Thioredoxin regulates human mercaptopyruvate sulfurtransferase at physiologically-relevant concentrations

Pramod Kumar Yadav1, Victor Vitvitsky1, Sebastián Carballal§1, Javier Seravalli†, and Ruma Banerjee†2

From the 1Department of Biological Chemistry, University of Michigan Medical School, Ann Arbor, Michigan 48109-0600, the 2Departamento de Bioquímica, Facultad de Medicina and Centro de Investigaciones Biomédicas (CEINBIO), Universidad de la República, Montevideo 11800, Uruguay, and the 3Department of Biochemistry and the Redox Biology Center, University of Nebraska, Lincoln, Nebraska 68588

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3-Mercaptopyruvate sulfur transferase (MPST) catalyzes the desulfuration of 3-mercaptoppyruvate (3-MP) and transfers sulfane sulfur from an enzyme-bound persulfide intermediate to thiophilic acceptors such as thioredoxin and cysteine. Hydrogen sulfide (H2S), a signaling molecule implicated in many physiological processes, can be released from the persulfide product of the MPST reaction. Two splice variants of MPST, differing by 20 amino acids at the N terminus, give rise to the cystolic MPST1 and mitochondrial MPST2 isofoms. Here, we characterized the poorly-studied MPST1 variant and demonstrated that substitutions in its Ser–His–Asp triad, proposed to serve a general acid–base role, minimally affect catalytic activity. We estimated the 3-MP concentration in murine liver, kidney, and brain tissues, finding that it ranges from 0.4 μmol·kg−1 in brain to 1.4 μmol·kg−1 in kidney. We also show that N-acetylcysteine, a widely-used antioxidant, is a poor substrate for MPST and is unlikely to function as a thiophilic acceptor. Thioredoxin exhibits substrate inhibition, increasing the KM for 3-MP ~15-fold compared with other sulfur acceptors. Kinetic simulations at physiologically-relevant substrate concentrations predicted that the proportion of sulfur transfer to thioredoxin increases ~3.5-fold as its concentration decreases from 10 to 1 μM, whereas the total MPST reaction rate increases ~7-fold. The simulations also predicted that cysteine is a quantitatively-significant sulfane sulfur acceptor, revealing MPST’s potential to generate low-molecular-weight persulfides. We conclude that the MPST1 and MPST2 isofoms are kinetically indistinguishable and that thioredoxin modulates the MPST-catalyzed reaction in a physiologically-relevant concentration range.

A toxin for higher organisms and a nutrient for some Bacteria and Archaea, hydrogen sulfide (H2S) is a product of mammalian sulfur metabolism (1–3). The steady-state levels of H2S are estimated to range from 10 to 30 nm in mammalian cells and tissues (4–6). Two enzymes, cystathionine β-synthase and γ-cystathionase, catalyze H2S elimination from the sulfur-containing amino acids, cysteine and homocysteine (1, 7). In contrast, 3-mercaptoppyruvate sulfur transferase (MPST) catalyzes the desulfuration of 3-mercaptoppyruvate (3-MP) forming pyruvate and a persulfide product from which H2S can be eliminated (7–10). Detailed kinetic analyses of cystathionine β-synthase (8) and γ-cystathionase (9) have provided estimates of their relative contributions to H2S-generation in mammalian tissues (11). In contrast, a similar assessment of MPST has been precluded by the following: (i) the absence of a complete kinetic dataset on mammalian MPSTs, which are commonly assayed at high pH (maximal activity is at pH 10.5) and with nonphysiologiical substrates (12, 13), and (ii) a lack of information on the intracellular 3-MP concentration. We report a detailed kinetic analysis of the mitochondrial form of recombinant human MPST at physiological pH (10), but a similar analysis of the cytoplasmic variant, discussed below, has been lacking.

MPST (also designated as MST and TUM1 for thioridazine modification protein 1) is implicated not only in cysteine catabolism but also in the thiolation of cytosolic tRNA (12). Two splice variants of human MPST, which differ by a 20-amino acid N-terminal extension immediately upstream of a mitochondrial targeting sequence, have been described (Fig. 1A) (12). The longer variant, hereafter designated as MPST1, is localized exclusively in the cytoplasm, whereas the shorter MPST2 variant has been detected in the cytoplasm and in mitochondria. Cleavage of the N-terminal mitochondrial targeting sequence results in MPST2 being ~45 residues shorter than MPST1. The two MPST isofoms reportedly exhibit very similar kinetic parameters in the presence of the nonphysiological acceptors, cyanide and DTT, at the pH optimum of 10.5 (12).

MPST belongs to the rhodanese superfamily whose members contain one to four rhodanese-like domains (14). Human MPST has two rhodanese-like domains, and Cys268/Cys248 in the C-terminal domain of MPST1/MPST2 serves as the persulfide carrier (10). 3-MP is derived via transamination of cysteine and α-ketoglutarate catalyzed by glutamate dehydrogenase; DHLA, dihydrolipoic acid; Trx, thioredoxin; GOT, glutamine oxoglutarate transaminase; PDB, Protein Data Bank.

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This article contains Fig. S1.

1 These authors contributed equally to this work.
2 To whom correspondence should be addressed. Tel.: 734-615-5238; E-mail: rbanerje@umich.edu.
3 The abbreviations used are: H2S, hydrogen sulfide; MPST, mercaptopyruvate sulfur transferase; 3-MP, 3-mercaptoppyruvate; GSH, glutathione; NAC, N-acetylcysteine; DHLA, dihydrolipoic acid; Trx, thioredoxin; GOT, glutamine oxoglutarate transaminase; PDB, Protein Data Bank.

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mine oxoglutarate transaminase (GOT, Fig. 1A) involved in the malate-aspartate shuttle, which also has cysteine oxoglutarate transaminase activity.

