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PURIFICATION AND CHARACTERIZATION OF 3-MERCAPTOPYRUVIC ACID S-CONJUGATE REDUCTASES

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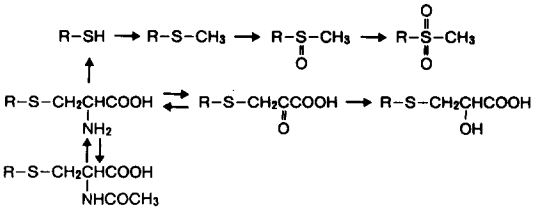
Abstract—Three kinds of 3-mercaptopyruvic acid S-conjugate reductase (MPR-I, MPR-II and MPR-III) were purified from rat liver cytosol. These enzymes reduced 3-mercaptopyruvic acid S-conjugates derived from cysteine conjugates and some endogenous α-keto acids to the corresponding α-hydroxy acids in the presence of either NADH (for MPR-I and MPR-II) or NADPH (MPR-III), while simple aldehydes or ketones did not significantly induce substrate activity. The molecular weight of the present enzymes was about 80 kDa composed of two subunits of the same molecular weight. K_m values of MPR-I, MPR-II and MPR-III were 0.38, 0.06 and 0.29 mM for *S*-(4-bromophenyl)-3-thiopyruvic acid, respectively, and 0.15 mM for NADH (MPR-I, MPR-II) and NADPH (MPR-III). V_{max} values of MPR-I, MPR-II and MPR-III for this substrate were 5.3, 20 and 13 nmol/min/mg, respectively. The sulphydryl-modifying agents inhibited the enzyme activities of all the three reductases. Based on the properties including substrate selectivity for α-keto acids derived from aromatic amino acids, we assumed that MPR-II and aromatic α-keto acid reductase are the same enzyme, while enzymes similar to MPR-I and MPR-III have not been reported. From the viewpoints of metabolism of xenobiotics, these enzymes are likely to be important in biotransformation of cysteine conjugates to 3-mercaptolactic acid S-conjugates.

In general, cysteine conjugates of xenobiotics are not excreted as such and are subjected to further metabolic conversion prior to excretion in mammals. Three pathways have been reported for biotransformation of the cysteine conjugates: (1) N-acetylation reaction to give mercapturic acid conjugates, (2) cleavage reaction of C—S bond to form thiol- or methylthio-containing metabolites [1–5], and (3) transamination reaction to yield 3-mercaptopyruvic acid S-conjugates which are further converted to 3-mercaptolactic acid S-conjugates [6–8] (Scheme 1).

The enzymes responsible for the former two reactions have been extensively studied by many investigators due to their important roles in detoxication and metabolic activation. Thus, the formation of mercapturic acid conjugates means detoxication and facilitates rapid excretion of xenobiotics in most cases, while the C—S bond cleavage reaction has been identified as an important bioactivation mechanism that is likely to be associated with the nephrotoxicity and nephrocarcinogenicity of some halogenated hydrocarbons [9–16]. On the contrary, not so much attention has been paid to the third reaction pathway in spite of the reports that certain kinds of xenobiotics gave rise to these types of conjugates as major metabolites [8, 17–19].

In order to understand the significance of the transamination pathway in the metabolism of xenobiotic cysteine conjugates, firstly we have examined the enzymes participating in this pathway. In the previous paper we reported the purification and properties of three cysteine conjugate aminotransferases which generate 3-mercaptopyruvic acid S-conjugates from corresponding cysteine conjugates [8].



Scheme 1. Metabolic pathway of cysteine conjugates.

In the present paper we describe the purification and characterization of the rat liver enzymes responsible for reduction of 3-mercaptopyruvic acid S-conjugates to form 3-mercaptolactic acid S-conjugates and discuss the biological roles of the enzymes.

MATERIALS AND METHODS

Materials

S-(4-Bromophenyl)-3-thiopyruvic acid was synthesized by a reaction of 4-bromobenzenethiol with 3-fluoropyruvic acid (Aldrich, U.S.A.) according to the procedure described by Parrod [20]. *S*-(4-Bromophenyl)-3-thiolactic acid was obtained by reduction of *S*-(4-bromophenyl)-3-thiopyruvic acid by sodium borohydride in ethanol by the usual

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method. Various cysteine conjugates, i.e. *S*-ethyl-L-cysteine sulphoxide, *S*-phenyl-L-cysteine sulphoxide, *S*-methyl-L-cysteine, *S*-ethyl-L-cysteine, *S*-(1-propyl)-L-cysteine, *S*-(1-butyl)-L-cysteine, *S*-(*tert*-butyl)-L-cysteine, *S*-(1,2-dichlorovinyl)-L-cysteine, *S*-benzyl-L-cysteine, *S*-phenyl-L-cysteine, which were used for preparing corresponding α-keto acids were obtained synthetically or commercially [8]. *S*-(2-Chloroethyl)-L-cysteine was synthesized by the method of Green *et al.* [21]. *S*-Carboxymethyl-L-cysteine, 3-iodo-L-tyrosine, 3,3′-diiodo-L-tyrosine dihydrate, acetaldehyde, phenylacetaldehyde, 3-nitrobenzaldehyde, 4-chloroacetophenone and 4-nitroacetophenone were purchased from Wako Pure Chemicals (Japan). Benzyl methyl ketone was prepared from 1-phenyl-2-propanol (Sigma, U.S.A.) by oxidation with chromium trioxide. Methyl phenylpyruvate was obtained by methylation of the corresponding acid with diazomethane by the usual method. Disodium α-ketoglutarate, sodium pyruvate, sodium α-ketobutyrate, sodium α-keto-isocaproate, sodium glyoxylate, sodium α-keto-methiolbutylate, sodium phenyl pyruvate, cis-oxalacetic acid, indole-3-pyruvic acid were purchased from Sigma (U.S.A.). Standard proteins (i.e. bovine thyroglobulin, bovine γ-globulin, chicken ovalbumin and horse myoglobin) used for determination of molecular weight of proteins were products of Bio-Rad (U.S.A.). All other reagents and amino acids used in the present study were purchased and were of analytical reagent grade. DEAE-cellulose (DE-53) and CM-cellulose (CM-52) were obtained from Whatman (U.K.). Hydroxyapatite (Biogel-HTP) and Sephadex G-25 and G-200 were purchased from Bio-Rad (U.S.A.) and Pharmacia Fine Chemicals (Sweden), respectively.

