A Purified Cysteine Conjugate β-Lyase from Rat Kidney Cytosol

REQUIREMENT FOR AN α-KETO ACID OR AN AMINO ACID OXIDASE FOR ACTIVITY AND IDENTITY WITH SOLUBLE GLUTAMINE TRANSAMINASE K*

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Cysteine conjugate β-lyase has been purified from rat kidney cytosol. The enzyme is a 100,000-dalton dimer of two 55,000-dalton subunits and has an absorption maximum at 432 nm. The enzyme is phenylalanine α-keto-γ-methylbutyrate transaminase activity and appears to be identical to rat kidney cytosolic glutamine transaminase K.

Metabolism of S-1,2-dichlorovinyl-1-cysteine (DCVC) by the purified enzyme was dependent on the presence of either α-keto-γ-methylbutyrate or a protein factor which is present in the cytosolic fraction of rat kidney cortex. The protein factor was identified as a flavin containing L-amino acid oxidase which oxidized DCVC to S-(1,2-dichlorovinyl)-3-mercapto-2-oxopropionic acid. S-(1,2-Dichlorovinyl)-3-mercapto-2-oxopropionic acid has not been previously reported as a metabolite of DCVC.

The data also show that rat kidney cytosolic glutamine transaminase K catalyzes both a-elimination and a transamination reaction with DCVC when α-keto-γ-methylbutyrate is present and that amino acid oxidase and α-keto-γ-methylbutyrate stimulate the enzyme activity by providing amino acceptors. When incubations were done with DCVC as substrate in the presence of excess α-keto-γ-methylbutyrate, the β-lyase catalyzed β-elimination and transamination in a ratio of 1:1.3, respectively. Under conditions where most of the α-keto-γ-methylbutyrate was consumed, the β-elimination predominated indicating that the S-1,2-dichlorovinyl-3-mercapto-2-oxopropionic acid pool was consumed by transamination after the α-keto-γ-methylbutyrate had been depleted. The data are discussed with regard to the importance of these pathways as regulators or participants in the toxicity of S-cysteine conjugates.

Glutathione S-transferases catalyze the formation of a pantheon of xenobiotic S-glutathione conjugates (1), the first step in the detoxication scheme known as mercapturic acid biosynthesis (2). The detoxication function of mercapturic acid biosynthesis is completed by the formation of an S-cysteine conjugate from an S-glutathione conjugate and the excretion of the S-cysteine conjugate after N-acetylation, i.e. as the mercapturate (3, 4). Though mercapturic acid biosynthesis functions largely as a detoxication system, the formation of glutathione conjugates from some xenobiotics can also initiate toxic processes rather than detoxication (5–11).

Cysteine conjugate β-lyase is an enzyme which cleaves some S-cysteine conjugates by β-elimination and has been implicated in the renal toxicity of some S-glutathione conjugates (5–9). We have reported that the majority of the rat liver cysteine conjugate β-lyase activity is contributed by an enzyme which is in fact a kynureninase (12). In addition, cysteine conjugate β-lyase activity is present in both the cytosolic and mitochondrial fractions of rat kidney cortex (13–15), but little is known about the nature of the kidney enzymes. The proteins responsible for rat kidney cytosolic cysteine conjugate β-lyase activity do not cross-react with antibody raised against the rat liver enzyme (13), suggesting that the cysteine conjugate β-lyase enzymes present in rat kidney cytosol are different from the hepatic enzyme(s).

In a model kidney cell line, LLC-PK1 cells, the toxicity of S-1,2-dichlorovinyl-L-glutathione is dependent on both metabolism to its S-cysteine conjugate, S-1,2-dichlorovinyl-L-cysteine (DCVC), and the metabolism of DCVC, probably by a cysteine conjugate β-lyase (6). Furthermore, the cysteine conjugate β-lyase activity in LLC-PK1 cells is stimulated by the addition of pyruvate to cell homogenates, suggesting that a transamination reaction regulates the β-elimination activity in this model system. Stimulation of β-elimination activity by pyruvate resulted in an increase in the covalent binding of a sulfur-containing fragment from DCVC to cellular macromolecules. Inhibition of β-lyase activity by aminoxyacetic acid, a general inhibitor of pyridoxal phosphate-dependent enzymes (16), blocked both the covalent binding and toxicity of DCVC in the LLC-PK1 model. Aminoxyacetic acid also blocks the toxicity of DCVC in the rat (5).

Preliminary results from the investigation of DCVC metabolism in rat kidney cytosol indicated that α-keto acids stimulated β-lyase activity 2–3-fold. In addition, a protein factor which separated from the β-lyase during purification, and appeared to be an amino acid oxidase, was able to stimulate DCVC metabolism. After the amino acid oxidase was separated from the β-lyase, the β-lyase activity became almost totally dependent on the presence of an α-keto acid. If the cytosolic cysteine conjugate β-lyase were a pyridoxal phosphate-dependent transaminase, the stimulation of activity by α-keto acids and an amino acid oxidase could be explained by the hypothesis represented schematically in Fig. 1. According to the hypothesis, a single enzyme might catalyze both β-elimination and transamination reactions, in accord with the proposal for the enzyme in LLC-PK1 cells (6). In the absence of an α-keto acid and an amino acid oxidase, the enzyme might catalyze transamination reactions, in accord with the proposal for the enzyme in LLC-PK1 cells (6). In the absence

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1 The abbreviations used are: DCVC, S-(1,2-dichlorovinyl)-L-cysteine; DMOP, S-(1,2-dichlorovinyl)-2-oxo-3-mercaptopropionic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.
protein was dialyzed against three changes of 5 mM potassium phosphate (pH 6.5) containing 1 mM diethiothreitol, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride (Buffer C), and charged onto a 1.5 × 15-cm column of hydroxyapatite (Bio-Rad) equilibrated with Buffer C. The enzyme was eluted with a 500-ml linear gradient of 5–250 mM potassium phosphate (pH 6.5) in Buffer C. The enzyme eluted at 3.4 mmho and was concentrated by vacuum dialysis against Buffer C adjusted to pH 7.5 (Buffer D). The enzyme was charged onto a 0.4 × 25-cm Sepharose Q300 anion exchange column (Sephacryl, Linden, NJ) equilibrated with Buffer D and operated at a flow rate of 1 ml/min with a Gilson programmable high pressure liquid chromatograph (Gilson Medical Electronic, Middleton, WI). The enzyme was eluted with a 30-ml gradient of 5–200 mM potassium phosphate (pH 7.5) in Buffer D. The purified enzyme was stored at a concentration of 1 mg/ml at 4°C for at least 12 weeks with no loss of activity. Protein was determined according to the method of Bradford (19) using the Bio-Rad kit and bovine IgG as standard.