The reaction catalyzed by MPST comprises two successive sulfur transfer steps (Fig. 1B). In the first half-reaction, the sulfur atom is transferred from 3-MP to MPST, resulting in a cysteine persulfide intermediate in the active site. The crystal structure of MPST2 revealed that the outer sulfur in the Cys248–SS/H11002 intermediate is encircled by the signature active-site hexapeptide loop (Cys248–Gly–Ser 250–Gly–Val–Thr) and stabilized via electrostatic interactions with four backbone amides (Fig. 2A) (10). Cysteine residues on analogous active–site loops are found in other sulfur transferases, e.g. rhodanese (15), TSTD1 (16), and PRF (17). Sequence differences in the cysteine–bearing active–site loop are important determinants of substrate specificity as demonstrated by mutagenesis studies (18). Ser250 within the MPST2 loop is part of a triad comprising His74 and Asp63 (Fig. 2A) (10). 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Results

Kinetic comparison of MPST1 and MPST2 isoforms with cysteine and NAC

MPST1 was obtained in a similar yield and purity (Fig. 1C) as described previously for recombinant human MPST2 (10). The kinetic data for MPST1 is shown in Fig. 3, and the kinetic parameters for the two isoforms are compared in Table 1. The kinetic plots showed a sigmoidal dependence as reported previously for MPST2 with cysteine and 3-MP (10). Overall, the kinetic constants for H₂S synthesis by the MPST isoforms were identical within experimental error in the presence of L-cysteine and NAC. Because non-Michaelis-Menten behavior was observed with cysteine and NAC (Fig. 3, A, B, D, and E), the data were fitted to the Hill equation yielding values for the Hill coefficient (Table 1). The $k_{cat}/K_m$(Cys) value is substantially lower than the $k_{cat}/K_m$(NAC) value, due to the ~4-fold higher $K_m$ and 2-fold lower $k_{cat}$ values with NAC compared with cysteine. Our data indicate that NAC is unlikely to be a significant co-sub-
strate for the MPST reaction in cells. In contrast, a prior study (23) reported a 1.3-fold higher catalytic efficiency with NAC versus cysteine. Because the MPST activity was reported in arbitrary fluorescence units in this study (23), a direct comparison with our data are not possible.

Regulation of MPST activity by thioredoxin

The $k_{cat}/K_{M(\text{Trx})}$ values for sulfur transfer from 3-MP to thioredoxin are identical for MPST1 and MPST2 within experimental error (Table 1) and >10-fold higher than with cysteine. The dependence of the reaction rate on the concentration of thioredoxin and 3-MP also showed sigmoidal behavior (Fig. 3, C and F). However, compared with cysteine and NAC, the $K_{M}$ for 3-MP is ∼15-fold higher in the presence of thioredoxin (Table 1). To understand the effect of thioredoxin on the $K_{M}$ for 3-MP, the dependence of the MPST reaction on thioredoxin concentration was examined at three 3-MP concentrations (Fig. 4A). At ≈300 μM 3-MP, the MPST reaction rate is inhibited above ∼8 μM thioredoxin. The extent of inhibition decreased with increasing 3-MP concentrations. From these data, a $K_{i}$ value of 3.6 μM for thioredoxin was estimated as described under “Experimental procedures.”

To further understand the potential physiological relevance of the inhibition of MPST by thioredoxin, kinetic simulations were employed. Using Equation 1 in Table 2, a reasonable correspondence was obtained between the experimental and sim-
inhibited in the presence of 3 mM 3-MP (Fig. 3).

In agreement with this prediction, the activity of MPST2 is not influenced by thioredoxin at high 3-MP concentrations (Fig. 4). The simulations predict that inhibition by thioredoxin can be alleviated only at unphysiologically high concentrations of 3-MP.

Table 1

Comparison of kinetic parameters for MPST1 and MPST2

MPST activity was measured in 200 mM HEPES buffer, pH 7.4, at 37 °C.

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>Parameter</th>
<th>MPST1</th>
<th>MPST2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Cysteine</td>
<td>$K_M^{(CB)}$</td>
<td>$3.8 \pm 0.4$ mm</td>
<td>$4.5 \pm 0.8$ mm</td>
</tr>
<tr>
<td></td>
<td>$n^a$ (Cys)</td>
<td>$1.4 \pm 0.1$</td>
<td>$1.6 \pm 0.1$</td>
</tr>
<tr>
<td></td>
<td>$K_{M3-MP}$</td>
<td>$24.0 \pm 0.8$ μM</td>
<td>$22 \pm 5$ μM</td>
</tr>
<tr>
<td></td>
<td>$n$ (3-MP)</td>
<td>$1.6 \pm 0.2$</td>
<td>$1.4 \pm 0.2$</td>
</tr>
<tr>
<td></td>
<td>$k_{cat}$</td>
<td>$0.44$ s$^{-1}$</td>
<td>$0.5$ s$^{-1}$</td>
</tr>
<tr>
<td></td>
<td>$k_{cat}/K_{M3-MP}$</td>
<td>$0.11$ mm$^{-1}$ s$^{-1}$</td>
<td>$0.11$ mm$^{-1}$ s$^{-1}$</td>
</tr>
<tr>
<td>NAC</td>
<td>$K_{MNC}$</td>
<td>$17 \pm 2$ mm</td>
<td>$16 \pm 2$ mm</td>
</tr>
<tr>
<td></td>
<td>$n$ (NAC)</td>
<td>$2.2 \pm 0.5$</td>
<td>$2.7 \pm 0.8$</td>
</tr>
<tr>
<td></td>
<td>$K_{M3-MP}$</td>
<td>$20.0 \pm 2$ μM</td>
<td>$15 \pm 2$ μM</td>
</tr>
<tr>
<td></td>
<td>$n$ (3-MP)</td>
<td>$1.3 \pm 0.1$</td>
<td>$1.1 \pm 0.1$</td>
</tr>
<tr>
<td></td>
<td>$k_{cat}$</td>
<td>$0.22$ s$^{-1}$</td>
<td>$0.27$ s$^{-1}$</td>
</tr>
<tr>
<td></td>
<td>$k_{cat}/K_{MNC}$</td>
<td>$0.013$ mm$^{-1}$ s$^{-1}$</td>
<td>$0.017$ mm$^{-1}$ s$^{-1}$</td>
</tr>
<tr>
<td>Thioredoxin</td>
<td>$K_{MTrx}$</td>
<td>$3.0 \pm 0.15$ μM</td>
<td>$3.1 \pm 0.7$ μM</td>
</tr>
<tr>
<td></td>
<td>$n$ (Trx)</td>
<td>$1.6 \pm 0.1$</td>
<td>$1.4 \pm 0.3$</td>
</tr>
<tr>
<td></td>
<td>$K_{M3-MP}$</td>
<td>$358 \pm 100$ μM</td>
<td>$360 \pm 40$ μM</td>
</tr>
<tr>
<td></td>
<td>$n$ (3-MP)</td>
<td>$2.0 \pm 0.6$</td>
<td>$2.1 \pm 0.4$</td>
</tr>
<tr>
<td></td>
<td>$k_{cat}$</td>
<td>$0.40$ s$^{-1}$</td>
<td>$0.64$ s$^{-1}$</td>
</tr>
<tr>
<td></td>
<td>$k_{cat}/K_{MTrx}$</td>
<td>$133$ mm$^{-1}$ s$^{-1}$</td>
<td>$142$ mm$^{-1}$ s$^{-1}$</td>
</tr>
</tbody>
</table>