Assay methods

Assay of 3-mercaptopyruvic acid S-conjugate reductase. For a standard assay of reductase activity, *S*-(4-bromophenyl)-3-thiopyruvic acid was used as substrate and the amount of *S*-(4-bromophenyl)-3-thiolactic acid formed was determined by HPLC (conditions described below). The standard assay mixture contained, in a final volume of 0.25 mL; *S*-(4-bromophenyl)-3-thiopyruvic acid, 0.125 μmol; NADH (or NADPH), 0.083 μmol; Tris–acetate buffer, pH 6.0, 50 μmol, and enzyme solution, 0.001–0.040 mL (protein concentration, 0.05–10 mg/mL). The mixture was incubated at 37° for 30 min and the reaction was terminated by addition of 0.25 mL of acetonitrile. The remaining substrate in the mixture, which interfered with HPLC analysis of the product, was converted to a corresponding Schiff-base by incubating with 100 mM of aminooxyacetic acid (50 μL) at 37° for 10 min. After centrifugation, 20 μL of the supernatant was applied to an HPLC system (L-6000, Hitachi, Japan) equipped with a reversed phase column (μ-Bondapak C_{18}, 3.9 × 300 mm, Waters). *S*-(4-Bromophenyl)-3-thiolactic acid and the Schiff-base of substrate were eluted at 7.0 and 9.1 min, respectively, with a solvent system of methanol–water–phosphoric acid (60:40:1, by vol.) at a flow rate of 1.2 mL/min and were detected at 254 nm (L-4000, Hitachi). Amounts of the α-hydroxy acid were calculated from the peak height. The calibration curve was linear over the range of 0.1–3.5 μg and the lower detection limit was 0.1 μg. The coefficient of variation of this assay was 8.8%. A unit of the reductase activity was defined as the quantity of enzyme catalysing the formation of 1 nmol of *S*-(4-bromophenyl)-3-thiolactic acid per min under the above conditions. The reaction was linear with respect to time up to 30 min and the purified enzyme-protein from 0 to 0.2 mg.

When compounds other than *S*-(4-bromophenyl)-3-thiopyruvic acid were examined as substrates, the enzyme activity was determined by monitoring the decrease in the amount of NADH (or NADPH) by measuring absorbance at 340 nm spectrophotometrically. Incubation conditions were identical to those described above except that the scale was enlarged by 4-fold. The substrate solution for some α-keto acids that were commercially unavailable was prepared as follows: the amino acids (including L-cysteine conjugates) (0.5 μmol) corresponding to the desired α-keto acids were incubated with an excess amount (1.6 units) of L-amino acid oxidase (*Crotalus adamanteus* venom, Worthington Biochemical Corp., U.S.A.) at 37° for 30 min at pH 6.0 in a total volume of 1.0 mL in the presence of Tris–acetate buffer (pH 6.0, 50 μmol) and 9700 units of catalase (Bovine liver, Sigma). According to this procedure, at least 70% of each amino acid substrate was converted to the corresponding α-keto acid, as was confirmed by determination of the liberated ammonia. The incubation mixture was subsequently subjected to ultrafiltration with Molcut II (Millipore Corp., U.S.A.) to remove proteins. To the filtrate (substrate solution) were added NADH or NADPH (each 0.33 μmol) and the purified reductase in this order. The mixture was subsequently incubated at 37° for 30 min. The reductase activity was calculated from the decrease in absorbance at 340 nm.

Assay of ammonia. Amounts of ammonia were determined by the enzymatic method on the basis of the reductive amination of α-ketoglutaric acid using Ammonia Assay Kit (Sigma) [22].

Determination of protein. Concentration of protein was determined by the method of Bradford [23] with catalase (50,000 units/mg, Sigma) as standard.

Electrophoresis

Proteins were analysed for purity by polyacrylamide gel electrophoresis (PAGE) in the presence of SDS (sodium dodesylsulphate) on 12.5% polyacrylamide slab gels (Phast Gel 12.5, Pharmacia) by automated electrophoresis system (Phast System, Pharmacia). Proteins were stained with Phast Gel Blue R (Pharmacia).

Determination of molecular weight by HPLC

The apparent molecular weight was determined by gel permeation chromatography (GPC) with HPLC equipped with a GPC column (TSK Gel G3000 SWXL 7.8 × 500 mm, Tosoh, Japan). The molecular weight standards used were: bovine thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa) and horse myoglobin (17 kDa). Proteins were eluted with 200 mM potassium phosphate buffer, pH 7.4, at a flow rate of 1 mL/min and were detected at 280 nm.

Table 1. Purification of 3-mercaptopyruvic acid S-conjugate reductases from rat liver

Step	Protein (mg)	Total activity (units)	Yield (%)	Specific activity (units/mg)	Purification (fold)
1. Liver cytosol					
MPR-I + MPR-II	4260	465	100	0.11	1
MPR-III		116	100	0.027	1
2. Ammonium sulphate fractionation					
MPR-I + MPR-II	2880	548	118	0.19	1.7
MPR-III		63	54.3	0.022	0.81
3. DEAE-cellulose					
MPR-I	1210	292	62.8	0.24	2.2
MPR-II	65.2	167	35.9	2.6	24
MPR-III	77.2	50	43.1	0.65	24
4. CM-cellulose					
MPR-I	63.7	149	32.0	2.3	21
5. Hydroxyapatite					
MPR-I	32.7	118	25.4	3.6	33
MPR-II	14.5	98	21.1	6.8	62
MPR-III	3.6	14	12.1	3.9	35
6. Gel filtration					
MPR-II	7.4	82	17.6	11.1	101
MPR-III	1.8	12	10.3	6.7	248

Purification of 3-mercaptopyruvic acid S-conjugate reductase from rat liver

All steps of the purification were carried out at 0–4°. Table 1 shows a typical example of purification of the reductases.

Step 1: preparation of rat liver cytosol. Male Sprague–Dawley rats (7 weeks old, N = 5) were killed by decapitation and the liver (60 g) was removed, minced with scissors, and homogenized in 2 volumes of 10 mM potassium phosphate buffer, pH 7.5, containing 1.15% KCl and 10 μM (4-amidinophenyl)-methanesulphonyl fluoride with a glass homogenizer with a teflon pestle (2000 rpm, 3 strokes). The homogenate was centrifuged at 10,000 *g* for 20 min. The pellet, in which the reductase activity did not exist, was discarded. The 10,000 *g* supernatant was subsequently centrifuged at 105,000 *g* for 60 min to separate cytosol and microsomal fractions. The microsomal fractions contained no reductase activity.

