Purification and characterization of human hepatic cysteine-conjugate β -lyase

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Cysteine-conjugate β -lyase (EC 4.4.1.13) was purified about 880-fold from human liver obtained *post mortem*. The purification procedure included $(\text{NH}_4)_2\text{SO}_4$ precipitation, chromatography on DEAE-cellulose and hydroxyapatite, gel filtration on Sephadex G-200, and chromatofocusing. The purified enzyme cleaves the C–S bond of several *S*-aryl-L-cysteines to yield equimolar amounts of thiols, pyruvic acid and ammonia via an $\alpha\beta$ -elimination reaction. The M_r of the enzyme was estimated to be 88 000 by gel filtration. The enzyme is thermolabile, has a pH optimum of 8.5, and an apparent K_m of 0.7 mM towards *S*-(*p*-bromophenyl)-L-cysteine. The enzyme requires pyridoxal 5'-phosphate as a cofactor, and hence the enzyme activity was completely abolished by hydroxylamine. No effect of EDTA or thiol-blocking reagents was observed on the activity of the enzyme.

INTRODUCTION

In various mammals a number of xenobiotics (including drugs, herbicides, aromatic hydrocarbons and environmental contaminants) that do not originally contain a sulphur atom are biotransformed into metabolites bearing the methylthio group (Tateishi & Shimizu, 1980; Stillwell, 1981; Tateishi, 1983; Tateishi *et al.*, 1984; Jakoby *et al.*, 1984).

From investigations in our laboratory (Tateishi *et al.*, 1978*a,b*; Suzuki *et al.*, 1983; Tomisawa *et al.*, 1984) and elsewhere (Bakke *et al.*, 1980; Stevens & Jakoby, 1983), it is now clear that formation of methylthio metabolites from cysteine conjugates, which are derived from GSH conjugates by a two-step hydrolysis (Tate, 1980) or from mercapturic acids by *N*-deacetylation (Suzuki & Tateishi, 1981), takes place by the two-step enzymic reaction shown in Scheme 1: the first step is mediated by cysteine-conjugate β -lyase (EC 4.4.1.13), which converts cysteine conjugates into equimolar amounts of corresponding thiols, ammonia and pyruvic acid via a C–S-bond-cleavage reaction (Tateishi *et al.*, 1978*b*; Tomisawa *et al.*, 1984). The second step is a methylation of the thiols by means of *S*-methyltransferase(s) in the presence of *S*-adenosylmethionine as a methyl donor (Tateishi *et al.*, 1978*b*; Weisiger & Jakoby, 1979, 1980).

The biological significance of the GSH conjugation reaction and its subsequent reactions leading to mercapturic acids has long been considered, at least in the field of drug metabolism, to lie in polarization and detoxication

of numerous xenobiotics. Our demonstrations (Tateishi *et al.*, 1978*a,b*; Tateishi & Shimizu, 1980; Tomisawa *et al.*, 1984) of this new pathway (where polar conjugates are converted into non-polar metabolites) gave a novel aspect in that the conjugation with GSH or cysteine is not a mere detoxication or polarization reaction, at least in some kinds of compounds. Thus, from a toxicological point of view, the thiol compounds generated by the action of cysteine-conjugate β -lyase are of great importance. The thiol compounds are highly reactive and become covalently bound to cellular macromolecules to cause cell injury (Elfarra & Anders, 1984). On the other hand, since several endogenous compounds such as 2-hydroxyoestrone and oestrol (Numazawa *et al.*, 1974), 3,4-dihydroxyphenylalanine (Prota *et al.*, 1977) and leukotrienes (Hammarström *et al.*, 1979) have been shown to be subject to conjugation with GSH, it is quite plausible that biologically active thiol- or methylthio-containing compounds are generated from such conjugates by the action of the β -lyase.