$^a$ Data denote the Hill coefficient (±S.D.) for the corresponding substrate obtained from fitting the experimental data to the Hill equation: $V = V_{max}[(S)^n/(S^n + K^n)]$, where S is the substrate concentration; $K = S_{50} (K_{cat})$, and n is the Hill coefficient.

Figure 4. Substrate inhibition of the MPST reaction by thioredoxin. A, inhibition of MPST2 activity (0.3 μM in 200 mM HEPES buffer, pH 7.4) at increasing concentrations of thioredoxin (1–20 μM) in the presence of thioredoxin reductase (3.5 μM) and 200 μM NADPH. The activity of MPST2 was assessed at three concentrations of 3-MP (50, 100, and 300 μM) as indicated. The data are representative of three independent experiments. B, simulations of the MPST reaction rate at the indicated 3-MP concentrations (in μM) show a reasonable correspondence with the experimental data in A and reveal that substrate inhibition by thioredoxin can be alleviated only at unphysiologically high concentrations of 3-MP.

Comparison of kinetic parameters for MPST1 and MPST2

The activity of MPST and the prevalence of its isoforms were assessed in murine liver, kidney, and brain. Because the triad residues are kinetically indistinguishable, the catalytic contribution of the triad residues decreases activity by at least 100-fold (27). Our results therefore argue against an essential role for the triad residues in the MPST reaction mechanism as suggested previously (20). Instead, Ser$^{250}$ is likely to function primarily as a determinant of substrate specificity as concluded from mutagenesis studies on rat MPST (18).

Comparison of kinetic parameters for MPST1 and MPST2

The activity of MPST and the prevalence of its isoforms were assessed in murine liver, kidney, and brain. Because the thiolphilic acceptors, cysteine, homocysteine, and GSH, can stimulate H$_2$S production (8, 9) or consumption (28, 29) by other enzymes present in tissue homogenates, DHLA was used as the thiolphilic acceptor to assess MPST activity. A 10-fold range in tissue MPST activity was seen using either the lead sulfide or gas chromatography (GC)-based sulfur chemiluminescence detection assay. The highest MPST activity was observed in liver, whereas kidney and brain were ~2- and 10-fold lower, respectively (Table 4). When the MPST activity was normalized to soluble protein concentration, the same liver > kidney >
brain activity order was seen. We note that enzyme activity normalized to tissue wet weight roughly corresponds to the enzyme concentration in the tissue and can be used to compare enzyme activities between tissues. Normalization to tissue protein concentration on the other hand can be misleading. For example, if the concentration of an enzyme were three times lower in brain than in liver, then normalization to tissue protein would yield similar specific activities for both tissues because the protein concentration in brain is \( \frac{1}{3} \) fold lower than in liver.

### MPST1 and MPST2 protein expression

The relative levels of MPST1 and MPST2 were assessed in different cell types. Three bands were detected in human red blood cell lysates from three donors (Fig. 5A). This is similar to the pattern reported previously in erythrocytes from a single
individual (30). In addition to MPST1 and MPST2, a third shorter and highly-abundant band was seen in these cells. We tentatively assign this band as MPST3, which is described in the UniProtKB database as a truncated variant extending from the mitochondrial leader sequence to residue 198. Lacking the C-terminal rhodanese-like domain, which contributes to the active site, MPST3 is predicted to be inactive. In contrast, MPST purified from rat erythrocytes reportedly migrates as a single band (31).

Western blot analysis was also used to detect MPST isoforms in four human and one murine cell line (Fig. 5B). Both MPST1 and MPST2 were seen in the human cell lines, but only MPST2 was observed in the murine cell line. The presence of a single MPST band in rat erythrocytes (31) and in the murine cell line (Fig. 5B) suggests that rodents might express a single MPST isoform. The small difference in band migration between the native human and murine MPST2 samples was due to the difference in their molecular mass (murine = 30,091 Da and human = 30,217 Da, both lacking the leader sequence). The molecular mass of native human MPST1 is 35,250 Da. The highest MPST levels were observed in SH-SY5Y and HepG2 cells, whereas the lowest levels were seen in the murine bEnd3 cells. The MPST1/MPST2 ratio varied in human cell lines (Fig. 5B). Although MPST1 predominated in the SH-SY5Y neuroblastoma cell line, MPST2 was the major isoform in HT29 colon adenocarcinoma cells and HepG2 hepatocellular carcinoma cells. HEK293 human embryonic kidney cells showed approximately equal amounts of the isoforms.

