Post-translational Regulation of Mercaptopyruvate Sulfurtransferase via a Low Redox Potential Cysteine-sulfenate in the Maintenance of Redox Homeostasis*

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3-Mercaptopyruvate sulfurtransferase (MST) (EC 2.6.1.2), a multifunctional enzyme, catalyzes a transsulfuration from mercaptopyruvate to pyruvate in the degradation process of cysteine. A stoichiometric concentration of hydrogen peroxide and of tetrathionate (S₄O₆⁻) inhibited rat MST (kᵣ = 3.3 min⁻¹, Kᵣ = 120.5 μM and kᵢ = 2.5 min⁻¹, Kᵢ = 178.6 μM, respectively). The activity was completely restored by dithiothreitol or thioredoxin with a reducing system containing thioredoxin reductase and NADPH, but glutathione did not restore the activity. On the other hand, an excess molar ratio of hydrogen peroxide inactivated MST. Oxidation with a stoichiometric concentration of hydrogen peroxide protected the enzyme against reaction by iodoacetate, which modifies a catalytic Cys²⁴⁷, suggesting that Cys²⁴⁷ is a target of the oxidants. A matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry analysis revealed that hydrogen peroxide- and tetrathionate-inhibited MSTs were increased in molecular mass consistent with the addition of atomic oxygen and with a thiosulfate (S₂O₃²⁻), respectively. Treatment with dithiothreitol restored modified MST to the original mass. These findings suggested that there was no nearby cysteine with which to form a disulfide, and mild oxidation of MST resulted in formation of a sulfenate (SO⁻) or a disulfide bond has been characterized (20–26). On the other hand, the cysteine-sulfenate or disulfide bond was reduced by dithiothreitol (DTT) or cellular reductants such as thioredoxin and glutathione to be restored (20–26). However, a physiological role of the rapid inhibition-reactivation cycle of these enzymes has not been focused.

To confirm sulfenate formation in these studies, the electrophilic reagents 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) (20–26) and dimedone (5,5-dimethyl-1,3-cyclohexanedione) (27, 28) were utilized. Further, the redox potential of cysteine-sulfenate was estimated to be higher than that of glutathione (29–32). The formation of a stable sulfenyl thiosulfate at the catalytic cysteine implied that a nearby cysteine to form a disulfide bond was not present. Peroxidase activity was another supporting evidence of a sulfenate formation (33–35).

We recently determined that MST is a housekeeping enzyme (36), and hydrogen peroxide did not change the amount of MST mRNA in Hep3B cells, suggesting that MST activity is regulated at the enzymatic level. Mosharov et al. (37) have reported that in the cysteine anabolic pathway hydrogen peroxide promotes the activity of cystathionine β-synthase and suppresses the activity of methionine synthase at the transcriptional level, resulting in a facilitation of the metabolic flow to cystathionine. Consequently the amount of cysteine increased, and cellular reductants such as glutathione and thioredoxin were overproduced (38).

In this study, we provide evidence that MST is rapidly regulated at the enzyme level by the redox state, which in turn is associated with the control of cysteine degradation. These results suggest that MST helps maintain cellular redox homeostasis.

**MATERIALS AND METHODS**

*Preparation of Wild Type and Mutant MSTs—Rat wild type and C247S (in which the catalytic site Cys²⁴⁷ is replaced with serine) MST cDNAs were prepared according to a procedure described previously (11). C247S was used only in SH group titration. Complementary DNAs coding single mutant MSTs (C64S, C154S, C254S, and C263S) were synthesized by PCR using TaKaRa LA Taq with GC buffer (TAKARA BIO Inc. Otsu, Japan); wild type MST cDNA inserted in pBluescript (SK+) vectors (Stratagene, La Jolla, CA) was used as templates for mutant MSTs.***

*This study was supported by the Naito Foundation. 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.

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2. The abbreviations used are: MST, mercaptopyruvate sulfurtransferase; DTNB, 5, 5′-di-thiobis(2-nitrobenzoic acid); DTT, dithiothreitol; MALDI-TOF-MS, matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole.

