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Determination of the isoelectric point value of 3-mercaptopyruvate sulfurtransferase and its shift by treatment with oxidized glutathione.

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Abstract

The isoelectric point (pI) value of 3-mercaptopyruvate sulfurtransferase (MST) from human erythrocytes was determined to be 6.3 at 10 degrees C by isoelectric focusing in horizontal slab polyacrylamide gel containing 2% carrier ampholyte (pH 3-10). The value was determined by comparison with the electrofocused bands of bovine pancreatic ribonuclease A-glutathione mixed disulfides (RNase-SG), which were composed of 8 species containing 1 (RNase-SG1) through 8 (RNase-SG8) moles of glutathione per mole of ribonuclease A with different pI values ranging from 5.3 (RNase-SG8) to 8.8 (RNase-SG1). The pI value of the same enzyme in a 110,000 X g supernatant of rat liver was 5.9, which was the same as that of rat erythrocyte enzyme. Treatments of rat hemolysate with oxidized glutathione or diamide resulted in a shift of the pI of MST to a lower value, 5.7-5.5. This shift was inhibited when these treatments were performed in the presence of dithiothreitol. These results indicate that the treatment of the enzyme with oxidized glutathione results in the formation of enzyme-glutathione mixed disulfide.

KEYWORDS: 3-mercaptopyruvate sulfurtransferase, isoelectric point, glutathione, mixed disulfide, isoelectric focusing

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Determination of the Isoelectric Point Value of 3-Mercaptopyruvate Sulfurtransferase and Its Shift by Treatment with Oxidized Glutathione

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The isoelectric point (pI) value of 3-mercaptopyruvate sulfurtransferase (MST) from human erythrocytes was determined to be 6.3 at 10°C by isoelectric focusing in horizontal slab polyacrylamide gel containing 2% carrier ampholyte (pH 3-10). The value was determined by comparison with the electrofocused bands of bovine pancreatic ribonuclease A-glutathione mixed disulfides (RNase-SG), which were composed of 8 species containing 1 (RNase-SG_1) through 8 (RNase-SG_8) moles of glutathione per mole of ribonuclease A with different pI values ranging from 5.3 (RNase-SG_8) to 8.8 (RNase-SG_1). The pI value of the same enzyme in a 110,000×*g* supernatant of rat liver was 5.9, which was the same as that of rat erythrocyte enzyme. Treatments of rat hemolysate with oxidized glutathione or diamide resulted in a shift of the pI of MST to a lower value, 5.7-5.5. This shift was inhibited when these treatments were performed in the presence of dithiothreitol. These results indicate that the treatment of the enzyme with oxidized glutathione results in the formation of enzyme-glutathione mixed disulfide.

Key words : **3-mercaptopyruvate sulfurtransferase, isoelectric point, glutathione, mixed disulfide, isoelectric focusing**

3-Mercaptopyruvate sulfurtransferase (EC 2.8.1.2) (MST) is a member of the transamination pathway of cysteine metabolism (1-5). The enzyme catalyzes the transfer of the sulfur atom of 3-mercaptopyruvate to thiophiles such as sulfite and cyanide (6). The activity of this enzyme is high in rat and human tissues, but low in guinea pig tissues (7, 8). The deficiency of this enzyme in humans is known as β-mercaptolactate-cysteine disulfiduria (9). Patients with this disorder excrete large amounts of β-mercaptolactate-cysteine and mercaptoacetate-cysteine disulfides (10), which are the normal constituents of human urine (11).

It has been reported that mammalian MST is highly unstable and requires 2-mercaptoethanol as a protective agent (12), and it has also been shown that this enzyme has reactive SH groups (12). We have reported (13, 14) that S-thiolation of reduced bovine pancreas ribonuclease A (EC 3.1.27.5) (RNase) with glutathione (GSH) resulted in a decrease in its isoelectric point (pI) and that the products, a mixture of ribonuclease A-glutathione mixed disulfides (RNase-SG), could be used as standards for the determination of pI values. Using these standards,

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the pI value of MST from rat erythrocytes was determined to be 5.9 at 10°C by isoelectric focusing.

