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Cysteine Conjugate β-Lyase

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SUMMARY

Cysteine conjugate β-lyase from rat liver, an enzyme participating in a shunt from mercapturic acid synthesis, has been purified and found to be active with a number of compounds that bear nonpolar leaving groups on the β-carbon of an amino acid substrate. Pyridoxal phosphate is considered to be a participant in the reaction. In addition to aromatic thioethers of cysteine, the enzyme is also active with two aliphatic amino acid derivatives, *S*-1,2-dichlorovinyl-L-cysteine and β-chloroalanine. Evidence is presented that catalysis results in "suicide" inhibition with a partition ratio of about 600 for each of the substrates.

INTRODUCTION

Thiol and thiomethyl metabolites in which sulfur is derived from glutathione have been identified during the last decade as excretion products of a variety of xenobiotics (1-3). Glutathione participates in the formation of thioethers by reaction with xenobiotics, or their metabolites, that bear an electrophilic center (4). Generally, the glutathione conjugate undergoes sequential enzymatic hydrolysis (5, 6) to yield the thioether of cysteine which, in turn, is acetylated to form the excretion product (7), a mercapturic acid. Tateishi and Shimizu (8) have demonstrated that metabolism of bromazepam results in the excretion of a urinary thiomethyl metabolite, the sulfur of which is derived from cysteine. Tateishi *et al.* (9) subsequently obtained an enzyme preparation, cysteine conjugate β-lyase, capable of cleaving between the β-carbon of cysteine and sulfur (Eq. 1). The respective thiomethyl metabolites of either 4-bromobenzene or 2,4-dinitrophenol were formed (9), presumably after catalysis by thiol *S*-methyltransferase (10, 11) (Eq. 2), when microsomes were added to incubation mixtures containing *S*-adenosylmethionine and the L-cysteine conjugates.

$$\text{Cysteine-S-R} + H_2O \rightarrow \text{RSH} + CH_3COCOOH + NH_3 \quad (1)$$

$$\text{RSH} + \textit{S}\text{-adenosyl-L-methionine} \rightarrow \text{RSCH}_3 + \textit{S}\text{-adenosyl-L-homocysteine} \quad (2)$$

Although the β-lyase was inactive with a number of aliphatic cysteine conjugates (9), the possibility that dichlorovinyl-L-cysteine might serve as a substrate was intriguing since this is the type of reaction that had been postulated as responsible for the high toxicity (12) of the compound. Such evaluation requires the availability of a highly purified enzyme, which we sought to obtain. This report is concerned with that purification, with the characterization of the enzyme, and with an account of suicide inactivation by each of the substrates.

MATERIALS AND METHODS

Livers from male Sprague-Dawley rats weighing 200-250 g (ARS Sprague-Dawley, Madison, Wisc.) were stored at −70° before use. CBZ[2] was synthesized by the method of Colucci and Buyshe (3) from 2-chlorobenzothiazole and solid sodium amide (Aldrich Chemical Company, Milwaukee, Wisc.); Dohn and Anders (13) have presented NMR and mass spectral data on CBZ which they prepared. *S*-1-propyl-L-cysteine, *S*-1-butyl-L-cysteine, and *S*-2-(*p*-dintrophenethyl)-L-cysteine were prepared as previously described (7). The following compounds were generously provided as gifts: *S*-2-chloroethyl-L-cysteine from M. W. Anders, of the University of Rochester; dichlorovinyl-L-cysteine from Gopal Krishna, of the National Heart, Lung and Blood Institute; and the L-cysteine derivative of propachlor (14) from J. E. Bakke, of the University of North Dakota. All other compounds tested as substrates were obtained from Sigma Chemical Company (St. Louis, Mo.).