For these toxicological and biological reasons, much attention has now been paid to cysteine-conjugate β -lyase. Although extensive studies have been performed on the β -lyase in rat liver (Tateishi *et al.*, 1978*b*; Tateishi & Shimizu, 1980; Stevens & Jakoby, 1983) and in *Fusobacterium varium*, populating mammalian gastrointestinal tract (Tomisawa *et al.*, 1984), and other intestinal micro-organisms (Larsen, 1985), little has been reported concerning the human enzyme. Our previous results with crude preparations of human origin (Tateishi & Shimizu,

Scheme 1. Pathway of formation of methylthio metabolites from cysteine conjugates

Abbreviation used: BrPh, *S*-(*p*-bromophenyl).

1980; Tateishi, 1983) did not permit a clear assignment of production of thiols to cysteine-conjugate β -lyase.

In the present study we describe the purification and characterization of human hepatic cysteine-conjugate β -lyase.

EXPERIMENTAL

Materials

BrPh-L-cysteine, BrPh-D-cysteine, BrPh-N-acetyl-cysteine and BrPh-GSH were prepared by the method of Saunders (1934). BrPh-cysteamine was synthesized by a reaction of diazotized *p*-bromoaniline and cuprous mercaptide with cysteamine (Parke & Williams, 1951). BrPh-thiopyruvic acid was prepared by a reaction of BrPh-SH with 3-fluoropyruvic acid (Aldrich Chemical Co.) by the procedure described by Parrod (1944). *S*-(2-Naphthyl)-DL-cysteine was obtained by the reaction of naphthalene-2-thiol with DL-chloroalanine (Aldrich Chemical Co.) by the method described by Ishikawa *et al.* (1973). *S*-(1-Propyl)-L-cysteine, *S*-(2-propyl)-L-cysteine, *S*-(1-butyl)-L-cysteine, *S*-(*t*-butyl)-L-cysteine, *S*-(1-hexyl)-L-cysteine and *S*-cyclohexyl-L-cysteine were synthesized by a reaction of L-cysteine with the corresponding alkyl iodides (Tokyo Kasei Kogyo Co., Tokyo, Japan) by the method described by us previously (Tomisawa *et al.*, 1984). *S*-Ethyl-L-cysteine sulfoxide and *S*-phenyl-L-cysteine sulfoxide were prepared by the oxidation of corresponding cysteine conjugates with H_2O_2 (Ishikawa *et al.*, 1973). *S*-Methyl-L-cysteine, *S*-ethyl-L-cysteine, *S*-benzyl-L-cysteine, DL-lanthionine, *S*-phenyl-L-cysteine and L-cystathionine were obtained from Tokyo Kasei Kogyo Co. BrPh-SH and *p*-fluorobenzenethiol were purchased from Aldrich Chemical Co. Bovine serum albumin (crystallized and freeze-dried) was a product from Sigma Chemical Co. All other amino acids and reagents used in the present study were purchased commercially and were of analytical special grade. DEAE-cellulose (DE-52) was obtained from Whatman, hydroxyapatite was from Bio-Rad Laboratories, and Sephadex G-200, Polybuffer exchanger and Polybuffers were from Pharmacia Fine Chemicals.

G.l.c.-m.s. analysis

The mass spectra were obtained with a JEOL DX-300 high-resolution mass spectrometer coupled to a Hewlett Packard M5710A gas chromatograph. The gas chromatograph was equipped with a glass column (2 mm $\times$ 2 m) packed with 10% GE SF96 (Gas-Chrom Q, 80–100 mesh). The oven temperature was programmed to rise to 180 °C at a rate of 30 °C/min with an initial setting at 100 °C. The temperatures of the injection port and chamber heater were kept at 210 °C and 200 °C respectively. Helium was used as carrier gas at a flow rate of 40 ml/min. The ionizing energy was 20 eV.

Polyacrylamide-gel electrophoresis

Proteins were analysed by polyacrylamide-gel electrophoresis on 7% polyacrylamide slab gels (1 mm thick) by the procedure described by Davis (1964). A constant voltage of 75 mV was applied until the tracking dye reached the bottom of the slab (about 24 h). Proteins were stained with Coomassie Brilliant Blue R.