3. N. Nagahara, unpublished data.
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The primers used for mutagenesis were as follows: rC64S-s (sense primer), GCATCGTACGCGCAGCATCGCCACATCGCC, and rC64S-A (antisense primer), GGGGCTTCTGCCTGAATCCGCAG, for the C64S mutant; rC154S-s, CTTGCGAAGTATGACGCGAGTGCGT, and rC154S-A, ATCGAGCTCCGGCGCTGAATCCGCAG, for the C154S mutant; rC254S-s, GTTGTCAAGGCCAGCGCACTGCGT, and rC254S-A, CAGGACACTGAGCCTGGCTGCTGGAACC, for the C254S mutant; rC263S-s, TAGCCGCTTCCTCAGCAGGAGAAGGCCAC, and rC263S-A, CATCGGCTTGCAGCAGAGAGGAAGGCCAC, for the C263S mutant.

Each mutagenesis was performed with a QuickChange site-directed mutagenesis kit (Stratagene). The cycling parameters for PCR were as follows: for the first segment, one cycle of denaturation at 95 °C for 30 s and for the second segment, 13 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min, and extension at 68 °C for 8.5 min.

The PCR product was treated with DpnI and introduced into Escherichia coli XL1-Blue according to the usual protocol except for a pre-culture in LB medium without antibiotics at 37 °C for 1 h before plating. Sequencing was performed to select each mutagenized cDNA using the synthesized antisense primers GCAGGTTCTGGCTCAGCCA, TGGGCGATGTGTGGTCGCTGCTACGATCG, and rC263S-A, ATCGAGCTCCGGCGCTGAATCCGCAG, for the C263S mutant.

Sequencing was performed to select each mutagenized cDNA using the antisense primer, GGGCGATGTGTGGTCGCTGCTACGATCG, and rC263S-A, ATCGAGCTCCGGCGCTGAATCCGCAG, for the C263S mutant.

Inhibition and Inactivation of MSTs—Tetrathionate must be mixed with cyanide because the addition of cyanide to tetrathionate causes its decomposition. Thus, MST was first incubated with tetrathionate or hydrogen peroxide, and a 5-

The kinetic analysis was performed basically according to the Kitz and Wilson method (39). Each t½ value (time when the remaining activity is 50% of the untreated control activity) was determined from a semilog plot of v/v₀ versus time (v₀ being the reaction rate when inhibitor = 0 mM). The kᵢ value (inhibition rate constant) was determined from a replot of the t½ value versus 1/[I] (inhibitor).

This relationship can be represented by the following equations,

\[ K_i = \frac{[I][E]}{[EI]} \] (Eq. 1)

where \( K_i \) = the dissociation constant of the EI complex, \( E \) = enzyme, and \( EI \) = enzyme-inhibitor noncovalent complex and

\[ [E] = \frac{[e]}{1 + \frac{[I]}{K_i}} \] (Eq. 2)

where \([e]\) = the concentration of MST when time is t, and \([e] = [E] + [EI]\).

The inhibition velocity equation is as follows.

\[ \frac{d[e]}{dt} = k_i[E] = \frac{k_i}{K_i}[1][E] \] (Eq. 3)

Equations 2 and 3 can be rearranged to the following.

\[ -\frac{d[e]}{[E]} = \frac{k_i}{K_i}[I]dt = -\frac{d[e]}{[e]}\left(1 + \frac{[I]}{[I]}\right) \] (Eq. 4)

Integration of Equation 4 leads to the following.

\[ \ln \frac{[e]}{[E]} = -\frac{k_i}{K_i} \cdot \frac{1}{[I]} \cdot t \] (Eq. 5)

where \([e]₀ = \) the initial concentration of the enzyme.

The ratio of the remaining enzyme activity can be represented as follows.

\[ 1 + \ln \frac{[e]}{[E]} = 1 - \frac{k_i}{K_i} \cdot \frac{1}{[I]} \] (Eq. 6)

Equation 6 can be rearranged to the following.

\[ t^{1/2} = \frac{\ln 0.5}{k_i} - \frac{\ln 0.5}{k_i} \cdot \frac{1}{[I]} \] (Eq. 7)

Reactivation of Hydrogen Peroxide- and Tetrathionate-inhibited MSTs by DTT—After incubation of 20 μM wild type and mutant MSTs with 20 μM or 0.5 mM hydrogen peroxide, or 20 μM or 0.5 mM tetrathionate in 60 μl of 20 mM potassium phosphate buffer, pH 7.4, on ice for 20 min, free oxidants were removed from each sample and a control with a NAP5 column (Amersham Biosciences), and each mixture was concentrated to 20 μM with a VIVASPIN (10,000MWCO. PES, Sartorius, Goettingen, Germany). A 5-μl aliquot was taken from the mixture and mixed with 1 μl of 10 mM (1.6 mM at a final concentration) DTT (Fluka, Osaka, Japan) solution. Each mixture was incubated at 25 °C for 20 min.
Comparative Study for Reactivation of MST by DTT, Cysteine, Thioredoxin, or Glutathione—After incubation of 20 μM wild type MST with 20 μM tetrahionate or hydrogen peroxide on ice for 20 min in 60 μl of 20 mM potassium phosphate buffer, pH 7.4, free oxidants were removed from each sample and a control with a NAP5 column. The enzyme-containing fractions were collected and concentrated to 20 μM with a VIVASPIN.