Enzyme Assays

Cysteine conjugate β-lyase activity was measured by either of two methods. The standard assay for the enzyme utilized CBZ as substrate and measured the appearance of 2-mercaptobenzothiazole as product at 316 nm (ε = 19,600 at pH 7.0) with a dual-beam Cary 219 spectrophotometer at 30°. The standard incubation mixture of 1 ml contained 50 μM CBZ, 50 mM potassium phosphate at pH 7.0, and an appropriate amount of enzyme; the reaction was initiated by the addition of enzyme, which was not added to a reference cell. Under the conditions described, the reaction was linear for 3 min with respect to time and protein concentration when less than 0.3 nmole of product was formed per minute. A unit of enzyme activity is defined as that amount of enzyme catalyzing the formation of 1 nmole of product per minute under standard assay conditions; specific activity is defined in terms of units of activity per milligram of protein. Protein was measured by the method of Bradford (15) with bovine serum albumin (Armour) as a standard.

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[2] The abbreviations used are: CBZ, *S*-2-benzothiazolyl-L-cysteine: SDS, sodium dodecyl sulfate.

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All other substrates were assessed by measuring the rate of formation of pyruvate in an assay in which the β-lyase reaction (Eq. 1) was coupled with lactate dehydrogenase and NADH; the resultant decrease at 340 nm is a function of pyruvate formation. Incubation mixtures of 1 ml at 30° contained 50 mM potassium phosphate at pH 7.0, varying amounts of substrate, 100 μM NADH, 0.1 unit of lactate dehydrogenase (Sigma Chemical Company), and an appropriate amount of enzyme that was used to initiate the reaction. Rates were linear with time and protein concentration when less than 0.3 nmole of product were formed per minute. With each of the substrates, deviations from linearity were present that became obvious at higher rates of product formation and at higher pH values; data are presented that the effect is due to suicide inactivation by substrate.

Inactivation was followed by using higher concentrations of substrates, as noted, and measuring product formation by either the standard assay or that for pyruvate. Nonlinear regression analysis was performed with an interactive curve-fitting program (16) on a Decasystem-10 computer. Product formation curves were fitted to the expression, $A = Be^{-kt}$, where A is proportional to enzyme inactivated at time, t, and B is proportional to the initial concentration of enzyme. Partition ratios were calculated by dividing A, the total product formed, by the molar concentration of enzyme. Substrate concentrations yielding half-maximal inactivation, K_m^{inact}, and the reciprocal of the maximal rate of inactivation, $t_{1/2}^{max}$, were calculated from double-reciprocal plots of first-order constants versus substrate concentration (17).

Electrophoresis and Molecular Weight

Electrophoresis was performed in gels, 10% in acrylamide, in Tris-glycine at pH 8.3 (18). Protein was visualized with 0.04% Coomassie Blue G-250 in 3.5% (w/v) perchloric acid (19). For SDS gels, 1% SDS and 1% 2-mercaptoethanol were added to protein samples and the solution was boiled for 5 min prior to application to the gel (18). Trypsin inhibitor (21,500), carbonic anhydrase (31,000), ovalbumin (45,000), albumin (66,000), phosphorylase B (93,000), β-glactosidase (116,000), and myosin (200,000) served as standard globular proteins for estimation of M_r. SDS gels were stained with Coomassie Blue as described by Maizel (18). Cross-linking of protein prior to electrophoresis was performed with bis-2-(succinimidooxycarbonyloxy)-ethylsulfone as described by Zarling *et al.* (20); the product was dialyzed against 10 mM sodium phosphate at pH 7.5 prior to application to an SDS gel.

After electrophoresis, activity of the protein was assessed by cutting the cylindrical polyacrylamide gels into 2-mm slices which were then crushed in tubes containing 1 ml of 50 mM potassium phosphate at pH 7.0 and 40 μM CBZ. Incubation of the suspensions at 30° for 15 min allowed determination of absorbance at 316 nm as a function of product formation; duplicate gels were stained for protein.

M_r was also determined with columns of Ultragel ACA 34 (1.5 × 90 cm), equilibrated with 50 mM potassium phosphate at pH 7.0 containing 60 μM pyridoxal phosphate. Under identical conditions, catalase, ovalbumin, hemoglobin (66,000), and alcohol dehydrogenase (141,000) served as standards.

