

STUDIES ON L-METHIONINE
 α -LYASE OF *PSEUDOMONAS*
OVALIS



1979

NOBUYOSHI ESAKI

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INTRODUCTION

Two different metabolic pathways have been proposed for the production of methanethiol from methionine. One of them is a pathway where methionine is first deaminated and then dethio-methylated with the release of methanethiol as reported for various aerobic bacteria and soil fungi (Segal & Starkey, 1969; Ruiz-Herrera & Starkey, 1969a). Alternatively, methionine is deaminated-dethio-methylated simultaneously to form methanethiol. The first evidence for the occurrence of this pathway was obtained by Onitake (1938) using the dried cells of *Escherichia coli* and *Proteus vulgaris*. Later the enzyme catalyzing the conversion of methionine into methanethiol, ammonia, and α -ketobutyrate was named methioninase (L-methionine γ -lyase, L-methionine methanethiollyase (deaminating) (EC 4.4.1.11)) (Ohigashi et al., 1951). L-Methionine γ -lyase was subsequently demonstrated in extracts of a soil bacterium (Mitsuhashi & Matsuo, 1950), *Pseudomonas* sp. (Miwatani et al., 1954; Kallio & Larson, 1955), *Clostridium sporogenes* (Wiesendanger & Nisman, 1953), rumen bacteria (Merrick & Salsbury, 1974), and *Ps. putida* (Ito et al., 1975). The highly purified enzyme, homogeneous judged from disc gel electrophoresis, was obtained from *Cl. sporogenes* by Kreis and Hession (1973) to show the antitumor activity. Little effort, however, has been devoted to characterization of the enzyme.

In this thesis, the bacterial distribution, the purification and the characterization, the enzymological and physicochemical properties of L-methionine γ -lyase are described.

CHAPTER I^{a,b}

DISTRIBUTION AND PURIFICATION OF L-METHIONINE γ -LYASE

In this chapter, the bacterial distribution of L-methionine γ -lyase, and purification of the enzyme from *Pseudomonas ovalis* are described.

EXPERIMENTAL PROCEDURE

Microorganisms and conditions of culture. *Ps. ovalis* IFO 3738 and other organisms were grown in a medium containing 0.25% L-methionine, 0.1% urea, 0.1% glycerol, 0.1% KH_2PO_4 , 0.1% K_2HPO_4 , 0.01% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.025% yeast extract. The pH was adjusted to 7.2 with NaOH. Cultures were grown at 28 °C for about 18 h under aeration. The cell harvested by centrifugation was washed twice with 0.85% NaCl solution, and subsequently with 0.01M potassium phosphate buffer (pH 7.2) containing 10^{-5} M pyridoxal 5'-phosphate (pyridoxal-P) and 0.01% 2-mercaptoethanol. The washed cells were frozen at -20 °C until used.

Enzyme assay. L-Methionine γ -lyase was routinely assayed by determining α -ketobutyrate formed. The standard reaction system contained 100 μmol of potassium phosphate buffer (pH 8.0), 100 μmol of L-methionine or other amino acids, 0.02 μmol of pyridoxal-P and enzyme in a final volume of 2.0 mL. Enzyme was replaced by water in a blank. Incubation was carried out at 30 °C for 10 min, and the reaction was terminated by addition of 0.25 mL of 50% trichloroacetic acid. After centrifugation, α -ketobutyrate was determined with 3-methyl-2-benzothiazolone hydrazone according to the procedure previously described (Soda, 1968).

Protein determination. Protein was determined by the method of Lowry et al. (1951) using crystalline egg albumin as a standard; with most column fractions, protein elution patterns were estimated by the 280-nm absorption. Concentrations of the purified enzyme

were derived from the absorbance at 278 nm. The absorbance coefficient ($A_{1\text{cm}}^{1\%} = 6.58$) was used throughout, obtained by absorbance and dry weight determinations.

Definition of units and specific Activity. One unit of enzyme is defined as the amount of enzyme that catalyzes the formation of 1 μmol of α -ketobutyrate per min. The specific activity is expressed as units per milligram of protein.

RESULTS AND DISCUSSION

Bacterial distribution of L-methionine γ -lyase. Screening was carried out in order to find out bacterial strains that would produce a high activity of L-methionine γ -lyase. Enzyme activity was determined by measuring the amount of α -ketobutyrate formed under aerobic or anaerobic conditions. The anaerobic incubation was carried out in a Thunberg test tube, the air in which was replaced by N_2 gas. Of the 17 strains of bacteria (8 *Pseudomonas*, 2 *Escherichia*, 1 *Aerobacter*, 1 *Alkaligenes*, 1 *Agrobacterium*, 1 *Bacillus*, 1 *Bacterium*, 1 *Brevibacterium*, and 1 *Proteus*) capable of deaminating L-methionine under aerobic conditions, only *Ps. ovalis*, *Ps. taetrolens*, *Ps. striata*, and *Ps. desmolytica* were found able to deaminate L-methionine under anaerobic conditions. *Ps. ovalis* in which L-methionine γ -lyase occurs most abundantly was chosen for the purpose of purification of the enzyme.

Purification of L-methionine γ -lyase. All operations were performed at 0 - 5 °C, unless otherwise stated all the buffers used contained 0.01% 2-mercaptoethanol.

Step 1: The washed cells (about 2.2 kg, wet weight) were suspended in 11 L of 0.01 M potassium phosphate buffer (pH 7.2), containing 10^{-5} M pyridoxal-P, and disrupted continuously with a Dyno-Mill (Willy A, Switzerland) at a flow rate of about 2 L per hour followed by centrifugation. The supernatant solution was dialyzed for 24 h against 1,000 volumes of the above-mentioned buffer. The

precipitate formed during dialysis was discarded.

