Role of Amino Acid Residues in the Active Site of Rat Liver Mercaptoipyruvate Sulfurtransferase
cDNA CLONING, OVEREXPRESSION, AND SITE-DIRECTED MUTAGENESIS*

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A complete amino acid structure of rat liver mercaptoipyruvate sulfurtransferase (MST, EC 2.8.1.2) was determined by sequence analysis of cDNA and purified enzyme. The enzyme consists of 296 amino acid residues with a calculated molecular mass of 32,808 Da. Sequence identity in cDNA and the deduced amino acid sequence are 65 and 60% respectively, between rat MST and rhodanese. By their entire sequence similarity MST and rhodanese are confirmed to be evolutionarily related enzymes (Nagahara, N., Okazaki, T., and Nishino, T. (1995) J. Biol. Chem. 270, 16230–16235). The conversion of MST to rhodanese was attempted, and the role of amino acid residues was studied by site-directed mutagenesis with the isolated cDNA of rat liver MST. There is a strong possibility that Cys 247 is a catalytic site of MST. Arg 187 is suggested to be a binding site of both mercaptoipyruvate and thiosulfate in MST. Arg 186, which is missed in rhodanese, is important for catalysis in MST. On the other hand, the substitution of Arg for Gly 248 or Lys for Ser 249 facilitates catalysis of thiosulfate in MST. It is concluded that Arg 187 and Arg 196 of rat MST are critical residues in determining substrate specificity for mercaptoipyruvate. On the other hand, Arg 185, Arg 247, and Lys 248 of rat rhodanese are critical residues in determining substrate specificity for thiosulfate.

Rhodanese (thiosulfate sulfurtransferase, EC 2.8.1.1) catalyzes the following reaction (1).

\[ \text{S}_2\text{O}_3^{2-} + \text{CN}^- \rightarrow \text{SO}_3^{2-} + \text{CNS}^- \]

**REACTION 1**

In the reaction the outer sulfur atom of thiosulfate is transferred to the Cys residue of the enzyme molecule to form a persulfide intermediate, which is subsequently attacked by cyanide anion to give thiocyanide. However, the enzyme has rather wide substrate specificity. Certain sulfur compounds such as thiosulfonate (2) or persulfdies (3, 4) may substitute for thiosulfate; and sulfite (5), sulfinates (5), or various thiol compounds (6) may substitute for cyanide. This enzyme was reported to be widely distributed in prokaryote and eukaryote mitochondria (7–9). Although the physiological role of this enzyme was not well understood, the enzyme is well characterized. The enzyme was first isolated from bovine liver (10) and subsequently from rat liver (11). Primary structures of the enzyme from various sources were determined from protein or deduced from cDNA, e.g. bovine liver (12) and adrenal (13), chicken liver (14), human liver (15), rat liver (16), hamster ovary (17), and mouse liver (18). Further, recombinant bovine liver (13) and adrenal (19), rat liver (20), hamster ovary (17), and mouse liver (18) rhodanese were overexpressed in Escherichia coli and characterized. The enzyme from bovine liver was crystallized (10, 21), and the three-dimensional structure was determined. (22–24). On the other hand, mercaptoipyruvate sulfurtransferase (MST, EC 2.8.1.2) catalyzes the following reaction.

\[ \text{HSCH}_2\text{COCOO}^- + \text{CN}^- \rightarrow \text{CH}_3\text{COCOO}^- + \text{CNS}^- \]

**REACTION 2**

The enzyme was discovered in rat liver quite a long time ago (25–28), but compared with rhodanese the enzyme was not well characterized. Although the enzyme responsible for this reaction was at first assumed to be rhodanese, subsequent investigation with crystallized rhodanese showed that rhodanese did not catalyze this reaction and that another enzyme, mercaptoipyruvate sulfurtransferase, was responsible for the reaction (28). Similar to rhodanese, compounds other than cyanide may function as sulfur acceptors in the reaction catalyzed by this enzyme (29–32), and its physiological role is a matter of discussion (33, 34). This enzyme was reported to be distributed in both prokaryote and eukaryote mitochondria and to be located in the cytosol of eukaryotic cells (26, 27, 34, 35), but the existence of this enzyme in mitochondria is also reported (36). Recently rat liver MST was purified to homogeneity, and the partial amino acid sequence around the active site of the enzyme was determined (20). Although the two enzymes were considered to be different, it was found that MST and rhodanese possessed both MST and rhodanese activities, but the ratios of their activities differed greatly. Further, a partial amino acid sequence of MST and the deduced primary structure of rhodanese showed striking similarity in sequence around the active site, but two amino acid residues following a catalytic site Cys were substituted. In a mutagenesis study with rat rhodanese cDNA, replacement of the two amino acid residues in the active site of rhodanese (Arg and/or Lys) with MST type residues (Gly and/or Ser) increased MST activity and decreased rhodanese activity, indicating that rhodanese was partly converted to MST (20). Based on these findings, it was proposed that MST and rhodanese were evolutionarily related enzymes. In this paper, to confirm the proposal, we attempted to convert MST to rhodanese by site-
directed mutagenesis of some amino acid residues around the proposed active site. The complete primary structure of the rat liver MST was determined after cDNA cloning. Wild type and six mutant MSTs were then constructed and overexpressed in E. coli. Based on kinetic studies for purified wild type and mutant MSTs, some amino acid residues that participate in the chemistry of mercaptopyruvate are discussed.