In the experiment using l-cysteine (Kanto Kagaku, Tokyo, Japan) or DTT, 1.6, 3.2, 6.4, 12.8, 25.6, 51.2, or 102.4 mM cysteine or 1.6 mM DTT was added to 30 μl of the mixture taken from the concentrated sample, and the total volume was adjusted to 60 μl in 20 mM potassium phosphate buffer, pH 7.4. The mixture was incubated on ice for 20 min. After gel filtration with a NAP5 column, the enzyme-containing fractions were collected and concentrated to 20 μM with a VIVASPIN. Then a 5-ml aliquot taken from the mixture was used for the assay of rhodanese activity.

In the experiment using recombinant E. coli thioredoxin (kindly provided by Dr. Abe, Department of Biochemistry and Molecular Biology, Nippon Medical School) or yeast glutathione (Sigma) with the reducing system, 30 μl of the mixture taken from the concentrated MST sample was added to the reducing system. The reducing system contained 50 μM NADPH (Sigma), 10 μM thioredoxin or glutathione, and 0.2 μM recombinant E. coli thioredoxin reductase (Dr. Abe) or 0.2 μM glutathione reductase (Roche Applied Science) in 20 mM potassium phosphate buffer, pH 7.4. The total volume was adjusted to 60 μl, and the mixture was incubated on ice for 20 min. After gel filtration, each activity was assayed in the same way as described above.

In the experiment using reduced thioredoxin or reduced glutathione, 2 μl of 50 mM DTT was added to 198 μl of 50 μM thioredoxin and glutathione, and the mixture was incubated on ice for 20 min. After gel filtration with a NAP5 column, each sample was concentrated to 30 μM with a Microcon-3 (Millipore). 30 μl of the concentrated MST sample was mixed with 10 μM reduced thioredoxin or reduced glutathione in 60 μl of 20 mM potassium phosphate buffer, pH 7.4, and each mixture was incubated on ice for 20 min. After gel filtration, each activity was assayed in the same way as described above.

SH Group Titration—In the titration of the oxidized enzymes, after incubation of the wild type and five mutant MSTs (300 μM) with a 300 μM or 1.5 mM hydrogen peroxide in 30 μl of 33 mM potassium phosphate buffer, pH 8.0, on ice for 20 min, free oxidants were removed from each sample with a NAP5 column.

In the titration of the reduced enzymes, oxygen in the solvent was removed using a glass apparatus by sequential evacuation and re-equilibration with oxygen-free argon. Oxygen-free argon was prepared by passing commercially obtained pure argon through a column of a Chromtopack Gas-Clean Oxygen Filter CP17970 (Varian, Inc., Palo Alto, CA). After incubation of 300 μM wild type and the five mutant MSTs with 5 mM DTT in 30 μl of 33 mM potassium phosphate buffer, pH 8.0, on ice overnight under anaerobic conditions, free DTT was removed from each sample with a NAP5 column before analysis.

Each enzyme (20 – 60 μl) was incubated with 0.5 mM 5,5’-dithiobis(2-nitrobenzoic acid) (DTNB) or 0.6 mM NBD-Cl at 25 °C for 60 min, and the change in absorbance at 412 nm (ε412 = 13,600 M⁻¹ cm⁻¹) or at 420 nm (ε420 = 13,000 M⁻¹ cm⁻¹) (21), respectively, was measured. The number of SH groups/subunit was calculated.

A Target Residue of Oxidants—To investigate the protection of Cys²⁴⁷ by oxidants against inactivation via carboxymethylation by iodoacetate (Nakalai Tesque), after incubation of 17 μM MST with 17 μM hydrogen peroxide or tetrahionate in 60 μl of 20 mM potassium phosphate buffer, pH 7.4, on ice for 20 min, free oxidants were removed from each mixture and a control group without oxidation with a NAP5 column.