In the present study, using this technique, we determined the pI values of MST from human erythrocytes and rat liver, and compared them with that of rat erythrocyte enzyme. It has been reported that various enzymes undergo S-thiolation with GSH, and the possible significance of enzyme S-thiolation on metabolic regulation was discussed (15). In order to examine the possibility of S-thiolation of MST with GSH, effects of treatments with oxidized glutathione (GSSG) and diamide (azodicarboxylic acid bis(dimethylamide)), an oxidizing agent of GSH (16, 17), on this enzyme were studied by examining the pI shift.

Materials and Methods

Materials. Ammonium 3-mercaptopyruvate was synthesized according to the method of Kun (18). RNase-SG was synthesized as reported, and a mixture containing eight species of RNase-SG, RNase and bovine serum albumin was used as the standard for pI determination (14). The GSH/RNase ratios (mol/mol) and pI values of eight RNase-SG species are summarized in Table 1. GSSG was obtained from Kohjin Co., Ltd., Tokyo, Japan. Pharmalyte (pH 3-10) was purchased from Pharmacia LKB Biotechnology, Uppsala, Sweden, Lactate dehydrogenase (EC 1.1.1.27) and GSH were from Boeringer Manheim Yamanouchi Co., Ltd., Tokyo. Nicotinamide adenine dinucleotide, reduced form (NADH), was obtained from Oriental Yeast Co., Ltd., Tokyo. Acrylamide and methylene-bis-acrylamide were products of Bio-Rad Laboratories, Richmond, CA, USA. Diamide was obtained from Sigma Chemical Co., St. Louis, MO, USA.

Human blood was obtained from the cubital vein using heparin as an anticoagulant. Erythrocytes, obtained by centrifugation at $1,200 \times g$ for 10 min at 4°C, were washed three times with ice-cold 0.9% sodium chloride containing 1 mM EDTA. White blood cells floating on the surface of the erythrocyte layer after centrifugation were removed using a Pasteur pipet. The washed erythrocytes were hemolyzed by the addition of 2 volumes of ice-cold water. The hemolysate was centrifuged at $15,000 \times g$ for 20 min. The resultant supernatant was used as the enzyme solution. In some experiments, hemolysate was prepared in the presence of dithiothreitol at a final concentration of 10 mM, and this preparation was subjected to the determination of the pI value after standing at 25°C for 60 min.

Table 1 Molecular species and isoelectric points (pI) of bovine pancreatic ribonuclease A-glutathione mixed disulfides (RNase-SG)

Species	GSH/RNase[a]	pI (10°C)[b]
RNase-SG1	1	8.8
RNase-SG2	2	8.2
RNase-SG3	3	7.7
RNase-SG4	4	7.3
RNase-SG5	5	6.9
RNase-SG6	6	6.4
RNase-SG7	7	5.8
RNase-SG8	8	5.3

a: Molar ratio of glutathione (GSH) and ribonuclease A (RNase) (ref. 13).
b: Values were determined by isoelectric focusing (ref. 13, 14).

Blood of male Wistar rats weighing about 300 g was obtained by decapitation, and hemolysate was obtained as above. The hemolysate was stored frozen at −20°C overnight. After thawing, the hemolysate was centrifuged at $15,000 \times g$ for 20 min to eliminate precipitated hemoglobin. The resulting supernatant was used for the study of the pI. As rat erythrocyte is a good source of this enzyme (19), rat hemolysate was used for the study of the effect of treatments with GSSG and diamide.

Livers of the same rats as those used for erythrocyte preparation were homogenized with 3 volumes of 25 mM potassium phosphate (pH 7.4) with or without 5 mM dithiothreitol. Homogenates were centrifuged at $110,000 \times g$ at 4°C for 60 min. The resultant supernatant was used for the determination of the pI value of MST.

Treatment of enzyme solutions with GSSG, diamide and dithiothreitol. Eight tenths ml of enzyme solution was incubated at 0°C for 60 min with 0.2 ml of GSSG at a final concentration of 0.2,

2.0, or 20.0 mM. In some experiments, enzyme solution was incubated as above with 40 mM GSH and 40 mM diamide or with 5 mM diamide and 20 mM dithiothreitol.