Amino acid oxidase was purified from the S-300 column eluate by pooling the fractions which had amino acid oxidase activity and concentrating them by precipitation with ammonium sulfate at 60% saturation. The protein was dialyzed against two changes of Buffer D and applied to a 1 × 25-cm Sepharose Q300 column equilibrated with Buffer D and operated at 3 ml/min. Enzyme was eluted with a 75-ml 5–200 mM gradient of potassium phosphate in Buffer D.

**Enzyme Assays**—Cysteine conjugate β-lyase activity was assayed by the extraction method previously reported (6) using [14C]DCVC as the substrate. Since [14C]cysteine was used as the label of [14C]DCVC, the assay detects those extractable products containing the cysteine carbon skeleton. As will be shown later, metabolism of DCVC can yield two α-keto acid products, pyruvate and the α-keto acid of DCVC, S-(1,2-dichlorovinyl)-3-mercapto-2-oxopropionic acid. The extraction assay does not differentiate between the two products. Separation of the two products is achieved with the HPLC assay described below. Enzyme was incubated in a volume of 250 µl for 10 min at 37°C with 1 mM [14C]DCVC and 100 mM Tris·HCl (pH 8.0) with 0.1 mM α-keto-γ-methylbutyrate, unless stated otherwise. The reaction was stopped by the addition of 125 µl of 2 M HCl and extracted with 2 ml of ethyl acetate. Radioactivity in 1 ml of ethyl acetate was determined by liquid scintillation counting. Glutamine transaminase K was assayed by the method of Cooper and Meister (18) using phenylalanine and α-keto-γ-methylbutyrate as the substrates. Amino acid oxidase activity was assayed with leucine (20 mM) using the dinitrophenylhydrazine method of Greenberg et al. (20) to determine α-keto acid product with α-ketocisaprico acid as the standard. Incubations with amino acid oxidase also included 50 mM sodium pyrophosphate (pH 9.0) and 40 µg/ml catalase.

**HPLC Analysis of Incubation Mixtures**—Products from reaction mixtures were separated by HPLC and quantitated by the radiochromatic detection and absorbance at 210 nm. Incubations were in a final volume of 150 µl and contained 1 mM DCVC; other additions are as described in the figure legends. Following incubation, 10 µl of phosphoric acid was added to stop the reaction, and 50 µl of the mixture were spotted onto a 0.4-µm glass fiber filter through a 6-mm diameter phase column (Waters Associates, Milford, MA) operating at a flow rate of 1 ml/min. Products were eluted with one of the following solvent programs where solvent A is 0.1 M potassium phosphate buffer (pH 3.0) and solvent B is methanol: System A, 0–5 min 100% A, 5–15 min ramp to 40% B, plateau at 40% B through 30 min; or System B, 0–10 min 100% A, 10–20 min ramp to 40% B, plateau at 40% B through 30 min.

**Identification of S-1,2-Dichlorovinyl-3-mercapto-2-oxopropionic Acid**—In order to gain structural information about the products from the amino acid oxidase and cysteine conjugate β-lyase-dependent metabolism of DCVC, the product from the amino acid oxidase reaction was isolated as a 2,4-dinitrophenylhydrazine derivative. Amino acid oxidase (0.36 mg), isolated as described above, was incubated with DCVC (9 µmol) catalase (24 µg), and 25 µmol of sodium pyrophosphate (pH 9.0) in a final volume of 0.5 ml. After 1 h at 37°C, 2% solution of 2,4-dinitrophenylhydrazine (4 ml of 25% w/v in 2 M HCl) was added and allowed to react for 15 min at room temperature. The unreacted dinitrophenylhydrazine and any hydrazine derivatives were extracted into 2 ml of ethyl acetate and the solvent removed in vacuo. The yellow residue was dissolved in 500 µl of methanol, and 200 µl of 0.1 M potassium phosphate (pH 6.5) was added in Buffer C. The enzyme was added to the solution as previously described and applied to a 0.4 × 30-cm Water’s Bondapak C18 column, and the column was eluted with 60% methanol in 0.1 M potassium phosphate (pH 3.0) while monitoring both 280- and 360-nm wavelengths with a Gilson model 116 detector. Unreacted dinitrophenylhydrazine eluted
just after the front, and the derivative of S-1,2-dichlorovinyl-3-mercapto-2-oxopropionic acid was recovered as two peaks which eluted at 19 and 24 min. The two peaks are believed to correspond to cis and trans (E and Z) isomers of the hydrazone derivative. The large peak from the HPLC analysis of the product at 19 min was collected and the solvent removed in vacuo with the aid of additional methanol. The residue was extracted into 2 ml of ethyl acetate and exchanged two times with a mixture of 1 ml of acetone and 0.5 ml of H2O. The sample was dried overnight under high vacuum, dissolved in 0.5 ml of CH3COONa, and the 400-MHz 1H-NMR spectrum was collected with a JEOL GX-400 spectrometer.