**Concentration of 3-MP in murine tissues**

To our knowledge, the intracellular concentration of 3-MP has not been reported. To address this gap, we used monobromobimane to derivatize tissue thiols and separated them by HPLC. The following retention times were seen for the standards: 3-MP (8 min), cysteine/homocysteine (4 min), and GSH (12 min). In tissue samples, a small albeit clear peak with a retention time corresponding to the monobimane adduct of 3-MP was observed and increased in intensity when spiked with standard 3-MP (data not shown). The fraction containing 3-MP was collected, and its identity was verified by LC/MS analysis, which revealed an m/z of 311.1 for the parent ion and 223.1 and 192.2 for the major fragments, as seen with an authentic 3-MP sample. The concentration of 3-MP in tissue samples was quantified using a calibration curve generated with known quantities of 3-MP. From this analysis, the following estimates for 3-MP concentration in murine tissue were obtained: 0.8 ± 0.3 μmol·kg tissue⁻¹ in liver, 1.4 ± 0.5 μmol·kg tissue⁻¹ in kidney, and 0.4 ± 0.4 μmol·kg tissue⁻¹ in brain (Table 4). For comparison, the concentration of 3-MP in rabbit plasma was reported to be 0.05—0.1 μM (32).

**Estimation of murine liver MPST reaction rate**

The MPST reaction rate was simulated using the kinetic parameters for recombinant human MPST2 and the physiologically relevant substrate concentrations of 3-MP and the thiophilic acceptors cysteine, homocysteine, and GSH (Tables 2 and 5). The simulations (using Equations 1–4 in Table 2) predicted that the total MPST reaction rate exhibits a bimodal dependence on the concentrations of thioredoxin (Fig. 6A). The reaction rate is maximal (626 μmol h⁻¹ kg tissue⁻¹) at 0.1 μM thioredoxin and declines to 34 μmol h⁻¹ kg tissue⁻¹ at 10 μM thioredoxin. Because thioredoxin increases the $K_M$ for 3-MP, the first substrate, the MPST reaction rate of the other thiophilic acceptors also declines as the concentration of thioredoxin increases.

The simulations predict that cysteine and thioredoxin are the major thiophilic acceptors at physiologically-relevant substrate concentrations. At 0.8 μM 3-MP, approximating the concentration in murine liver (Table 4), and 1 μM thioredoxin, cysteine

**Table 4**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Assay</th>
<th>Liver</th>
<th>Kidney</th>
<th>Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPST activity (mmol h⁻¹ kg tissue⁻¹)</td>
<td>PbS</td>
<td>265 ± 31</td>
<td>114 ± 20</td>
<td>27 ± 2</td>
</tr>
<tr>
<td>MPST activity (mmol h⁻¹ kg tissue⁻¹)</td>
<td>GC</td>
<td>205 ± 12</td>
<td>107 ± 19</td>
<td>20 ± 5</td>
</tr>
<tr>
<td>MPST-specific activity (mmol h⁻¹ g protein⁻¹)</td>
<td>PbS</td>
<td>1.1 ± 0.2</td>
<td>0.69 ± 0.13</td>
<td>0.31 ± 0.02</td>
</tr>
<tr>
<td>Soluble protein concentration (g/kg tissue⁻¹)</td>
<td></td>
<td>238 ± 11.0</td>
<td>166 ± 4</td>
<td>86 ± 4</td>
</tr>
<tr>
<td>3-MP concentration (μmol/kg tissue⁻¹)</td>
<td></td>
<td>0.8 ± 0.3</td>
<td>1.4 ± 0.5</td>
<td>0.4 ± 0.4</td>
</tr>
</tbody>
</table>

**Table 5**

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>$K_{M(Ac)}$ (μM)</th>
<th>$K_{M(3-MP)}$ (μM)</th>
<th>$V_{max(DHLA)}/V_{max(Ac)}$</th>
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<tbody>
<tr>
<td>Dihydrolipoic acid</td>
<td>4.4 ± 0.3</td>
<td>25 ± 6</td>
<td>1</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>4.1 ± 0.6</td>
<td>22 ± 2</td>
<td>1.55</td>
</tr>
<tr>
<td>L-Homocysteine</td>
<td>12.5 ± 1.6</td>
<td>30 ± 0.2</td>
<td>1.94</td>
</tr>
<tr>
<td>Glutathione</td>
<td>28 ± 2</td>
<td>20 ± 0.4</td>
<td>5.1</td>
</tr>
<tr>
<td>Thioredoxin</td>
<td>0.0025 ± 0.0004</td>
<td>350 ± 62</td>
<td>1.35</td>
</tr>
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</table>

**Figure 5. Expression of MPST isoforms in human and murine cells.** A. Western blot analysis of MPST in human red blood cells (RBC). Cell lysates (200 μg of hemoglobin/lane) from three donors (lanes 1–3) were separated by SDS-PAGE and detected using MPST antibody as described under “Experimental procedures.” The migration of MPST1 and MPST2 is indicated on the right. B. Western blot analysis of MPST in extracts (100 μg of protein per lane) from human hepatoma (HepG2), colon adenocarcinoma (HT29), human embryonic kidney (HEK293), and neuroblastoma (SH-SY5Y) cells and from mouse endothelial cells (bEnd3).
and thioredoxin account for 51 and 37% of the total reaction rate, respectively, with GSH contributing 12% (Fig. 6C). Increasing the concentration of thioredoxin to 10 μM decreases the total MPST reaction rate 12-fold and increases the contribution of cysteine to 80%. Under these conditions, thioredoxin accounts for 19% of the overall rate, while GSH and homocysteine make negligible contributions (Fig. 6B). The rate of the thioredoxin-dependent MPST reactions increases as the concentration of 3-MP increases, whereas the reaction rates with the other acceptors are less sensitive above 0.5 μM 3-MP (Fig. 6, B and C).