Antibody Preparation

Antibody to the β-lyase was raised in goats by injection of 100 μg, 60 μg, and 50 μg of the enzyme on days 1, 18, and 32, respectively. In each instance the purified enzyme was subjected to acrylamide gel electrophoresis as described above, and the single band was sliced and homogenized together in 1 ml of saline prior to injection; the initial dose was supplemented with Freund's adjuvant. Antibody was obtained by plasmaphoresis on day 50. Preimmune serum served as control in all assays with the antibody. Determination of enzyme activity after addition of the antibody was performed in both the presence and absence of Pansorbin (Calbiochem). Interaction of the enzyme with antibody was also examined by Ouchterlony immunodiffusion (21).

Purification of Cysteine Conjugate β-Lyase

Livers (400 g) were partially thawed and rinsed in distilled water at room temperature. Thawing was completed at 4° in 800 ml of 0.25 M sucrose containing 10 mM Tris-HCl at pH 7.5. Homogenization and all subsequent steps were carried out at 4°.

Step 1: extraction. Livers were homogenized for 30 sec in two batches of 200 g each in a Waring Blendor. After centrifugation at 9,000 × *g* for 30 min, the supernatant liquid was filtered through a plug of glass wool and centrifuged at 100,000 × *g* for 60 min. The supernatant fluid was collected and retained.

Step 2: DEAE-cellulose I. A column of DEAE-cellulose (5 × 50 cm) was equilibrated with 10 mM Tris-HCl at pH 7.5 containing 50 mM potassium chloride (Buffer A) and charged with the cytosol from Step 1. After washing with 1 liter of Buffer A, a 3-liter linear gradient from 0.05 to 0.35 M KCl in Buffer A was applied, and fractions of 23 ml were collected (Fig. 1A). Active fractions, eluted at a conductivity of approximately 6 mmho, were pooled and concentrated 10-fold with an Amicon ultrafiltration apparatus and a PM 30 filter. All subsequent concentration steps were performed in an entirely similar manner.

Step 3: DEAE-cellulose II. A column of DEAE-cellulose (2 × 40 cm) was equilibrated with 10 mM Tris-HCl at pH 7.5 (Buffer B) and charged with the concentrate from Step 2 which had been diluted 2-fold in Buffer B. After washing with 100 ml of Buffer B, a 1-liter gradient of 0–0.3 M potassium chloride in Buffer B was applied. Fractions of 15 ml were collected in tubes containing 50 μl of 15 mM pyridoxal phosphate adjusted to pH 6.5. Active fractions were concentrated 10-fold.

Step 4: hydroxylapatite I. A column of hydroxylapatite (2 × 20 cm) was equilibrated with 10 mM potassium phosphate at pH 7.0 containing 50 μM pyridoxal phosphate (Buffer C), after which the concentrated