The enzyme-containing fractions were collected and concentrated to 60 μl with a VIVASPIN. Iodoacetate was added to the two experimental groups with oxidation and a control group without oxidation at a concentration of 1 mM, and the mixtures were incubated on ice for 20 min. After gel filtration of each mixture with a NAP5 column, the enzyme-containing fractions were collected and concentrated to 40 μl with VIVASPIN. 5 μl of the mixture was incubated with 5 μl of 2 mM DTT or the same buffer at 25 °C for 20 min. The values of the rhodanese activity remaining were determined.

MALDI-TOF Mass Spectrometric Analysis for Oxidized MSTs—After 12 μM MST was incubated with 12 μM tetrahionate, 12 μM hydrogen peroxide, or 0.6 mM hydrogen peroxide in 20 mM potassium phosphate buffer, pH 7.4, on ice for 20 min, each mixture was treated with gel filtration with a NAP5 column to remove free oxidants. For a control experiment, 12 μM MST was incubated without tetrathionate or hydrogen peroxide, and the mixture was treated with gel filtration with a NAP5 column. Each enzyme-containing fraction was collected and concentrated to 60 μl with VIVASPIN. For reduction of each oxidized enzyme, 30 μl of each sample was treated with 0.6 mM DTT at 25 °C for 20 min, and free DTT was removed with a NAP5 column. The enzyme-containing fractions were collected and concentrated to 20 μl with a VIVASPIN.

To examine whether the hydrogen peroxide-inhibited MST was modified with dimedone (Tokyo Kasei Co. Ltd., Tokyo, Japan), 1.5 mM MST was incubated with 1.5 mM hydrogen peroxide in 20 mM potassium phosphate buffer, pH 7.4, on ice for 20 min. After gel filtration of the mixture with a NAP5 column, the enzyme-containing fractions were collected and concentrated to 36 μl with a VIVASPIN. The solution was incubated with 50 mM dimedone on ice for 30 min. After gel filtration of the solution with a NAP5 column, the enzyme-containing fractions were collected and concentrated to 43 μl with a VIVASPIN.

Each sample was desalted with Zip Tip C₁₈ (Millipore), and 0.5 μl of the sample in 70% acetone (Wako Pure Chemicals) containing 0.1% trfluoroacetic acid (Wako Pure Chemicals) was mixed with 0.5 μl of sinapinic acid (Bruker Daltonics, Brumen, Germany) saturated in 50% acetone containing 0.1% trfluoroacetic acid. The mixture was dried at room temperature on the target plate.

MALDI-TOF mass spectrometry was performed on a Reflex III (Bruker Daltonics) mass spectrometer equipped with a SCOUT 384 ion source laser, operating in the linear positive mode at a 20-kV acceleration voltage. Mass spectra were obtained by averaging 300 individual laser shots. External mass calibration was performed using the protein mixture of protein calibration standard II (Bruker Daltonics). In the mass spectrometric analysis of a chemical modification of 30 – 40 of kDa protein using our system, mass measurement error was 0.03%.

Peroxidase Activity of MST—The assay mixture contained 0.2 mM NADPH, 40 μM recombinant E. coli thioredoxin, and 1 μM recombinant E. coli thioredoxin reductase in 500 μl of 50 mM potassium phosphate buffer, pH 7.4. With monitoring the decrease in absorbance at 340 nm, 10 μM hydrogen peroxide was added to the mixture, and 300 min later, 10, 20, 40, or 60 μM wild type MST was added. The rate of decrease in NADPH was calculated (ε₃₄₀ = 6220 M⁻¹ cm⁻¹). For a control study, C₂⁴⁷S was used instead of wild type MST.

Assay for Rhodanese Activity of MST—A procedure to measure the activity catalyzing a transsulfuration from mercaptopyruvate to β-mercaptoethanol would not be appropriate for this experiment, because β-mercaptoethanol would be able to reduce a sulfenate or a sulfenyl thiosulfate formed at a catalytic site cysteine during incubation in the...
assay mixture, as MST possesses rhodanese activity catalyzing transulfuration from thiosulfate to cyanide (10, 11) and it is this rhodanese activity of MST that was measured.

On the other hand, the amount of thiosulfate that is a derivative of tetrathionate was negligible in the rhodanese activity assay because only a very small amount of the compound was diluted further in the assay mixture.

**Protein Determination**—The protein concentrations were determined with a Coomassie protein assay kit (Pierce Biotechnology) with crystalline bovine serum albumin (ICN Biochemicals) as the standard.