Assay methods

Assay of cysteine-conjugate β -lyase. For a routine assay of C-S-bond lyase activity, BrPh-cysteine was used as substrate and the amount of BrPh-SH formed was determined by h.p.l.c. by the method described by Tomisawa *et al.* (1984). The assay mixture contained, in a final volume of 0.40 ml: BrPh-cysteine, 0.20 μ mol; dithiothreitol, 2 μ mol; pyridoxal 5'-phosphate, 0.02 μ mol; Tris/HCl buffer, pH 8.5, 40 μ mol; enzyme solution (0.01–0.175 ml). The reaction mixture was incubated at 37 °C for 1 h under anaerobic conditions. The incubation was terminated by addition of 0.4 ml of acetonitrile containing *p*-fluorobenzenethiol (60 μ g) as internal standard.

A unit of the C-S-bond lyase activity was defined as the quantity of enzyme catalysing the formation of 1 μ mol of the thiol compound/min under the conditions of the assay. The reaction was linear with respect to time (0–90 min) and protein concentration (0–113 μ g).

Assay of α -oxo acid. A spectrophotometric method with 3-methyl-2-benzothiazolinone hydrazone hydrochloride was used for the quantitative determination of α -oxo acid by the procedure of Soda (1967).

Assay of anthranilic acid. Kynureninase activity was determined with kynurenine as a substrate. Anthranilic acid was assayed by h.p.l.c. equipped with a reversed-phase column (AM-312 ODS; 6 mm $\times$ 150 mm; Yamamura Chemical Laboratories, Osaka, Japan). The acid emerged at 3.8 min (at a flow rate of 1.5 ml/min) by elution with 30% (v/v) methanol in 5 mM-potassium phosphate buffer, pH 5.0.

Assay of ammonia. Ammonia in reaction mixtures was determined by the method of Levitzki (1970).

Determination of protein. Protein content was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Determination of M_r by gel filtration on h.p.l.c.

The apparent M_r was determined by h.p.l.c. with a gel-filtration column (Asahi-pak GS-510; Asahi Kasei Kogyo, Tokyo, Japan). The standards used were: bovine thyroglobulin (M_r 670000), bovine γ -globulin (M_r 158000), fructose-bisphosphate aldolase (M_r 158000), bovine serum albumin (M_r 68000), ovalbumin (M_r 44000), chymotrypsinogen A (M_r 25000) and horse myoglobin (M_r 17000).

Purification of human hepatic cysteine-conjugate β -lyase

Liver was obtained from the cadaver of a Japanese male (48 years old) who died in a traffic accident. Permission was granted by next of kin for removal of the liver for investigational purposes. The liver was removed within 18 h after death, sliced and stored at –80 °C. All steps of the purification were carried out at 0–4 °C. The purification of the enzyme is shown in Table 1.

Step 1: preparation of 105000 g supernatant. The frozen human liver slices (696 g) were rinsed and thawed in 1400 ml of ice-chilled 200 mM-potassium phosphate buffer, pH 7.4, containing 0.5 mM-dithiothreitol. The

Table 1. Purification of cysteine-conjugate β -lyase from human liver

For experimental details see the text.

Step	Volume (ml)	Total protein (mg)	Total activity (munits)	Specific activity (munits/mg)	Purification (fold)	Yield (%)
1. Cytosol	1030	54400	762	0.014	1	100
2. $(\text{NH}_4)_2\text{SO}_4$ fractionation	200	14400	844	0.058	4.1	111
3. Negative absorption on DEAE-cellulose	460	11000	925	0.084	6.0	121
4. Hydroxyapatite	150	1520	597	0.392	28	78
5. Sephadex G-200 I	123	606	533	0.880	63	70
Sephadex G-200 II	50	285	468	1.64	117	61
6. Chromatofocusing	52	13	157	12.3	876	21

liver was minced with scissors and homogenized with a mechanical homogenizer (Polytron; Kinematica) for about 5 min at 4 °C. The homogenate was centrifuged at 9000 g for 20 min. The supernatant fluid was subsequently centrifuged at 105 000 g for 60 min to separate supernatant (cytosol) and microsomal fractions. The microsomal fraction contained virtually no cysteine-conjugate β -lyase activity.