Step 2: The enzyme solution was applied to a DEAE-cellulose column (12 X 60 cm) equilibrated with the dialysis buffer. After the column was washed thoroughly with the buffer, and then with the buffer containing 0.12 M NaCl. The enzyme was eluted with the buffer supplemented with 0.15 M NaCl. The active fractions were combined and brought to 20% saturation with ammonium sulfate. The precipitate was removed by centrifugation and ammonium sulfate was added to the supernatant to 50% saturation. The resultant precipitate was dissolved in 0.1 M potassium phosphate buffer (pH 7.2) containing 10^{-5} M pyridoxal-P and 20% glycerol, and dialyzed against 10,000 volumes of 0.01 M potassium phosphate buffer, pH 7.2.

Step 3: The enzyme was re-chromatographed with a DEAE-cellulose column (6 X 60 cm) in the same manner as described above. The active fractions were combined and concentrated by addition of ammonium sulfate (50% saturation). The precipitate was dissolved and dialyzed as described in Step 2.

Step 4: The enzyme was concentrated by ultrafiltration through a Diaflow membrane (Amicon, Cambridge, Mass., USA) to approximately 100 mg of protein per mL, and the resulting solution was supplemented with pyridoxal-P (final concentration, 10^{-4} M). To the enzyme solution was added 1 M potassium phosphate buffer (pH 7.2) under stirring to bring its final concentration to 0.1 M. After heating at 60 °C for 10 min, cooled rapidly and centrifuged to remove the precipitate.

Step 5: The supernatant solution was applied to a hydroxyapatite column (6 X 60 cm) equilibrated with 0.01 M potassium phosphate buffer (pH 7.2) containing 10^{-5} M pyridoxal-P. The enzyme was eluted with 0.03 M potassium phosphate buffer (pH 7.2) containing 10^{-5} M pyridoxal-P. The active fractions were collected and concentrated by ultrafiltration.

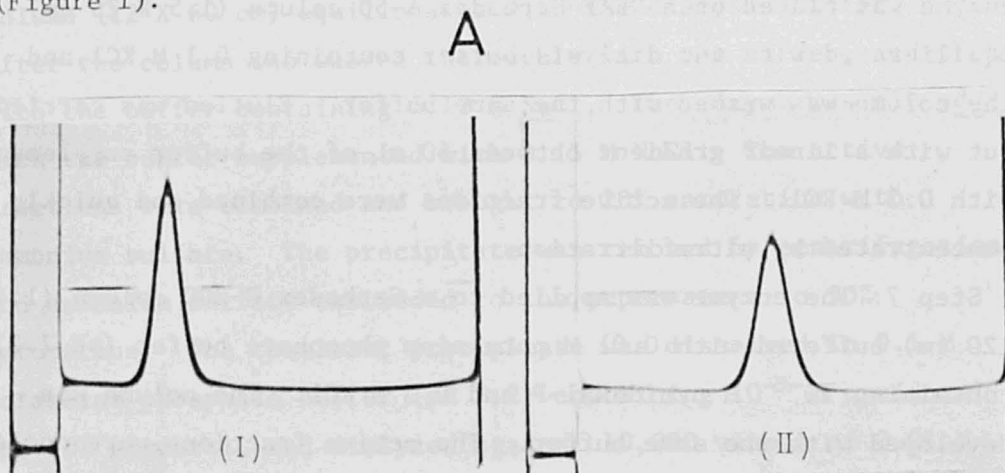
Step 6: The enzyme solution was dialyzed for 10 h against 1,000 volumes of 0.02 M potassium pyrophosphate buffer (pH 8.3). The enzyme was placed on a DEAE-Sephadex A-50 column (1.5 X 25 cm) equilibrated with the dialysis buffer containing 0.1 M KCl and the column was washed with the same buffer. Elution was carried out with a linear gradient between 60 mL of the buffer supplemented with 0.3 M KCl. The active fractions were combined and quickly concentrated by ultrafiltration.

Step 7: The enzyme was applied to a Sephadex G-200 column (1.4 X 120 cm) buffered with 0.01 M potassium phosphate buffer (pH 7.2) containing 10^{-5} M pyridoxal-P and 0.3 M KCl. The column was developed with the same buffer. The active fractions were pooled and concentrated. A summary of the purification is given in Table I.

TABLE I: Purification of L-Methionine γ -Lyase.

Step	Fraction	Total protein (mg)	Total units	Specific activity	Yield (%)
1.	Crude extract	132,000	5,600	0.040	100
2.	First DEAE-cellulose chromatography	17,700	3,800	0.220	68
3.	Second DEAE-cellulose chromatography	3,500	1,300	0.380	24
4.	Heat treatment	1,060	1,300	0.800	24
5.	Hydroxyapatite chromatography	550	900	1.640	17
6.	DEAE-Sephadex A-50 chromatography	360	650	1.810	12
7.	Sephadex G-200 chromatography	230	410	1.800	7

Purity. The purified enzyme was shown to be homogeneous by the criteria of ultracentrifugation and disc gel electrophoresis (Figure 1).



B

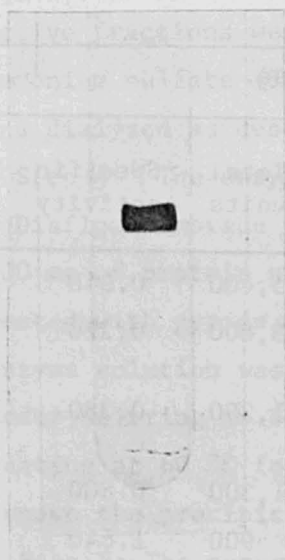


FIGURE 1. Sedimentation pattern (A) and disc gel electrophoresis (B) of L-methionine γ -lyase. (A) Sedimentation pattern was obtained at 0.96% of protein concentration in 0.01 M potassium phosphate buffer (pH 7.2). Pictures were taken at a bar angle of 70°, 40 (I) and 60 (II) min after achieving top speed (59,780 rev/min). (B) A sample of the enzyme preparation (50 μ g) was electrophoresed under the conditions of Davis (1964).

CHAPTER II^b

PROPERTIES OF L-METHIONINE γ -LYASE FROM *PSEUDOMONAS OVALIS*

Preparation of L-methionine γ -lyase from the cell-free extract of *Ps. ovalis* was described in CHAPTER I.

