-181, and Arg-185. Almost directly opposite is a hydrophobic area formed by a side-on contribution from Tyr-35 and the side chains of Leu-37, Met-108, and Val-257. Above this hydrophobic area, on the periphery of the active-site cleft, lie Glu-104 and Tyr-210. There are numerous hydrogen bonding interactions involving the amino acid side chains in and around the active site, which contribute to the structure of the cleft and placement of important functional groups. The interactions involving the catalytic Cys-253 have already been described. Other associations of note are the interactions between Tyr-210 and Glu-104, His-75 and Tyr-35, and Asp-61 and Ser-255 (see below); Asp-179 and Asp-196 both interact with Arg-181, and Asp-196 also interacts with Arg-185. The guanidinium group of Arg-74 is the only polar side chain component lacking an interaction with some other functional group of the enzyme. This arginine, in conjunction with Arg-185, provides electrostatic interactions to bind a well-ordered sulfite ion in the active site. The sulfite would prevent access to the catalytic center and is actually a potent inhibitor of rhodanese (15). A crystallographic study showed that metal cyanides bind at the entrance to the rhodanese active site in a similar fashion to the sulfite, thereby suggesting a common mechanism of action (58).

As mentioned above, a noteworthy difference between MSTs and rhodaneses occurs in the active-site consensus sequence. Now that structures are available for both types of enzyme, we can describe the structural consequences of such differences; and, for comparative purposes, an overlay of the LmMST and bovine rhodanese active sites is presented in Fig. 6. This overlay identifies a number of structural features that are conserved in the active sites of the two enzymes. These include the loop that forms the floor of the active site and also a number of...
hydrophobic and acidic residues that line one side of the cleft. In \textit{LmMST}, Tyr-35, Met-108, and Tyr-210 are equivalent to and overlay well with Trp-35, Phe-106, and Phe-212 of rhodanese. Near to the hydrophobic patch in \textit{LmMST} are Asp-61 and Glu-104, which are equivalent to Glu-61 and Asp-101 in rhodanese. Opposite the hydrophobic patch toward the sulfite-binding site is a highly conserved area of structure that, in \textit{LmMST}, consists of Arg-181 and Arg-185 clustered with Glu-179 and Asp-196. In rhodanese, the equivalent residues are Arg-183, Arg-186, Asp-180, and Glu-196. A network of hydrogen bonding interactions involving these residues serves to place one of the arginines (\textit{LmMST} Arg-185 or rhodanese Arg-186) to interact with ligands.

There are significant amino acid differences between the \textit{LmMST} and rhodanese active sites that warrant discussion. Arg-74, His-75, Gly-109, Gly-254, and Ser-255 in \textit{LmMST} are replaced by Glu-71, Val-72, Tyr-107, Arg-248, and Lys-249, respectively in bovine rhodanese. The Gly-109/Tyr-107 and Ser-255/Lys-249 differences reduce the size of the active-site cleft on one side, which is partially compensated for by the His-75/Val-72 difference. Three changes are relevant to differences in the active-site hexapeptide motif. To recap, in rhodanese, the consensus sequence is Cys-Arg-Lys-Gly-Val-Thr, whereas in \textit{LmMST}, Arg-185 or rhodanese Arg-186 interacts with Glu-71. In \textit{LmMST}, Arg-74 is the equivalent of Glu-71 and occupies the space that is filled by Arg-248 in rhodanese and thereby compensates for the Gly-254/Arg-248 difference. The replacement of Arg-248 and Lys-249 of bovine liver rhodanese with glycine and serine, respectively, by site-directed mutagenesis decreases the TST activity and increases the MST activity, showing that these two amino acid positions are critical determinants of rhodanese/MST activity (6).

A molecular model of 3-mercaptopyruvate in the \textit{LmMST} active site (data not shown), constructed on the basis that the carboxylate oxygen atoms would bind in the same position as the sulfite oxygen atoms, suggests that Arg-74 and Arg-185 are well placed to interact with the substrate. Indeed, the alteration of Arg-187 in rat liver MST (the equivalent of Arg-185 in \textit{LmMST}) reduces binding of 3-mercaptopyruvate significantly (24). Mutation of \textit{LmMST} Arg-74 might have a similar effect.

Ser-255 could contribute to the binding of 3-mercaptopyruvate by interaction with the carbonyl group. Such an interaction could polarize the carbonyl group and serve to enhance nucleophilic attack by the Cys-253 thiolate, allowing the enzyme to then attain the persulfide form by turnover of the appropriate sulfur donor. Ser-255 in \textit{LmMST} is equivalent to Lys-249 in rhodanese, a residue that directly interacts with thiosulfate. Significantly, alteration by site-directed mutagenesis of the equivalent serine in rat liver MST (Ser-249) (24) or in \textit{E. coli} SseA (Ser-240) (25) to lysine directs specificity away from 3-mercaptopyruvate toward thiosulfate.

