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Cytosolic Mercaptopyruvate Sulfurtransferase Is Evolutionarily Related to Mitochondrial Rhodanese

STRIKING SIMILARITY IN ACTIVE SITE AMINO ACID SEQUENCE AND THE INCREASE IN THE MERCAPTOPYRUVATE SULFURTRANSFERASE ACTIVITY OF RHODANESE BY SITE-DIRECTED MUTAGENESIS*

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Rat liver mercaptopyruvate sulfurtransferase (MST) was purified to homogeneity. MST is very similar to rhodanese in physicochemical properties. Further, rhodanese cross-reacts with anti-MST antibody. Both purified authentic MST and expressed rhodanese possess MST and rhodanese activities, although the ratio of rhodanese to MST activity is low in MST and high in rhodanese. In order to compare the active site regions of MST and rhodanese, the primary structure of a possible active site region of MST was determined. The sequence showed 66% homology with that of rat liver rhodanese. An active site cysteine residue (Cys^{246}; site of formation of persulfide in catalysis) and an arginine residue (Arg^{185}; substrate binding site) in rhodanese were also conserved in MST. On the other hand, two other active site residues (Arg^{247} and Lys^{248}) were replaced by Gly and Ser, respectively. Conversion of rhodanese to MST was tried by site-directed mutagenesis. After cloning of rat liver rhodanese, recombinant wild type and three mutants (Arg^{247} → Gly and/or Lys^{248} → Ser) were constructed. The enzymes were expressed in *Escherichia coli* strain BL21(DE3) with a T7 promoter system. The mutation of these residues decreases rhodanese activity and increases MST activity.

Mercaptopyruvate sulfurtransferase (MST, EC 2.8.1.2),[1] which catalyzes the transfer of sulfur ion from mercaptopyruvate to mercaptoethanol, was first discovered in rat liver (Meister, 1953; Wood and Fiedler, 1953; Meister *et al.*, 1954; Sörbo, 1954). This enzyme is widely distributed both in prokaryotes and eukaryotes and is located mainly in the cytosol of eukaryotic cells (Wood and Fiedler, 1953; Meister *et al.*, 1954; Kun and Fanshier, 1959; Jarabak and Westley, 1978). MST has been proposed to play a role in cysteine degradation (Meister *et al.*, 1954) or in the biosynthesis of thiosulfate (Fasth & Sörbo, 1973). Because eukaryotic MST has not been purified, its molecular properties are unknown. Rhodanese (thiosulfate sulfurtransferase, EC 2.8.1.1) is better understood. It was first found in rat liver (Lang, 1933) and catalyzes the transfer of sulfur ion from thiosulfate to potassium cyanide. Bovine liver rhodanese was isolated and crystallized (Sörbo, 1953; Horowitz and DeToma, 1970), and the rat liver enzyme has also been purified (Wasylewski *et al.*, 1979). Rhodanese exists as a monomer or dimer of identical subunits (Volini *et al.*, 1967) and is widely distributed in prokaryote and eukaryote mitochondria (Ludwig and Chanutin, 1950; Sörbo, 1951; Duve *et al.*, 1955). Primary structures of bovine liver (Russell *et al.*, 1978) and adrenal (Miller *et al.*, 1991), chicken liver (Kohanski and Heinrikson, 1990), human liver (Pallini *et al.*, 1991), and rat liver (Weiland and Dooley, 1991) rhodanese were determined from protein or deduced from cDNA. The molecular mass of these enzymes is about 33 kDa. The crystal structure of bovine liver rhodanese has been reported (Ploegman *et al.*, 1978a, 1978b, 1979), and its reaction mechanism has been clarified (Westley and Nakamoto, 1962; Wang and Volini, 1968; Westley and Heyse, 1971; Schlesinger and Westley, 1974). Recombinant bovine liver and adrenal rhodanese enzymes were overexpressed in *Escherichia coli* (Miller *et al.*, 1991, 1992). Several biological functions of rhodanese have been postulated: detoxification of cyanide (Lang, 1933), prevention of the formation of inorganic sulfide (Koj and Frendo, 1962), and incorporation of sulfur into iron-sulfur protein (Finazzi *et al.*, 1971). Although rhodanese and MST were believed to be different enzymes, Nishino *et al.* (1983, 1985) reported that rhodanese or MST could transfer a sulfur ion from thiosulfate or mercaptopyruvate to the molybdenum ligand of xanthine oxidase, showing that these enzymes could catalyze similar reactions. This paper compares the catalytic and structural properties of cytosolic MST and mitochondrial rhodanese and shows that these enzymes are evolutionarily related.

MATERIALS AND METHODS

Chemicals—Mercaptopyruvate was synthesized essentially by the method of Kun (1957). The amount of thiol group in mercaptopyruvate was confirmed to be nearly one:one molar by titration of thiol group with 5,5'-dithiobis(2-nitrobenzoic acid). Other chemicals were analytical grade.