Unlike the other thiophilic acceptors, the dependence of the MPST rate on the concentration of DHLA exhibits Michaelis-Menten behavior as reported previously for MPST2 (10) and also observed with MPST1 (Fig. S1). Although monomeric, MPST1 (Fig. 3) and MPST2 (10) both exhibit non-Michaelis-Menten kinetics. Although less common, kinetic cooperativity has been seen with other monomeric enzymes, including other sulfur transferases (16, 17). Operationally, the Hill coefficient for monomeric enzymes is a relative measure of the cooperative effect rather than the number of ligand-binding sites as with oligomeric enzymes (37). Slow substrate-induced conformational change often underlies monomeric cooperativity, such that substrate binding to the enzyme does not reach equilibrium on the timescale of catalytic turnover (37). Although the molecular mechanism underlying monomeric cooperativity in MPST is not known, it is interesting that the only substrate with which it exhibits Michaelis-

Discussion

MPST is expressed in many cell types and tissues (34), and its physiological significance in sulfur metabolism is revealed by the autosomal-recessive disorder, mercaptolactate-cysteine disulfiduria, which results from mutations in MPST (35, 36). This study leads to several major conclusions. (i) The MPST1 and MPST2 isoforms are kinetically indistinguishable, and their relative levels vary in human cell lines. (ii) NAC is unlikely to serve as an acceptor in the MPST-catalyzed reaction in contrast to what was previously a proposal (23). (iii) MPST can be a source of the low-molecular-weight cysteine persulfide. (iv) Thioredoxin can modulate MPST activity due to pronounced substrate inhibition in a physiologically-relevant concentration range.

Although monomeric, MPST1 (Fig. 3) and MPST2 (10) both exhibit non-Michaelis-Menten kinetics. Although less common, kinetic cooperativity has been seen with other monomeric enzymes, including other sulfur transferases (16, 17). Operationally, the Hill coefficient for monomeric enzymes is a relative measure of the cooperative effect rather than the number of ligand-binding sites as with oligomeric enzymes (37). Slow substrate-induced conformational change often underlies monomeric cooperativity, such that substrate binding to the enzyme does not reach equilibrium on the timescale of catalytic turnover (37). Although the molecular mechanism underlying monomeric cooperativity in MPST is not known, it is interesting that the only substrate with which it exhibits Michaelis-

Figure 6. Simulation of the murine liver MPST reaction rate at physiological substrate concentrations. A, dependence of total MPST reaction rate and the component reactions with different acceptors on the concentration of thioredoxin at physiologically-relevant concentrations of 3-MP and other thiophilic acceptors (as specified in Table 2). B and C, dependence of the total MPST reaction rate and of the component reactions with different acceptors on the concentration of 3-MP at 10 μM (B) or 1 μM (C) thioredoxin. D, dependence of total MPST reaction rate and the component reactions with different acceptors, including DHLA (6 μM), on the concentration of 3-MP at 1 μM thioredoxin. Because of the hyperbolic dependence of the MPST reaction on DHLA concentration, its contribution to the total rate is predicted to be significant. However, as discussed in the text, the intracellular pool of free DHLA available for the MPST reaction is believed to be very low.
This model helps explain the free enzyme, decreasing its availability for 3-MP (Fig. 7).}

...ping-pong mechanism in which thioredoxin can also bind to inhibition of MPST by thioredoxin, we propose a modified reaction cycle (Fig. 1). Transfer and release of the persulfide product complete the...ment behavior is a relatively hydrophobic one, i.e. DHLA (10).

The MPST reaction is proposed to occur via a ping-pong mechanism in which 3-MP binds first and forms an enzyme-bound persulfide intermediate (38). This mechanism is consistent with the crystal structures of murine and human MPST, which reveal that the active site is too small to simultaneously accommodate 3-MP and a thiophilic acceptor (10, 38). Following release of pyruvate, the second substrate binds, and sulfur transfer and release of the persulfide product complete the reaction cycle (Fig. 1B) (10). To explain the observed substrate inhibition of MPST by thioredoxin, we propose a modified ping-pong mechanism in which thioredoxin can also bind to the free enzyme, decreasing its availability for 3-MP (Fig. 7). This model helps explain the ~15-fold increase in the $K_{i,v}$ value for 3-MP in the presence of thioredoxin (Table 5) (10). Substrate inhibition is not observed with the other (low molecular weight) acceptors, and the $K_M$ value for 3-MP is similar in their presence. Our study thus reveals that thioredoxin functions both as a persulfide acceptor in the MPST reaction and as a potential regulator, lowering the reaction rate as its concentration rises within a physiologically-relevant concentration range.

Cysteine is estimated to account for 50–80% of the hepatic MPST reaction rate depending on the thioredoxin concentration. Furthermore, an increase in intracellular cysteine is expected to stimulate catabolic pathways, including the GOT-dependent transamination reaction enhancing 3-MP synthesis (Fig. 1B). Thus, the actual effect of cysteine elevation on H$_2$S production could be more significant than predicted by the simulations if 3-MP levels rise.

Unexpectedly, our kinetic data predict that MPST activity will increase if the concentration of thioredoxin decreases.

Although we were unable to assess the effect of oxidized thioredoxin on the MPST reaction rate, we speculate that the MPST reaction could be enhanced under oxidative stress conditions, which decrease thioredoxin availability. Examples of redox-sensitive regulation by thioredoxin that is independent of its oxidoreductase activity are known. For example, the interaction of T7 DNA polymerase with thioredoxin in the dithiol but not in the disulfide state increases its processivity (39). Similarly, the interaction of reduced thioredoxin with apoptosis-signaling kinase 1 inhibits apoptosis signaling (40).