Step 2: $(\text{NH}_4)_2\text{SO}_4$ fractionation. To 1030 ml of the cytosol fraction was added 250 g of powdered $(\text{NH}_4)_2\text{SO}_4$ to give 40% saturation. The mixture was stirred for 90 min, followed by centrifugation at 20 000 g for 30 min. To the supernatant (1100 ml) was added 145 g of powdered $(\text{NH}_4)_2\text{SO}_4$ to give 60% saturation. After being stirred for 90 min, the suspension was centrifuged at 20 000 g for 30 min to precipitate the active enzyme pellet. The pellet was redissolved in 200 ml of 200 mM-potassium phosphate buffer, pH 7.4, containing 0.5 mM-dithiothreitol. $(\text{NH}_4)_2\text{SO}_4$ in the solution was removed with a column (4 cm $\times$ 25 cm) of Sephadex G-25 previously equilibrated with the same buffer.

Step 3: DEAE-cellulose. The $(\text{NH}_4)_2\text{SO}_4$ fraction was applied to a DEAE-cellulose column (5.0 cm $\times$ 25 cm) equilibrated with 200 mM-potassium phosphate buffer, pH 7.4, containing 0.5 mM-dithiothreitol. Proteins were eluted with the same buffer at a flow rate of 1 ml/min. The first 306 ml of the eluate was discarded; the subsequent 460 ml was collected and concentrated to 40 ml with Diaflo ultrafiltration apparatus (PM 10, Amicon).

Step 4: hydroxyapatite. To the concentrated solution obtained in Step 3 was added 120 ml of distilled water containing 0.5 mM-dithiothreitol. The solution was applied to a hydroxyapatite column (2.6 cm $\times$ 37 cm) equilibrated with 50 mM-potassium phosphate buffer, pH 7.4, containing 0.5 mM-dithiothreitol and first washed with 200 ml of the same buffer. The enzyme was then eluted at a flow rate of 0.7 ml/min with a linear gradient of 50–400 mM-potassium phosphate buffer, pH 7.4. Fractions (15 ml) were collected in fraction tubes, each containing 100 μ l of an aqueous solution of pyridoxal 5'-phosphate (0.5 μ mol) for stabilization of the enzyme. The enzyme activity emerged at about 150–220 mM-potassium phosphate buffer (Fig. 1). Fractions 38–47 were pooled and concentrated to 20 ml with a Diaflo membrane (PM-10; Amicon) as described above.

Fig. 1. Hydroxyapatite column chromatography (Step 4) in the purification of human hepatic cysteine-conjugate β -lyase

The concentrated β -lyase fraction (40 ml) from the negative absorption on the DEAE-cellulose column (see the text) was diluted with an aqueous solution (120 ml) of 0.5 mM-dithiothreitol and was applied to a hydroxyapatite column (2.6 cm $\times$ 37 cm) equilibrated with 50 mM-potassium phosphate buffer, pH 7.4, containing 0.5 mM-dithiothreitol. After the first washing with 200 ml of the buffer, the enzyme was eluted with a linear gradient of 50–400 mM-potassium phosphate buffer, pH 7.4 (---), at a flow rate of 0.7 ml/min; 15 ml fractions were collected. $\circ$, A_{280} ; $\bullet$, enzyme activity.

Step 5: gel filtration. One half (10 ml) of the solution obtained from the hydroxyapatite column chromatography was applied to a Sephadex G-200 column (2.6 cm $\times$ 81 cm) previously equilibrated with 200 mM-potassium phosphate buffer, pH 7.4. Elution was effected with the same buffer at a flow rate of 0.07 ml/min

Fig. 2. Gel filtration I (Step 5)

A 10 ml portion of the concentrated enzyme solution (fractions 38–47 at Step 4; see the text) was applied to a Sephadex G-200 column (2.6 cm $\times$ 81 cm). The column was previously equilibrated and then eluted with 200 mM-potassium phosphate buffer, pH 7.4, containing 0.5 mM-dithiothreitol at a flow rate of 0.07 ml/min; 5.1 ml fractions were collected. $\circ$, A_{280} ; $\bullet$, enzyme activity.