In this chapter, the physicochemical and enzymological properties of the enzyme are described.

EXPERIMENTAL PROCEDURE

Materials. S-Methyl-L-methionine (Toennis, 1940), S-(β -amino ethyl)-L-cysteine (Cavallini et al., 1955; Rothfus & Crow, 1968), S-(β -aminoethyl)-L-homocysteine (Hope & Horncastle, 1966), α -keto- γ -methiolbutyrate (Meister, 1952), lead methylmercaptide (Sliwinski & Doty, 1958), and N^E-pyridoxyllysine (Dempsey and Christensen, 1962) were prepared according to the methods given in the literature. L-Methionine and the other amino acids were products of Ajinomoto Co., Tokyo. Alanine dehydrogenase was purified from a cell-free extract of *Bacillus sphaericus* IFO 3525 to homogeneity and crystallized¹. L-Methionine γ -lyase was purified to homogeneity from a cell-free extract of *Ps. ovalis* as described in CHAPTER I. Thiols were obtained from Tokyo Chemical Industry Co., Tokyo, S-methyl-L-cysteine and S-ethyl-L-cysteine from Fluka AG, Buchs, pyridoxal-P was from Dainippon Seiyaku Co., Osaka, D-cycloserine from Shionogi Seiyaku Co., Osaka, D- and L-penicillamine were from Calbiochem, Calif., 3-methyl-2-benzothiazolone hydrazone HCl was from Aldrich Chemical Co., Inc. Pyridoxal-P was chromatographically purified by the method of Peterson & Sober (1954). Sodium lauryl sulfate was a specifically prepared reagent for protein research (Nakarai Chemical, Kyoto). The other chemicals were analytical grade reagents.

¹Ohshima, T., and Soda, K., manuscript in preparation.

Enzyme assay. Method A. The enzymatic α,γ -elimination reaction was routinely followed by determining α -ketobutyrate formed. The standard assay systems and conditions are described in CHAPTER I.

Method B. For the replacement reaction, the reaction system consisted of 40 μ mol of potassium phosphate buffer (pH 8.0), 20 μ mol of L-methionine or other sulfur amino acids, 20 μ mol of alkanethiols or substituted thiols, 0.02 μ mol of pyridoxal-P, and enzyme in a final volume of 0.5 mL. After incubation in a test tube sealed with a rubber plug at 30 °C for 10 min, the reaction was stopped by heating for 5 min at 100 °C, followed by centrifugation. The enzyme activity was determined by measuring the amount of sulfur amino acids formed with ninhydrin after separation by paper chromatography (Soda et al., 1961).

Ultracentrifugal analysis. The purity of the purified enzyme and its sedimentation coefficient were determined with a Spinco Model E ultracentrifuge equipped with a phase plate as a schlieren diaphragm. The molecular weight of the enzyme was determined by the ultracentrifugal sedimentation equilibrium method according to the procedure of Van Holde and Baldwin (1958). The experiments were carried out in a Spinco Model E ultracentrifuge equipped with Rayleigh interference optics. Multicell operations were employed in order to perform the experiment on four samples of different initial concentrations ranging from 0.37 to 0.94% with the use of An-G rotor and double cells of different side-wedge angles. The rotor was centrifuged at 6,166 rpm for 20 h at 20 °C. Interference patterns were photographed at intervals of 30 min to compare and make sure that the equilibrium was established. The relation between the concentration of the enzyme and the fringe shift was determined using the synthetic boundary cell.

Other analytical method. Spectrophotometric measurements were

made with a Shimadzu MPS-50L recording spectrophotometer or with a Carl Zeiss PMQ II spectrophotometer with a 1.0-cm light path. Amino acids in the incubation mixture were identified by chromatography and cochromatography with authentic materials on an amino acid analyzer (Yanagimoto LC-5S, Kyoto) by the method of Spackman et al. (1958). Sulfur amino acids on a paper chromatogram were visualized with a platinum reagent (Toennis & Kolb, 1951). Thiols such as methanethiol and ethanethiol were identified and determined with a Shimadzu Gas Chromatograph GC-4BM-PE equipped with a digital integrator ITG-4A using a 20% PEG-6000 on Gas-Chrom P column. Hydrogen and air were streamed in at 0.7 and 0.8 kg/cm², respectively. Both the injection port and the ionizing detector oven were heated at 150 °C. The column oven was kept at 90 °C. Infrared spectra were taken with a Hitachi EPI-S2 spectrophotometer and ¹H-NMR spectra with a Varian Associates recording spectrometer (A 60) at 60 MHz in deuterium oxide and deuteriotrifluoroacetic acid with sodium 4,4-dimethyl-4-silapentane-5-sulfonate and trimethylsilane, respectively, as an internal standard. Chemical shifts are reported in δ values (ppm).

RESULTS

Stability of enzyme. The purified enzyme can be stored in 0.01 M potassium phosphate buffer (pH 7.2) containing 10⁻⁵ M pyridoxal-P at 4 °C for 1 week without loss of activity. The enzyme is stable in a deep freeze (-20 °C), although repeated freezing and thawing cause a slight decrease in activity. The enzyme was found stable in the pH range of 6.5 - 9.0 when the enzyme solution (0.5 mg/mL) was heated at 50 °C for 5 min in the following buffers (a final concentration, 50 mM): Tris-maleate buffer, pH 5.5 - 6.5; Tri-HCl buffer, pH 8.0; potassium phosphate buffer, pH 7.0 - 8.0; potassium pyrophosphate buffer, pH 8.5 - 9.0; glycine-KOH buffer, pH 10; and K₂HPO₄-KOH, pH 11.5.

Molecular weight. The sedimentation coefficient ($S_{20,w}^0$) of the enzyme is 8.5 S. The molecular weight was determined to be about 180,000 by the Sephadex G-200 gel filtration method of Andrews (1964), with ovalbumin (43,000), bovine serum albumin (dimer, 136,000), bovine heart lactate dehydrogenase (140,000), bovine liver catalase (240,000) and bovine liver glutamate dehydrogenase (332,000) as standard proteins. A molecular weight of $173,000 \pm 2,000$ was also obtained by the sedimentation equilibrium method, assuming a partial specific volume of 0.74.