Based on our model of substrate inhibition, we speculate that regulation of MPST by thioredoxin might be important for promoting target persulfidation under oxidizing conditions where it is postulated to serve a protective function (41–44). Thus, conditions that decrease the concentration of reduced thioredoxin might promote sulfur transfer from MPST to other low-molecular-weight acceptors or to proteins while also enhancing the overall reaction rate (Fig. 7). This model suggests the hypothesis that sulfur transfer from 3-MP to acceptors increases under oxidizing conditions and warrants testing. The active-site cysteine in MPST renders it potentially susceptible to inactivation by oxidants. However, the relatively high $K_i$ (120 ± 10.5 μM) for H$_2$O$_2$-dependent inhibition of rat MPST (45) indicates that the active-site cysteine is well-protected. This is consistent with crystallization of the persulfide intermediate in mouse and human MPST (10, 38). Rat MPST is also susceptible to inhibition via an intersubunit disulfide bond formation between two surface-exposed cysteines (Cys$^{154}$ and Cys$^{263}$) (46). These cysteines are, however, not conserved in human MPST.

The pattern of MPST activity is similar to that reported previously for CBS and CSE, being highest in liver then followed by kidney and brain (11). Simulations using the experimentally-determined kinetic parameters in liver predicted a rate of MPST-dependent H$_2$S (or persulfide) production of 0.03–0.41 mmol h$^{-1}$ kg tissue$^{-1}$ at physiologically-relevant substrate concentrations and 10–1 μM thioredoxin. At the upper limit, the rate is comparable with the experimentally-determined rate of H$_2$S production (0.48 mmol h$^{-1}$ kg tissue$^{-1}$) predominantly by CSE in murine liver homogenate at 100 μM cysteine (6).

**Experimental procedures**

**Materials**

3-MP (sodium salt) was purchased from Research Organics (Cleveland, OH); DHLA was from Santa Cruz Biotechnology (Dallas, TX); monobromobimane (FluoroPure grade) was from Molecular Probes (Grand Island, NY); and l-cysteine, NAC, and NADPH were from Millipore-Sigma. Lead nitrate was from Acros Organics (Pittsburgh, PA). HepG2, HT29, HEK293, bEnd3, and SH-SY5Y lines were purchased from ATCC (Camden, NJ). Red blood cell lysates were prepared as described previously (30) using blood from three healthy volunteers provided by Dr. David Fox (University of Michigan).

**MPST purification**

Recombinant human MPST2, thioredoxin, and thioredoxin reductase were purified as described previously (10). The cDNA-encoding recombinant human MPST1 was cloned from
Regulation of MPST

pBF15 (12) into pET28b (Millipore-Sigma) using NdeI and EcoRI restriction sites. The pET28b_MPST1 expression construct incorporated a thrombin-cleavable His\textsubscript{6} tag. BL21(DE3) Escherichia coli was transformed with pET28b_MPST1 and pTrc99A-EL (expressing the molecular chaperones GroES/ GroEL, as described previously (10)). Overnight 10-ml cultures grown at 37 °C in Luria Bertani (LB) medium with 50 μg/ml kanamycin and 100 μg/ml ampicillin were used to inoculate six 1-liter cultures in the same medium. Growth was continued at 28 °C until the OD\textsubscript{600 nm} was ∼0.45, at which point the temperature was lowered to 15 °C. Induction of protein expression and purification of MPST1 was performed essentially as described previously for MPST2 (10). The protein was judged to be >90% pure by SDS-PAGE analysis. If desired, MPST1 was further purified using a DEAE-Sepharose column as described previously (10). All purified proteins were concentrated and stored at −80 °C.

Thioredoxin and thioredoxin reductase purification

Expression constructs for human thioredoxin (cytoplasmic) and thioredoxin reductase were the generous gifts from Dr. Vadim Gladyshev (Harvard Medical School). The cDNAs were expressed in *E. coli* and purified as described previously (47, 48).

Recombinant human thioredoxin was expressed in BL21(DE3), as described previously (47). Briefly, BL21(DE3) *E. coli* cells transformed with pET-20b-hTrx constructs were grown in LB medium at 37 °C until the OD\textsubscript{600 nm} was ∼0.8. IPTG (0.1 mM) was added to induce protein expression, and cells were grown for 5 h at 37 °C, then harvested by centrifugation, and stored at −80 °C until further use. To purify the protein, the cells were suspended in 50 mM HEPES, pH 7.4, containing 500 mM NaCl, 20 mM imidazole, EDTA-free protease inhibitor mixture (Roche Applied Science), 2 mM tris(2-carboxyethyl)phosphine (TCEP), lysozyme (50 mg), MgCl\textsubscript{2} (5 mM), DNase (1 mg). Furthermore, the cell lysate was sonicated and centrifuged at 18,000 rpm. The supernatant was loaded onto a nickel-NTA–agarose column, washed with 50 mM sodium phosphate buffer, pH 7.4, containing 500 mM NaCl and 40 mM imidazole in the same buffer. Fractions containing thioredoxin were pooled, concentrated, and further purified on a Superdex 200 column eluted with PBS. Fractions containing thioredoxin reductase were pooled, concentrated, and stored at −80 °C.

Recombinant WT thioredoxin reductase in which the selenocysteine is substituted by cysteine was expressed in BL21(DE3), as described previously (48). Briefly, BL21(DE3) *E. coli* cells transformed with pET-28b-hTR were grown in LB medium at 28 °C, and protein expression was induced with 0.5 mM IPTG when OD\textsubscript{600 nm} = 0.6. Cells were grown overnight at 28 °C, harvested by centrifugation, and stored at −80 °C. To purify the protein, cells were suspended in 50 mM sodium phosphate, pH 7.4, containing 500 mM NaCl, 20 mM imidazole, EDTA-free protease inhibitor mixture (Roche Applied Science), lysozyme (50 mg), MgCl\textsubscript{2} (5 mM), and DNase (1 mg). Cells were sonicated; the lyse was centrifuged at 18,000 rpm; and the supernatant was loaded onto a nickel-NTA–agarose column, washed with 50 mM sodium phosphate buffer, pH 7.4.