MST Purification—Liver MST was isolated from 7-week-old male Wistar rats. The rats were anesthetized with ether, and their livers were excised. 200 g of livers were cut into small pieces and homogenized with a Polytron (KINEMATICA) for 3 min on ice in 600 ml of 5 mM potassium phosphate buffer containing 0.2 mM EDTA, pH 7.4. The homogenate was centrifuged at 130,000 × *g* for 1 h at 4 °C. The 35–60% ammonium sulfate precipitate was collected and dissolved in a minimal volume of 10 mM Tris-HCl buffer containing 0.2 mM EDTA, pH 7.8 (buffer A). After repeating this procedure with a second batch of liver, the combined solution (obtained from 400 g of liver) was dialyzed against three changes of 5 liters of the same buffer at 4 °C. The dialyzed enzyme was loaded onto a Q-Sepharose (Pharmacia Biotech Inc.) column (3 × 24 cm) equilibrated with the same buffer. The column was washed with 340 ml of the same buffer and then with 600 ml of 60 mM Tris-HCl buffer containing 0.2 mM EDTA, pH 7.8. The enzyme was

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[1] The abbreviations used are: MST, mercaptopyruvate sulfurtransferase; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; bp, base pair(s); pBs, pBluescript.

eluted with linear gradient of 60–500 mM Tris-HCl containing 0.2 mM EDTA, pH 7.8. MST-containing fractions were collected, and ammonium sulfate was added to 60% saturation. The precipitate was dissolved in a minimal volume of buffer A (final volume, 75 ml), dialyzed against three changes of 2 liters of the same buffer at 4 °C, and loaded onto a DEAE-cellulose (Whatman DE52) column (3 × 16.5 cm) equilibrated with the same buffer. The column was washed with 420 ml of buffer and then with 580 ml of 50 mM Tris-HCl buffer containing 0.2 mM EDTA, pH 7.8. The enzyme was eluted with a linear gradient of 50–200 mM Tris-HCl containing 0.2 mM EDTA, pH 7.8. The enzyme-containing fractions were collected, and ammonium sulfate was added to 60% saturation. The precipitate was dissolved in the minimal volume of 5 mM potassium phosphate buffer containing 0.2 mM EDTA, pH 6.5 (final volume, 7 ml). The enzyme solution was then applied to a G25-Sephadex (Pharmacia Biotech Inc.) column (3 × 16.5 cm) equilibrated with the same buffer. The enzyme-containing fractions were collected and loaded onto a hydroxylapatite (Bio-Gel HTP, Bio-Rad) column (2.4 × 13 cm) equilibrated with the same buffer. The column was washed with 180 ml of buffer and developed with a linear gradient of 400 ml of buffer to 400 ml of 25 mM potassium phosphate buffer containing 0.2 mM EDTA and 0.1 M ammonium sulfate, pH 7.4. The enzyme-containing fractions were collected, and ammonium sulfate was added to 65% saturation. The precipitate was dissolved in a minimal volume of 25 mM potassium phosphate buffer, pH 6.5 (final volume, 1.5 ml) and concentrated with a Centricon 10 (final volume, 200 μl). The enzyme solution was desalted with Ampure™SA (Amersham Corp.) and concentrated with a Centricon 10, (final volume, 320 μl). 160 μl of the solution was applied in two runs to a HCA (hydroxylapatite for HPLC, Mitsui Toatsu Chemicals Inc.) column (0.8 × 10 cm). The column was washed with 5 mM potassium phosphate buffer, pH 6.5, for 5 min at a flow rate of 0.5 ml/min and eluted with a 30-min linear gradient of the same buffer to 104 mM potassium phosphate buffer, pH 8. The enzyme-containing fractions were collected and concentrated with a Centricon 10. 280 μl of the concentrated enzyme solution was applied to two TSK (gel filtration for HPLC, TOSOH Corp.) columns, which were connected in a series (3,000 SW, 2.15 × 60 cm and 2,000 SW_{XL}, 0.6 × 40 cm) and were equilibrated with 0.2 M potassium phosphate buffer containing 10 mM ammonium sulfate, pH 7.4. The enzyme-containing fractions were concentrated with a Centricon 10 and frozen until use.

Molecular Mass Determination—Purified enzyme was applied to TSK 3,000 SW and 2,000 SW_{XL} columns that were connected in a series equilibrated with 0.2 M potassium phosphate buffer containing 10 mM ammonium sulfate, pH 7.4, and eluted with the same buffer at a flow rate of 0.5 ml/min. The column was calibrated with bovine serum albumin (67 kDa), ovalbumin (43 kDa), β-lactoglobulin (36 kDa), and carbonic anhydrase (28 kDa).