MATERIALS AND METHODS

Chemicals—Mercaptopyruvate for kinetic study was synthesized essentially by the method of Kun (37) as described previously (20). Other chemicals were of analytical grade.

Isolation of a cDNA Clone for Rat Liver MST and Construction of an Expression Vector for Wild Type MST—PCR primers were designed with reference to the cDNA sequence of human rhodanese (14), because the deduced amino acid sequence of human rhodanese was found to be strikingly similar to the primary structure of purified rat MST as predicted by partial sequencing (20). 5'-Sense primer (CAACGAGTTCCGACCCGCCCTTCATCAAG; 506-523 bp of human rhodanese cDNA with an EcoRI restriction site) and 3'-antisense primer (GATCAAGGCTCTAGTAGCACG, designated pBs; 940 bp) was inserted into pET-15b vector (NOVAGEN) between the NcoI and XhoI sites. The construct full-length cDNA for rat liver MST containing 3'-untranslated region of MST between the NcoI and XhoI sites for expression.

Expression and Purification of Wild Type and Mutant MST—These constructs were transformed into E. coli strain BL21 (DE3). Transformed cells were cultured in 2 liters of LB medium containing 50 mg/ml of 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 h at 37 °C, the cells were harvested by centrifugation at 6,000 g for 10 min. Lysate was obtained essentially by the method of Sambrook et al. (41) except for the addition of 0.5 mg/ml trypsin inhibitor. The lysate was centrifuged, and the supernatant was fractionated with ammonium sulfate (40–60% saturation). The precipitate was dissolved in 0.1 M Tris-HCl buffer (pH 8.0) containing 150 mM sodium chloride, dialyzed against 5 mM sodium acetate containing 100 mM sodium chloride, and ammonium sulfate was added to 65% saturation. The precipitate was dissolved in a minimal volume of 5 mM sodium acetate buffer (pH 8.0) containing 10 mM β-mercaptoethanol. The solution was dialyzed overnight against 1 liter of the same buffer at 4 °C. 30 ml of the dialyzed enzyme was loaded onto an Express-ion™ exchanger (Diaion anion, Whatman) column (1.5 x 17 cm) equilibrated with the same buffer. After the column was washed with 45 ml of the same buffer and then with 45 ml of 50 mM Tris-HCl buffer, pH 7.8, containing 0.2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 10 mM β-mercaptoethanol, the enzyme was eluted with a linear gradient to 200 mM Tris-HCl buffer, pH 7.8, containing 0.2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 10 mM β-mercaptoethanol. 25 ml of the enzyme-containing fractions eluted at approximately 90 mM Tris-HCl were collected, and the ammonium sulfate was added to 65% saturation. The precipitate was dissolved in a minimal volume of 5 mM sodium acetate containing 100 mM sodium chloride, dialyzed against 5 mM sodium acetate containing 400 mM sodium chloride. 12 ml of recombinant MST-containing fractions eluted at approximately 14 mM sodium acetate containing 200 mM sodium chloride, were collected and concentrated with Centriprep-10 (Amicon) to more than 10 mg/ml. To the concentrated sample was added one-third volume of 100% saturated ammonium sulfate solution, and it was stored at −80 °C. Under these conditions, no significant loss of activity was observed for at least 6 months.