The separation of two isomers of dinitrophenylhydrazones by thin layer chromatography is well known (21). To confirm this behavior in the HPLC system described here, the dinitrophenylhydrazone of pyruvate was prepared by adding 2.0 g of pyruvate to 50 ml of 85% phosphoric acid and recrystallizing the product from ethanol/water. The recrystallized dinitrophenylhydrazone of pyruvate gave a single peak by HPLC analysis. Upon heating an aqueous solution of the hydrazone, two peaks were observed, both of which were found to be the dinitrophenylhydrazone of pyruvate by NMR analysis (data not shown). When the dinitrophenylhydrazone of pyruvate was prepared from incubations containing [35S]DCVC and amino acid oxidase, two peaks, both of which contained [35S] label, were found. These data confirm the formation of cis and trans isomers of dinitrophenylhydrazone, two peaks were observed, both of which were found to be the dinitrophenylhydrazone of pyruvate by HPLC analysis prepared from incubation mixtures spiked with authentic pyruvate, using the method described above, two peaks were seen by HPLC as well. Furthermore, when dinitrophenylhydrazone derivatives were prepared from incubations containing [35S]DCVC and amino acid oxidase, two peaks, both of which contained [35S] label, were found. These data confirm the formation of cis and trans isomers of dinitrophenylhydrazones from α-keto acids and their separation using the HPLC system described here.

Electrophoresis—SDS-PAGE and nondenaturating gel electrophoresis were done by the method of Maizel (22). Enzyme activity was determined after nondenaturing gel electrophoresis by incubating gel slices in 200 μl of 10 mM potassium phosphate buffer at 4°C (pH 7.5) and assaying aliquots of the supernatant for enzyme activity. The Rp values for enzyme activity were compared to the Rp for protein which was determined by staining an identical gel with Coomassie Brilliant Blue R-250 (22).

RESULTS

Purification and Characterization of Rat Kidney Cysteine Conjugate β-Lyase—Cysteine conjugate β-lyase activity was purified 890-fold from rat kidney cytosol (Table I). Phenylalanine-α-keto-γ-methylthiobutyrate transamination activity copurified with the β-lyase activity, and both activities comigrated during ion exchange chromatography by HPLC (Fig. 2A) and nondenaturating gel electrophoresis (Fig. 2B). When phenylalanine was included in incubation mixtures containing the purified enzyme, α-keto-γ-methylthiobutyrate and [14C]DCVC, the formation of product from DCVC was inhibited with kinetic behavior characteristic of competitive inhibition (Fig. 3). The Km and Vmax for DCVC metabolism in the absence of inhibitors were 0.26 mM and 22.0 μmol/mg-10 min, respectively. The dashed line in Fig. 2 illustrates that substrate inhibition was observed with high DCVC concentrations. The data show that both the phenylalanine-α-keto-γ-methylthiobutyrate transaminase and cysteine conjugate β-lyase activities are the property of the same protein and suggest that both reactions are catalyzed at the same active site on the enzyme.

The protein has an M, of about 100,000, as determined by gel filtration chromatography (data not shown), and two subunits of M, = 55,000 determined by SDS-PAGE (Fig. 2A). A minor band which was not seen by nondenaturing gel electrophoresis (Fig. 2B) was apparent by SDS-PAGE.

Although pyridoxal phosphate is not required for stability during the purification, the purified enzyme has absorption maxima at 432 and 282 nm (Fig. 4), a spectrum characteristic of some pyridoxal phosphate-containing proteins (23). Furthermore, agents that are known to inhibit pyridoxal phosphate-dependent enzymes inhibited the purified enzyme (Table II).

During purification (Table I), the ability of the enzyme to metabolize DCVC became increasingly dependent on the presence of α-keto-γ-methylthiobutyrate. Fig. 5 shows the dependence of the purified enzyme on the α-keto-γ-methylthiobutyrate concentration. Activity reaches a plateau at about 0.5 mM α-keto-γ-methylthiobutyrate and then decreases; increasing the concentrations (1–5 mM) of α-keto-γ-methylthiobutyrate actually resulted in a further decrease in enzyme activity. In initial experiments with enzyme from Step 5, maximal stimulation was seen with 0.1 mM α-keto-γ-methylthiobutyrate, and this concentration was chosen for the standard assay. Taken together, the substrate inhibition kinetics (Fig. 3) and the inhibition of the enzyme at high α-keto-γ-methylthiobutyrate concentrations suggest that substrate inhibition occurs.

The structure-activity relationship for the simulation of DCVC metabolism by α-keto acids was also investigated (Table III). Those α-keto acids with more hydrophobic substituents at the β-carbon were most effective in maintaining β-elimination activity. The ability to stimulate enzyme activity increased at higher concentrations (5 mM) for those α-keto acids which, at a concentration of 0.1 mM, were not as effective as α-keto-γ-methylthiobutyrate (data not shown).

An Amino Acid Oxidase Is Required for the Expression of Rat Kidney Cysteine Conjugate β-Lyase Activity in Vitro—Addition of α-keto-γ-methylthiobutyrate produced only

**Table 1**

| Protein                | Specific activity | Total units | Specific activity | Total units | +mth/-mth | TRA/CBL |
|------------------------|------------------|-------------|------------------|-------------|-----------|---------|
| Cytosol                | 30.673           | 0.2         | 6.135            | 0.02        | 601       | 2.5     |
| pH treatment           | 10.054           | 0.3         | 3.016            | 0.04        | 402       | 2.5     |
| Ammonium sulfate       | 7.830            | 0.4         | 3.132            | 0.06        | 470       | 2.7     |
| DAE-Sepharose          | 300              | 7.2         | 2.160            | 1.2         | 360       | 2.0     |
| Sepharyl S-300         | 44               | 18.8        | 827              | 4.1         | 180       | 16.3    |
| Hydroxyapatite         | 5                | 86.2        | 377              | 16.3        | 78        | 170     |
| Synchronus Q300        | 1                | 98.8        | 94.8             | 17.8        | 17.1      | 30      |
Fig. 2. Co-migration of phenylalanine-α-keto-γ-methylbutyrate transaminase and cysteine conjugate β-lyase activities by HPLC anion exchange chromatography. A shows the co-chromatography of phenylalanine-α-keto-γ-methylbutyrate transaminase activity (●—●) and cysteine conjugate β-lyase (○—○) activity on a Synchropak Q300 anion exchange column and SDS-PAGE analysis of the purified protein; the major band has an M, of 55,000. Protein (——) is expressed as absorbance at 280 nm relative to the absorbance of the major peak, and nmol refers to the amount of product produced from a 10-min incubation. B shows the comigration of transaminase and cysteine conjugate β-lyase activity with protein (13 µg) upon nondenaturing gel electrophoresis of the purified protein. An identical gel was stained to show protein.