Step 2: ammonium sulphate fractionation. To 92 mL of the cytosol fraction was added 19.0 g of powdered ammonium sulphate to give 35% saturation. The mixture was stirred for 90 min followed by centrifugation at 20,000 *g* for 20 min. To the supernatant (92 mL) was added 25.6 g of powdered ammonium sulphate to give 75% saturation. After being stirred for 90 min, the suspension was centrifuged at 20,000 *g* for 20 min to precipitate the active enzyme pellet. The pellet was redissolved in 30 mL of 10 mM potassium phosphate buffer, pH 7.5. Ammonium sulphate in the solution was removed with a column (5 × 30 cm) of Sephadex G-25 previously equilibrated with the buffer.

Step 3: DEAE-cellulose. The ammonium sulphate fraction was applied to a DEAE-cellulose column (2.5 × 40 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.5. The column was first washed with 260 mL of the same buffer and proteins were subsequently eluted with a linear gradient of 0–200 mM KCl in 10 mM potassium phosphate buffer. Fractions of 8.4 mL were collected. NADH-dependent reductase activities were eluted in the void volume of the initial washing (tentatively designated as MPR-I) and at KCl concentrations of 70–80 mM (MPR-II), while NADPH-dependent activities gave a single peak at KCl concentrations of 50–70 mM (MPR-III) (Fig. 1). Fractions 11–18 (MPR-I), 57–60 (MPR-II) and 49–56 (MPR-III) were collected separately, concentrated to appropriate volumes (5–10 mL) with Diaflo ultrafiltration apparatus (PM-30, Amicon, U.S.A.), and desalted with a column of Sephadex G-25 equilibrated with 10 mM potassium phosphate buffer, pH 7.5.

The three reductases thus obtained were further purified by the following steps separately.

Step 4: CM-cellulose. The concentrated solution of MPR-I obtained in Step 3 was applied to a CM-cellulose column (1.5 × 42 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.5, and the column was washed with 154 mL of the same buffer. The enzyme was then eluted with a linear gradient of 0–100 mM KCl in 10 mM potassium phosphate buffer (360 mL) (Fig. 2). Active fractions (each 5.3 mL) 57–62 were collected, pooled, and concentrated with Diaflo ultrafiltration apparatus.

Step 5: hydroxyapatite. The three concentrated solutions obtained in Step 3 (MPR-II and MPR-III) and Step 4 (MPR-I) were applied separately to a hydroxyapatite column (1.5 × 20 cm) equilibrated with 10 mM potassium phosphate buffer, pH 6.8, and the columns were washed with the same buffer (about 130 mL for the MPR-I and 100 mL for the MPR-II and MPR-III). The enzymes were then eluted with a linear gradient of 10–400 mM (for MPR-I) or 10–200 mM (for MPR-II and MPR-III)

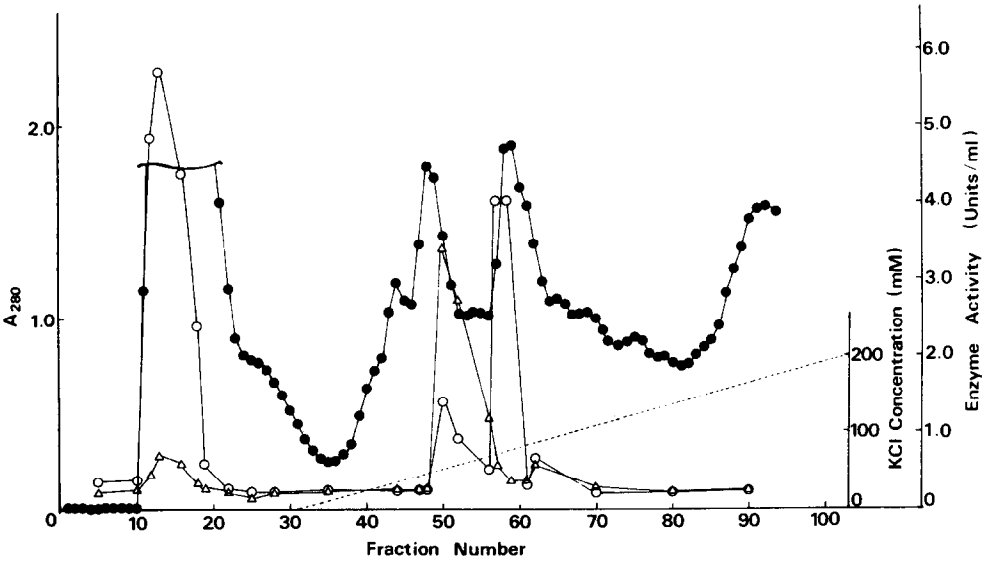

Fig. 1. DEAE-cellulose column chromatography (Step 3) in the purification of rat liver MPRs. The ammonium sulphate fraction was applied to a DEAE-cellulose column (2.5 × 40 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.5. The column was first washed with 260 mL of the buffer and proteins were subsequently eluted with a linear gradient of 0–200 mM KCl in the same buffer (----). Fractions of 8.4 mL were collected. ●——●, A_{280}; ○——○, NADH-dependent reductase activity; △——△, NADPH-dependent reductase activity.

potassium phosphate buffer, pH 6.8. Fractions (5.5 mL) were collected. MPR-I, MPR-II, and MPR-III gave a single active peak at buffer concentrations of 200–220, 90–105 and 75–85 mM, respectively (Fig. 3a–c). Each active fraction (fraction 48–51 for MPR-I, 43–47 for MPR-II and 37–39 for MPR-III) was pooled and concentrated with Diaflo ultrafiltration-apparatus. Active fractions of MPR-I were combined, sub-divided into small portions, and stored at −20° until use.