Protein Sequencing—Amino acid sequencing of recombinant enzymes was performed by the automated Edman degradation method with a gas phase protein sequencer model 477A (Applied Biosystems). The protein was denatured with 6 M urea and precipitated with trichloroacetic acid (TCA). Amino acid sequencing of recombinant enzymes was performed on a gas phase protein sequencer model 477A (Applied Biosystems). The protein was denatured with 6 M urea and precipitated with trichloroacetic acid (TCA). Amino acid sequencing of recombinant enzymes was performed on a gas phase protein sequencer model 477A (Applied Biosystems). The protein was denatured with 6 M urea and precipitated with trichloroacetic acid (TCA). Amino acid sequencing of recombinant enzymes was performed on a gas phase protein sequencer model 477A (Applied Biosystems). The protein was denatured with 6 M urea and precipitated with trichloroacetic acid (TCA). Amino acid sequencing of recombinant enzymes was performed on a gas phase protein sequencer model 477A (Applied Biosystems).
zyme) (12) rhodaneses shows 60, 60, 60, 59, and 59% identity, respectively (Fig. 2). In cDNA, identity of the nucleotide sequence of rat liver MST compared with that of rat liver, bovine adrenal (13), hamster ovary, and mouse liver rhodaneses is around 65%.

It should be noted that the sequence of human rhodanese is very similar to that of rat MST. The entire sequence identity between rat MST and human rhodanese (15) is 82 and 84% in cDNA and deduced primary structure, respectively. Around the active center, Gly248 (Fig. 2, #4) and Ser249 (Fig. 2, #5) for rat MST are conserved in human rhodanese. As described previously, replacement of the two amino acid residues (Arg and/or Lys) of rat liver rhodanese with MST type residues (Gly and/or Ser) increased MST activity and decreased rhodanese activity (20). Furthermore, Arg196 (Fig. 2, #2) of rat MST is a unique residue that is absent in all rhodaneses except human rhodanese. This observation supports the previous proposal that the reported human rhodanese may in fact be human MST (20). It is intriguing that the primary structure of SseA deduced from the sseA gene of E. coli (43) is similar to those of MST and rhodaneses (Fig. 2). SseA protein was reported to enhance growth inhibition of E. coli when serine was added to the medium. SseA protein lacks the first 50 amino acid residues in the N-terminal region and six amino acid residues in the C-terminal region, compared with sequences of rat MST (Fig. 2). Sequence identity between SseA and rat MST is 53 and 38% for nucleotide and amino acid sequences, respectively, but that between SseA and rat rhodanese is 50 and 35% for nucleotide and amino acid sequences, respectively. Further, SseA contains the typical Cys-Gly-Ser sequence found in the active site of MST, as mentioned above (Fig. 2). These findings suggest that SseA is possibly an E. coli MST and an evolutionary prototype of the two family enzymes. It was reported that rhodanese was sorted to mitochondria in eukaryotic cells (7, 8, 10), but MST was localized in cytosol and mitochondria (35, 36). Bovine rhodanese was reported to contain a noncleavable signal in the N-terminal region for mitochondrial import (13). Residues 11–22 are seen to form an α-helix of bovine rhodanese upon x-ray analysis (22, 23). This is consistent with the fact that a synthetic peptide (residues 3–20) of the N-terminal region of rat rhodanese is found to form an α-helix by means of two-dimensional NMR (44). This α-helix of these family enzymes is considered to be a signal for mitochondrial import. It is reasonable that SseA, a possible E. coli MST, does not contain this α-helix in the N-terminal region (Fig. 2). Furthermore, positively charged Lys13 and Arg20 of bovine rhodanese are closely positioned at the outside of the α-helix (22, 23). Edmundson wheel analysis for rat rhodanese reveals that positively charged Lys 12 and Arg19 are also positioned very close on one side of the amphipathic helix. Arg 19 of rat rhodanese is conserved in other rhodaneses and MST. On the other hand, Lys12 of rat rhodanese is conserved in other rhodaneses but is replaced by Gln in MST (Fig. 2).

Expression and Purification of Wild Type and Mutant Rat Liver MST—To clarify the role of amino acid residues of Arg187
the cDNA sequences. All expressed proteins were preceded by
methionine at the N terminus of all of the expressed proteins agreed with
the sequence of the cDNA coding for E. coli sseA, the homolog of the rat liver enzyme.