Both \textit{LmMST} and rhodanese active sites are positively charged (Fig. 5) to attract and then bind negatively charged ligands. The active-site cleft and immediate vicinity of \textit{LmMST} are positively charged because of the basic patch described above and contributions from Lys-38, Lys-40, and Lys-197 together with Arg-289. These last four residues are not conserved in rhodanese, which in part explains why the TST active site appears less positively charged. It seems likely that the electrostatic properties of physiological substrates would complement those of the individual sulfurtransferases.

\textit{A Serine Protease-like Triad}—Two residues, His-75 and Ser-255 (discussed above in the context of differences between MSTs and bovine rhodanese), in conjunction with Asp-61, are arranged in a serine protease-like triad at the active site (Fig. 6). The distance between Ser-255 O-γ and His-75 N-ε1 is 2.9 Å, and that between His-75 N-ε2 and Asp-61 O-ε1 is 2.8 Å. His-75 participates in a three-center hydrogen bond with Asp-61 and the hydroxyl group of Tyr-35 (3.0 Å distant). The triad is aligned perpendicular to the active-site entrance at the surface of the enzyme. Sequence alignments (discussed above) indicate that this triad is a common and, we predict, defining feature of the MST family, distinguishing MSTs from TSTs. Ser-255 and His-75 are strictly conserved, whereas Asp-61 is more variable, present as glutamate or asparagine. MST family members retain the aspartate/glutamate equivalents, but lack the histidine and serine equivalents.

As a result of the structure determination of \textit{LmMST} and our sequence-structure analysis, we now know that the characteristic combination of an acidic residue, a histidine, and a serine that form a serine protease-like triad is conserved in the MST sequences and therefore can be used as an extension of the Cys-Gly-Ser-Gly-Val-Thr/Ser motif to identify members of the MST family of enzymes. The triad presumably exists as a means of activating the serine for its role in binding and polarizing the carbonyl group of 3-mercaptopyruvate to assist thiophilic attack.

\textit{Is \textit{LmMST} a Protease?—}Once a serine protease-like Asp-His-Ser triad was noted, we decided to investigate whether \textit{LmMST} does indeed display protease activity and whether classical serine protease inhibitors affect the protein. Peptidyl-pNA substrates with arginine at the P_1 position were used to test for protease activity. In the presence of β-mercaptoethanol, \textit{LmMST} hydrolyzed Bz-Arg-pNA, albeit at a low rate (3.6 nmol/min/mg of protein) relative to the control enzyme trypsin (150 μmol/min/mg of protein). Peptidyl substrates with phenylalanine and valine or with proline and phenylalanine occupying the P_3 and P_2 positions, respectively, were also hydrolyzed, but at a rate ~50% of that observed for Bz-Arg-pNA, whereas Bz-Arg-Arg-pNA was not hydrolyzed at all. All activities were inhibited by 0.5 mM TLCK. TLCK and phenylmethanesulfonyl fluoride, known inhibitors of trypsin-like serine proteases, also effectively inhibited the sulfurtransferase activity of \textit{LmMST}, with IC_{50} values of 44 and 700 μM, respectively (Fig. 7). In contrast, TPCCK, an inhibitor of chymotrypsin-like serine proteases, and E-64, an inhibitor of cysteine proteases, had no
of the enzyme to inhibition by TLCK and phenylmethanesulfonyl fluoride. Binding of these molecules would affect both access of the substrate to the active site, through steric hindrance, and the path played by the activated serine in polarizing the carbonyl group of the substrate as part of the catalytic process. A higher level of protease activity could exist for help with the PPIase assays, and the European Synchrotron Radiation Facility for mass spectrometry, Charles Bond for help with the PPIase assays, and the European Synchrotron Radiation Facility for access.

Concluding Remarks—With this high resolution crystal structure determination of LmMST, we now have accurate models for the two distinctive subclasses of sulfurtransferases. This, in conjunction with biochemical analyses, has provided important insights into the structure-activity relationships for a widely distributed enzyme. Most interestingly, it has been shown that MSTs contain a serine protease-like catalytic triad in their active site. Although such triads exist in esterases and lipases, this is the first demonstration that such a distinctive structural feature also occurs in some sulfurtransferases.

The analysis provides information on how TST and MST differ and also on how they are adapted to interact with and process different substrates. Understanding the significance of these differences in terms of physiological functions of the enzymes is hindered by the limited information available about the biological roles of the distinct sulfurtransferases. A contributing factor to this problem may be the diverse roles that this widely distributed class of enzymes can play. Nevertheless, a picture is starting to emerge of an enzyme activity that is widely distributed class of enzymes can play. Nevertheless, a picture is starting to emerge of an enzyme activity that is widely distributed enzyme. Most interestingly, it has been reported previously, although this is the first example...