Partial Primary Structure of MST—30 nmol of the carboxymethylcysteinyl MST was digested with 10 μg of endoprotease Lys-C (Boehringer Mannheim) in 25 mM Tris-HCl buffer containing 1 mM EDTA, pH 8.5, at 37 °C for 8.5 h. 3 nmol of alkylated enzyme was digested with 20 pmol of V8 protease (Takara Shuzo Co., Ltd.) in 50 mM ammonium bicarbonate buffer, pH 7.8, at 37 °C for 8.5 h. The digested samples were applied to a reverse-phase C18 column (Capcellpak, Shiseido) and separated with a linear gradient of acetonitrile between 15 and 80% in 0.1% trifluoroacetic acid solution. Rechromatography was also performed for some fractions.

Amino acid sequencing was performed with a gas-phase protein sequencer (ABI). Sequence homology was examined by means of GENETYX (Software Development Co.).

Antibody Preparation and Western Blotting—The highly purified enzyme solution (1.5 mg of protein) was injected subcutaneously into a rabbit 5 times over 2 months. The antiserum obtained from the immunized rabbit was precipitated with ammonium sulfate and purified by DEAE-cellulose chromatography.

A supernatant of the rat liver homogenate after ultracentrifugation, purified MST, and recombinant wild type rhodanese described below were loaded on a SDS-polyacrylamide gel (10%) according to the method of Laemmli (1970). Separated proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell) with an electrotransfer apparatus (Sartorius) by the multiphasic buffer method according to Hirano (1989). The first antibody was diluted (1:300) for use, and alkaline phosphatase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., 1:5,000) was used as a second antibody. Alkaline phosphatase activity was detected with the usual color development system.

cDNA Cloning of a Rat Liver Rhodanese and Construction of Expression Vector of Wild Type Rhodanese—5′ sense primer (CAAAGCTTGTATGGTGGATGTTTCGTGTG; 328–348 bp of rat liver rhodanese cDNA with *Hin*dIII restriction site) and 3′ antisense primer (TCGAATTCTGTCAGGAAGTTCATGAAGGG; 628–648 bp with *Eco*R I site) were synthesized according to the DNA sequence of the rat liver rhodanese. A 323-bp product was obtained by polymerase chain reaction with rat liver single-stranded cDNA as a template. Polymerase chain reaction was performed with AmpliTaq DNA polymerase (Perkin-Elmer). The probe was labeled with [α-^{32}P]dCTP by using a random-primed DNA labeling kit (Boehringer Mannheim). After 5.5 × 10^5 clones of rat liver λgt11 cDNA library (CLONTECH) were screened by plaque hybridization, 15 positive clones were obtained. A 970-bp clone contained the longest reading frame of rhodanese cDNA in the positive clones, but it showed a deletion of 29 base pairs in the 5′ region. On the other hand, the reading frame was followed by 115 bp of 3′-untranslated sequence. The deleted double-stranded cDNA in the 5′ region was reconstructed with four synthetic oligonucleotides (between the *Pst*I and *Rsr*II sites) and added to the *Pst*I-*Sph*I sites at the 5′ end. The reconstructed rhodanese cDNA was subcloned to pBluescript (pBs) at the *Pst*I/*Eco*RI site (pBs/Rho).

To insert rhodanese cDNA into the pET-15b vector (Novagen) between the *Nco*I and *Bam*HI sites, each restriction site was synthesized at sides of rhodanese cDNA by polymerase chain reaction with pBs/Rho plasmid as a template. No mutation of polymerase chain reaction product was confirmed by DNA sequencing with synthetic oligonucleotide primers. Val was added to authentic rhodanese at the N terminus in this study. This chimeric enzyme was designated wild type.

Site-directed Mutagenesis—Replacement of Arg^{247} (Arg^{248} in recombinant wild type) by Gly (R248G) and/or Lys^{248} (Lys^{249} in recombinant wild type) by Ser (K249S) was performed according to the method of Kunkel (1985). A fragment of rhodanese cDNA between *Kpn*I (from 559 bp) and *Bam*HI site (to the last) was excised from the pBs/Rho plasmid. The fragment was inserted to pBs between the same restriction sites. In the present study for mutagenesis, three mutagenic oligonucleotides, CGCCACATGCGGCAAAGGGGTCA, CACATGCCGCAGTGGGGTCACTG, and CGCCACATGCGGCAGTGGGGTCACTG, were synthesized for R248G, K249S, and double mutants, respectively (underlined codons indicate mutagenized sites). The accuracy of mutagenesis was checked by DNA sequencing of the mutagenized rhodanese cDNA with synthetic oligonucleotide primers. Each mutagenic fragment (*Kpn*I/*Bam*HI) was replaced with a fragment between *Kpn*I and *Bam*HI sites of pET/Rho plasmid.