Structure of subunits. The subunit structure of the enzyme was examined by disc gel electrophoresis. The enzyme was treated with 6.5 M guanidine·HCl in 1.5 M Tris-HCl buffer (pH 8.1) containing 50 mM dithiothreitol for 2 h at room temperature followed by addition of monoiodoacetate (a final concentration, 0.1 M). The pH of the solution was kept at 8.5 with 6 N NaOH. After standing at room temperature for 1 h, the solution was dialyzed against 0.01 M sodium phosphate buffer (pH 7.0) and then incubated with sodium lauryl sulfate (a final concentration, 1.0%) at 50 °C for 1 h. The treated enzyme preparation (10 μ g) were subjected to electrophoresis in the presence of 0.1% sodium lauryl sulfate (Weber & Osborn, 1969). There were two bands of stained protein (Figure 1). To determine the molecular weight of the polypeptide

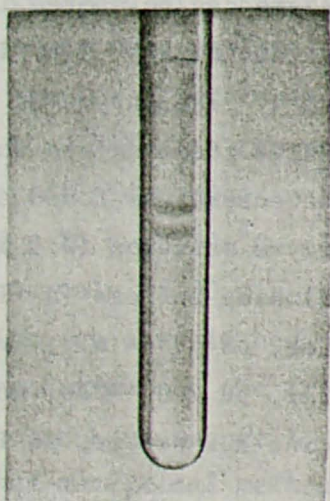


FIGURE 1: Sodium lauryl sulfate disc gel electrophoresis of carboxymethylated L-methionine γ -lyase. The conditions of carboxymethylation and sodium lauryl sulfate disc gel electrophoresis are given in the text. The direction of migration is from the cathod (top) to the anode.

in these bands, I ran a series of marker proteins treated in the same manner: chymotrypsinogen A (25,000), carboxypeptidase A (34,600), D-amino acid oxidase (37,000), ovalbumin (45,000), glutamate dehydrogenase of bovine liver (50,000), pyruvate kinase (57,000), catalase (60,000), and L-amino acid oxidase of snake venom (63,000). The molecular weights were calculated to be approximately 40,000 and 48,000, respectively, from a semilog plot of molecular weight against mobility.

Absorption spectrum of the enzyme and reduction with sodium borohydride. The enzyme exhibits absorption maxima at 278 (ϵ 118,000) and 420 nm (ϵ 28,000), and a slight shoulder peak around 330 nm (Figure 2A). No appreciable spectral shift occurred by varying the pH (6.0 to 10.0). The occurrence of the absorption peak at 420 nm suggests that the formyl group of the bound pyridoxal-P forms an azomethine linkage with an amino group of the protein, as in other pyridoxal-P enzymes thus far studied.

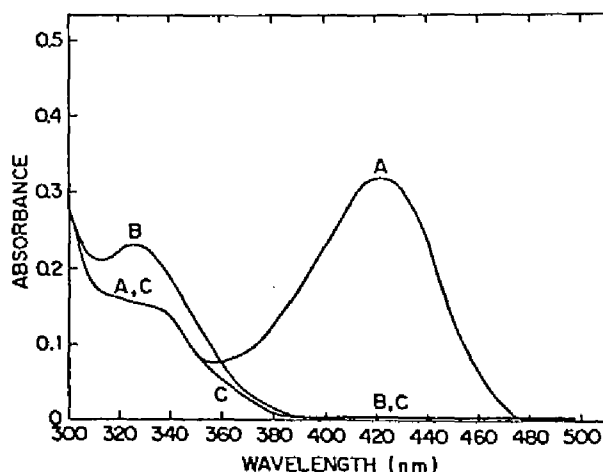


FIGURE 2: Absorption spectra of L-methionine γ -lyase. (Curve A) Holoenzyme in 0.01 M potassium phosphate buffer (pH 7.2); (curve B) holoenzyme reduced with NaBH_4 and dialyzed against the same buffer; (curve C) apoenzyme.

Reduction of the enzyme with sodium borohydride by the dialysis method of Matsuo & Greenberg (1959) affects both the absorption spectrum (Figure 2B) and the activity. Reduced enzyme was cata-

lytically inactive and the addition of pyridoxal-P did not reverse the inactivation. These results suggest that the borohydride reduces the aldimine linkage formed between the 4-formyl group of pyridoxal-P and an amino group of the protein to yield the aldamine bond. To identify the amino acid residue to which pyridoxal-P binds in the enzyme, the hydrolyzate of the NaBH_4 -reduced enzyme was examined by amino acid analysis and paper chromatography according to the method of Moriguchi et al. (1973). The fluorescent amino acid derivative in the hydrolyzate was identified with the authentic N^{ϵ} -pyridoxyllysine.

Resolution and reconstitution of L-methionine γ -lyase.

Pyridoxal-P was required for maximum activity of the enzyme. Pyridoxal-P is removed approximately 55% from the enzyme by dialysis against 0.01 M potassium phosphate buffer (pH 7.2) for 12 h. Full resolution of the enzyme was carried out as follows. The enzyme was incubated with 1 mM hydroxylamine solution (pH 7.2), followed by dialysis against three changes of the same buffer for 12 h. The enzyme thus treated had no detectable activity in the absence of added pyridoxal-P and no longer exhibited absorption maximum at 420 nm, but has a shoulder in the range of 330 nm (Figure 2C). Activity is about 70% restored by addition of pyridoxal-P. The Michaelis constant was estimated as 1.4 μM for pyridoxal-P.

Pyridoxal-P content. The amount of pyridoxal-P bound with the enzyme was examined by the phenylhydrazine method (Wada & Snell, 1961) and the 3-methyl-2-benzothiazolone hydrazone method (Soda et al., 1969), after the enzyme solution (1.4 mg in 0.5 mL) was desalted through a Sephadex G-25 column with deionized water, and treated with 1.0 mL of 0.1 N HCl at 37 °C for 30 min to release the bound pyridoxal-P. An average pyridoxal-P content of 4 mol/173,000 g of protein was obtained, indicating that 4 mol of pyridoxal-P are bound to 1 mol of the enzyme protein in the holoenzyme.