**Statistical Analysis**—All values are expressed as the mean ± the S.E. The significance of difference between values was estimated with Student’s t test.

**Data Fitting**—The fitting of the data obtained from the kinetic studies was done with Kaleidagraph (Synergy Software, Reading, PA).

### RESULTS

**Inhibition Kinetics of Wild Type MST Using Inhibitors Hydrogen Peroxide and Tetrathionate**—Inhibition of MST by hydrogen peroxide ($E^{'-'} = 1.76$ mV) and tetrathionate ($E^{'-'} = 1.07$ mV (40)) proceeded in a dose- and time-dependent manner and exhibited pseudo first-order kinetics with $k_1 = 3.3 ± 0.2$ and $2.5 ± 0.2$ min$^{-1}$, respectively (n = 3, mean ± S.E.) and $k_2 = 120.5 ± 10.5$ and $178.6 ± 12.2$ μM, respectively (n = 3) (Fig. 1).

**Inhibition and Inactivation of MSTs by Hydrogen Peroxide and Tetrathionate and Reactivation by DTT**—20 μM hydrogen peroxide decreased the activity ratios of the 20 μM wild type and mutant (C64S, C154S, C254S, and C263S) MSTs for each control activity to 6.0 ± 0.6, 11.5 ± 0.9, 12.0 ± 1.1, 10.3 ± 0.9, and 8.3 ± 0.7% (n = 3), respectively. The addition of 1.6 mM DTT restored them to 90.1 ± 8.7, 88.1 ± 7.9, 84.0 ± 6.2, 98.1 ± 7.7, and 93.3 ± 9.1% (n = 3) of each control activity, respectively, within 10 min. Each inhibition-reactivation pattern was not significantly different among these enzymes.

The activity of oxidized wild type MST was restored by DTT in a time-dependent manner, and the time course was formulated as $y = 62.9 + 27.7 \log t$ (where y is the percent of control enzyme activity, and t is incubation time in min). An excess molar dose of hydrogen peroxide (0.5 mM) inactivated MSTs, and the activity ratios relative to control activity were decreased to $4.1 ± 0.2, 7.3 ± 0.5, 8.8 ± 0.6, 6.2 ± 0.4, and 4.5 ± 0.3% (n = 3), respectively. There was no significant difference among these enzymes. The addition of 1.6 mM DTT did not restore activity for any of these enzymes (7.1 ± 0.3, 6.5 ± 0.5, 6.4 ± 0.4, and 5.8 ± 0.4%, respectively (n = 3) to the level of the control activity).

20 μM tetrathionate decreased the activity ratios of the 20 μM wild type and mutant MSTs (C64S, C154S, C254S, and C263S) for each control activity to 1.8 ± 0.1, 12.9 ± 1.1, 11.3 ± 1.0, 12.3 ± 1.2, and 11.9 ± 0.9% (n = 3), respectively. Incubation with 1.6 mM DTT at 25 °C restored activity (98.0 ± 9.3, 92.0 ± 9.1, 97.2 ± 9.3, 98.0 ± 9.1, and 96.2 ± 8.1% (n = 3) of each control activity, respectively) within 10 min. It was noteworthy that the 20 μM MSTs were inhibited by 0.5 mM tetrathionate (4.8 ± 0.4, 9.4 ± 0.9, 9.8 ± 0.8, 11.3 ± 1.2, and 4.6 ± 0.4% (n = 3) in terms of the control activity, respectively), and 1.6 mM DTT restored the activity to 95.5 ± 9.3, 95.2 ± 9.2, 97.6 ± 9.8, 95.5 ± 9.3, and 89.2 ± 8.3% (n = 3) of each control activity, respectively. Each inhibition-reactivation pattern was not significantly different among these enzymes.

The activity of wild type MST was restored by in a time-dependent manner, and the time course is formulated as $y = 90.8 + 10.1 \log t$ (where y is the percent of control enzyme activity, and t is incubation time in min). An excess molar dose of DTT restored the tetrathionate-inhibited MST activity faster than hydrogen peroxide-inhibited one.

These findings suggest that the redox potentials are different among the tetrathionate- and hydrogen peroxide-inhibited MSTs. The data suggest that a disulfide bond is not formed at the catalytic site Cys247, but rather a sulfenate is formed.