Isoelectric focusing and determination of the pI value of MST. Isoelectric focusing was performed as described previously (14) with some modifications. Isoelectric focusing is an excellent method for separating proteins according to their isoelectric point, and as reported previously for the determination of the pI value of MST, the method gave the same results for a crude extract as for a partially purified enzyme (14). Therefore, supernatants of hemolysates and of liver homogenates were used as enzyme solutions in the present study. Horizontal slab polyacrylamide gel (10×11 cm, 0.8 mm thick) containing 2% Pharmalyte (pH 3-10) was subjected to prefocusing using an Ultrophor apparatus (Pharmacia LKB Biotechnology) with a cooling unit regulated at about 6°C. The temperature of the gel was measured at the start and at the end of the prefocusing using a thermometer with a surface sensor (Model 2455, Yokogawa Hokushin Electric Co., Tokyo). After prefocusing for 40 min, 4 μl of the above standard solution was applied to a piece of filter paper (Whatman 3MM, 2×6 mm) which was placed 12.5 mm from the anode in the center lane (lane B of Fig. 1). Thirty μl of each sample solution was applied to pieces of filter paper (Whatman 3MM, 2×40 mm) placed in the lanes on both sides of the filter paper for the standard (lanes A and C of Fig. 1). When the pI values of two different samples were compared, these samples were applied to lanes A and C in the same gel. In some experiments, a mixture of two sample solutions was applied. Electrofocusing was performed for 100 min. The temperature of the cooling unit was adjusted in order to maintain the temperature of the gel surface at 10±1°C. When the isoelectric focusing was completed, lanes A and C of the gel were cut into 2.5-mm sections. Fig. 1 shows an example, in which more than half of lane C has been cut into sections. Enzyme assay and pH determination of gel sections were performed as reported (14).

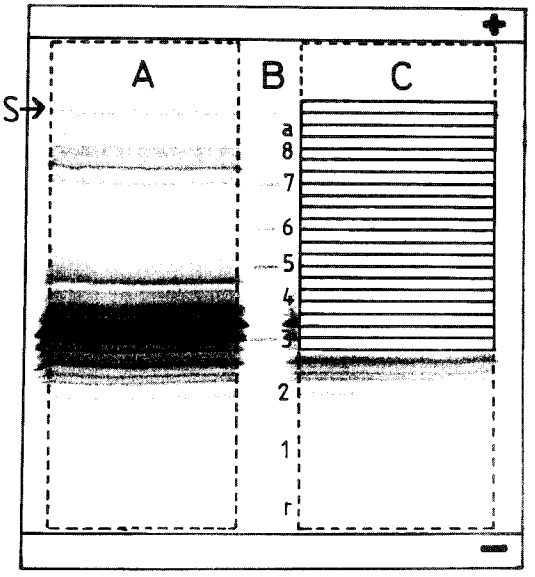

Fig. 1 The pattern of isoelectric focusing and gel sections for the determination of the isoelectric point (pI) value of 3-mercaptopyruvate sulfurtransferase. A hemolysate of human erythrocytes was applied to pieces of filter paper in lanes A and C (shown with an arrow and letter s). A solution of pI standards consisting of bovine serum albumin (a), ribonuclease A (r) and ribonuclease A-glutathione mixed disulfides (RNase-SGn) was applied to a piece of filter paper in lane B. Numbers indicate n of RNase-SGn, which means glutathione content (mol/mol of ribonuclease A). After 100 min of electrofocusing at 10°C, lanes A and C (shown with dotted lines) were cut into 2.5×40-mm strips for enzyme assay. In this figure, the major portion of lane C has been cut into strips and removed. The remaining part of the gel was stained with Coomassie brilliant blue R-250 and dried on a sheet of filter paper.

Results and Discussion

Fig. 2 shows the result of the pI determination of human erythrocyte MST. The figure shows that MST was electrofocused between RNase-SG_7 and RNase-SG_6, and that the pI value is 6.2 at 10°C. This value is 0.3 pH unit higher than that of rat erythrocytes. There was a small band of MST activity with a pI of 5.6. This band was reduced when the enzyme solution was prepared in the presence of dithiothreitol.