Incorporating 500 mM NaCl and 40 mM imidazole. The protein was eluted with a linear gradient of 40–400 mM imidazole. Fractions containing thioredoxin reductase were pooled, concentrated, and further purified on a Superdex 200 column, which was eluted with PBS. Fractions containing thioredoxin reductase were pooled, concentrated, and stored at −80 °C.

**Animals and tissue collection**

Male BALB/c mice, 8 weeks old, were purchased from Charles River Laboratories (Wilmington, MA). Animals were sacrificed in a CO\textsubscript{2} atmosphere, and liver, kidney, and brain were collected immediately, frozen in liquid nitrogen, and stored at −80 °C until further use. All procedures for animal handling were performed in accordance with the protocols approved by the University’s Committee on Use and Care of Animals.

**Tissue sample preparation**

Each frozen organ was pulverized in liquid nitrogen using a porcelain mortar and pestle, and the frozen tissue powder was collected in pre-weighed sample tubes containing either (i) 400 μl of 200 mM HEPES buffer, pH 7.4 for MPST activity assay, or (ii) 500 μl of metaphosphoric acid solution (16.7 mg/ml) for 3-MP analysis. The tubes were then re-weighed to obtain an estimate of the weight of the tissue in each sample. The final tissue dilution (w/v) was ∼1:4 for MPST activity assays and 1:5–1:9 for 3-MP analysis. Protein concentration was determined using the Bradford method using BSA as a standard.

**MPST activity assays**

Tissue homogenates were divided into two parts. The first part was used as is to measure MPST activity in crude homogenates. The second part was centrifuged at 15,000 × g, 4 °C, for 10 min, and the supernatant was used to measure soluble MPST activity. The following methods were used to determine MPST activity in murine tissue homogenates.

(i) Tissue MPST activity using a colorimetric assay—The lead sulfide method was used to measure MPST activity in soluble tissue lysates (10). Briefly, the reaction mixture (1.0 ml final volume) containing 200 mM HEPES buffer, pH 7.4, BSA (100 μg/ml), 20 mM DHLA, 0.4 mM lead nitrate, and tissue homogenate (20 μg (liver or kidney) or 50 μg (brain)) was preincubated at 37 °C for 5 min, and the reaction was initiated by addition of 0.5 mM 3-MP. Formation of lead sulfide was monitored by the increase in absorbance at 390 nm, and a molar extinction coefficient of 5500 M\textsuperscript{−1} cm\textsuperscript{−1} was used to determine its concentration (9). Control reactions lacking substrate or tissue homogenate were performed in parallel, and the value was subtracted to obtain the final rate of the MPST reaction in the tissue samples.

(ii) Tissue MPST activity using GC coupled to sulfur chemiluminescence detection—H\textsubscript{2}S accumulation was measured directly using a GC equipped with a 355 sulfur chemiluminescence detector (Agilent, CA) as described previously (6, 11). Briefly, 0.5 ml of the reaction mixture described above, but lacking lead nitrate, was incubated at 37 °C in polypropylene syringes flushed with nitrogen in a total volume of 20 ml and with gentle agitation on a platform shaker. The rate of H\textsubscript{2}S production was determined by measuring
the concentration of H$_2$S in the gas phase in the syringe after 10 and 20 min of incubation.

(iii) Kinetic analysis of purified recombinant human MPST1—H$_2$S synthesis by MPST was assayed using the colorimetric assay described above. Briefly, the reaction mixture (1.0-ml final volume) containing 200 mM HEPES buffer, pH 7.4, BSA (100 $\mu$g/ml), variable concentrations of 3-MP and either cysteine (0–12 mM), NAC (0–40 mM), DHLA (0–10 mM), or thioredoxin (0–10 $\mu$M)/thioredoxin reductase (3.5 $\mu$M) and NADPH (200 $\mu$M) was incubated at 37 °C for 5 min. The reaction was started by addition of 0.3 $\mu$M human recombinant MPST1 and was monitored as described above. The kinetic data were subjected to either the Michaelis-Menten or the Hill equation analysis.

**Kinetic analysis of MPST reaction rate on thioredoxin concentration**

The dependence of the MPST3 reaction rate on the concentration of thioredoxin was assayed at different 3-MP concentrations using the coupled thioredoxin reductase/NADPH assay to recycle oxidized thioredoxin as described previously (10). Briefly, a 1-ml reaction mixture containing 200 mM HEPES buffer, pH 7.4, 200 $\mu$M NADPH, 3.5 $\mu$M thioredoxin reductase, 1–20 $\mu$M thioredoxin, 100 $\mu$g/ml BSA, and variable concentrations of 3-MP (0–3 mM) was preincubated for 5 min at 37 °C. The reaction was started by addition of 0.3 $\mu$M human recombinant MPST1, and the rate of NADPH oxidation was monitored at 340 nm using the extinction coefficient, 6,200 M$^{-1}$ cm$^{-1}$.

**Purification of MPST antibodies**

Polyclonal antibodies against human MPST2 were raised in chicken by Aves Labs, Inc. (Tigard, OR). For this, purified recombinant human MPST2 was separated on a 10% polyacrylamide gel under denaturing conditions and the 33-kDa protein band was excised and used as the antigen. Total IgY from eggs was purified using an MPST2 affinity column prepared using the Actigel-ALD kit (Sterogene, Carlsbad, CA) according to the manufacturer’s protocol. The purified antibodies were validated by Western blot analysis (described below) using purified MPST as an antigen.

**Western blot analysis**

Lysates from human (HepG2, HEK293, and SH-SY5Y) and murine (bEnd3) cell lines were prepared as described previously (49). Red blood cell lysates were prepared as described previously (30), and the concentration of hemoglobin in each sample was assessed by the absorption at 415 nm using an extinction coefficient of 128 mm$^{-1}$ cm$^{-1}$ (50). The protein amount loaded per lane for Western blot analysis is indicated in the figure legends.