Substrate specificity. The ability of L-methionine γ -lyase to catalyze elimination reaction of various amino acids is presented in Table I. In addition to L-methionine, which is the preferred substrate, several derivatives of L-methionine and L-cysteine, e.g., L-ethionine, DL-methionine sulfone, L-homocysteine, and S-methyl-L-cysteine serve as the effective substrates. S-Methyl-L-methionine, S-(β -aminoethyl)-L-cysteine, and L-cysteine can be decomposed, though slowly, whereas D-methionine, D-cysteine, L-cystathionine, and L-norleucine are inert. These results provide evidence that the enzyme catalyzes both α, γ - and α, β -elimination reactions. The enzyme has the maximum reactivity at about pH 8.0 for all the substrates. The K_m values were calculated to be 1.33×10^{-3} M for L-methionine, 0.77×10^{-3} M for S-methyl-L-cysteine, 0.45×10^{-3} M for L-ethionine and 0.43×10^{-3} M for L-homocysteine.

TABLE I: Substrate Specificity.

Substrates ^a	Rel Act.
L-Methionine	100
D-Methionine	0
L-Ethionine	90
DL-Methionine sulfone	87
DL-Methionine sulfoxide	35
L-Methionine-DL-sulfoximine	27
S-Methyl-L-methionine	8
L-Homocysteine ^b	71
L-Norleucine ^c	0
L-Cystathionine ^c	0
L-Norvaline ^c	0
DL- α -Aminobutyrate	0
L-Cysteine	11
D-Cysteine	0
L-Cystine ^c	0
S-(β -Aminoethyl)-L-cysteine	14
S-Methyl-L-cysteine	67

^aThe concentration of the amino acid: 25 mM; ^b12.5 mM; ^c5 mM. The enzyme activity was determined by method A.

Reaction products. The products from L-methionine by the enzymatic α,γ -elimination were identified as α -ketobutyrate, ammonia, and methanethiol as follows. α -Ketobutyrate was identified by paper chromatography of its 2,4-dinitrophenyl-hydrazone using several solvent systems (e.g., 1-butanol-water-ethanol (5:1:1, v/v/v) (R_f 0.63), methanol-benzene-1-butanol-water (4:2:2:2, v/v/v/v) (R_f 0.80)). The identity of the compound was further confirmed with alanine dehydrogenase. The reaction mixture containing 100 μ mol of potassium phosphate buffer (pH 8.0), 100 μ mol of L-methionine, 0.02 μ mol of pyridoxal-P, and enzyme in a final volume of 2.0 mL was incubated at 37 °C for 60 min, followed by addition of 4 mL of 99.5% ethanol. The deproteinized solution was evaporated to dryness under reduced pressure. To the residue were added 500 μ mol of $\text{NH}_4\text{Cl-NH}_4\text{OH}$ buffer (pH 9.0), 0.1 μ mol of NADH, and 10 units of purified alanine dehydrogenase to make a final volume of 1 mL. After incubation at 37 °C for 60 min, the solution was subjected to an amino acid analyzer. The amino acid formed was identified as α -aminobutyrate and determined to be 6.27 μ mol. Formation of 6.30 μ mol of α -ketobutyrate was also observed separately by method A. Values obtained by both the methods were closely similar. α -Ketobutyrate was produced enzymatically also from L-ethionine, S-methyl-L-methionine, DL-methionine sulfone, DL-methionine sulfoxide, L-methionine-DL-sulfoximine, or L-homocysteine. When S-methyl-L-cysteine was employed as a substrate, pyruvate was formed, which was identified paper-chromatographically and enzymatically in the same manner.

Ammonia was identified and determined to be 6.25 μ mol with Nessler reagent (Thompson & Morrison, 1951). Methanethiol was identified by gas chromatography. Retention time of the authentic and enzymatically formed methanethiol was essentially the same under the conditions employed (0.94 min). The sulfur-containing products from L-ethionine and S-methyl-L-cysteine were identified

as ethanethiol (retention time: 1.52 min) and methanethiol (retention time: 0.94 min), respectively, in the same manner. The product from L-homocysteine and L-cysteine is volatile under acidic conditions and reacts with *N,N*-dimethyl-*p*-phenylenediamine to develop a blue color after addition of ferric chloride as an oxidizing agent. The colored compound had the same absorption spectrum as the product from sodium sulfide and *N,N*-dimethyl-*p*-phenylenediamine (λ_{max} 685 nm), indicating that hydrogen sulfide is produced from L-homocysteine and L-cysteine.

Stoichiometry of reaction. The reaction mixture (4 mL) containing 160 μmol of potassium phosphate buffer (pH 8.0), 50 μmol of L-methionine, 0.02 μmol of pyridoxal-P, and enzyme was incubated in an air-tight, two-necked tube at 37 °C for 1 h. After the reaction was terminated by addition of 0.5 mL of 50% trichloroacetic acid, one neck was provided with a capillary and the other was connected to a trapping chamber containing 6 mL of 5% mercuric acetate solution. Nitrogen gas was then introduced through the capillary into the incubation mixture for 20 min. Trapped methanethiol was measured by a modification of the method of Sliwinski & Doty (1958). α -Ketobutyrate and ammonia were determined as described above. The results indicate that α -ketobutyrate, ammonia, and methanethiol are formed stoichiometrically with a consumption of L-methionine (Table II).

TABLE II: Stoichiometry of Reaction.