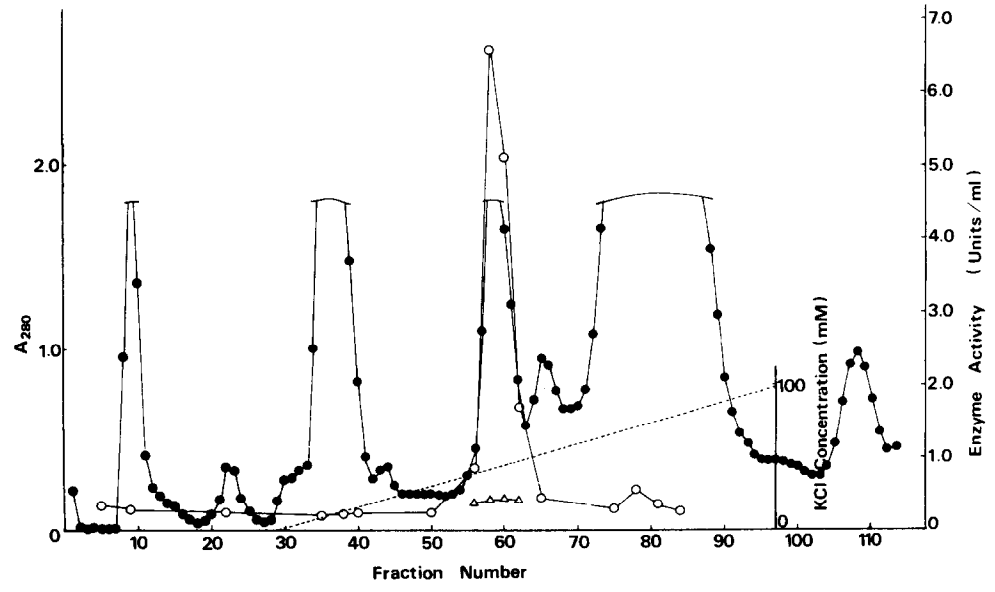

Fig. 2. CM-cellulose column chromatography (Step 4) in the purification of rat liver MPR-I. The concentrated solution of MPR-I obtained in Step 3 was applied to a CM-cellulose column (1.5 × 42 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.5, and the column was washed with 154 mL of the buffer. MPR-I was then eluted with a linear gradient of 0–100 mM KCl in the same buffer (----). Fractions of 5.3 mL were collected. ●——●, A_{280}; ○——○, NADH-dependent reductase activity; △——△, NADPH-dependent reductase activity.

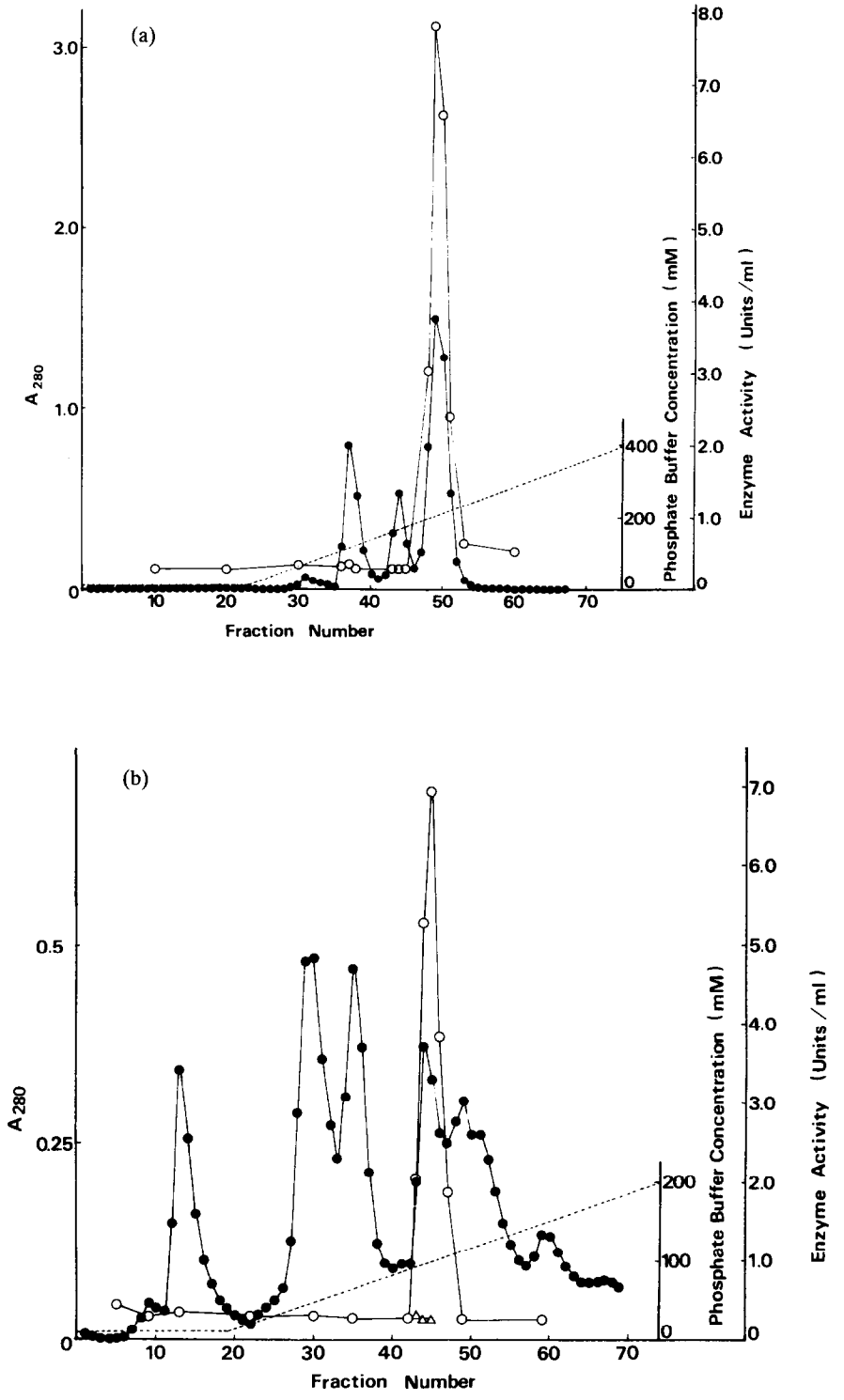

Fig. 3. Hydroxyapatite column chromatography (Step 5) in the purification of rat liver MPRs. The three concentrated solutions obtained in Step 3 (MPR-II and MPR-III) and Step 4 (MPR-I) were applied separately to a hydroxyapatite column (1.5 × 20 cm) equilibrated with 10 mM potassium phosphate buffer, pH 6.8, and the columns were washed with the buffer. MPRs were then eluted with a linear gradient of 10–400 mM (MPR-I) or 10–200 mM (MPR-II and MPR-III) potassium phosphate buffer, pH 6.8 (----). Fractions of 5.5 mL were collected. ●——●, A_{280}; ○——○, NADH-dependent reductase activity; △——△, NADPH-dependent reductase activity. (a) MPR-I; (b) MPR-II; (c) MPR-III.