**Effect of Mutagenesis, Possible Role of Amino Acid Residues in Catalysis**—The double reciprocal plots of recombinant wild type MST show a mixed inhibition pattern when MST activity is measured (Fig. 5) as observed for the native rat liver MST (20). It is suggested that recombinant MST follows a sequential kinetic pattern (45). There is no significant variation in kinetic pattern among wild type and mutant MSTs except for the C247S mutant. On the other hand, the reaction mechanism of bovine liver rhodanese was reported to follow a ping-pong pattern (46, 47). An intermediate product (persulfide formation at the catalytic site) was identified (22, 24). In the present study, however, the double reciprocal plot of MST (molecular mass 32,808 Da) shows a straight line when MST activity is measured (Fig. 5) as observed for the native rat liver MST (20). Ammonium sulfate was therefore added to purified MST to stabilize the enzyme before performing experiments. The amount of the overexpressed enzymes obtained from 1 liter of culture varied from 0.2 to 2 mg depending on the mutant. The specific activity of the purified wild type enzyme shows 3015 units/mg of protein and about a 20-fold increase compared with that of the native enzyme. SDS-polyacrylamide gel electrophoresis during the purification of wild type and mutant MST is shown in Fig. 3. Because neither MST nor rhodanese activity was detected in the C247S mutant (see below), this purified protein was confirmed to be a MST mutant by cross-reacting with anti-MST antibody (Fig. 4). SDS-polyacrylamide gel electrophoresis shows that wild type and mutant enzymes are 34-kDa molecules (Fig. 3), which is in reasonable agreement with the subunit size calculated from the deduced primary structure of rat MST (molecular mass 32,808 Da).

**Fig. 3.** SDS-polyacrylamide gel electrophoresis of purification steps for the wild type MST and purified mutant MST. M, molecular weight marker. Lanes 1–4 show the purification steps for wild type MST. Lane 1, lysate; lane 2, ammonium sulfate fraction (40–60%); lane 3, DEAF fraction; lane 4, CM fraction. (Data from: lane 5, purified S249K enzyme; lane 6, purified 1887G enzyme; lane 7, purified C247S enzyme; lane 8, purified G248R enzyme; lane 9, purified S249K enzyme; lane 10, purified S249A enzyme.)

**Fig. 2.** Comparison of partial primary structure of rat liver MST with sequences of rhodanases of human, rat, hamster, bovine and avian liver, and E. coli. A, HUM RHOD, deduced primary structure of human liver rhodanese (15); B, RAT RHOD, deduced primary structure of rat liver rhodanese (16); HAM RHOD, deduced primary structure of hamster ovary rhodanese (17); BOV RHOD, deduced primary structure of bovine liver rhodanese (18); AVI RHOD, deduced primary structure of avian liver rhodanese (19, 20); E. coli sseA, deduced primary structure of E. coli sseA gene (22, 23); shaded box, identical amino acid residues: arginine (47), arginine (48, 49), cysteine (49) (4), arginine (48, 49) (49), and lysine (48, 49) (49). AAG, the order of these three amino acid residues was not determined (49). 5G5A, these three amino acids were not detected by protein sequencing of purified mature rhodanese from bovine liver (12) but were contained in the deduced primary structure of bovine adrenal rhodanese cDNA (13).
could increase its MST activity. In the present study, to attempt to convert MST to rhodanese reversely, G248R, S249K, and R196G mutants were constructed. Arg substituted for Gly248 (Fig. 2, #4) and Lys substituted for Ser249 (Fig. 2, #5) are typical residues for rhodanese, whereas Arg196 is a unique residue for MST that is absent from rhodanese, as mentioned above. These mutant MSTs, the kinetic properties of which are discussed below, show an increase in the ratio of rhodanese to MST activity (Table II). This indicates that MST is partly converted to rhodanese, confirming the previous conclusion that the difference in the ratio of their activities is caused by specific differences in amino acid residues (20).