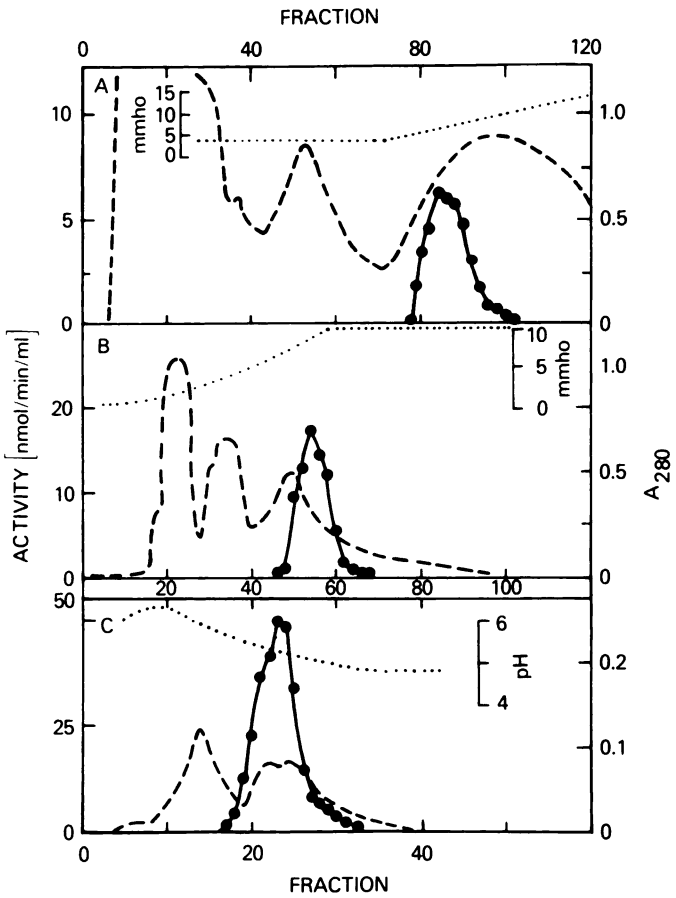

FIG. 1. *Elution patterns upon chromatography from the DEAE-cellulose (A), hydroxylapatite I (B), and chromatofocusing steps (C) in the purification of cysteine conjugate β-lyase*

Enzyme activity is indicated by the *solid lines*, A_{280} by the *dashed lines*, and the pH or conductivity by the *dotted lines*.

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protein solution was applied to the column. The column was washed with 200 ml of Buffer C, and an 800-ml linear gradient of Buffer C, 0–0.2 M in potassium phosphate at the same pH was applied. Active fractions (Fig. 1B), eluting at approximately 7 mmho, were pooled and concentrated 10-fold.

Step 5: hydroxylapatite II. The concentrate was diluted 2-fold and applied to a column of hydroxylapatite (1 × 10 cm) equilibrated with Buffer C. After washing with 50 ml of Buffer C, the column was developed with a 0–0.3 M linear gradient of potassium phosphate in 400 ml of Buffer C. Active fractions were pooled, concentrated 10-fold, and dialyzed against 1 liter of potassium phosphate at pH 7.0 that contained 50 μM pyridoxal phosphate.

Step 6: chromatofocusing. A column of Polybuffer exchanger gel (Pharmacia PBE 94) (0.5 × 20 cm) was washed and equilibrated with 25 mM histidine HCl (pH 6.0) as described (22). After 5 ml of Polybuffer 96, a 10-fold dilution of the supplied concentrate at pH 5.0 was run onto the column, and the dialysate from Step 5 (9 ml) was applied. Elution was with the diluted Polybuffer, allowing collection of fractions of 5 ml. In initial experiments, active fractions eluted at pH 5.3 (Fig. 1C), a pH at which the enzyme was unstable. Subsequently, each fraction was collected in a tube containing 0.5 ml of 0.5 M potassium phosphate (pH 7.0) and 50 μl of 50 μM pyridoxal phosphate. Active fractions were pooled and concentrated to approximately 2 ml.

Step 7: Sepharose 6B. The concentrate was applied to a column of Sepharose 6B (1.5 × 90 cm) equilibrated with 0.1 M sodium phosphate/50 μM pyridoxal phosphate at pH 7.5, and washed with the same buffer. Fractions of 2 ml were collected, and those containing lyase activity were pooled and concentrated to 1.5 ml.

Step 8: AH-Sepharose. A column of AH-Sepharose (0.5 × 5 cm) was equilibrated with 10 mM sodium phosphate at pH 7.8 (Buffer D) and then charged with the concentrate from Step 7 after the protein solution was diluted 3-fold. After washing with 20 ml of Buffer D, enzyme was eluted in 2-ml fractions with 100 ml of a 10–200 mM linear gradient of sodium phosphate at pH 7.0. As in the second DEAE-cellulose step, sufficient pyridoxal phosphate was added to each collection tube to yield a final concentration of 5 μM in each fraction. Active fractions were pooled and concentrated to 2 ml.

RESULTS

The product of extensive series of steps for purification of cysteine conjugate β-lyase was a protein with specific activity increased about 1500-fold (Table 1). Polyacrylamide disc gel electrophoresis of the product revealed a single concomitant band of protein and catalytic activity (Fig. 2A). That band could be excised and eluted from the gel, and subjected to treatment with SDS followed by SDS/polyacrylamide gel electrophoresis. Enzyme that had been cut from a disc gel, as well as the purified enzyme which had not undergone electrophoresis, disclosed two major bands with M_r estimated at 39,000 and 47,000, respectively (Fig. 2B), on the basis of comparison with standard proteins in SDS gels. Electrophoresis in detergent also revealed two additional bands of slightly larger size that, together, were estimated as constituting less than 5% of the stained proteins (Fig. 2B); the minor bands showed no change in concentration during Steps 6, 7, and 8, nor after using Sephadex G-100 when gel filtration was added as an additional procedure. After the purified enzyme was cross-linked by action of bis-(2-(succinimidoxycarbonyloxy)-ethylsulfone (20), SDS gel electrophoresis disclosed the presence of a protein species estimated at 94,000 daltons (Fig. 2C). In accord, gel filtration on Ultragel ACA-34 with globular protein standards allowed estimation of M_r as 100,000.