(Fig. 2). Fractions (5.1 ml each) 45–56 were combined and concentrated to about 10 ml with a Diaflo membrane (PM 10). The other half of the hydroxyapatite fraction was subjected to gel filtration in the same manner. Both concentrated fractions were combined and then subjected to a second Sephadex G-200 column under the same conditions. Upon elution with the buffer (0.04 ml/min), the initial 230 ml of the eluate was discarded and the following 50 ml (fractions 47–56) collected.

Step 6: chromatofocusing. A column of Polybuffer exchanger PBE 94 (1.5 cm $\times$ 14.2 cm) (Pharmacia) was equilibrated with 25 mM-Tris/acetate buffer, pH 8.5. A portion (12 ml) of the sample obtained by gel filtration (Step 5) was introduced after first running off 5 ml of the eluent, Polybuffer 96 plus Polybuffer 74 (7:3, v/v) diluted with distilled water 1:10 and adjusted to pH 5.0 with acetic acid. The elution was continued at a flow rate of 0.3 ml/min, and 2.2 ml of the sample was collected. The enzyme activity emerged from fractions 110–115 (corresponding to pH 5.7–5.5) (Fig. 3). After concentration with a Diaflo membrane, the buffer was replaced with 200 mM-Tris/HCl buffer, pH 8.5, by gel filtration with a short column of Sephadex G-25. The enzyme in the remaining portion (38 ml) obtained in Step 5 was purified by the same procedure on a larger scale.

RESULTS

Purification of human hepatic cysteine-conjugate β -lyase

The human hepatic cysteine-conjugate β -lyase was purified 876-fold over the 105000 g supernatant by the six-step procedure, with an overall yield of 21% (Table 1). Polyacrylamide-gel-electrophoretic monitoring of the enzyme preparation at several steps of the purification procedure gave evidence of the stepwise purification: the

Fig. 3. Chromatofocusing (Step 6) in the purification of human hepatic cysteine-conjugate β -lyase

A 12 ml portion of the β -lyase fraction from Step 5 (see the text) was introduced to a column (1.5 cm $\times$ 14.2 cm) of Polybuffer exchanger PBE 94 after first running off 5 ml of the eluent. The column was previously equilibrated with 25 mM-Tris/acetate buffer, pH 8.5. Elution was performed with Polybuffer 96 plus Polybuffer 74 (7:3, v/v) diluted 1:10 with distilled water and adjusted to pH 5.0 with acetic acid at a flow rate of 0.3 ml/min (...., pH). The enzyme emerged at fractions 110–115 (2.2 ml fractions). —, A_{280} ; $\bullet$, enzyme activity.

Fig. 4. Semi-logarithmic plot of M_r against retention time on h.p.l.c. of human hepatic cysteine-conjugate β -lyase

The purified enzyme from Step 6 (see the text) was injected into a gel-filtration column (Asahi-pak GS-510; 76 mm $\times$ 50 cm), which was calibrated with bovine thyroglobulin (A), bovine γ -globulin (B), fructose-bisphosphate aldolase (C), bovine serum albumin (D), ovalbumin (E), chymotrypsinogen A (F) and horse myoglobin (G). Proteins were eluted isocratically with 100 mM-potassium phosphate buffer, pH 7.4, containing 0.5 mM-dithiothreitol at a flow rate of 1.0 ml/min. The elution point of the β -lyase is indicated (O). Each value indicates the mean $\pm$ S.E.M. of three experiments.

enzyme solution at step 6 gave one main and one minor band. During storage of the purified enzyme for 3 months at -20°C , the enzyme activity did not decrease to any significant extent. The M_r of the β -lyase was approx. 88000 as determined by h.p.l.c. (Fig. 4).