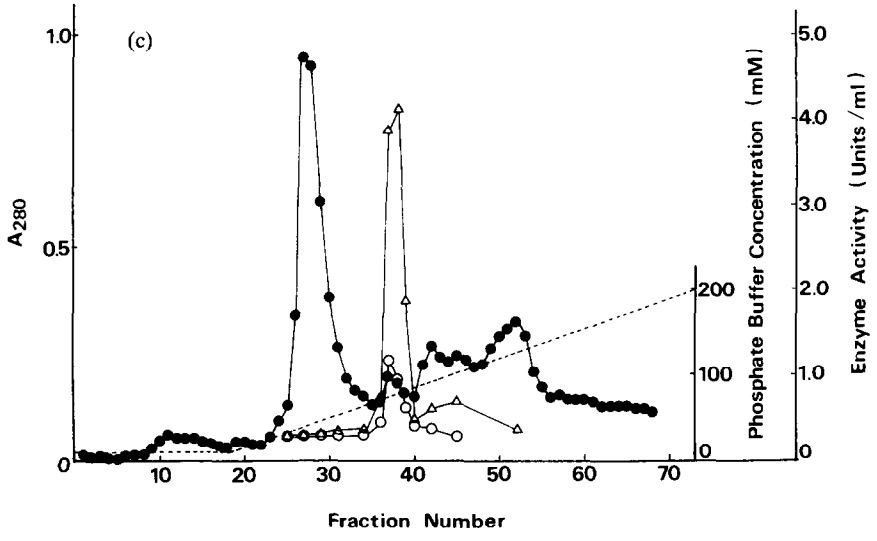

Fig. 3. Continued.

Step 6: gel filtration. Each solution for MPR-II and MPR-III obtained in Step 5 was applied in portions to HPLC equipped with a GPC column (TSK Gel G3000 SWXL, 7.8 × 500 mm, Tosoh). Each enzyme was eluted as a single peak with 200 mM potassium phosphate buffer, pH 7.5, at a flow rate of 0.5 mL/min. The elution profiles for MPR-II and MPR-III are shown in Fig. 4. Active fractions were combined, subdivided into small portions, and stored at −20° until use.

Identification of the product

S-(4-Bromophenyl)-3-thiopyruvic acid was incubated with the reductases according to the procedure described in *Assay methods*. After termination of the reaction, the medium was applied to HPLC under the conditions described in *Assay methods* except that the column was Capcell pak C_{18} (4.6 × 250 mm, Shiseido, Japan) and that the product was eluted with a mixture of methanol–water–acetic acid (60:40:1, by vol.). The product emerged at 8.9 min at a flow rate of 1.0 mL/min, was collected, pooled, concentrated under nitrogen gas stream, and extracted with ethylacetate at pH 1. The organic layer was washed with distilled water, dried over sodium sulphate anhydride, and concentrated under reduced pressure. To the residue was added an excess amount of etherial diazomethane at 0° followed by evaporation to dryness. The methylated product was subsequently subjected to GC–MS analysis with a high resolution mass spectrometer (JMS-DX-303, JEOL Co. Ltd, Japan) and a gas chromatograph (Model 5890, Hewlett Packard) equipped with a column (2 mm × 2 m) packed with 10% GE SF96 (Gas-chrom Q, 80–100 mesh). The column temperature was elevated from 150 to 240° at a rate of 20° per min. Helium (20 mL/min) was used as carrier gas.

RESULTS

GC–MS analysis of the metabolite

Methylated product of *S*-(4-bromophenyl)-3-thiolactic acid emerged at 11 min on the gas chromatograph under the conditions described in Materials and Methods. The mass spectra of the methylated product gave a molecular ion peak at *m/z* 290 ($C_{10}H_{11}O_3SBr$, isotope peak at *m/z* 292) and two main fragment peaks at *m/z* 272 ($M-H_2O$, isotope peak at *m/z* 274) and at *m/z* 201 ($M-CH(OH)CO_2CH_3$, base peak, isotope peak at *m/z* 203) (Fig. 5). The spectrum was identical with that of the authentic methyl *S*-(4-bromophenyl)-3-thiolactate.

Tissue distribution

Several tissues and caecal content of rats were homogenized in 2 volumes of potassium phosphate buffer (10 mM, pH 7.5) containing 1.15% KCl and centrifuged at 700 *g* for 10 min. A portion of the supernatant was subsequently passed through a short column of Sephadex G-25 which had been previously equilibrated with the same buffer. 3-Mercaptopyruvic acid S-conjugate reductase activities in the 700 *g* supernatant and its G-25-treated fraction—700 *g* supernatant (G-25)—were measured by HPLC as described in *Assay methods*, either in the presence or absence of NADH or NADPH (1 mM each) (Table 2).

The highest reductase activity was observed in the liver followed by testis, brain and kidney, while small intestinal mucosa and lung contained low activity. The activity was negligible in the blood and caecal contents, which were mostly composed of intestinal flora. None of the tissues showed the activity after removing the endogenous low molecular weight substances including NADPH and NADH, whereas the