The pI of rat liver MST was examined in the presence (Fig. 3A) and absence (Fig. 3B) of dithiothreitol. As shown in the figure,

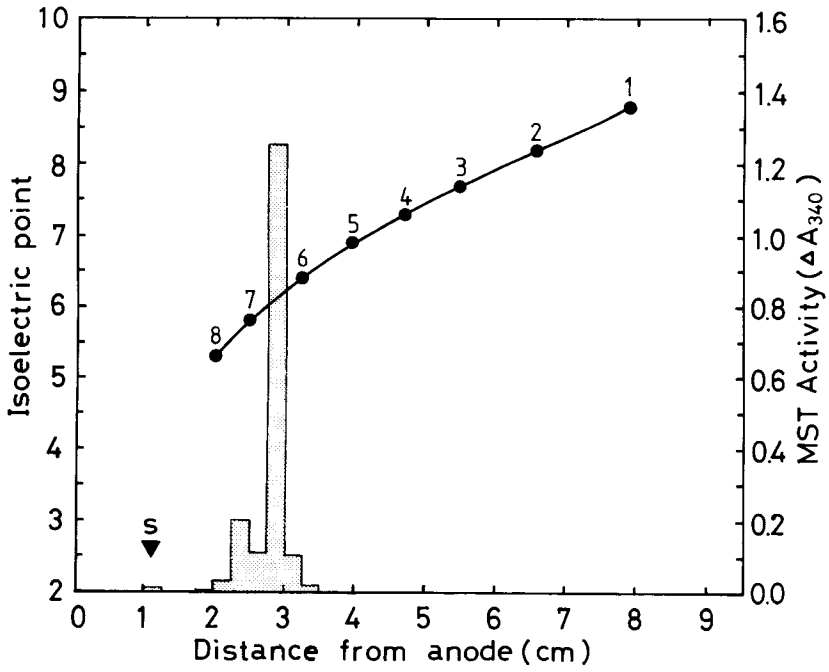

Fig. 2 Determination of the isoelectric point (pI) value of 3-mercaptopyruvate sulfurtransferase (MST) of human erythrocytes by isoelectric focusing. Numbers indicate n of RNase-SGn. For details, see the legend to Fig. 1 and the text. The distance from the anode in cm was plotted against the pI value of RNase-SGn. MST activity in gel sections was determined by determining pyruvate formed using lactate dehydrogenase and NADH. The decrease in absorbance at 340 nm/section is shown (▨). Letter s indicates the position of sample application.

rat liver MST focused at pH 5.9, indicating that its pI is 5.9 at 10°C. This value is the same as that of rat erythrocyte MST (14). The presence of dithiothreitol in the enzyme solution increased the total MST activity to some extent, but did not affect the isoelectric focusing profile.

MST activity is detected in soluble and mitochondrial fractions of rat liver (2) and bovine adrenal cortex (20). The present experiment dealt with MST in the soluble fraction. It seems necessary to examine the properties of mitochondrial enzyme in order to study the physiological function of MST in the cell.

The effect of the treatment with various concentrations of GSSG on the pI of rat erythrocyte MST was studied. As shown in Fig. 4 and in our preliminary report (21), when the enzyme solution was treated with 0.2 mM GSSG, MST activity corresponding to pI 5.9 decreased and the activity corresponding to pI 5.7 and 5.5 appeared. When concentrations of GSSG were raised to 2.0 or 20 mM, almost all the activity at pI 5.9 disappeared and the activity at 5.5 increased. The decrease in the pI value by GSSG treatment suggests that MST was modified by S-thiolation with an acidic tripeptide GSH as occurred with RNase-SG. The fact that electrofocusing profiles of enzyme solutions treated with GSSG of 2.0 and 20 mM were similar indicates that S-thiolation of this enzyme with GSH was completed with GSSG at a concentration of 2 mM under the present conditions. When the enzyme solution was treated with 0.2 and 2.0 mM GSSG, a very low, but distinct MST activity was detected in a gel section corresponding to pI 6.9. This activity was also detected in the enzyme

solution treated with 5mM diamide as shown in Fig. 5C. The nature of this band is unknown and is under investigation.