**Reactivation of Oxidant-inhibited Wild Type MST by Glutathione, Thioredoxin, and Cysteine**—The values of the activity of the hydrogen peroxide-inhibited and tetrathionate-inhibited wild type MSTs were restored by incubation with 1.6 mM DTT to 89.8 ± 6.6 and 94.4 ± 5.2% (n = 3) of the control, respectively for 20 min (Fig. 2).

Cysteine (1.6, 3.2, 6.4, 12.8, 25.6, 51.2, or 102.4 mM) partially restored the activity of tetrathionate-inhibited MST to 31.5 ± 2.4 (p = 0.003, n = 3) (Fig. 2), 32.3 ± 3.1, 32.9 ± 3.3, 33.2 ± 3.1, 33.4 ± 3.2, 33.6 ± 3.4 (p = 0.0025), 31.2 ± 2.4% of the control MST, respectively for 20 min. On the other hand, 1.6, 3.2, 6.4, 12.8, 25.6, 51.2, or 102.4 mM cysteine did not
restore the activity of hydrogen peroxide-inhibited MST (11.1 ± 0.8\% (p = 0.06, n = 3) (Fig. 2), 12.2 ± 1.4, 12.3 ± 1.3, 14.3 ± 1.5, 17.7 ± 1.9, 18.1 ± 2.0 (p = 0.011), 12.1 ± 1.4\% of the control MST, respectively for 20 min.

Glutathione together with the reducing system significantly restored the values of the activity of hydrogen peroxide- and tetrathionate-inhibited MSTs to 22.1 ± 1.1 and 38.2 ± 2.3\% of each control value, respectively (p = 0.005 and 0.002, respectively, n = 3) for 20 min (Fig. 2). Furthermore, reduced glutathione also restored activity reaching only 20.9\% ± 1.8 and 33.2 ± 2.9\% of each control value, respectively, (p = 0.009 and 0.006, respectively, n = 3). The Restoration rate was slower than that by glutathione with the reducing system (data not shown).

Thioredoxin together with the reducing system completely restored the values of the activity of hydrogen peroxide- and tetrathionate-inhibited MSTs to 106.2 ± 9.8 and 113.6 ± 8.6\% (n = 3) of each control value, respectively (Fig. 2). Furthermore, reduced thioredoxin restored activity to 91.5 ± 6.3 and 98.6 ± 7.2\% of each control value, respectively. These results showed that these oxidized MSTs differ in terms of their redox potential, and the mid-redox potential of the hydrogen peroxide-inhibited MST was close to that of thioredoxin and lower than that of glutathione.

SH Group Titration and the Target Residue of Oxidants—The results of SH titration of the reduced and oxidized MSTs using DTNB and NBD-Cl (TABLE ONE) determined that Cys\(^{154}\), Cys\(^{247}\), and Cys\(^{263}\) were exposed cysteines. Cys\(^{154}\) and Cys\(^{263}\) were outside cysteines, which was estimated from the data of the ternary structure of Leishmania MST (9), and partly contributed to a dimer formation via a disulfide bond. Iodoacetate inactivated MST (TABLE TWO) via carboxymethylation of Cys\(^{247}\). When MST had been inhibited by a stoichiometric concentration of hydrogen peroxide or tetrathionate prior to the treatment with iodoacetate, the activity was restored by DTT (TABLE TWO). These findings suggested that oxidants protect a catalytic Cys\(^{247}\) against inactivation by iodoacetate, and therefore, Cys\(^{247}\) is a target of oxidants. However, Cys\(^{247}\) of the hydrogen peroxide-inhibited MST was not modified by NBD-Cl or DTNB, and no spectrophotometric change at 237 nm was observed. These findings show that the sulfinate in this case was not modified by NBD-Cl.

The Mass Spectrometric Analyses of the Inhibited and Inactivated MST: Oxidized Forms of Cys\(^{247}\)—After MST was inhibited by tetrathionate, the main peak was shifted (m/z 32,882.4; [M + H]\(^{+}\) and 16,444.4; [M + 2H]\(^{2+}\)), which was different from that of untreated control MST (m/z 32,778.6; [M + H]\(^{+}\) and 16,398.6; [M + 2H]\(^{2+}\)) (Fig. 3). Further, incubation with DTT shifted the main peak (m/z 32,779.4; [M + H]\(^{+}\) and 16401.5; [M + 2H]\(^{2+}\)) (Fig. 3). The difference in mass number (104 amu) was consistent with that of a thiosulfate (-SO\(_3\)) (32,890.6 ± 9.9 (the expected mass number ± mass measurement error)), suggesting that a sulfenyl thiosulfate was formed at Cys\(^{247}\) by incubation with tetrathionate.