Expression and Purification of Wild Type and Mutant Rhodanese—These constructs were transformed into *E. coli* strain BL21(DE3). Transformed cells were cultured in LB medium containing 50 mg/ml ampicillin at 27 °C. At an absorbance of 0.8 at 600 nm, expression was induced by adding 1 mM isopropyl β-D-thiogalactopyranoside and by increasing the culture temperature to 37 °C. After 3.5 h at 37 °C, cells were harvested by centrifugation at 6,000 × *g* for 5 min. Wild type and mutant rhodaneses were purified by a major modification of the method of Miller *et al.* (1992). After adjusting the pH to 5.4 with 2 M glycine sulfate, pH 2.5, the lysates were centrifuged, and the supernatant was fractionated with ammonium sulfate (35–65% saturation). The precipitate was dissolved in a minimal volume of 10 mM Tris-HCl buffer, pH 7.8 (buffer B), (final volume, 2 ml) and dialyzed overnight against 2 liters of the same buffer containing 2 mM sodium thiosulfate.

Dialyzed enzyme was loaded onto a DE52 column (1.5 × 17 cm) equilibrated with buffer B. The enzyme was eluted with the same buffer. Enzyme fractions were collected and concentrated with a FILTRON-10 and a Centricon 10 to a final volume of 10 ml. The enzyme solution was dialyzed overnight against 2 liters of 5 mM sodium acetate buffer containing 2 mM sodium thiosulfate, pH 5.0, at 4 °C. The enzyme was then loaded onto a CM52 (Whatman) column (0.5 × 12.7 cm) equilibrated with 5 mM sodium acetate, pH 5.0. The column was washed with the same buffer, and the enzyme was eluted with a linear gradient to 1.05 M sodium acetate. Enzyme-containing fractions were collected, concentrated in 5 mM sodium acetate, pH 5.0, and stored at −20 °C after the addition of one-third volume of 100% saturated ammonium sulfate solution.

Enzyme Assays—Rhodanese activity was measured by following the rate of thiocyanate formation by a modification of the method of Sörbo (1953). The assay mixture contained 60 mM sodium thiosulfate, 60 mM potassium cyanide, 40 mM NaH_2PO_4, and 5 μl of enzyme solution in a final volume of 0.5 ml at pH 5.0. The mixture was incubated for 10 min at 25 °C. One unit of the enzyme was defined as 1 μmol of pyruvate formed per minute.

MST activity was measured by the rate of pyruvate formation by a modification of the method of Vachek and Wood (1972). The assay mixture contained 5 mM ammonium mercaptopyruvate, 25 mM mercaptoethanol, 0.1 mg/ml of bovine serum albumin, and 225 mM 2-amino-2-

TABLE I
Purification of MST from rat liver

Step	Total activity	Total protein	Specific activity	Yield	Purification ratio
	units	*mg*	*units/mg*	%	
120,000 × *g* supernatant	86,512	48,753	1.77	100	1.0
Ammonium sulfate fraction (35–60%)	80,772	24,312	3.37	93	1.9
Q-Sepharose	25,583	5,085	5.03	30	2.8
DEAE-cellulose	21,340	919.8	23.20	25	13
Hydroxylapatite	15,529	12.2	1272.87	18	719
HCA-HPLC	6,876	2.7	2546.67	8	1439
TSK G3000 SW-G2000 SW_{XL}	1,517	0.4	3792.96	2	2143

methyl-1,3-propanediol-HCl, and 5 μl of enzyme solution in a final volume of 0.55 ml at pH 9.55. One unit of MST activity was defined as 1 μmol of thiocyanate formed per minute.

Protein Determination—The protein concentration was determined with a Coomassie protein assay kit (Pierce) with crystalline bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Purification of MST—MST is unstable during purification, but it has been found that ammonium sulfate stabilizes the enzyme. This allowed rat liver MST to be purified to homogeneity by including 10 mM ammonium sulfate in the late steps of the purification procedure. A representative purification is summarized in Table I. The specific activity of the purified enzyme shows a more than 2,000-fold increase compared with that of a rat liver supernatant. SDS-PAGE indicated that MST is composed of a 34-kDa subunit (Fig. 1). Gel filtration shows that the enzyme elutes as two peaks having apparent molecular masses of 34.5 kDa and 53.5 kDa (data not shown). The larger molecular weight fraction was converted to the smaller one by treatment with 5 mM dithiothreitol in 0.2 M potassium phosphate buffer, pH 7.4, containing 10 mM ammonium sulfate (data not shown). Because both fractions from gel filtration were identical on SDS-PAGE only after reduction with dithiothreitol (data not shown), it was suggested that MST exists in monomer and homodimer forms and that dimer formation involves an intersubunit disulfide bond. Because the calculated molecular mass of rat liver rhodanese is 33,176 kDa (Weiland and Dooley, 1991) and bovine liver rhodanese was reported to exist in monomer-dimer equilibrium mediated via disulfide bond formation (Volini *et al.*, 1967), MST seems to have similar physicochemical properties to rhodanese.