Goat immunoglobulin raised against the purified enzyme produced a single line of identity when tested by immunodiffusion against the enzyme and an extract of rat liver; no line of precipitation was evident with an extract of rat kidney or with a β-lyase preparation from that organ which had been taken through the first three purification steps outlined for liver, to yield 100-fold purification. Solutions of the liver β-lyase treated with the antibody retained their activity, which could be removed by centrifugation following addition of Pansor-

TABLE 1

Purification of cysteine conjugate β-lyase from 400 g of rat liver

Step	Volume	Total protein	Total activity	Specific activity
	ml	*mg*	*nmoles/min*	*nmoles/min/mg*
1. Extract	800	25,600	5,120	0.2
2. DEAE-cellulose I	202	1,480	5,590	3.8
3. DEAE-cellulose II	190	266	5,080	19
4. Hydroxylapatite I	80	72	3,380	47
5. Hydroxylapatite II	88	62	3,280	53
6. Chromatofocusing	1.85	7.0	2,090	297
7. Sepharose 6B	1.35	3.5	1,100	314
8. AH-Sepharose	1.4	2.1	668	318

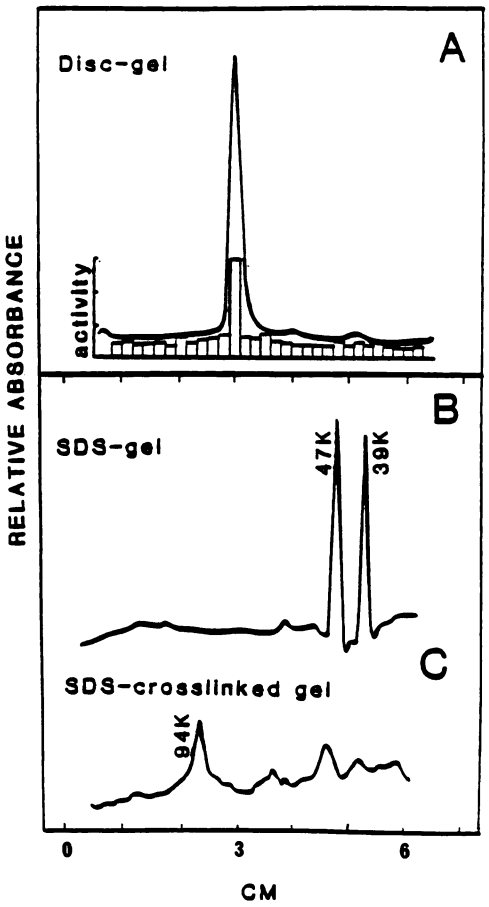

FIG. 2. *Electrophoresis of purified enzyme preparations*

A, Densitometric scan of a disc gel stained for protein; a duplicate gel, assayed for enzyme activity, is shown in arbitrary units on the bar graph. B, Densitometry of an SDS gel of the purified protein and after treatment (20) with a cross-linking reagent.

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TABLE 2

Apparent kinetic constants for cysteine conjugate β-lyase

Substrate	Activity		Inactivation		
	K_m	V_{max}	K_m^{intact}	$t_{1/2}^{max}$	Partition ratio
	mM	*μmoles/min/mg*	*mM*	*min*	
CBZ	0.1	2.0	0.17	2.3	580
S-2,4-Dinitrophenyl-L-cysteine	0.08	1.8	0.08	1.5	620
S-1,2-Dichlorovinyl-L-cysteine	0.11	1.5	0.20	1.6	630
β-Chloroalanine	2.6	1.7	2.9	4.1	680

bin[3]; the kidney enzyme was unaffected by these procedures.