A 2.5-fold stimulation of β-lyase activity in rat kidney cortex cytosol. Dialysis of kidney cortex cytosol or purification through Step 4 (Table I) did not result in an increase in the ratio of independent to α-keto-γ-methylbutyrate-dependent cysteine conjugate β-lyase activity with DCVC as the substrate, suggesting that the presence of α-keto acids in the cytosol was not responsible for the modest dependence on α-keto-γ-methylbutyrate. Since gel filtration on Sephacryl S-300 (Step 5, Table I) resulted in an increase in the ratio of α-keto-γ-methylbutyrate stimulated to unstimulated activity from 2- to 10-fold, we investigated the possibility that a factor which stimulates β-lyase activity was separated by gel filtration from the α-keto-γ-methylbutyrate-stimulated β-lyase activity. Fig. 6 shows that a small peak (Peak 1) of apparent β-lyase activity, which was insensitive to α-keto-γ-methylbutyrate, eluted prior to the main peak of α-keto-γ-methylbutyrate-stimulated activity and phenylalanine-α-keto-γ-

Fig. 3. Lineweaver-Burk plot showing competitive inhibition kinetics for phenylalanine inhibition of cysteine conjugate β-lyase activity. Incubations were performed with 0.26 µg of purified enzyme and 1 mM DCVC using the standard assay conditions, in the absence (A) or presence of 0.1 (●) or 0.6 mM (○) phenylalanine. Reaction rates (V) are expressed as µmol of product/mg·10 min × 10⁻¹ and substrate (S) is mM.

Fig. 4. UV-visible spectrum of purified cysteine conjugate β-lyase. The enzyme concentration was 1.28 mg/ml in Buffer D (pH 7.5, see "Experimental Procedures"). The spectrum was obtained with a Hewlett-Packard 8451A spectrophotometer (Hewlett-Packard, Gaithersburg, MD).

metabolism butyrate transaminase activity. When aliquots from Peak 1 were added back to the fractions which contained the α-keto-γ-methylbutyrate-dependent β-lyase, activity with DCVC was restored in the absence of α-keto-γ-methylbutyrate (data not shown).

The protein in Peak 1 was purified further by ion exchange HPLC. The purified protein oxidized leucine to an α-keto acid (specific activity, 30 nmol/min·mg) and had a spectrum which was characteristic of a flavin-containing protein (Fig. 7 (24)), suggesting that the factor from Peak 1 is a flavin
TABLE II

Inhibition of cysteine conjugate β-lyase activity

Inhibition studies were done using enzyme with a specific activity of 0.8 μmol/mg·min. The enzyme was incubated in the standard assay mixture with the inhibitor for 15 min prior to the addition of 1 mM DCVC and 0.1 mM α-keto-γ-methylbutyrate.

| Inhibitor                  | mM  | Inhibition % |
|----------------------------|-----|--------------|
| Potassium cyanide          | 0.1 | 79           |
|                            | 0.01| 24           |
| Hydroxylamine              | 0.1 | 94           |
|                            | 0.01| 87           |
| Aminooxyacetic acid        | 0.1 | 95           |
|                            | 0.01| 91           |

Fig. 5. Stimulation of cysteine conjugate β-lyase activity by α-keto-γ-methylbutyrate. Enzyme (0.26 μg) and 1 mM DCVC were incubated for 10 min at 37 °C in 50 mM Tris-HCl (pH 8.0) in the absence or presence of various α-keto-γ-methylbutyrate (MTB) concentrations.

TABLE III

Structure-activity relationship for the stimulation of enzyme activity

Metabolism was assayed with DCVC as described under “Experimental Procedures.” All α-keto acids were added at 0.1 mM, and the stimulation is expressed as a percentage of the stimulation seen with α-keto-γ-methylbutyrate.

| α-Keto acid (0.1 mM) | Activity % |
|----------------------|------------|
| α-Keto-γ-methylbutyrate | 100        |
| Phenyl pyruvate       | 127        |
| α-Ketoocatoate        | 45         |
| α-Ketovalerate        | 27         |
| α-Ketoisocaproate     | 22         |
| Glyoxalate            | 14         |
| Oxaloacetate          | 13         |
| Pyruvate              | 10         |
| None                  | 9          |

containing amino acid oxidase (25). SDS-PAGE (Fig. 7) revealed a major diffuse band of about 38,000 daltons suggesting a high molecular weight protein with multiple subunits, consistent with data reported for renal amino acid oxidase (26).

The ability of the amino acid oxidase to stimulate enzyme activity was compared to α-keto-γ-methylbutyrate stimulation (Fig. 8). Stimulation by high concentrations of amino acid oxidase was almost as effective as α-keto-γ-methylbutyrate (0.1 mM; single point in Fig. 8). The stoichiometry, on a molar basis, of amino acid oxidase to β-lyase which produced maximal stimulation was 36:1, but ratios as low as 3:1 stimulated activity at a level which was 25% of the level achieved with 0.1 mM α-keto-γ-methylbutyrate. The calculations are based on an M₆ of 314,000 (25) for amino acid oxidase and 100,000 for the cysteine conjugate β-lyase.