System	L-Methionine Disappeared (μmol)	α -Ketobutyrate formed (μmol)	NH ₃ formed (μmol)	CH ₃ SH formed (μmol)
Complete	2.55	2.70	2.70	2.59
Complete minus substrate		0	0	0
Complete minus enzyme	0	0	0.10	0

Inhibitors. The various compounds were investigated for their inhibitory effects on enzyme activity. The enzyme is inhibited 70 - 100% after a 10-min incubation at concentrations of 0.1 - 1 mM hydroxylamine, L-penicillamine, and L-cycloserine, which were typical inhibitors for pyridoxal-P enzymes. The D enantiomers of penicillamine and cycloserine are much weaker inhibitors (inhibition: 24 and 9%, respectively). L-Methionine γ -lyase displays a high sensitivity to thiol reagents (0.1 - 1 mM) e.g., N-ethylmaleimide, HgCl₂, iodoacetate, and p-chloromercuribenzoate, which show 80 - 100% inhibition.

γ -Replacement reactions catalyzed by L-methionine γ -lyase.

(1) *Formation of ethionine.* It has been known that some pyridoxal-P enzymes have multiple catalytic functions, e.g., tryptophanase, tyrosine phenol-lyase (Kumagai et al., 1969), and cystathionine γ -synthase, which catalyze both elimination and replacement reactions as reviewed by Davis & Metzler (1972). Therefore, attempts were made to elucidate whether L-methionine γ -lyase shows replacement activity as well. An incubation was carried out at 30 °C with a reaction mixture (10 mL) containing L-methionine (0.4 mmol), ethanethiol (20 mmol), pyridoxal-P (0.2 μ mol), potassium phosphate buffer, pH 8.0 (0.2 mmol), and enzyme (1 mg). The enzyme and ethanethiol were added successively. Aliquot samples of the reaction mixture were withdrawn at intervals to follow the reaction. Methionine disappeared almost completely in 20 h. After deproteinization by addition of 0.5 mL of 50% trichloroacetic acid and centrifugation, the supernatant solution was applied to a column (1 X 24 cm) of Dowex 50-X8 (H⁺), washed thoroughly with water, and eluted with 0.3 N NH₄OH. The fractions containing the product were pooled and concentrated to a small volume, followed by evaporation to dryness under reduced pressure. The residues were dissolved in a small volume of hot 80% ethanol and allowed to crystallize at 4 °C. Recrystallization was per-

formed in the same way. The product was identified as ethionine based on the physicochemical analyses as follows. The $^1\text{H-NMR}$ spectrum of the product in $^2\text{H}_2\text{O}$ was demonstrated to be identical with that of authentic ethionine (Table III). The infrared spectra and the paper chromatographic behaviors of the enzymatic product and authentic ethionine were also identical. On elemental analysis of the enzymatic product, the following result was obtained. Anal. Calcd for ethionine ($\text{C}_6\text{H}_{13}\text{NO}_2\text{S}$): C, 44.15; H, 8.02; N, 8.58%. Found: C, 44.10; H, 8.05; N, 8.43%. The enzymatic product was quantitatively deaminated by L-amino acid oxidase, but not by D-amino acid oxidase, indicating that the enzymatically synthesized ethionine is the L isomer. The synthesis of ethionine proceeded as a function of enzyme concentration and incubation time (0 - 40 min). Thus, the enzyme catalyzes also the γ -replacement reaction between the thiomethyl group of methionine and ethanethiol to form L-ethionine.

L-Ethionine was also produced by the γ -replacement reaction between the derivatives of methionine (e.g., homocysteine, methionine sulfone and methionine sulfoxide) which are substrates for the elimination reaction and ethanethiol, and was identified in the same manner.

(2) *Formation of other S-substituted homocysteines.* When ethanethiol was replaced by various alkanethiols ($\text{C}_3 - \text{C}_7$) and arylthio alcohols (benzenethiol and β -naphthalenethiol) in the reaction system, new sulfur amino acids corresponding to the substrates, which are positive to both the ninhydrin and platinum reagent tests, were enzymatically synthesized. Longer chain alkanethiol ($\text{C}_8 - \text{C}_{10}$), however were not substrates. The amino acids produced from L-methionine with 1-propanethiol, 1-butanethiol, α -toluenethiol, and benzenethiol were purified in the same way as L-ethionine and were identified as the corresponding S-substituted homocysteine as follows. The $^1\text{H-NMR}$ spectra of the respective enzymatic products were consistent with the assigned

structures as presented in Table III. The infrared spectra of the products also were identical with those of the corresponding compounds prepared by the method of Kolenbrander (1969).

The derivatives of ethanethiol, e.g., 2-mercaptoethanol and cysteamine, also serve as the substituent donors to methionine. The products from 2-mercaptoethanol and cysteamine were purified in the same manner as described above and identified as S-(β -hydroxyethyl)homocysteine and S-(β -aminoethyl)homocysteine, respectively, on the basis of the following examinations. The proton magnetic resonance spectrum of the product from 2-mercaptoethanol gave at δ 2.30 (2H, triplet, $>\text{C}-3-\text{H}_2$), at 2.31 - 2.78 (4H, multiplet, $-\text{CH}_2-\text{S}-\text{CH}_2-$), at 3.60 (2H, triplet, $\text{OD}-\text{CH}_2-$), and 4.23 (1H, triplet, $>\text{C}-2-\text{H}$) in deuteriotrifluoroacetic acid. The infrared spectrum of the product using KBr pellets was demonstrated to be almost identical with that of L-ethionine, except that the absorption bands at 3,200 and 1,060 cm^{-1} , which arise from a hydroxy group. On elemental analysis of the product, the following result was obtained. Anal. Calcd for S-(β -hydroxyethyl)homocysteine ($\text{C}_6\text{H}_{13}\text{O}_3\text{NS}$): C, 40.22; H, 7.31; N, 7.82%. Found: C, 40.83; H, 7.28; N, 7.89%. These results reveal that the compounds is S-(β -hydroxyethyl)homocysteine.

The product from cysteamine was analyzed with an amino acid analyzer. It emerged between ammonia and histidine. Cochromatography of the product with authentic S-(β -aminoethyl)homocysteine confirmed their identity. The infrared spectra of the product and authentic S-(β -aminoethyl)homocysteine were also identical.