Proteins were separated on a 10% polyacrylamide gel under denaturing conditions and transferred to a PVDF membrane. Blots were probed using polyclonal anti-human MPST2 chicken antibodies described above (1:1000 dilution). Secondary anti-chicken antibodies conjugated to horseradish peroxidase (Aves Labs, Inc.) were used at a 1:500,000 dilution, and signals were visualized using the chemiluminescent peroxidase substrate kit SuperSignal West Dura (Thermo Fisher Scientific, Pittsburgh, PA). The MPST antibody detects both MPST1 and MPST2.

**Quantitation of tissue 3-MP**

For 3-MP analysis, tissue homogenate obtained as described above was centrifuged for 5 min at 15,000 × g at 4 °C. The supernatant was treated with monobromobimane to block free thiols as described previously (51). Briefly, 100 $\mu$l of supernatant was mixed with 20 $\mu$l of 1 M Tris base solution and 3 $\mu$l of 60 mM monobromobimane in acetonitrile, and the mixture was incubated in the dark at room temperature for 10 min. Then, 30 $\mu$l of metaphosphoric acid solution (16.7 mg/ml) was added, and the mixture (10–50 $\mu$l) was injected into a $\mu$-Bondapak–NH$_2$ column (10 $\mu$m, 4 × 300 mm (Waters, Milford, MA)). The sample was eluted using solutions A and B at a flow rate of 1 ml/min using the following gradients: 0–5 min, 10% B; 5–15 min, linear from 10 to 20% B; 15–20 min, linear from 20 to 100% B; 20–25 min, 100% B, 25–35 min, 10% B. Solution A was 20% water and 80% methanol. Solution B was 66% solution A and 34% ammonium acetate solution prepared by mixing 154 g of ammonium acetate, 300 ml of acetic acid, and 100 ml of water. The eluant was monitored by fluorescence excitation at 390 nm (emission at 490 nm). A calibration curve was generated using known concentrations of 3-MP derivatized with monobromobimane, which was stable for at least 2 h at 25 °C. For LC-MS analysis fractions containing 3-MP were collected, concentrated in a SpeedVac instrument, and stored at −80 °C until analysis.

**Mass spectroscopic analysis**

The derivatized 3-MP samples were thawed and kept at 4 °C during the LC-MS experiments. A BioBasic C-18 column (Thermo Fisher Scientific, Waltham, MA) was used to separate the derivatized compounds. The mobile phase was 0.1% formic acid in water (A) or acetonitrile (B). A 5-$\mu$l aliquot of each sample was injected into the column using an LC1200 HPLC from Agilent at a flow rate 0.2 ml/min. The eluate was attached to a 4000 QTrap (Sciex, Framingham, MA) operating in the MS, MS/MS, or MRM mode. The parameters for the electrospray ion-source were as follows: curtain gas = 25 p.s.i.; ionization spray = 5000 V; temperature = 650 °C; source gas 1 = 70 p.s.i.; source gas 2 = 40 p.s.i. The parameters after compound optimization were as follows: declustering potential = 80 V; entrance potential = 10 V; exit potential = 15 V; collision energy = 30 V. The transitions for the monobimine adduct of 3-MP were obtained by fragmentation of an authentic standard (311.1/223.1 and 311.1/192.2), and the second transition was used for quantification.

**Kinetic simulations**

Mathematical simulations were used to estimate H$_2$S production by murine liver MPST2 at physiologically-relevant substrate concentrations using Microsoft Excel software. The following metabolite concentrations were used as representative of their respective physiological values in murine liver: 3-MP (0.8 $\mu$m, this study) and DHLA (6 $\mu$m (33)); cysteine (100 $\mu$m (52, 53)); homocysteine (4 $\mu$m (54, 55)); GSH (7 $\mu$m (52, 53));
Regulation of MPST

and thioredoxin (10 μM (25)). The remaining parameter values used for the simulations are described in Table 2.

The total rate of the MPST2 reaction (Equation 2 in Table 2) is the sum of the component reactions with various thiophilic acceptors (cysteine, homocysteine, GSH, and thioredoxin ± DHLA). A ping-pong mechanism was used to describe the MPST reaction (Equation 2 in Table 2). The reaction rate with each acceptor included the influence of the other acceptors as competitive inhibitors. Because the $K_v$ values were not known, the inhibition constant for each acceptor was set as a first approximation as equal to its $K_M$ value. For example, Equation 4 in Table 2 describes the rate of the MPST reaction with cysteine, and it includes the inhibitory effects of thioredoxin, GSH, and homocysteine on the reaction rate. The $K_M$ values for the substrates (Table 2) were reported previously for MPST2 (10). The Hill coefficient for each substrate is included in the reaction.

The $K_M$ values for 3-MP in the presence of cysteine, GSH, and homocysteine are very similar (20–30 μM). In contrast the $K_M$ for 3-MP is 15-fold higher in the presence of 20 μM thioredoxin (Table 1). As described by Equation 1 in Table 2, the $K_M$ value for 3-MP increases in proportion to the concentration of thioredoxin. A value of 25 μM was used for $K_M^{(3MP)}$ in Equation 1, which represents the average value for the $K_M$ for 3-MP with the other acceptors, i.e. in the absence of substrate inhibition. This equation incorporates the Hill coefficient for thioredoxin ($n = 1.5$). Taking into account that at 20 μM thioredoxin, $K_M^{(3MP)} = 350$ μM (10), a value of 3.6 μM for the $K_v$ for thioredoxin was obtained using Equation 1.

MPST activity in murine liver with DHLA as an acceptor ($V_{max(DHLA)}$) was determined in this study to be 265 mmol h$^{-1}$ kg tissue$^{-1}$ (Table 4). Murine liver MPST reaction rates with the other acceptors were estimated from the $V_{max(DHLA)}/V_{max(Acc)}$ ratio, using values obtained with purified MPST2 in vitro (Table 5). The remaining parameter values used for the simulations are described in Table 2.

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