Characterization of the Products from the Metabolism of DCVC by Amino Acid Oxidase and Cysteine Conjugate β-Lyase—It seemed possible that in the crude extracts from rat kidney cortex, an amino acid oxidase provides the necessary α-keto acid required to maintain β-lyase activity by oxidizing substrate to an α-keto acid. Walsh et al. (27) have shown that with β-chloro-D-alanine as substrate, D-amino acid oxidase from pig kidney can catalyze a β-elimination reaction producing ammonia, pyruvate, and chloride as the products or oxidize β-chloro-D-alanine to chloropyruvate and ammonia with the concomitant reduction of molecular oxygen. The ratio of pyruvate to chloropyruvate produced is dependent on the availability of oxygen as an electron acceptor, and a mixture of pyruvate and chloropyruvate was produced at 20% oxygen.

Our data suggested that pyruvate was not particularly effective in stimulating the β-lyase activity indicating that the stimulation might result from the oxidation of DCVC by rat kidney amino acid oxidase to the corresponding α-keto acid, S-(1,2-dichlorovinyl)-3-mercapto-2-oxopropionic acid.
under identical conditions in the presence of 0.1 mM \( \alpha \)-keto-7-conjugate \( \beta \)-lyase. The activity of cysteine conjugate \( \beta \)-lyase incubated with DCVC by rat kidney amino acid oxidase and cysteine conjugate \( \beta \)-lyase activity contributed by the amino acid oxidase alone from the total activity in the presence of the amino acid oxidase and cysteine conjugate \( \beta \)-lyase. The activity of cysteine conjugate \( \beta \)-lyase incubated under identical conditions but in the presence of 0.1 mM \( \alpha \)-keto-\( \gamma \)-methiolbutyrate (DMOP) by rat kidney cytosolic cysteine conjugate \( \beta \)-lyase.

Fig. 9. Reversed phase HPLC analysis of the metabolism of DCVC by rat kidney amino acid oxidase and cysteine conjugate \( \beta \)-lyase. Incubations were performed with (---) and without (- - -) enzyme and were analyzed by HPLC. A, analysis of a 30-min incubation at 37 °C with amino acid oxidase (65 \( \mu \)g) containing 40 \( \mu \)g/ml catalase, 0.05 M sodium pyrophosphate, and 1 mM DCVC in a 150-\( \mu \)l volume. B, analysis of a 10-min incubation at 37 °C which contained 1.3 \( \mu \)g of purified cysteine conjugate \( \beta \)-lyase, 1 mM DCVC, 0.5 mM \( \alpha \)-keto-\( \gamma \)-methiolbutyrate and 50 mM Tris-HCl (pH 8.0) in a 150-\( \mu \)l volume.

Fig. 10. Time course of \( S \)-(1,2-dichlorovinyl)-2-oxo-3-mercaptopropionic acid production by amino acid oxidase. Rat kidney amino acid oxidase (65 \( \mu \)g) was incubated with 1 mM [\(^{14} \)C]DCVC (20 cpm/nmol), 50 mM sodium pyrophosphate (pH 9.0), and 40 \( \mu \)g of catalase in a 150-\( \mu \)l volume. Radioactivity which was lost from either \([\text{\textsuperscript{14}}} \text{C}]\)DCVC or \([\text{\textsuperscript{35}}} \text{S}]\)DCVC indicating that all of the label from DCVC is in the product. Furthermore, when the \( \text{\textsuperscript{35}}} \text{S}\)-labeled product was derivatized with 2,4-dinitrophenylhydrazine, the radioactivity shifted to two peaks which eluted at a much higher methanol concentration (65%) and had absorbance at 380 nm, suggesting that the product had formed a 2,4-dinitrophenylhydrazone derivative of an \( \alpha \)-keto acid (data not shown). The 400-MHz \( ^1 \)H-NMR spectrum of the dinitrophenylhydrazones isolated from incubations (Fig. 11) was consistent with the structural assignment shown. The intensity of the vinyl proton signal increased when the acquisition time was increased from 4 to 30 s. This is consistent with the behavior of a proton which resides on a carbon with chlorine substituents and no nearby protons. The long relaxation time of the chlorovinyl proton accounts for the 1:0.8

with DCVC for 30 min at 37 °C, pyruvate has a retention time of 3.5 min on this column. When identical incubations were done using either \( \text{\textsuperscript{35}}} \text{S}\)- or \( \text{\textsuperscript{14}}} \text{C}\)-labeled DCVC and radioactivity in the peaks was quantitated, both labels were associated with the DMOP peak. Fig. 10 shows that the product in the DMOP peak is produced in a time-dependent manner concomitant with the loss of DCVC. The combined recovery of radioactivity in the DCVC and DMOP peaks was 97, 95, 96, and 97% at 10, 20, 30, and 60 min, respectively, suggesting that DMOP was the only major product and that the product had lost none of the radioactivity present in the parent DCVC. Table IV summarizes results from incubations with \( \text{\textsuperscript{35}}} \text{S}\)- and \( \text{\textsuperscript{14}}} \text{C}\)-labeled DCVC and shows that similar results are obtained with either \( \text{\textsuperscript{35}}} \text{S}\)- or \( \text{\textsuperscript{14}}} \text{C}\)-labeled DCVC indicating that all of the label from DCVC is in the product. Furthermore, when the DMOP peak was derivatized with 2,4-dinitrophenylhydrazine, the radioactivity shifted to two peaks which eluted at a much higher methanol concentration (65%) and had absorbance at 380 nm, suggesting that the product had formed a 2,4-dinitrophenylhydrazone derivative of an \( \alpha \)-keto acid (data not shown). The 400-MHz \( ^1 \)H-NMR spectrum of the dinitrophenylhydrazones isolated from incubations (Fig. 11) was consistent with the structural assignment shown. The intensity of the vinyl proton signal increased when the acquisition time was increased from 4 to 30 s. This is consistent with the behavior of a proton which resides on a carbon with chlorine substituents and no nearby protons. The long relaxation time of the chlorovinyl proton accounts for the 1:0.8

(DMOP) which then serves as a cosubstrate for transamination (Fig. 1). We investigated this possibility by HPLC analysis of the metabolites produced from incubations with DCVC and the purified rat kidney cytosolic amino acid oxidase. In addition, we characterized the products from the metabolism of DCVC by rat kidney cytosolic cysteine conjugate \( \beta \)-lyase.