Identification of BrPh-SH

BrPh-cysteine (2 μmol) was incubated with the enzyme from Step 6. Incubation conditions were the same as those used for the enzyme assay, except that the scale was 5 times as large. Thiol compound in the reaction mixture was extracted with 4 ml of diethyl ether containing 0.2 ml of acetonitrile. After evaporation of ether, an excess amount of ethereal diazomethane was added to the residue, and the mixture was kept at room temperature for 5 min for methylation of the thiol group. After evaporation of diazomethane, a portion of the solution was injected into the g.l.c.-mass spectrometer. BrPh-SMe appeared at 5.0 min under the conditions described in the Experimental section. The mass spectrum of BrPh-SMe showed a molecular-ion peak at m/z 202 (M^+ , base peak) with its isotope peak at m/z 204, m/z 187 ($M^+ - \text{CH}_3$, intensity relative to the base peak 23%, isotope peak at m/z 189), m/z 169 ($M^+ - \text{SH}$, 10%, isotope peak at m/z 171), m/z 156 ($M^+ - \text{SCH}_2$, 8%, isotope peak at m/z 158), m/z 123 ($M^+ - \text{Br}$, 13%) and m/z 108 ($M^+ - \text{Br} - \text{CH}_3$, 9%). The fragmentation pattern was identical with that of the authentic sample.

In a control incubation mixture, from which the

enzyme was omitted, formation of the thiol compound was not observed.

Stoichiometry study

BrPh-[U- ^{14}C]cysteine (0.2 μCi , 2 μmol) was incubated with Step-6 enzyme in 2 ml of 200 mM-potassium phosphate buffer, pH 8.5, containing 0.5 mM-dithiothreitol. Formation of the thiol and ammonia was quantitatively determined in portions of the incubated mixture by the method described in the Experimental section. Identification and quantitative analysis of the [^{14}C]pyruvic acid formed were performed by the procedure described by Tomisawa *et al.* (1984). The relative rates of formation of the three products (i.e. BrPh-SH, pyruvic acid and ammonia) on a molar basis were 1.0:0.98:0.96, thus indicating equimolar formation of the three products. Neither [^{14}C]serine nor [^{14}C]alanine nor [^{14}C]BrPh-thiopyruvic acid was detectable in the reaction mixture.

pH optimum and kinetic studies

The pH profile of cysteine-conjugate β -lyase activity was examined with BrPh-cysteine as substrate over the pH range 6.0–9.0 by using 200 mM-potassium phosphate buffer, pH 6.0–7.0, and 200 mM-Tris/HCl buffer, pH 7.0–9.0. Activity of the enzyme was optimal at about pH 8.5 and virtually absent at pH 6.0. The apparent K_m was 0.7 mM, as determined from a Lineweaver–Burk plot (Fig. 5).

pH-stability and thermostability

The pH-stability was determined at 4°C in 140 mM-sodium barbital/sodium acetate buffer in the pH range 3.2–8.9. Most (over 95%) of the initial activity was retained after 20 h in the pH range 5.3–8.9, but 70 and 20% of the initial activity was lost at pH 3.2 and 4.3 respectively.

The activity was completely abolished by thermal treatment at 60°C for 2 min. This thermolability of human hepatic enzyme was quite different from that of the rat enzyme (Tateishi *et al.*, 1978b).

Fig. 5. Lineweaver–Burk plot showing the effect of substrate concentration on human hepatic cysteine-conjugate β -lyase activity

BrPh-cysteine at various concentrations was used as substrate under the assay conditions described in the text. Each value indicates the mean $\pm$ S.E.M. of three experiments.

Effect of pyridoxal 5'-phosphate and other reagents on enzyme activity

Pyridoxal 5'-phosphate stimulated the activity of the purified enzyme by 100% at a concentration of 0.01 mM and by 130% at 0.05–1.0 mM. Preincubation of the enzyme in the absence of pyridoxal 5'-phosphate at 37 °C for 3 h showed a slow but steady decrease of the activity (30%), whereas no significant loss was observed in the presence of 1 mM-pyridoxal 5'-phosphate under the same incubation conditions. This result suggests that pyridoxal 5'-phosphate is of importance for both activation and stabilization of the enzyme.

Cysteine-conjugate β -lyase was preincubated (for 30 min before the enzyme assay) with iodoacetic acid, KCN and hydroxylamine (each 1 mM), each in the presence or in the absence of pyridoxal 5'-phosphate. The activity was inhibited completely by hydroxylamine and partially (about 30%) by KCN, whereas almost all the activity (97%) was retained after treatment with iodoacetic acid. EDTA and bivalent metal ions such as Ca^{2+} , Zn^{2+} , Mg^{2+} and Fe^{2+} (at 1 mM) showed neither inhibitory nor stimulatory effect on the activity of this enzyme, though Co^{2+} (1 mM) inhibited 50% of the activity.