In order to examine further the possibility of S-thiolation of MST with GSH, the enzyme solution was treated with diamide. Fig. 5A shows the result of an experiment, in which the enzyme solution from rat erythrocytes was treated with 20 mM GSSG. As shown in Fig. 5B, when the enzyme solution was treated with 40 mM GSH and 40 mM diamide, the electrofocusing profile was similar to that with 20 mM GSSG. Fig. 5C shows that the treatment of the enzyme solution with 5 mM diamide resulted in an increase in MST activity focusing at pI 5.7. This increase was inhibited by the presence of dithiothreitol, a potent reducing agent of disulfide bonds, as shown in Fig. 5D. These results indicate that GSH contained in the hemolysate was oxidized with diamide, and

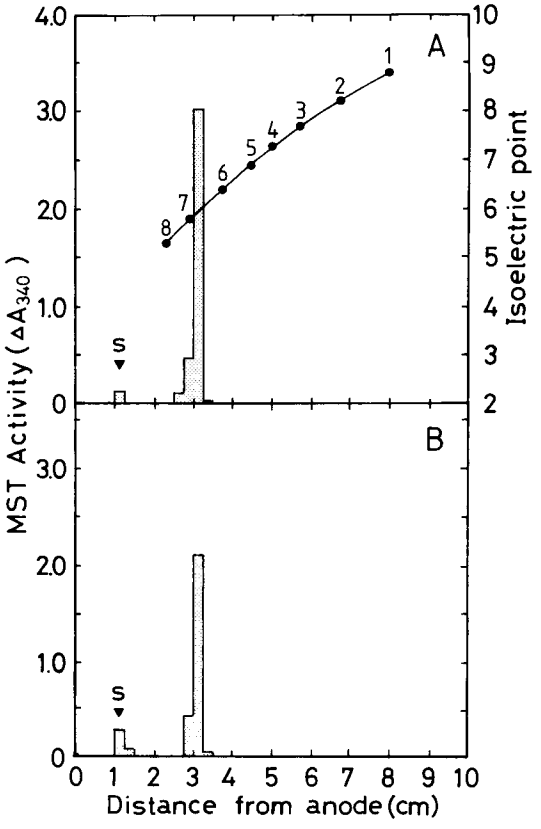

Fig. 3 Determination of the isoelectric point value of 3-mercaptopyruvate sulfurtransferase of rat liver by isoelectric focusing. Isoelectric focusing was performed as described under Materials and Methods and in the legends to Figs. 1 and 2. A 110,000×g supernatant of liver homogenate prepared in the presence (A) or absence (B) of dithiothreitol was analyzed.