Fig. 9A shows the HPLC chromatogram from a reaction mixture which contained the purified rat kidney amino acid oxidase and DCVC. A new peak, labeled DMOP, with a retention time of 19 min, is seen when the enzyme is incubated

![Graph](image1)

**Fig. 8. Stimulation of cysteine conjugate \( \beta \)-lyase activity by rat kidney amino acid oxidase.** Purified cysteine conjugate \( \beta \)-lyase (0.26 \( \mu \)g; 2.6 pmol) was incubated in the presence or absence of purified rat kidney amino acid oxidase (1 pmol = 0.31 pmol) and 1 mM DCVC. The cysteine conjugate \( \beta \)-lyase activity (■■■) and amino acid oxidase activity (O—O) were determined by subtracting the activity contributed by the amino acid oxidase alone from the total activity in the presence of the amino acid oxidase and cysteine conjugate \( \beta \)-lyase. The activity of cysteine conjugate \( \beta \)-lyase incubated under identical conditions but in the presence of 0.1 mM \( \alpha \)-keto-\( \gamma \)-methiolbutyrate (■■■) is shown for comparison.

![Graph](image2)

**Fig. 9. Reversed phase HPLC analysis of the metabolism of DCVC by rat kidney amino acid oxidase and cysteine conjugate \( \beta \)-lyase.** Incubations were performed with (---) and without (- - -) enzyme and were analyzed by HPLC. A, analysis of a 30-min incubation at 37 °C with amino acid oxidase (65 \( \mu \)g) containing 40 \( \mu \)g/ml catalase, 0.05 M sodium pyrophosphate, and 1 mM DCVC in a 150-\( \mu \)l volume. B, analysis of a 10-min incubation at 37 °C which contained 1.3 \( \mu \)g of purified cysteine conjugate \( \beta \)-lyase, 1 mM DCVC, 0.5 mM \( \alpha \)-keto-\( \gamma \)-methiolbutyrate and 50 mM Tris-HCl (pH 8.0) in a 150-\( \mu \)l volume.

![Graph](image3)

**Fig. 10. Time course of \( S \)-(1,2-dichlorovinyl)-2-oxo-3-mercaptopropionic acid production by amino acid oxidase.** Rat kidney amino acid oxidase (65 \( \mu \)g) was incubated with 1 mM [\(^{14} \)C]DCVC (20 cpm/nmol), 50 mM sodium pyrophosphate (pH 9.0), and 40 \( \mu \)g of catalase in a final volume of 150 \( \mu \)l as described under "Experimental Procedures." Aliquots of the incubation mixture (50 \( \mu \)l) were analyzed by HPLC, and the amount of \( S \)-(1,2-dichlorovinyl)-3-mercapto-2-oxopropionic acid (DMOP) produced and DCVC metabolized (■) were quantitated by HPLC. Data are expressed as cpm of \( ^{14} \)C label recovered or lost, and the total recovery at each time point is shown (C).

**TABLE IV**

| Isotope        | DMOP produced | DCVC metabolized |
|---------------|---------------|-----------------|
| \([\text{\textsuperscript{35}}} \text{S}]\)DCVC | 686 ± 5       | 661 ± 5         |
| \([\text{\textsuperscript{14}}} \text{C}]\)DCVC | 648 ± 4       | 632 ± 3         |

**Metabolism of \( S \)-(1,2-dichlorovinyl)-L-cysteine by amino acid oxidase** Radioactivity which was lost from either \([\text{\textsuperscript{14}}} \text{C}]\)DCVC or \([\text{\textsuperscript{35}}} \text{S}]\)DCVC as well as radioactivity appearing in DMOP was measured by reversed phase HPLC analysis as described under "Experimental Procedures." Amino acid oxidase (0.36 mg) was incubated for 30 min at 37 °C in a final volume of 150 \( \mu \)l with 1 mM DCVC, 50 mM sodium pyrophosphate buffer (pH 9.0), and 40 \( \mu \)g/ml catalase. Reactions were stopped by the addition of 16 \( \mu \)l of 5 M phosphoric acid, and 50 \( \mu \)l were injected onto the column. n = 2.
**DISCUSSION**

Cysteine conjugate β-lyase was purified from rat kidney cortex cytosol and found to have β-lyase activity with S-cysteine conjugates and phenylalanine-α-keto-γ-methylbutyrate transamination activity. Cooper (28) has shown that phenylalanine-α-keto-γ-methylbutyrate transamination activity in rat kidney is a property of glutamine transaminase K. The size and subunit composition of rat kidney cysteine conjugate β-lyase activity, as well as the predilection for α-keto acids with hydrophobic substituents at the β-carbon, suggests that the cysteine conjugate β-lyase reported here is identical to the soluble form of rat kidney glutamine transaminase K. Our results agree with those of Cooper and Meister (17) in that the enzyme is not stabilized by pyridoxal phosphate during the purification, unlike the hepatic form of cysteine conjugate β-lyase which has been shown to be a kynureninase and is stabilized by pyridoxal phosphate during purification (12). However, the inhibition of the enzyme by agents which inhibit pyridoxal phosphate enzymes and the UV-visible spectrum of the purified enzyme suggests that pyridoxal phosphate is a cofactor. Odum and Green (29) have reported a 24-fold purification of a soluble form of cysteine conjugate β-lyase from rat kidney. They include pyridoxal phosphate in some of the purification steps but do not report whether this stabilized the enzyme. In addition, these authors do not mention a role for either α-keto acid or an amino acid oxidase.