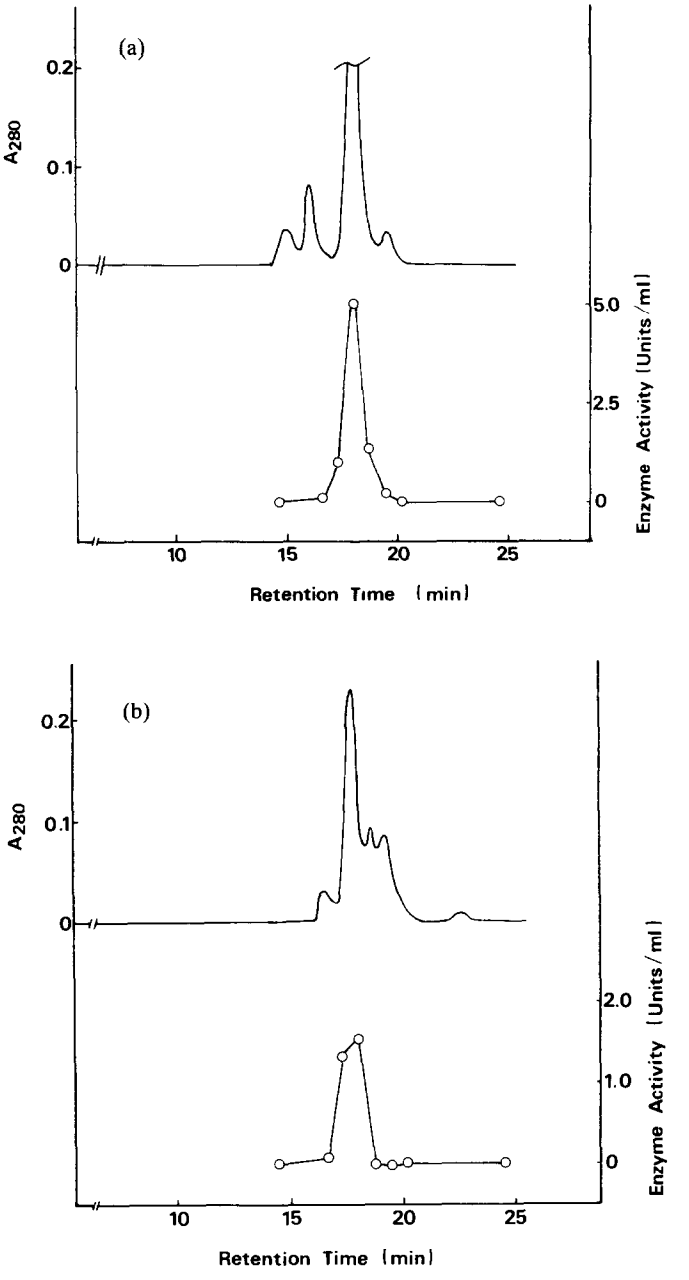

Fig. 4. Gel filtration column chromatography (Step 6) in the purification of rat liver MPRs. MPR-II and MPR-III solutions obtained in Step 5 were applied in portions to HPLC equipped with TSK Gel G3000 SWXL. Each enzyme was eluted with 200 mM potassium phosphate buffer, pH 7.5, at a flow rate of 0.5 mL/min. ——, A_{280}; O——O, reductase activity. (a) MPR-II; (b) MPR-III.

activity was remarkably enhanced by adding either NADH or NADPH at a concentration of 1 mM.

Purification

3-Mercaptopyruvic acid S-conjugate reductases, MPR-I, MPR-II and MPR-III, were purified from rat liver cytosol by the five- (MPR-I) or six- (MPR-II and MPR-III) step procedures (Table 1). The respective purification factors of the three reductases were 33, 101 and 248 over the cytosol. The purified MPR-I gave a single band in the SDS–PAGE, while MPR-II and MPR-III gave one major and one minor band (Fig. 6).

Stability and molecular weight

The enzymes from Step 5 (MPR-I) and Step 6 (MPR-II and MPR-III) were stable for at least 6 months during storage at −20°. Thermotreatment at 60° for 3 min resulted in 70% loss of the activities of the three reductases. The molecular weight of each enzyme was approximately 80 kDa, composed of two subunits with an identical molecular weight of about

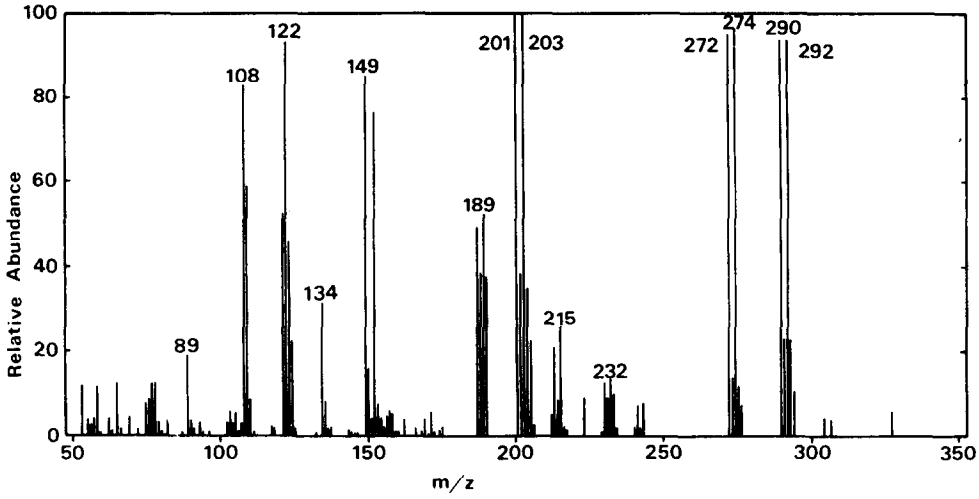

Fig. 5. GC–MS spectrum of methylated *S*-(4-bromophenyl)-3-thiolactic acid. *S*-(4-Bromophenyl)-3-thiopyruvic acid was incubated with MPR and the product formed was purified by HPLC according to the procedure described in Materials and Methods. The purified product was then methylated and was subsequently subjected to GC–MS analysis. Detailed conditions were described in Materials and Methods.

39 kDa as estimated by HPLC and SDS–polyacrylamide gel electrophoresis, respectively.

pH Optimum

The pH dependence of the reductase activity was examined in acetate buffer (pH 4.0–6.1) and Tris–acetate buffer (pH 5.8–8.2) with *S*-(4-bromophenyl)-3-thiopyruvic acid as substrate in the presence of NADH for MPR-I and MPR-II or NADPH for MPR-III (each 1 mM). Enzyme activity was optimal at around pH 6 for the three reductases.

Effect of various reagents on enzyme activity

3-Mercaptopyruvic acid S-conjugate reductase activity was assayed in the presence of iodoacetic acid and *N*-ethylmaleimide (sulphydryl-modifying agents), dithiothreitol (a thiol-protecting agent), and ethylenediamine-tetraacetic acid (EDTA, metal reagent) (each 10 mM). The sulphydryl-modifying agents inhibited the enzyme activities of all the three reductases, although the inhibitory effects were different in each reductase; MPR-I was most susceptible to these agents and virtually inactive in the presence of iodoacetic acid, while the activity of MPR-II and MPR-III was less susceptible to these agents. The results indicate that in the present enzymes, especially in MPR-I, thiol group(s) are essential to exert the activities. Dithiothreitol, EDTA, and bivalent metal ions (Ca^{2+} and Mg^{2+}) had no effect on the activity of these reductases.