β -Replacement reaction catalyzed by L-methionine γ -lyase.
Formation of S-ethyl-L-cysteine. In addition the enzyme catalyzes the β -replacement reaction between S-methyl-L-cysteine and ethanethiol to yield S-ethyl-L-cysteine and methanethiol. The solutions of the product and authentic S-ethyl-L-cysteine were subjected to an amino acid analyzer. The elution time of the enzymatic product was identical with that of the authentic S-ethylcysteine. The

TABLE III: Proton Magnetic Resonance Spectra of the Enzymatically Synthesized *S*-Substituted Homocysteines.*

RSH, R =	Homocysteine Protons		<i>S</i> -Substituent Protons	Solvents
	α	β and γ		
Ethyl	3.35 t	1.63-2.86 m	<i>S</i> (α), 2.42-2.88 m <i>S</i> (β), 1.23 t	D ₂ O
<i>n</i> -Propyl	4.22 t	1.78-2.73 m	<i>S</i> (α), 2.30-2.69 m <i>S</i> (β), 0.83-1.52 m <i>S</i> (γ), 0.62 t	CF ₃ COOD
<i>n</i> -Butyl	4.67 t	2.34-3.11 m	<i>S</i> (α), 2.80-3.09 m <i>S</i> (β), 1.21-1.81 m <i>S</i> (γ), 0.75-1.13 m <i>S</i> (δ), 0.46 t	CF ₃ COOD
Benzyl	4.79 t	2.24-3.29 m	<i>S</i> (α), 4.09 s Phenyl, 7.74 s	CF ₃ COOD
Phenyl	4.90 t	2.34-2.89 m 3.42 t	Phenyl, 7.73 s	CF ₃ COOD

* The notations m, s, and t refer to multiplets, singlets, and triplets, respectively.

product was quantitatively deaminated with L-amino acid oxidase. Methanthiol was identified by the gas chromatographic method as described above.

DISCUSSION

Various elimination and replacement reactions play important roles in amino acid metabolism as reviewed by Davis & Metzler (1972). Although the mechanism of α,β -elimination and β -replacement reactions of amino acids catalyzed by pyridoxal-P-dependent enzymes have been extensively studied and well established (Snell & DiMari, 1970), those of α,γ -elimination and γ -replacement reactions have not been fully elucidated. A few pyridoxal-P-dependent enzymes catalyzing α,γ -elimination reaction have been reported, e.g., cystathionine γ -synthase (Kaplan & Flavin, 1966; Kerr & Flavin, 1970) and γ -cystathionase (Matsuo & Greenberg, 1959; Flavin & Segal, 1964). L-Methionine γ -lyase is unique in catalyzing α,γ and α,β -elimination reactions to produce alkanethiols

The studies described here deal with the enzymological and physicochemical characterization of the enzyme. The absorption spectrum of the enzyme ($\lambda_{\max}$ 420 nm with a shoulder at about 330

nm) closely resembles those of some other pyridoxal-P enzymes; e.g., L-leucine aminotransferase ($\lambda_{\max}$ 326 and 414 nm, Taylor & Jenkins, 1966), L-ornithine aminotransferase ($\lambda_{\max}$ 330 and 420 nm, Peraino et al., 1969), tyrosine phenol-lyase ($\lambda_{\max}$ 340 and 430 nm, Kumagai et al., 1970), and kynureninase ($\lambda_{\max}$ 337 and 430 nm, Moriguchi et al., 1973). L-Methionine γ -lyase behaves like tyrosine phenol-lyase (Kumagai et al., 1970) in that its spectrum does not appear to change with pH in contrast with tryptophanase (Morino & Snell, 1967), the spectrum of which shifts on varying pH, among pyridoxal-P enzymes catalyzing elimination and replacement reactions.

The determination of pyridoxal-P shows that 4 mol of the cofactor are bound per mol of enzyme (173,000 g). The value is close to those obtained for cystathionine γ -synthase of *Salmonella* (mol wt 160,000), γ -cystathionase of rat liver (190,000), and tryptophanase of *E. coli* (220,000). Although these enzymes consist of four identical subunits, L-methionine γ -lyase is composed of two nonidentical subunits. Further work is needed to elucidate which subunit pyridoxal-P binds to, and the structure of the binding site.

L-Methionine γ -lyase can decompose various β - and γ -substituted amino acids in addition to L-methionine. Conversion of the thioether of methionine into sulfoxide, sulfone, or sulfonium leads to a decrease in susceptibility to the enzyme. An ω -carboxyl group of cystine and cystathionine probably prevents the enzyme from binding the substrates, but the enzyme acts on S-(β -aminoethyl)-L-cysteine having an ω -amino group, though slowly. The enzyme is different from γ -cystathionase and cystathionine γ -synthase in this regard. Norleucine, norvaline, and D enantiomers of methionine and cysteine are not substrates, indicating that the enzyme cannot cleave a C-C bond, and the L configuration of α carbon of the substrates is necessary to bind the active site.

Ruiz-Herrera & Starkey (1969b) reported that an enzyme from *Aspergillus* catalyzes the γ -elimination of D-methionine, and α -keto and α -hydroxy analogues of methionine, but the L enantiomer is inert. α -Keto- γ -methiolbutyrate, however, is not a substrate for L-methionine γ -lyase. L-Methionine γ -lyase of *Ps. ovalis* is strikingly different from *Cl. sporogenes* enzyme (Kreis & Hession, 1973) in the substrate specificity. The enzyme of *Cl. sporogenes* can decompose, in addition to methionine, homocysteine, ethionine, cysteine, cystine, and certain other methionine analogues. The reactivity of methionine corresponds to only about 14 and 36% of those of homocysteine, which is the preferred substrate, and ethionine, respectively. The enzyme also catalyzes the γ -replacement reaction between methionine and its derivatives and alkane-thiols ($C_2 - C_7$, probably also C_1), arylthio alcohols, and the derivatives of ethanethiol.