The results show that the enzyme is capable of catalyzing both transamination and β-elimination reactions with DCVC as substrate. In fact the ability to catalyze a β-elimination is dependent on a source of α-keto acids which act as amino group acceptors and complete the transamination reaction. According to the scheme in Fig. 1, when a source of α-keto acids is absent the enzyme would accumulate in the inactive pyridoxamine form which cannot accept another substrate. The requirement for an α-keto acid is fulfilled by the presence of an amino acid oxidase in the cytosol which complements the transamination activity by oxidizing DCVC to S-(1,2-dichlorovinyl)-3-mercapto-2-oxopropionic acid which then serves as the amino acceptor to complete the transamination.

A key feature of the scheme is that both reactions can occur through the formation of a common intermediate, the Schiff base between the pyridoxal phosphate and its amino acid substrate. Following Schiff base formation, pyridoxal phosphate enzymes are thought to abstract the α-proton of the substrate amino acid and stabilize the carbanion through resonance with the pyridine ring of the pyridoxal phosphate. The resulting quinoneimine intermediate (not shown in Fig. 1) is common to enzymes which catalyze β-elimination, β-replacement, and transamination reactions (30). If a good leaving group is present at the β-carbon, the quinoneimine can collapse via β-elimination to an enzyme-bound aminoacrylate, which is released from the enzyme and hydrolyzes to pyruvate nonenzymatically (Fig. 1 (30, 31)). Alternatively,
addition of water to the \( \alpha \)-carbon of the quinoneimine results in the formation of an \( \alpha \)-keto acid and an enzyme-bound pyridoxamine, completing the first half of a transamination reaction. The partitioning between \( \beta \)-elimination and transamination from a common quinoneimine intermediate can be involved in the mechanism-based inactivation of some pyridoxal phosphate-dependent enzymes (31).

A similar scheme was proposed for the stimulation of DCVC metabolism by pyruvate in LLC-PK1 cells (6). In LLC-PK1 cells, DCVC metabolism to pyruvate was stimulated 2-3-fold by the addition of 10 mM pyruvate to incubation mixtures containing cell homogenate and DCVC. The data presented here are consistent with this model. The 2-3-fold stimulation of activity in LLC-PK1 cell homogenates is similar to the stimulation seen in rat kidney cytosol (Table I).

At present we cannot determine how such S-1,2-dichlorovinyl-3-mercapto-2-oxopropionic acid might be produced in vivo from the metabolism of DCVC by glutamine transaminase K and/or amino acid oxidase, since it is both a substrate and a product. The data in Table V show that the ratio of S-1,2-dichlorovinyl-3-mercapto-2-oxopropionic acid to pyruvate is 1:4, respectively, when the incubation was done under conditions where the \( \alpha \)-keto-\( \gamma \)-methiobutyrate was limiting (0.1 mM). At this concentration, at least 80% of the \( \alpha \)-keto-\( \gamma \)-methiobutyrate was metabolized, while at a concentration of 0.5 mM, only 30% of the \( \alpha \)-keto-\( \gamma \)-methiobutyrate was metabolized (data not shown). Therefore, the apparent decrease in the ratio of S-1,2-dichlorovinyl-3-mercapto-2-oxopropionic acid to pyruvate in the presence of 0.1 mM \( \alpha \)-keto-\( \gamma \)-methiobutylate is probably due to the reutilization of the S-1,2-dichlorovinyl-3-mercapto-2-oxopropionic acid as a co-substrate for transamination when \( \alpha \)-keto-\( \gamma \)-methiobutyrate becomes limiting due to metabolism. The recycling of S-1,2-dichlorovinyl-3-mercapto-2-oxopropionic acid to DCVC and further metabolism via \( \beta \)-elimination and transamination would eventually result in the accumulation of pyruvate. Therefore, the ratio of products in vivo may also depend on the availability of \( \alpha \)-keto acid co-substrates. This recycling hypothesis is supported by the observation that amino acid oxidase stimulates the metabolism of DCVC in the absence of another amino acid or \( \alpha \)-keto acid substrate.

When the metabolism of DCVC by rat kidney amino acid oxidase was measured by the formation of a dinitrophenylhydrazine derivative, a product was formed which, under basic conditions, had absorption at 440 nm (data not shown), a wavelength at which, under basic conditions, the dinitrophenylhydrazine of pyruvate and \( \alpha \)-ketocisocaproate absorb. Therefore, since 2,4-dinitrophenylhydrazine will form a hydrazone with any \( \alpha \)-keto acid, it may not be appropriate for the detection of an \( \alpha \)-keto acid metabolite produced from an S-cysteine conjugate if structural information is not available.

The observation that S-1,2-dichlorovinyl-3-mercapto-2-oxopropionic acid can constitute 60% of the product from the metabolism of DCVC by glutamine transaminase K and is the sole product from the amino acid oxidase-catalyzed metabolism of DCVC raises the obvious question. Do \( \alpha \)-keto acids of S-cysteine conjugates play any role in toxicity? At this point, we have no information on the toxicity of \( \alpha \)-keto acid products from any cysteine conjugates. However, in this regard it is interesting to note that Kacorzowski and Walsh (32) have suggested that chloropyruvate produced from \( \beta \)-chloroalanine may be the species which inhibits active transport in Escherichia coli membrane vesicles.

Whether the oxidation of DCVC by the kidney amino acid oxidase plays a role in toxicity is not clear. However, aminoxyacetic acid inhibits DCVC toxicity in the rat (5) and in LLC-PK1 cells (6) but does not inhibit the amino acid oxidase isolated from rat kidney cytosol (data not shown). This suggests that the cysteine conjugate \( \beta \)-lyase arm of the metabolic scheme is necessary. It is possible, however, that inhibition of the amino acid oxidase would produce a similar blockade of toxicity if the metabolic cooperation between amino acid oxidase and cysteine conjugate \( \beta \)-lyase operates in the whole cell. Amino acid oxidase is also present in the mitochondrial fraction of rat kidney (25) along with cysteine conjugate \( \beta \)-lyase activity (14, 15). Mitochondria have been proposed as a site of S-cysteine conjugate toxicity; therefore, the purification of the mitochondrial \( \beta \)-lyase is an important issue. Moreover, it is possible that metabolic cooperation between amino acid oxidase and mitochondrial cysteine conjugate \( \beta \)-lyase occurs as well.