Substrate specificity

Regarding the substrate specificity of a hydrogen donor, both MPR-I and MPR-II required NADH but not NADPH, and vice versa in MPR-III. Neither xanthine, 2-hydroxypyrimidine nor reduced glu-

Table 2. Relative activity of 3-mercaptopyruvic acid S-conjugate reductases in the rat

		700 *g* Supernatant (G-25)		
	700 *g* Supernatant	None	NADH (1 mM)	NADPH (1 mM)
Liver	100	4	790	910
Kidney	33	0	1340	780
Small intestinal mucosa	17	0	1670	1120
Brain	38	0	870	100
Lung	6	0	880	190
Testis	81	0	870	720
Blood	0	0	48	0
Caecal content	0	0	370	324

700 *g* Supernatant of tissue homogenate was passed through Sephadex G-25 column. Assay conditions were described in Materials and Methods. Values were the average from two experiments and were expressed in % relative activity compared to that in liver 700 *g* supernatant (0.01 unit/μL).

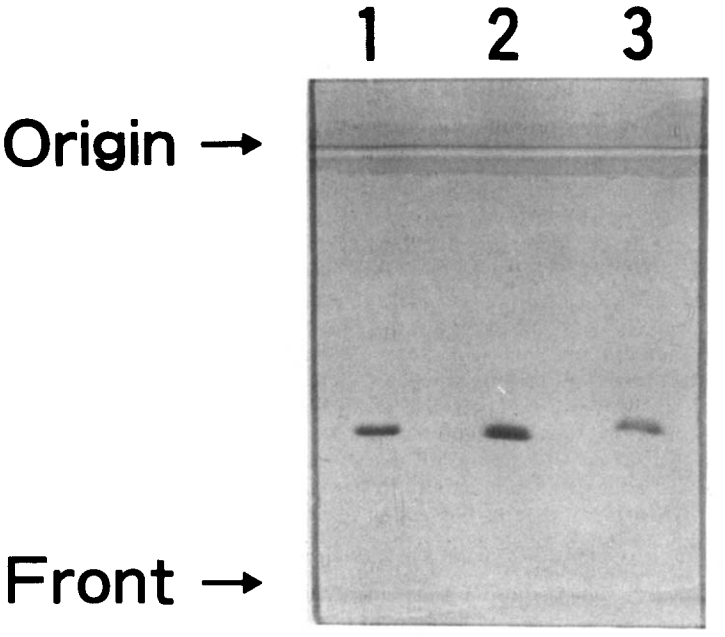

Fig. 6. Electrophoresis of purified MPR. MPR-I (Step 5), MPR-II (Step 6) and MPR-III (Step 6) were analysed for purity by SDS–polyacrylamide gel electrophoresis on a 12.5% slab gel. Proteins were stained with Phast Gel Blue R (Pharmacia). Lane 1, MPR-III; lanc 2, MPR-III; lanc 3, MPR-I.

Table 3. Substrate specificity of 3-mercaptopyruvic acid S-conjugate reductases

Substrate	Relative activity of MPR (%) I	II	III
S-(4-Bromophenyl)-3-thiopyruvic acid	100	100	100
S-Phenyl-3-thiopyruvic acid	172	160	38
S-Phenyl-3-thiopyruvic acid sulphoxide	ND	ND	29
S-Benzyl-3-thiopyruvic acid	25	41	ND
S-Methyl-3-thiopyruvic acid	30	ND	ND
S-Ethyl-3-thiopyruvic acid	58	ND	ND
S-Ethyl-3-thiopyruvic acid sulphoxide	ND	ND	37
S-(1-Propyl)-3-thiopyruvic acid	10	10	ND
S-(n-Butyl)-3-thiopyruvic acid	34	22	ND
S-(*tert*-Butyl)-3-thiopyruvic acid	ND	ND	ND
S-(2-Chloroethyl)-3-thiopyruvic acid	ND	ND	ND
S-(1,2-Dichlorovinyl)-3-thiopyruvic acid	69	126	31
S-Carboxymethyl-3-thiopyruvic acid	ND	ND	ND
Phenylpyruvic acid	75	105	ND
4-Hydroxyphenylpyruvic acid	16	103	ND
4-Hydroxy-3-iodophenylpyruvic acid	162	98	ND
4-Hydroxy-3,5-diiodophenylpyruvic acid	133	104	ND
Indole-3-pyruvic acid	10	68	ND
α-Keto-γ-methiolbutyric acid	54	ND	ND
α-Keto-γ-mercaptobutyric acid	16	ND	ND
Glyoxylic acid	124	ND	84
α-Ketoglutaric acid	14	ND	ND
Pyruvic acid	114	ND	ND
cis-Oxalacetic acid	57	82	ND
α-Ketobutyric acid	113	ND	ND

Step 5 (MPR-I) and Step 6 preparation (MPR-II and MPR-III) were used. Activity of each preparation (MPR-I, MPR-II and MPR-III) was 3.6, 11.1 and 6.7 unit/mg, respectively.

The following substances were inactive as substrates: imidazole-4-pyruvic acid, bis(2-oxo-2-carboxyethyl)sulphide, bis(2-oxo-2-carboxyethyl)disulphide, 3-mercaptopyruvic acid, 3,3′-methylenedithiobis(2-oxo-propanoic acid), α-ketoisocaproic acid, acetaldehyde, 3-nitrobenzaldehyde, phenylacetaldehyde, 4-chloroacetophenone, 4-nitroacetophenone, benzyl methyl ketone, and methylphenylpyruvate. Values were the average from three experiments.

ND, lower than the detection limit (<5%).

tathione worked as a hydrogen donor. K_m values of NADH or NADPH were 0.15 mM for the three reductases.