Reactivity of Rhodanese with Anti-MST Antibody—In order to know the immunological similarity between rat liver MST and rhodanese, Western blot analyses were performed after SDS-PAGE of the purified rhodanese and MST, as shown in Fig. 2. It was found that anti-MST polyclonal antibody cross-reacts weakly with recombinant wild type rat liver rhodanese (Fig. 2, *lane 1*). These results suggest that there is a similarity between MST and rhodanese in protein structure. In the soluble fraction of the rat liver homogenate (Fig. 2, *lane 3*), the main band and an additional faint band were observed. The fact that the main band has a molecular weight corresponding to MST suggested that it was MST. Another minor band shows a larger molecular weight than that of MST but is slightly smaller than that of recombinant liver rhodanese. It is possible that this minor band is either mature rhodanese that leaked from mitochondria during homogenization or another cross-reacting protein.

Partial Primary Structure of MST around the Active Site and Comparison with the Sequences of Rhodanese—In order to elucidate the structural difference between the active sites of MST and rhodanese, a partial amino acid sequence of the rat liver MST was determined by protein sequencing. Although the N terminus of MST is blocked, 204-amino-acid sequences of internal peptides were determined after proteolytic cleavage of the protein and purification of the peptides (see "Materials and Methods"). In a comparison of the obtained sequence of MST with that of rat liver rhodanese, which was deduced from the cDNA sequence (Weiland and Dooley, 1991), it was found that the sequences of rat liver MST and rhodanese are highly homologous and that the obtained sequence of MST covered the sequence corresponding to that of the active site region of rhodanese (from Gln^{169} to Pro^{265}). The active site structure of rhodanese was well characterized at the tertial structure level by x-ray crystallographic studies with bovine liver rhodanese (Ploegman *et al.*, 1978a, 1978b, 1979). The sequence of MST covering the sequences around the active site of rhodanese are shown in Fig. 3 in comparison with the sequences of rhodanese from various sources. The degrees of sequence identity in these regions were 66.0%, 63.9%, 68.1%, and 87.5% between rat MST and rat, bovine, chicken, and human rhodanese, respectively. In this part, one amino acid of rat MST is deleted at the position corresponding to Val^{196} of rat rhodanese. The remaining part of the obtained sequence of MST has 57% identity with the corresponding part of the sequence of rat rhodanese (data not shown). These findings on the striking similarity between MST and rhodanese in the amino acid sequence suggest that MST is evolutionarily related to the enzyme family of rhodanese.

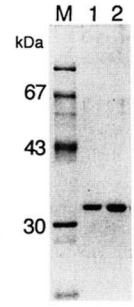

FIG. 1. **SDS-PAGE of purified rat liver MST.** MST shows a subunit molecular mass of 34 kDa. *Lane 1*, 3 μg of MST; *lane 2*, 6 μg of MST; *M*, molecular mass markers.

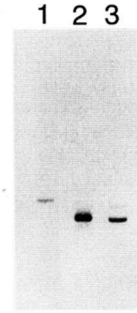

FIG. 2. **Western blotting with anti-MST antigen.** *Lane 1*, 1 μg of recombinant wild type rhodanese; *lane 2*, 0.28 μg of purified rat liver MST; *lane 3*, 16.4 μg of supernatant of the rat liver.