Substrates. Substrates for the β-lyase include those compounds that bear a good leaving group on the β-carbon of the amino acid substrate. Thus, three thioethers of L-cysteine that fit this generalization serve as substrate, including the nonaromatic derivative, *S*-2-dichlorovinyl-L-cysteine (Table 2). The following compounds, each tested at approximately 1 mM within the limits of solubility, were not active as substrates: cystathionine, 4-nitrophenethyl, benzyl, methyl, ethyl, 2-chloroethyl, and butyl thioethers of L-cysteine, L-aspartate, L-phenylalanine; L-serine; and L-tryptophan. Compounds that were not substrates were not, in the same concentration range, inhibitors of the β-lyase reaction under standard assay conditions.

Inactivation. The time course of the β-lyase reaction is a hyperbolic curve, although the standard enzyme assay, over a limited time period and product concentration, approaches linearity. This phenomenon was not due to inhibition by reaction products, metal ions, or thiol oxidation, since 100 mM NH_4Cl, 10 mM pyruvate, 100 μM 2-mercaptorenzothiazole, 10 mM EDTA, or 10 mM 2-mercaptoethanol had no effect on reaction rates or nonlinearity. Additional substrate resulted in no further product formation, suggesting that enzyme was being inactivated. The departure from linearity was first-order, with higher rates of inactivation noted as substrate concentration or pH (Fig. 3) were increased.

The effect is achieved with all substrates, and the partition ratio (i.e., the rate of product formed per inactivation event) is essentially the same for each of them (Table 2), as it is over the pH range tested despite a 3-fold increase in the *rate* of inactivation with pH.

pH Optimum. As noted, the rate of inactivation during the enzyme-catalyzed reaction increases with increasing pH (Fig. 3), resulting in difficulty in obtaining accurate rate data. The results of testing pH activity relationships must be presented with the caveat that all rates were curvilinear, even during the first 2 min of reaction. Estimation of initial rates for otherwise standard assay systems in 50 mM Tris-HCl disclosed a pH optimum at 8.8 with half-maximal activity at pH 7.5 and 9.5. In order to approach linearity, the standard enzyme assay was conducted at pH 7.0 in potassium phosphate rather than at

[3] The compound was added to the otherwise standard assay system in 10 μl of acetone; the solvent had no effect on the reaction rate.

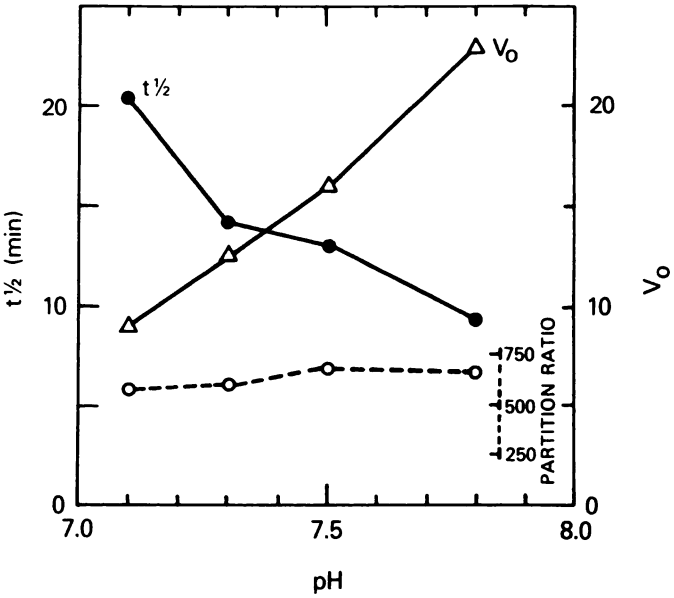

FIG. 3. *Effect of pH on the inactivation of the enzyme with substrate*

The β-lyase (10 units) was incubated with 41 nmoles of CBZ in the presence of 0.1 M potassium phosphate. The data shown are based on initial rates (V_o) in nanomoles per minute.

the pH optimum observed for Tris; Tris-HCl was actually inhibitory at pH 7.0 in the presence of 50 mM potassium phosphate buffer, resulting in 50% inhibition at 100 mM Tris-HCl. When several buffers were compared at pH 7.5 and at 50 mM in an otherwise standard assay system, the following relative rates were attained: potassium phosphate, 1.0; sodium pyrophosphate, 1.8; Tris-HCl, 0.8; and sodium borate, 0.6. The addition of 2 or 10 mM EDTA had no effect on the rate with phosphate buffer.