When *S*-(4-bromophenyl)-3-thiopyruvic acid was subjected to reduction by MPR-I, MPR-II and MPR-III, the corresponding α-hydroxy acid was stoichiometrically formed with disappearance of equimolar amounts of both the α-keto acid and the hydrogen donors. K_m values of *S*-(4-bromophenyl)-3-thiopyruvic acid were 0.38, 0.06 and 0.29 mM for MPR-I, MPR-II and MPR-III, respectively. The respective V_{max} values of MPR-I, MPR-II and MPR-III for this substrate were 5.3, 20 and 13 nmol/min/mg. Among the 3-mercaptopyruvic acid S-conjugates examined in the present study, the conjugates in which the aromatic ring or vinyl moiety is directly attached to the sulphur atom showed highest substrate activity with all three reductases (Table 3). MPR-I and MPR-II had a significant activity for some 3-mercaptopyruvic acid conjugates possessing aralkyl or alkyl group as the S-substituent, while MPR-III did not. It is notable that only MPR-III exerted its activity towards 3-mercaptopyruvic acid S-conjugate sulphoxides like *S*-ethyl- and *S*-phenyl-3-mercaptopyruvic acid sulphoxide (Table 3).

Since the present reductases showed relatively low substrate specificity for various kinds of 3-mercaptopyruvic acid S-conjugates, we examined, in the next step, whether these enzymes reduced endogenous α-keto acids generated from corresponding L-amino acids. Both MPR-I and MPR-II reduced various endogenous α-keto acids, but MPR-III only reduced glyoxylic acid among the endogenous α-keto acids (Table 3). When MPR-I and MPR-II were compared, MPR-I showed lower substrate specificity than MPR-II, i.e. in addition to some kinds of aromatic α-keto acids, the keto acids such as glyoxylic acid, α-keto glutaric acid, α-keto-γ-methiolbutyric acid, pyruvic acid, oxalacetic acid and α-keto butyric acid, acted as substrate for MPR-I, while MPR-II exerted its activity only towards aromatic α-keto acids and oxalacetic acid. The profile of this substrate specificity of MPR-II closely resembled that of aromatic α-keto acid reductase purified from rat kidney [24].

When simple aldehydes or ketones (e.g. acetaldehyde, 3-nitrobenzaldehyde, 4-chloroacetophenone, 4-nitroacetophenone or phenylacetaldehyde) were incubated with either of the three reductases, decrease in NADPH or NADH could not be detected to any extent. In addition, methylation of the carboxylic acid group or its lack of α-keto acids like benzyl methyl ketone abolished the substrate activity. These results together indicate that the existence of a carboxylic acid moiety probably at the adjacent position of carbonyl carbon is essential as a substrate for the present reductases.

DISCUSSION

In the tissue distribution study in the rat, we found that enzyme activities to reduce *S*-(4-bromophenyl)-3-thiopyruvic acid to the corresponding α-hydroxy acid were largely distributed in the liver. Together with our previous observation that the liver is an important organ for transamination of cysteine conjugates [8], this finding prompted us to use rat liver as the enzyme source for purification of the reductases. By the conventional method three reductases were isolated and tentatively designated as 3-mercaptopyruvic acid S-conjugate reductase I, II and III (MPR-I, MPR-II and MPR-III).

Although these purified reductases had the same molecular weight of approximately 80 kDa composed of two subunits (39 kDa), several distinct properties were observed among them: (a) MPR-I was retained on CM-cellulose but not on DEAE-cellulose and vice versa for MPR-II and MPR-III, the results suggesting that MPR-I was positively charged in the buffer at neutral pH, while MPR-II and MPR-III were negatively charged; (b) both MPR-I and MPR-II required NADH but not NADPH as cosubstrate, but NADPH served as cosubstrate in the case of MPR-III; and (c) MPR-I and MPR-II reduced carbonyl moiety of not only 3-mercaptopyruvic acid S-conjugates but also endogenous α-keto acids, while MPR-III exerted its activity only towards the S-conjugates and glyoxylic acid (Table 2).

Conspicuous and common property of the present three reductases was observed in the substrate specificity. All the carbonyl compounds, which exerted substrate activity towards MPR-I, MPR-II and MPR-III, possessed α-keto carboxylic acid moiety in the structure; neither simple aldehyde nor ketone (e.g. acetaldehyde, phenylacetaldehyde, 3-nitrobenzaldehyde, 4-nitroacetophenone or 4-chloroacetophenone) was reduced to the corresponding alcohol in the presence of either NADPH or NADH. In addition to this property, the requirement for NADH or NADPH as hydrogen donors and a molecular weight of about 80 kDa are useful to identify the present enzymes with carbonyl carbon reductases hitherto reported. Various kinds of NADPH- or NADH-linked carbonyl carbon reductases have been extensively studied and many of them were purified from mammalian tissues by many investigators. However, there are no reports on reductases with similar properties to MPR-I and MPR-III, the result indicating these two reductases are hitherto unknown enzymes. On the other hand, aromatic α-keto acid reductase isolated from the rat kidney by Nakano *et al.* [24] is very close to MPR-II in terms of its substrate specificity, cosubstrate requirement, inhibitory effect of thiol reagents, and molecular weight, although the molecular weights of the renal aromatic α-keto acid reductase and MPR-II were different, i.e. 71 and 80 kDa, respectively. Thus, the aromatic α-keto acid reductase was reported to show high activity towards α-keto carboxylic acids derived from aromatic L-amino acids such as 3-iodo-L-tyrosine, 3,3′-diiodo-L-tyrosine, L-tyrosine, L-phenylalanine and L-tryptophane as was observed for MPR-II. Based on these observations, we have assumed MPR-II and aromatic α-keto acid reductase were the same enzymes.

Up to now some kinds of xenobiotics were reported to be excreted as 3-mercaptopyruvic acid S-conjugates or 3-mercaptolactic acid S-conjugates or both [14–19]. Together with our previous report concerning characterization of transaminases for cysteine conjugates [8], the present study firstly provides

evidence for the formation mechanism of such S-conjugates; cysteine conjugates are the precursor of those conjugates and the transaminases and present reductases play important roles in this metabolic pathway.

While biological significance of the present enzymes is obscure at present, it is probable that the significance partly lies in maintaining endogenous α-keto acids at physiological levels. Higher levels of some α-keto acids (e.g. phenylpyruvic acid, 4-hydroxyphenyl pyruvic acid, α-keto-γ-methiolbutyric acid, and α-keto-γ-mercaptobutyric acid) are likely to exert certain kinds of toxicity [25]; indeed in diseases which are associated with accumulation of corresponding amino acids, significant amounts of these α-keto acids were also found to form and to be excreted into urine of such patients [26]. Additionally, Havass *et al.* stated that administration of phenylpyruvic acid caused memory impairment in newborn rats [27]. Thus, the present enzymes are likely to be essential to protect an organism from the toxicity of such α-keto acids.

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