After MST was inhibited by a stoichiometric concentration of hydrogen peroxide, the molecular mass (m/z 32,799.5; [M + H]\(^{+}\) and 16,410.6; [M + 2H]\(^{2+}\)) (Fig. 3) differed from that of the control and was consistent with the formation of a sulfinylate (32,794.6 ± 9.8). Further, incubation with DTT shifted the main peak (m/z 32,783.0; [M + H]\(^{+}\) and 16,392.6; [M + 2H]\(^{2+}\)), which was not significantly different from that of the untreated control (32,778.6 ± 9.8) (Fig. 3).

After incubation with an excess molar dose of hydrogen peroxide, the main peak was shifted (m/z 32,827.5; [M + H]\(^{+}\) and 16,414.6; [M + 2H]\(^{2+}\)) (Fig. 3). The difference in mass number was consistent with that of a sulfone (-O\(_2\)) (32,826.6 ± 9.8). The peak did not change after incubation with DTT (m/z 32,827.5; [M + H]\(^{+}\) and 16,412.2; [M + 2H]\(^{2+}\)) (Fig. 3), suggesting that a sulfone was formed at Cys\(^{247}\). Hydrogen peroxide-inhibited MST was not modified by dimedone (data not shown).

Peroxidase Activity of MST—When 10 \(\mu\)M hydrogen peroxide was added to the assay mixture containing 0.2 \(\mu\)M NADPH, 40 \(\mu\)M thioredoxin and 1 \(\mu\)M thioredoxin reductase, the rate of consumption of NADPH was 3.78 \(\mu\)M/min (Fig. 4); electrons were transferred from reduced thioredoxin directly to hydrogen peroxide (a scheme in Fig. 4). In the presence of 10, 20, 40, or 60 \(\mu\)M MST, the rate was increased to 2.65, 3.98, 6.72 (Fig. 4), or 7.96 \(\mu\)M/min, suggesting that the peroxidase reaction proceeded in a MST concentration-dependent manner. Further, this reaction also proceeded in a hydrogen peroxide concentration-dependent manner (data not shown). Electrons were passed from

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**TABLE ONE**

| SH group titration for wild type and mutant MSTs |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Wild type       | C64S            | C154S           | C247S           | C254S           | C263S           |
| Red             | Ox              | Red             | Ox              | Red             | Red             | Red             |
| x1             | x5              | x1             | x5              | x1             | x5              | x1             |
| DTNB            | 2.9             | 1.8             | 1.7             | 2.9             | 1.6             | 1.6             |
| NBDC            | 3.8             | 1.9             | 1.8             | 2.9             | 1.6             | 1.5             |

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**Note:** Details are described under “Materials and Methods.”
reduced thioredoxin to hydrogen peroxide via MST (a scheme in Fig. 4). On the other hand, C247S did not possess the peroxidase activity (data not shown).

DISCUSSION

A sulfenyl compound and a sulfenate formed at the cysteine residues in proteins or enzymes (12–19, 21, 22) are stable in the absence of a nearby cysteine (21, 22) or in a hydrogen bond-rich environment (21, 22). A sulfenyl thiosulfate in tetrahionate-inhibited MST and a cysteine-sulfenate in hydrogen peroxide-inhibited MST were stable on ice for at least 48 h, indicating that there was no cysteine residue close to Cys247 (data not shown). This was confirmed by DTNB and NBD-Cl titration of the SH-group.

In a rare case, protein-tyrosine phosphatase IB formed a sulfenyl amide with a main chain nitrogen atom of the adjacent amino acid (15, 16). Replacement of the adjacent amino acid, Gly248 with Arg or Ser249 with Ala in rat MST did not affect inhibition kinetics (data not shown), indicating that a sulfenyl amide was not formed in this case.

Rhodanese (34) and NADH peroxidase (33, 35) catalyzed the thioredoxin oxidase and peroxidase reaction, respectively, which strongly suggested cysteine-sulfenate formation. MST also possessed thioredoxin peroxidase activity only when the concentration of thioredoxin reductase was less than 1/20 that of MST. The peroxidase reaction should proceed via a sulfenic intermediate of thioredoxin (34).