Substrate specificity

The enzyme activity towards sulphur-containing conjugates (i.e. cysteine, GSH and *N*-acetylcysteine conjugate) of *p*-bromobenzene and towards BrPh-thiopyruvic acid was determined by the assay of BrPh-SH as described in the Experimental section. With regard to kynurenine, formation of anthranilic acid was examined by h.p.l.c. For other substrates, formation of pyruvic acid or α -oxo acid was assayed.

Table 2 shows the relative activity of human hepatic cysteine-conjugate β -lyase towards various *S*-substituted cysteines or its derivatives as well as several amino acids. The human hepatic β -lyase cleaved a C–S bond of cysteine conjugates, but not of BrPh-thiopyruvic acid, BrPh-cysteamine, BrPh-*N*-acetylcysteine or BrPh-GSH. This result indicates that both free amino and carboxy groups on the cysteinyl moiety are essential for the enzyme activity. The human hepatic β -lyase exhibited the highest activity towards the sulfoxide derivative of *S*-phenyl-L-cysteine conjugate, followed by *S*-aryl-L-cysteines. The substrate activity of cysteine conjugates possessing alkyl and aralkyl groups as *S*-substituents was virtually negligible. L-Methionine, DL-lanthionine, L-cystathionine, L-homoserine, L-kynurenine and L-cysteine did not serve as substrates of the human cysteine-conjugate β -lyase.

DISCUSSION

Our present results clearly demonstrate that most of the C–S bond-cleavage activity towards *S*-arylcysteine conjugates in human liver is attributable to the β -lyase purified in the present study. The β -lyase cleaves the C–S bond of *S*-aryl-L-cysteine conjugates according to the

Table 2. Substrate specificity of human hepatic cysteine-conjugate β -lyase

Formation of anthranilic acid was measured by h.p.l.c. for L-kynurenine. For other substrates, pyruvic acid or other α -oxo acid was assayed. Each substrate was incubated at 0.5 mM concentration under the standard incubation conditions.

Substrate	Relative activity
BrPh-cysteine*	100
BrPh-D-cysteine*	0
BrPh- <i>N</i> -acetylcysteine*	0
BrPh-thiopyruvic acid*	0
BrPh-glutathione*	0
BrPh-cysteamine*	0
<i>S</i> -Phenyl-L-cysteine	30
<i>S</i> -Phenyl-L-cysteine sulfoxide	260
<i>S</i> -Naphthyl-DL-cysteine	16
<i>S</i> -Benzyl-L-cysteine	0
<i>S</i> -Alkyl-L-cysteines†	0
L-Cysteine	0
DL-Lanthionine	0
L-Methionine	0
L-Homoserine	0
L-Cystathionine	0
L-Kynurenine	0

* The enzyme activities towards these substrates were determined by the h.p.l.c. assay of BrPh-SH as described in the text.

† Alkyl groups in *S*-alkyl-L-cysteines were methyl, ethyl, 1-propyl, 2-propyl, 1-butyl, *t*-butyl, 1-hexyl, and cyclohexyl.

equation in Scheme 2, as was observed for the cysteine-conjugate β -lyase isolated from rat liver (Tateishi *et al.*, 1978b; Stevens & Jakoby, 1983) and from *Fusobacterium varium*, populating the mammalian intestinal tract (Tomisawa *et al.*, 1984).

Neither serine nor alanine nor *S*-substituted thiopyruvic acid could be detected as an intermediate, although these compounds were stable under the assay conditions. Therefore it is unlikely that the $\alpha\beta$ -elimination reaction catalysed by the enzyme proceeds via a two-step mechanism involving the above-mentioned amino acids or thiopyruvic acid as intermediates as the result of a dual function of the enzyme. The reaction probably proceeds with the formation of pyridoxal phosphate Schiff bases of cysteine conjugates, because (1) pyridoxal 5'-phosphate is required for the enzyme activity (especially after the purification step by hydroxyapatite column chromatography), (2) a carbonyl reagent such as hydroxylamine causes complete inhibition of the enzyme activity, and (3) a free amino group on a cysteinyl moiety is obligatory for substrate activity (Table 2).