Role of pyridoxal phosphate. Despite inability of the enzyme to accept cystathionine as a substrate, the reaction that is catalyzed is analogous to that of a cystathionase for which pyridoxal phosphate is the prosthetic group (23). Attempts at depleting cysteine conjugate β-lyase of pyridoxal phosphate, while resulting in complete loss of activity, never allowed reactivation by the coenzyme. Nevertheless, when large losses in activity were incurred at early, relatively benign, purification steps, pyridoxal phosphate was added to each of the enzyme buffers in which this was practical and acted as a stabilizer for the enzyme. Purification with hydroxylapatite, for example, resulted in a 5-fold increase in the recovery of enzyme upon elution when pyridoxal phosphate was present in the eluting buffer. Because of the requirement for the coenzyme, the usual spectral and fluorescent methods could not be used to identify pyridoxal phosphate bound to the enzyme.

Reagents known to inhibit enzymes bearing pyridoxal phosphate (24) were effective inhibitors of the β-lyase in the standard reaction system. L-Cysteine and potassium cyanide were not inhibitory when added to enzyme in the presence of substrate in an otherwise standard incubation mixture; the enzyme was inhibited maximally after 5 min of preincubation of the enzyme with cysteine (I_{50} = 3 mM). Although hydroxylamine did inhibit in the

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presence of substrate (I_{50} = 50 μM), inhibition was 50-fold greater upon preincubation with enzyme alone.

DISCUSSION

In keeping with other enzymes of detoxication (25), cysteine conjugate β-lyase has markedly low specificity for its substrates despite the few compounds that have been found to serve that function. It is also clear that its designation is somewhat a misnomer since it is not specific for cysteine conjugates. β-Chloroalanine, which has a good leaving group at the β-carbon and an otherwise identical structure, also serves as substrate.

The inactivation phenomenon observed is consistent with that described as suicide inactivation (17) in which an enzyme-bound intermediate will react with the enzyme itself, thereby inactivating it. In analyzing the inhibition, none of the three products of the reaction (Eq. 1) was found to be inhibitory nor would soluble electrophiles be good candidates, since high concentrations of mercaptoethanol or dithiothreotol have no effect on the observed first-order inactivation. Each of the substrates acts in an apparently identical manner, and the partition ratio is between 580 and 680 for all of them. Indeed, over a 3-fold change in rate, because of differences in pH (Fig. 3), the partition ratio remains the same. These data suggest that, independent of the absolute rate of formation, the inactivating chemical species that is produced inactivates once in every 600 or so catalytic cycles regardless of the substrate. Despite the absence of data on covalent binding, we suggest that inactivation depends on the partitioning of an enzyme-bound eneamino acid between release into solution followed by hydrolysis to pyruvate, and covalent modification by Michaelis addition to an enzyme nucleophile that leads to inactivation. Since the eneamino acid, dehydroalanine in this case, is a common intermediate to pyridoxal phosphate-mediated α,β-elimination reactions at the β-carbon, any substrate leading to its formation by a susceptible enzyme will lead to inactivation. Indeed, β-haloalanines have been shown by elegant means to act in this manner with alanine racemase (26) and aspartate decarboxylase (27).

The presence of cysteine conjugate β-lyase represents a shunt mechanism for the handling of premercapturic acids. Although the need for such a shunt pathway is not clear, the products of the reaction, i.e., the thiols, would be expected to be toxic. Indeed, the cleavage catalyzed by β-lyase produces a reactive fragment from dichlorovinyl-L-cysteine that is known to bind covalently to protein (28) and to DNA (29), resulting in cell damage. It is only the presence of a second enzyme, thiol *S*-methyltransferase, that, with *S*-adenosylmethionine, completes the detoxication function.

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