Cooper and Meister (17, 18, 28) have characterized a mitochondrial form of glutamine transaminase K which is very similar to the cytosolic form of glutamine transaminase K. However, Lash et al. (15) have performed mitochondrial fractionation studies in which they find that glutamine transaminase K is in the matrix, as reported by Cooper and Meister (18), but that cysteine conjugate \( \beta \)-lyase activity is present only in the outer membrane fraction indicating that glutamine transaminase K in mitochondria is not a \( \beta \)-lyase. Our preliminary data suggest that a cysteine conjugate \( \beta \)-lyase which is active with DCVC and is present in the rat kidney mitochondrial matrix is also dependent on the presence of an \( \alpha \)-keto acid and that the enzyme cross-reacts with antibody raised against the cytosolic enzyme, which we have shown is in fact glutamine transaminase K (33). Moreover, when assayed with DCVC, the mitochondrial matrix enzyme is also dependent on the presence of \( \alpha \)-keto-\( \gamma \)-methiobutyrate. Therefore, the matrix enzyme could be missed if one does not assay DCVC activity in the presence of an \( \alpha \)-keto acid.

In conclusion, we have shown that metabolism of DCVC in rat kidney cytosol is the result of cooperation between an amino acid oxidase and glutamine transaminase K. A novel \( \alpha \)-keto acid metabolite of DCVC has been identified. The data suggest that metabolic cooperation between an amino acid oxidase and a cysteine conjugate \( \beta \)-lyase activity may be important as a regulator and/or participant in the bioactivation of S-cysteine conjugates to toxic species. This hypothesis is under investigation.

REFERENCES
1. Jakoby, W. B., and Habig, W. H. (1980) in Enzymatic Basis of Detoxication (Jakoby, W. B., ed) p. 63, Academic Press, Orlando, FL
2. Chasseaud, L. F. (1976) in Glutathione: Metabolism and Function (Jakoby, W. B., and Arias, I. M., eds) p. 77, Raven Press, New York
3. Duffel, M., and Jakoby, W. B. (1982) Mol. Pharmacol. 21, 444-448
4. Inoue, M., Okajima, K., and Morino, Y. (1982) Hepatology (Baltimore) 2, 311-316
5. Ellarla, A. A., Jakobson, I., and Anders, M. W. (1986) Biochem. Pharmacol. 35, 283-288
6. Stevens, J. L., Hayden, P., and Taylor, S. (1986) J. Biol. Chem. 261, 3325-3332
7. Locke, E. A., and Ishmael, J. (1985) Toxicol. Appl. Pharmacol. 81, 32-42
8. Derr, R. F., and Schultz, M. D. (1983) Biochem. Pharmacol. 12, 465-474
9. Hassall, C. D., Gamboli, A. J., and Brendel, K. (1983) Drug Chem. Toxicol. 6, 567-572
10. Ozawa, N., and Guengerich, F. P. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 5266-5270
11. Monks, T. J., Lau, S. S., Hight, R. J., and Gillette, J. R. (1985) Drug Metab. Dispos. 13, 553-559
12. Stevens, J. L. (1986) J. Biol. Chem. 260, 7945-7950
13. Stevens, J. L. (1985) *Biochem. Biophys. Res. Commun.* 129, 499–504
14. Stonard, M. D., and Parker, V. H. (1971) *Biochem. Pharmacol.* 20, 2429–2437
15. Lash, L. H., Elfarra, A. A., and Anders, M. W. (1986) *J. Biol. Chem.* 261, 5950–5955
16. Beeler, T., and Churchich, J. E. (1976) *J. Biol. Chem.* 251, 5267–5271
17. Cooper, A. J. L., and Meister, A. (1961) *Comp. Biochem. Physiol.* 69B, 137–145
18. Cooper, A. J. L., and Meister, A. (1985) *Methods Enzymol.* 113, 344–349
19. Bradford, M. M. (1976) *Anal. Biochem.* 72, 248–254
20. Greenberg, D. M., Mastalerz, P., and Nagabhushanam, A. (1964) *Biochim. Biophys. Acta* 81, 158–164
21. Meister, A. (1965) in *Biochemistry of the Amino Acids* (Meister, A., ed) p. 164, Academic Press, Orlando, FL
22. Maizel, J. V. (1971) *Methods Virol.* 5, 179–245
23. Walsh, C. (1979) in *Enzymatic Reaction Mechanisms* (Walsh, C., ed) p. 782, Freeman and Co., San Francisco, CA
24. Walsh, C. (1979) in *Enzymatic Reaction Mechanisms* (Walsh, C., ed) p. 364, Freeman Publications, San Francisco, CA
25. Nakano, M., and Danowski, T. S. (1966) *J. Biol. Chem.* 241, 2075–2083
26. Nakano, M., Tarutani, O., and Danowski, T. S. (1968) *Biochim. Biophys. Acta* 168, 156–157
27. Walsh, C., Schonbrunn, A., and Abeles, R. H. (1971) *J. Biol. Chem.* 246, 6855–6866
28. Cooper, A. J. L. (1978) *Anal. Biochem.* 89, 451–460
29. Green, T., and Odum, J. (1985) *Chem.-Biol. Interactions* 54, 15–31
30. Miles, E. W. (1985) in *Transaminases* (Christen, P., and Metzler, D., eds) p. 470, John Wiley and Sons, New York
31. Likos, J. J., Ueno, H., Feldhaus, W., and Metzler, D. E. (1982) *Biochemistry* 21, 4357–4366
32. Kaczorowski, B., and Walsh, C. (1975) *J. Biol. Chem.* 250, 8931–8937
33. Robbins, J. D., and Stevens, J. L. (1986) *Pharmacologist* 28, 196