Various compounds, most of which have a thioether moiety, have been treated to define the substrate specificity for the β -lyase. None of the endogenous amino acids, including kynurenine [which was reported to give anthranilic acid by the rat liver β -lyase (Stevens & Jakoby,

Scheme 2. Reaction of C–S-bond cleavage of cysteine conjugates by cysteine-conjugate β -lyase

1983)], was responsive to the human hepatic cysteine conjugate β -lyase. Whereas *S*-aryl-L-cysteine conjugates, in the structure of which an aromatic ring is directly attached to the sulphur atom, are good substrates, neither *S*-alkyl- nor *S*-aralkyl-L-cysteines (e.g. *S*-benzyl-L-cysteine) served as substrates. With respect to the amino acid moiety, L-cysteine with a free amino and a free carboxy group is conceivably the obligatory configuration.

It is interesting that the substrate specificity of human cysteine-conjugate β -lyase was somewhat higher than that of rat origin: neither kynurenine nor *S*-aralkyl-L-cysteine [both of which were substrates for the rat liver β -lyase (Tateishi *et al.*, 1978*b*; Stevens & Jakoby, 1983)] were acted on by the human β -lyase.

It is notable that the oxidation of the thioether moiety of *S*-arylcysteine conjugates to corresponding sulfoxides increased the reactivity as a substrate, probably owing to the high electronegativity of the oxygen atom on the sulfoxide group. The C-S bond of cysteine conjugate sulfoxides is readily cleaved by plant C-S lyases such as alliinase to give equimolar amounts of ammonia, pyruvic acid and chemically reactive sulphenic acid (Stoll & Seebeck, 1949; Virtanen & Spare, 1963). Although we have not yet attempted structural elucidation of the sulphur-containing product generated from the cysteine-conjugate sulfoxide in the present study, the human β -lyase was likely to yield sulphenic acid.

The reaction (Scheme 2) is quite similar to that catalysed by human cystathionase, which cleaves a C-S linkage of L-homocysteine, L-methionine, L-cystathionine, L-homoserine and L-cysteine by $\alpha\beta$ - or $\alpha\gamma$ -elimination reactions in the presence of pyridoxal 5'-phosphate, whereby the corresponding thiol product, α -oxo acid and ammonia are stoichiometrically produced. Several lines of evidence, however, clearly indicate that the present β -lyase is distinct from human hepatic cystathionase: (1) the activity of the β -lyase was completely abolished by means of thermal treatment (60 °C for 2 min), whereas a salient feature of the cystathionase is its higher thermostability (Frimpter *et al.*, 1969); (2) the substrate specificity of the β -lyase was different from that of human cystathionase; thus cystathionase acts on L-homoserine and L-cystathionine, neither of which is attacked by the present enzyme; (3) the β -lyase and the cystathionase displayed different distribution profiles in the $(\text{NH}_4)_2\text{SO}_4$ fractionation of the 105000 *g* supernatant fluids, i.e. most of the β -lyase activity was found in the pellet precipitated at 40–60% saturation, whereas cystathionase was not completely precipitated even at 73% saturation (Frimpter *et al.*, 1969); (4) thiol-blocking reagents such as iodoacetic acid had virtually negligible inhibitory effect on the β -lyase, whereas mammalian cystathionase contains an essential thiol group on the catalytic site (Brown & DeFoor, 1974).

Several investigators have implicated β -lyase in necrosis induced with cysteine conjugates of halogenated ethylene or buta-1,3-diene in rats (Bonhaus & Gandorfi, 1981; Nash *et al.*, 1983). Further investigation will, however, be essential to clarify the participation of human cysteine-conjugate β -lyase in such cytotoxicity, since, firstly, the GSH conjugation reaction is markedly

higher in the rat in comparison with man, and, secondly, the activity of the hepatic β -lyase in man is much lower (about one-tenth) than that in the rat.

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