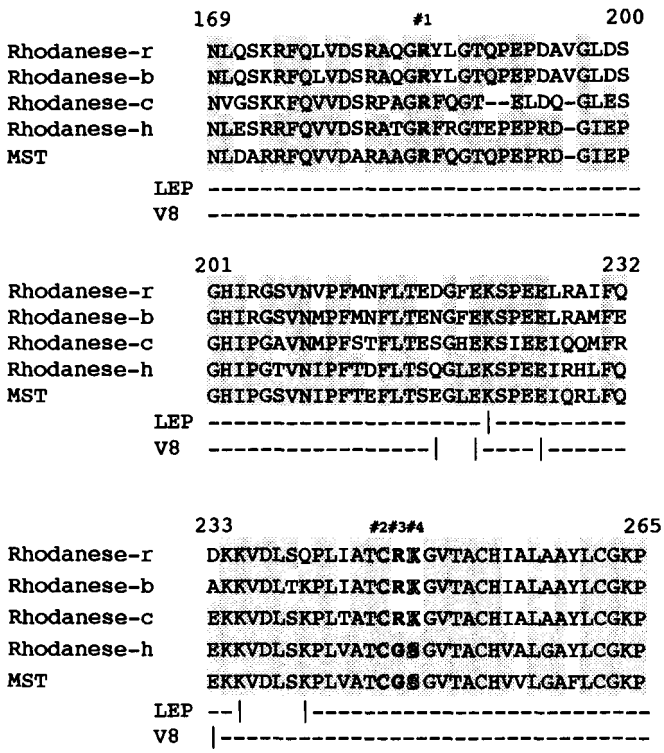

FIG. 3. **Comparison of partial primary structure of rat liver MST with sequences around active site of rhodaneses of rat, bovine, chicken, and human liver.** *LEP*, fragments obtained from digestion of authentic rat liver MST with lysine endopeptidase; *V8*, fragments obtained from digestion of authentic rat liver MST with V8 peptidase; *Rhodanese-r*, deduced primary structure of rat liver rhodanese (Weiland and Dooley, 1991); *Rhodanese-b*, primary structure of purified bovine liver rhodanese (Russell *et al.*, 1978); *Rhodanese-c*, amino acid sequence from purified chicken liver rhodanese (Kohanski and Heinrikson, 1990); *Rhodanese-h*, deduced primary structure of human liver rhodanese (Pallini *et al.*, 1991); *shaded regions*, identical amino acid residues; *#1*, arginine 185; *#2*, cysteine 246; *#3*, arginine 247; *#4*, lysine 248 in authentic rat liver rhodanese.

It is noteworthy that some catalytically important amino acid residues of rhodanese were conserved among all rhodaneses and even MST, whereas some residues were replaced by other amino acids in MST. Arg^{185} of rat rhodanese (Fig. 3, *#1*) is conserved in all of these sequences. This residue is located at the entrance of the pocket of the active center and substrate binding site in bovine rhodanese (Ploegman *et al.*, 1978a, 1978b, 1979). Cys^{246} of rat rhodanese (Fig. 3, *#2*) is also conserved among all these sequences. This residue is identified to form the persulfide and transfer sulfur ion between substrates in bovine rhodanese (Ploegman *et al.*, 1978a, 1978b, 1979; Weng *et al.*, 1978). On the other hand, Arg^{247} of rat rhodanese (Fig. 3, *#3*) is conserved in bovine and chicken rhodaneses but is replaced with a Gly in MST and human rhodanese. Lys^{248} of rat rhodanese (Fig. 3, *#4*) is also conserved in bovine and chicken rhodaneses but is replaced with a Ser in MST and human rhodanese. This residue is located at the entrance of the pocket of the active center and substrate binding site in bovine rhodanese (Ploegman *et al.*, 1978a, 1978b, 1979). It should be noted that the primary structure of human rhodanese deduced from cDNA (Pallini *et al.*, 1991) is very similar to that of rat liver MST and the four amino acid residues described above are identical with those in MST. As the purified MST has a weak rhodanese activity, as mentioned below, it is possible that the reported human rhodanese might in fact be a MST.

Expression and Purification of Wild Type and Mutant Rat Liver Rhodanese—We found that rhodanese and MST possess both rhodanese and MST activities, but their ratios are different. The ratio of the activity of rhodanese to MST is high in rhodanese and is low in MST as shown in Table III. To exclude the possibility of contamination of enzymes by each other, to elucidate the function of the two amino acid residues in rhodanese and MST activities ($Arg^{247} \rightarrow$ Gly and $Lys^{248} \rightarrow$ Ser) discussed above (Fig. 3), and to attempt to convert rhodanese to MST, we cloned rat liver rhodanese cDNA and constructed three recombinant mutants (see "Materials and Methods" and Table II). We confirmed that the nucleotide sequence was identical to that reported previously by Weiland and Dooley (1991). The wild type and three mutant rhodanese cDNAs were identified as described under "Materials and Methods." Protein sequencing showed that the amino acid sequences in the N terminus of all of the expressed rhodaneses agreed with the cDNA sequences. All are preceded by Val (not Met) as expected from construction of the expression system described under "Materials and Methods." These findings also confirm that the proteins obtained are recombinant rhodaneses and not authentic enzymes that possibly exist in *E. coli*.

TABLE II
Amino acid residues in recombinant rhodaneses and MST

Enzyme	Position	
	248	249
Wild type	R	K
R248G	G	K
K249S	R	S
R248G and K249S	G	S
MST	G[a]	S[b]

[a] Amino acid residue that corresponds to this one at position 248 in recombinant rhodanese.
[b] Amino acid residue that corresponds to this one at position 249 in recombinant rhodanese.