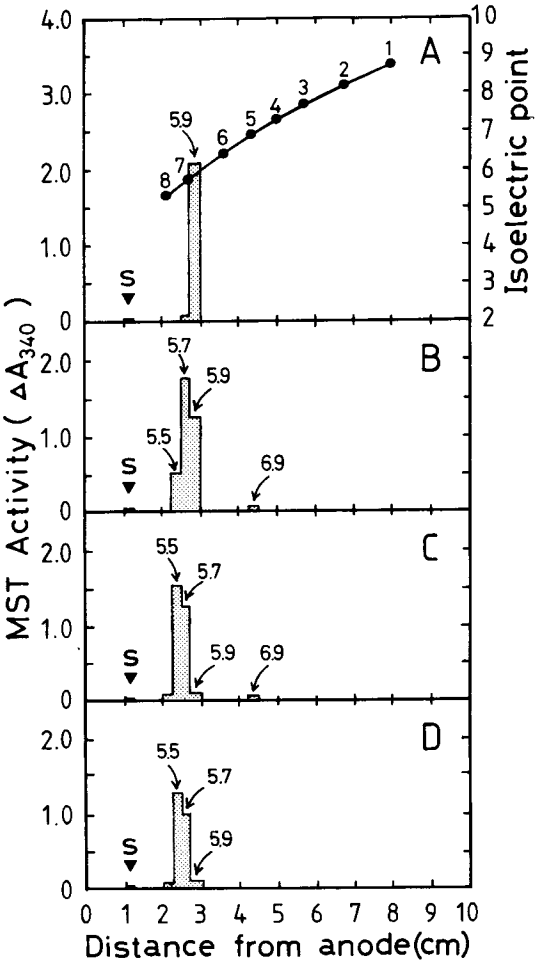

Fig. 4 Effect of treatment with oxidized glutathione (GSSG) on the isoelectric point (pI) of rat erythrocyte 3-mercaptopyruvate sulfurtransferase (MST). Fig. 4A shows MST focused at pI 5.9. Figs. 4B, C and D show that treatments of the enzyme solution with GSSG of 0.2 (B), 2.0 (C) or 20.0 mM (D) resulted in a shift to lower pI (5.7 to 5.5). For details, see the text and legends to Figs. 1 to 3.

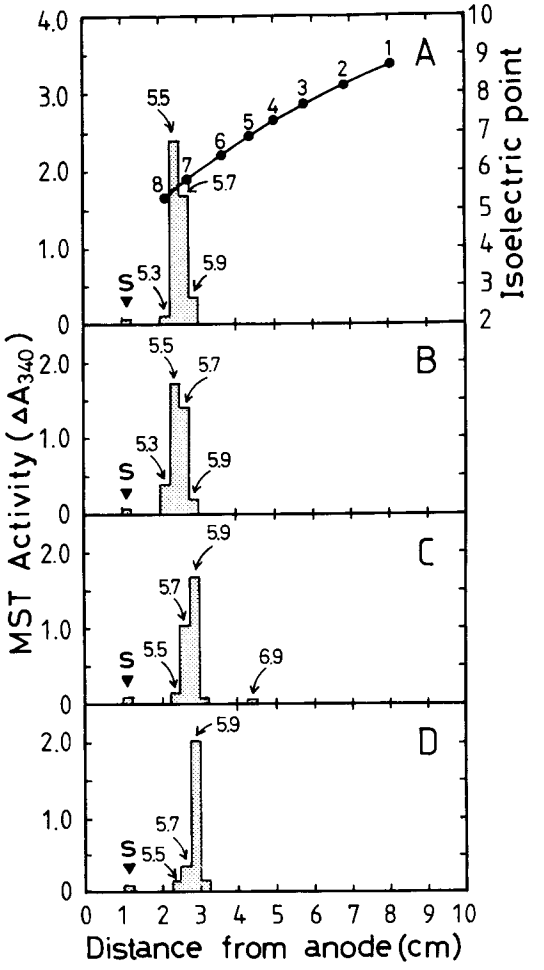

Fig. 5 Effect of diamide treatment on the isoelectric point (pI) of rat erythrocyte 3-mercaptopyruvate sulfurtransferase (MST). The enzyme solution was treated with 20 mM oxidized glutathione (A), 40 mM glutathione and 40 mM diamide (B), 5 mM diamide (C) or 5 mM diamide and 20 mM dithiothreitol (D). For details, see the text and legends to Figs. 1 to 4.

that the resulting GSSG reacted with MST.

The results obtained in the present study seem to indicate that S-thiolation of MST with GSH occurred, *i. e.*, GSH bound to MST with disulfide bonds. It has been reported that the treatment of cultured beating neonatal heart cells with 0.2 or 0.5 mM diamide resulted in an increase in the intracellular GSSG level and S-thiolation of several proteins (22). GSH is a ubiquitous constituent of mammalian cells, and the intracellular concentrations are especially high in the liver and erythrocytes. It is of interest that intracellular S-thiolation may regulate enzyme activities (12) and metabolic processes (12, 22). In the present study, electrofocused MST activity of enzyme solutions treated with GSSG and diamide was determined in the presence of dithiothreitol (8). Therefore, the possible change of MST activity could not be detected. Further experiments using purified MST and an assay system without reducing agent is needed for the study of S-thiolation and regulation of this enzyme.

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