To define the role of five amino acid residues of MST in utilization of mercaptopyruvate and thiosulfate, a kinetic study of wild type, R187G, R196G, G248R, S249K, and S249A mutant MST (Table I) was performed. Arg187 of MST corresponds to the residue of bovine rhodanese that was proposed to serve as a binding site of thiosulfate (22–24, 48, 49). As shown in Table II, for rhodanese activity in R187G, $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ are decreased to about 1⁄4 and 1⁄400, respectively, of those in wild type, and $K_m$ for thiosulfate is increased to about 5-fold that in wild type, consistent with the previously reported results for the corresponding substitution in rhodanese (20). On the other hand, R187G mutant shows a much greater decrease in $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ for MST activity to about 3-order lower values and a noticeable increase in $K_m$ for mercaptopyruvate to about 60-fold that in wild type (Table II). Arg187 (Fig. 2, #1) therefore appears to be a binding site of mercaptopyruvate and a critical residue for catalysis in MST. A possible role in catalysis is speculated to be that a positively charged side chain of Arg187 interacts with the oxygen atom in the carbonyl group of mercaptopyruvate to cause an electrostatic interaction, as shown in Fig. 6. Since cysteine is not a substrate of this enzyme (data not shown), the carbonyl group is essential for catalysis. Polarization of the carbonyl group may accelerate catalysis by introducing electron strain in mercaptopyruvate. A nucleophilic attack on the sulfur of mercaptopyruvate by Cys247 may then be facilitated. An alternative explanation is that this Arg residue stabilizes a persulfide intermediate as postulated for Arg248 in rhodanese (22–24). However, this is unlikely, because much more pronounced effects on MST activity than on rhodanese activity have been observed, findings inconsistent with the possible common mechanism that persulfide forms as an intermediate. Arg196 of MST is a unique residue (Fig. 2, #2), which is absent from rhodanese as described above. Based on the tertiary structure of bovine rhodanese (22–24), a bulky side chain of Arg196 of MST is modeled as covering the pocket of the active site by computer simulation analysis with QUANTA/CHARMM as described under “Materials and Methods” (data not shown). The R196G mutant shows an increase in $K_m$ for mercaptopyruvate to about 10-fold that in wild type and a decrease in $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ for MST activity to about 1⁄4 and 1⁄400, respectively, of those in wild type (Table II). For rhodanese activity, on the other hand, the R196G mutant shows almost the same $K_m$ for thiosulfate as that in wild type, but $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ for rhodanese activity are increased to about 3-fold those in wild type (Table II). These findings indicate that Arg196 facilitates catalysis of mercaptopyruvate in MST but hinders catalysis of thiosulfate in MST. A possible role in the catalytic mechanism is that the positively charged side chain of Arg196 interacts with the carboxyl group of mercaptopyruvate and assists in polarizing the substrate.

The G248R mutant shows only a small difference in $K_m$ for mercaptopyruvate from that of wild type but a marked decrease in $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ for MST activity to about 1⁄1000 and 1⁄10000, respectively, of those in wild type (Table II). This behavior may be explained by steric hindrance. Although Gly248 may not play an important role in the catalysis of mercaptopyruvate, such a small residue as a Gly may be advantageous for catalysis. A bulky side chain of replaced Arg probably hinders catalysis of mercaptopyruvate, and MST activity is decreased as a consequence. For rhodanese activity, the G248R mutant...
shows a decrease in $K_m$ for thiosulfate to about one-fifth of that in wild type, but the $k_{cat}$ and $k_{cat}/K_m$ values for rhodanese activity were observed to be about 1/16 and 1⁄10, respectively, of those in wild type (Table II). The decrease in rhodanese activity caused by substituting Arg for Gly is inconsistent with the previous finding indicating that this Arg facilitates catalysis of thiosulfate in rhodanese (20). Possibly, the substituted Arg cannot be positioned suitably for rhodanese activity in the pocket of an active site in this case, because the substituted Arg and Arg are modeled as interfering with optimal positioning of each other in the homology-modeled tertiary structure of MST (data not shown). The S249K mutant shows a $K_m$ for mercaptopyruvate that is almost the same as that in wild type, suggesting that Ser is not a binding site for mercaptopyruvate. Although the $k_{cat}$ and $k_{cat}/K_m$ values for MST activity of the same mutant are decreased to about 1/16 and 1⁄10, respectively, of those in wild type (Table II), the residue may not play a critical role in catalysis. Another mutant (S249A) shows no significant difference in $K_m$ for mercaptopyruvate from that in wild type and a smaller decrease in the $k_{cat}$ and $k_{cat}/K_m$ values for MST activity to about 1/16 and 1⁄10, respectively, of those in wild type (Table II). In the S249K mutant, a substituted Lys that contains a bulky side chain may interfere with the catalysis of mercaptopyruvate, and MST activity would be decreased as a consequence. In any case, the replacement of Ser for Arg with other residues causes a slight decrease in MST activity. It can be speculated that this Ser is of adequate residue size and maintains a suitable conformation of the enzyme for catalysis of mercaptopyruvate. For rhodanese activity, replacement of a Ser with a Lys (S249K) decreased $K_m$ for thiosulfate to about 1/16 of that in wild type and increased $k_{cat}/K_m$ for rhodanese activity to about 10-fold that in wild type (Table II). The smaller substrate of thiosulfate may not be influenced by a bulky side chain, but rather the positive charge of this group may be important for catalysis of rhodanese. This is also consistent with the conclusion that the substituted Lys is a binding site of thiosulfate in rhodanese (20, 22–24, 49, 50).

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