SDS-PAGE during purification of wild type and mutant rhodaneses is shown in Fig. 4. These expressed purified enzymes are also unstable and can be stabilized in an ammonium sulfate solution. The amount of the overexpressed enzymes obtained from 1 liter of culture varied from 3 to 7 mg. 54% of purified wild type rhodanese is recovered from the lysate. The specific activity of the purified wild type enzyme shows 579.4 units/mg protein and about a 30-fold increase compared with that of the lysate. SDS-PAGE shows that wild and mutant enzymes are 34.5-kDa molecules (Fig. 4), which is in reasonable agreement with that calculated from the deduced primary structure of rat liver rhodanese (molecular weight, 33,176; Weiland and Dooley, (1991)). Purified recombinant enzymes possess both rhodanese and MST activities. This is not likely due to contamination by endogenous activities, because the ammonium sulfate fraction (see "Materials and Methods") of control *E. coli* contains endogenous rhodanese and MST activities, 0.061 and 0.058 units/mg protein, respectively, which are only 0.26 and 29.3% of rhodanese and MST activities, respectively, in *E. coli* that overexpresses wild type rhodanese. The total endogenous activities are less than those of purified expressed enzymes. Furthermore, we failed to recover the activities from the control *E. coli* by the same purification procedures as recombinant rhodanese.

Effect of Mutagenesis and Kinetic Properties of Enzymes—In the study of bovine liver rhodanese (Westley and Heyse, 1971; Schlesinger and Westley, 1974), the reaction mechanism was reported to follow a ping-pong kinetic pattern. But this study showed that the double reciprocal plots of velocity *versus* KCN concentration did not show a straight line when rhodanese activities were measured with the wild type and mutant rhodaneses and the purified rat liver MST (data not shown). Apparent K_m values for thiosulfate were therefore determined with a constant concentration of potassium cyanide at 60 mM in

this study. On the other hand, the double reciprocal plots of all these rhodaneses and the purified rat liver MST show a sequential kinetic pattern when MST activity is measured (data not shown), as in the partially purified bovine kidney MST (Jarabak and Westley, 1978, 1980).

Replacement of Arg^{248} (corresponding to Arg^{247} in authentic rat rhodanese) with Gly in recombinant rhodanese (R248G in Table II) does not affect rhodanese activity; K_m and k_{cat} for thiosulfate are not essentially affected (Table III), suggesting that Arg^{248} is not critical for rhodanese activity. On the other hand, this replacement increases MST activity; k_{cat} for MST activity and k_{cat}/K_m are increased about 35-fold and about 30-fold of that in wild type, respectively, without significant change in K_m for mercaptopyruvate (Table III). Replacement of Lys^{249} (corresponding to Lys^{248} in authentic rat rhodanese) with Ser in recombinant rhodanese (K249S in Table II) decreases rhodanese activity, K_m for thiosulfate is increased to about 12 times that in wild type (Table III), and both k_{cat} and k_{cat}/K_m using thiosulfate are decreased to about one-fifth and about one-sixtieth of that in wild type, respectively (Table III). On the other hand, this replacement does not essentially affect MST activity; K_m and k_{cat} for mercaptopyruvate are not affected (Table III). Double replacement of Arg^{248} and Lys^{249} with Gly and Ser, respectively, in rhodanese (Table II) not only decreases rhodanese activity but also increases MST activity as shown in Table III. The ratio of the apparent k_{cat} for rhodanese activity/apparent K_m for thiosulfate to the k_{cat} for MST activity/K_m for mercaptopyruvate is the largest in mutant rhodaneses (about 340 times that in wild type) (Table III). These data indicate that the double mutagenesis is most effective in the conversion of rhodanese to MST.

This mutagenesis study shows that Arg^{248} and Lys^{249} are critical residues in determining the rhodanese/MST activity ratio of this family enzyme. In this study, replacement of Lys^{249} with Ser decreased rhodanese activity. Another report showed that the replacement of this Lys with Ala of bovine liver rhodanese completely eliminated rhodanese activity (Luo and Horowitz, 1994). It is possible that the replacement of Lys^{249} by an alanine residue changes the conformation around the active site to a higher degree than replacement by a serine residue. The facts that the structure of MST is similar to that of rhodanese in the active site region and that they both catalyze sulfurtransferase reactions suggest that the two enzymes should have some similarity in the reaction mechanism. The cysteine residue in the rhodanese is known to be involved in catalysis as a residue responsible for persulfide formation in the thiosulfate sulfurtransferase reaction (Ploegman *et al.*, 1979). Because this residue is also conserved in MST, it is not surprising that MST can catalyze the thiosulfate sulfurtransferase reaction. This cysteine residue might also be considered to play a role for persulfide formation between protein and mercaptopyruvate during catalysis. However, the facts that the residue of Lys^{249}, which was considered to be important for thiosulfate binding, is replaced by Ser in MST and that the site-directed mutagenesis of this residue of rhodanese decreased k_{cat} values without dramatic change of K_m for thiosulfate suggest that the electrostatic interaction between the minus charge of the oxygen atom of thiosulfate and the plus charge of lysine residue might be important for transferring outer sulfur atom of thiosulfate to form persulfide intermediate. On the other hand, in mercaptopyruvate sulfurtransferase reaction, the existence of a plus charge seems to disturb the formation of persulfide. However, the mutants of rhodanese do not possess the same degree of MST activity as the purified rat liver enzyme, suggesting that a factor(s) other than the residues mutated in this experiment may be involved in the higher catalytic activity in the authentic MST enzyme.