A sulfenyl cysteine-NBD adduct was successfully detected in oxidized forms of NADPH oxidase (20), alkyl hydroperoxide reductase (23), α1-antitrypsin (41), and serum albumin (28). The spectral property of sulfenate-NBD adducts showed maximal absorption at 347 nm, which

| TABLE TWO |
| Protection of MST by oxidants against iodoacetate inactivation |
| MST was incubated with (+) or without (−) 1 mM DTT. 17 μM MST was carboxymethylated with 1 mM iodoacetate (IA). After the removal of excess IA, MST was incubated with or without DTT, and the remaining activity was measured. 17 μM MST was oxidized by 17 μM hydrogen peroxide (HP) or 17 μM tetrahionate (TT) before treatment with iodoacetate. All data are shown as a percentage of the mean value of specific activity in each experimental group for the specific activity of each control group before treatment (mean ± S.E., n = 3). Details are described under “Materials and Methods.” |
| DTT | Treatment | IA | HP + IA | TT + IA |
| --- | --- | --- | --- | --- |
| − | % | 5.7 ± 0.2 | 1.5 ± 0.1 | 5.4 ± 0.2 |
| + | % | 6.1 ± 0.3 | 79.0 ± 3.1 | 94.2 ± 3.5 |

FIGURE 3. MALDI-TOF-MS data for oxidized forms of MST. Details are provided under “Results.”
was different from that of free NBD-Cl at 343 nm (344 nm in this study) and the cysteine-NBD adduct at 420 nm (416 nm in this study). On the other hand, a spectral change at 347 nm was not observed in the hydrogen peroxide-inhibited MST after incubation with NBD-Cl, even when the sample was treated with 5 M guanidine hydrochloride or 8 M urea. Other possible reagents for modification of a sulfenate, 2-nitro-5-thiobenzoic acid (23, 26) and 4-fluoro-7-nitrobenz-2-oxa-1,3-diazole, were also not able to modify Cys247 of the hydrogen peroxide-inhibited MST after incubation with NBD-Cl, even when other hand, a spectral change at 347 nm was not observed in the hydrogen peroxide-inhibited MST.

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A MALDI-TOF mass spectrometric analysis revealed that the hydrogen peroxide-inhibited MST increased in mass number in a manner consistent with the formation of a sulfenate (-SO$_3^-$), and DTT decreased the mass to that of control MST. An excess molar dose of hydrogen peroxide increased the molecular mass of MST consistent with that of the formation of a sulfonate (-SO$_4^{2-}$), and this mass was not significantly changed by DTT. This is consistent with the fact that DTT could not restore the activity. A crystallographic study of Leishmania MST confirmed a sulfonate formation at the catalytic cysteine (42). A peak indicating MST-sulfinate was not distinctly observable, probably because of being a minor constituent in oxidized MST species.

It is noteworthy that reduced thioredoxin or thioredoxin along with the reducing system completely restored the activity of the tetrathionate- and hydrogen peroxide-inhibited MSTs. An excess molar dose of cysteine partly restored tetrathionate-inhibited MST. An excess molar dose of cysteine restored the activity of tetrathionate-inhibited streptococcal proteinase via the formation of a sulfenyl thiosulfate at the catalytic cysteine (25). On the other hand, reduced glutathione or glutathione along with the reducing system partly restored the activity of tetrathionate- and hydrogen peroxide-inhibited MSTs. These findings suggest that the mid-redox potential of the cysteine-sulfenate in this case could be estimated as close to and lower than that of glutathione (−240 mV (43)) and higher than that of thioredoxin (−270 mV (43)).

In previous studies on the reduction of a cysteine-sulfenate, glutathione was used as an effective reductant (29–32), meaning that the mid-redox potential of a cysteine-sulfenate was higher than that of glutathione. On the other hand, the mid-potential of a MST-sulfenate was exceptionally close to and lower than that of glutathione, and the characteristics of the MST-sulfenate were different from those reported previously. The stability and reactivity of a sulfenate contained in an enzyme has not been studied precisely. It has been only reported that a well developed network of hydrogen bonding interactions stabilizes the sulfenate (21, 22, 44). The lower reactivity of the MST-sulfenate with NBD-Cl and dimedone is probably because of this reported well developed network of hydrogen bonding (42) interlacing with it.

Cytosolic and mitochondrial MST (45) plays physiological role in the protection against oxidative stress, and peculiarly contributes to the maintenance of cellular redox homeostasis via the metabolic regulation of cysteine degradation.

Acknowledgments—We thank Taro Yoshii, Tokyo University of Agriculture, for technical assistance.

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