The present study showed that cytosolic MST and mitochondrial rhodanese possess both MST and rhodanese activities, although the ratio of rhodanese to MST activities was low in MST and high in rhodanese. Further, these enzymes show striking similarity in amino acid sequences and immunological cross-reactivity. In a mutagenesis study, replacing two amino acids (Arg^{248} and Lys^{249} by Gly and Ser, respectively) converted rhodanese to MST most effectively. These findings strongly suggested that the two enzymes are evolutionarily related members of the same "family". Although rhodanese is well characterized in its properties and the enzyme is known to localize in mitochondria (Ludwig and Chanutin, 1950; Sörbo,

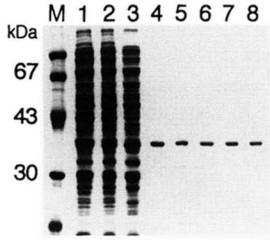

FIG. 4. **SDS-PAGE of purification steps of the wild type rhodanese and purified mutant rhodaneses.** *M*, molecular mass marker. *Lanes 1–5* show the purification step of wild type rhodanese. *Lane 1*, lysate; *lane 2*, supernatant after acid precipitation; *lane 3*, ammonium sulfate fraction (35–65%); *lane 4*, DE52 fraction; *lane 5*, CM52 fraction; *lane 6*, purified K249S enzyme; *lane 7*, purified R248G enzyme; *lane 8*, purified double mutant enzyme.

TABLE III
Kinetic properties of wild type and mutant rhodaneses and MST

All data except the ratio are shown as the mean ± standard error.

Enzyme	K_m		k_{cat}		k_{cat}/K_m		Ratio[f]
	TS[a]	MP	TST[b]	MST[c]	TST[d]	MST[e]	
	mM		*min*$^{-1}$		*mM*$^{-1}$ *min*$^{-1}$		
Rhodanese							
wild	4.4 ± 0.3	2.6 ± 0.2	(4.6 ± 0.1) × 10^4	(1.5 ± 0.1) × 10^3	(1.1 ± 0.1) × 10^4	(5.9 ± 0.1) × 10^2	5.6 × 10^{-2}
R248G	7.2 ± 0.4	2.8 ± 0.2	(2.4 ± 0.2) × 10^4	(4.9 ± 0.2) × 10^4	(3.6 ± 0.2) × 10^3	(1.8 ± 0.1) × 10^4	4.7
K249S	(5.2 ± 0.3) × 10	2.9 ± 0.1	(9.8 ± 0.4) × 10^3	(1.9 ± 0.1) × 10^3	(1.9 ± 0.1) × 10^2	(6.6 ± 0.1) × 10^2	3.5
R248G & K249S	(7.4 ± 0.4) × 10	2.8 ± 0.1	(7.3 ± 0.1) × 10^3	(5.3 ± 0.1) × 10^3	(9.9 ± 0.4) × 10	(1.9 ± 0.1) × 10^3	1.9 × 10
MST	(7.3 ± 0.2) × 10	1.2 ± 0.1	(5.4 ± 0.1) × 10^{2g}	(2.3 ± 0.1) × 10^{5g}	7.5 ± 0.1[g]	(1.9 ± 0.1) × 10^{5g}	2.5 × 10^4

[a] Apparent K_m for thiosulfate (TS) as a substrate using a constant concentration of KCN at 60 mM.
[b] TST, thiosulfate sulfurtransferase. Apparent k_{cat} for rhodanese activity.
[c] k_{cat} for MST activity.
[d] Apparent k_{cat} for rhodanese activity/apparent K_m for thiosulfate.
[e] k_{cat} for MST activity/K_m for mercaptopyruvate (MP).
[f] Ratio of mean value for k_{cat} for MST activity/K_m for mercaptopyruvate to that for apparent k_{cat} for rhodanese activity/apparent K_m for thiosulfate.
[g] Values are calculated assuming a molecular mass for MST of 34 kDa.

1951; Duve *et al.*, 1955), the physiological role of the enzyme is not well understood. On the other hand, MST is less characterized than rhodanese, although the enzyme was discovered quite a long time ago (Meister, 1953) and is known to localize in the cytosolic fraction (Wood and Fiedler, 1953; Meister *et al.*, 1954; Kun and Fanshier, 1959; Jarabak and Westley, 1978). This study provides new insights related to the investigation of the physiological roles of these enzymes and the mechanism of distribution of these enzymes in two different compartments of the cell.

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