Some multifunctional lyases have been demonstrated. Guggenheim & Flavin (1969) reported that cystathionine γ -synthase of *Salmonella* catalyzes α, γ -elimination and γ -replacement reactions of amino acids substituted at γ -C atom, and also α, β -elimination and β -replacement reactions of S-substituted amino acids. They showed as well that γ -cystathionase of *Neurospora* (Flavin & Segal, 1964) catalyzes both α, β - and α, γ -elimination reactions. The mechanism of α, β - or α, γ -elimination and β - or γ -replacement reactions by L-methionine γ -lyase is probably similar to that of cystathionine γ -synthase including formation of α -aminoacrylate or vinylglycine Schiff base as an intermediate (Guggenheim & Flavin, 1969) as reviewed by Snell & Di Mari (1970) and Davis & Metzler (1972).

CHAPTER III^C

CATALYTIC ACTION OF L-METHIONINE γ -LYASE ON SELENOMETHIONINE AND SELENOLS

Selenium is highly toxic but is also an essential trace element for animals and bacteria. Its biological role has been received considerable attention as reviewed by Stadtman (1973) and Shrift (1973). Proteins and enzymes which contain selenium as an essential component were isolated from animals and microorganisms, though the form of selenium is not known, except the selenium moiety of protein A of a glycine reductase complex from *Clostridium sticklandii* and glutathione peroxidase of rat liver-selenocysteine (2-amino-3-hydroselenopropionic acid) (Cone et al., 1976; Forstrom et al., 1978).

Several enzymatic processes that cannot distinguish between selenium and sulfur have been reported (Stadtman, 1973; Shrift, 1973). The selenium analogues of sulfur-containing amino acids such as methionine, cystathionine, and S-methylcysteine occur in higher plants and microorganisms. It is suggested that some of the selenium compounds, e.g., selenomethionine (2-amino-4-(methyl-seleno)butyric acid) and selenocysteine, are produced through the biosynthetic pathways of the corresponding sulfur amino acids, although the mechanism has not been investigated in detail. ATP: L-methionine S-adenosyltransferase (Greene, 1969) and S-adenosyl-methionine methyltransferase (Pegg, 1969) act on selenomethionine and Se-adenosylselenomethionine, respectively.

L-Methionine γ -lyase catalyzes α,γ -elimination and γ -replacement reactions of L-methionine and its derivatives, and also α,β -elimination and β -replacement reactions of S-substituted L-cysteines as described in CHAPTER II.

In this chapter, I describe the α,γ -elimination and γ -replacement reactions of selenomethionine, and also γ -replacement

reaction of methionine and its derivatives with selenols by L-methionine γ -lyase, the first proven mechanism for the incorporation of selenium into amino acids.

EXPERIMENTAL PROCEDURE

Materials. L-Methionine γ -lyase was purified to homogeneity from a cell-free extract of *Pseudomonas ovalis* (IFO 3738) as described in CHAPTER I. DL-Selenomethionine, sodium α -ketobutyrate, and crystalline lactate dehydrogenase of bovine heart were purchased from Sigma, crystalline glutamate dehydrogenase of bovine liver from Boehringer Mannheim GmbH, dimethyl diselenide from Alfa Division-Ventron, pyridine solution of *N,N*-dimethylformamide di-*n*-butyl acetal (Butyl 8) from Pierce Chemical, and D₂O (99.75%) from Merck. Alanine dehydrogenase was purified from a cell-free extract of *Bacillus sphaericus* (IFO 3525) to homogeneity.

Benzeneselenol was synthesized by the borohydride reduction of benzeneselenocyanate which was prepared by the method of Uemura et al. (1975). Sodium borohydride (0.68 g, 18 mmol) in ethanol was added dropwise over 40 min to benzeneselenocyanate (2.73 g, 15 mmol) in ethanol (a final volume of 20 mL) in a stream of N₂. Concentrated HCl (5 mL) was added to the mixture, followed by addition of 200 mL of water. After extraction with ether and drying over Na₂SO₄, the product was distilled under reduced pressure and N₂. The product was identified as benzeneselenol by ¹H-NMR. Thus, we obtained 0.9 g of benzeneselenol, whose boiling point is 63 °C (20 mmHg). 1-Hexaneselenol was synthesized in the same way. Sodium borohydride (0.57 g, 15 mmol) and 1-hexaneselenocyanate (2.5 g, 13 mmol) were allowed to react in 20 mL of ethanol under N₂. The product was identified as 1-hexaneselenol by ¹H-NMR. The yield was 0.5 g. Methaneselenol was prepared in a form of gas, since it is very volatile as reported by Baroni (1930) and Coates (1953). To 10 mL of dimethyl diselenide (3 g, 16 mmol) in ethanol,

10 mL of sodium borohydride (1.8 g, 48 mmol) in ethanol was added slowly at 30 °C. Evolved methaneselenol was introduced into the enzyme reaction mixture with N₂ as a carrier gas. The other chemicals were analytical grade reagents.

Enzyme assay. The enzymatic α,γ -elimination of selenium or sulfur amino acids was routinely followed by determining α -ketobutyrate, a product, with 3-methyl-2-benzothiazolone hydrazone HCl (MBTH) (Soda, 1968). The γ -replacement reaction was determined by measuring the amount of amino acids formed with ninhydrin after separation by paper chromatography (Soda et al, 1961). The standard assay systems and conditions were described in CHAPTER II. DL-Selenomethionine and selenols were substituted for L-methionine and thiols, respectively. Reactions with selenols were carried out in sealed tubes in which air was displaced with N₂. The pH dependence of the α,γ -elimination and γ -replacement reactions were examined in the following buffers (a final concentration of 50 mM): sodium acetate, pH 5.0 - 6.0; potassium phosphate, pH 6.5 - 8.0; sodium pyrophosphate, pH 8.0 - 9.5; NaHCO₃-Na₂CO₃, pH 9.5 - 10.5; and K₂HPO₄-KOH, pH 11.0 - 12.0.

The incubation mixture for the stoichiometric studies consisted of 20 μ mol of potassium phosphate buffer (pH 8.0), 0.02 μ mol of pyridoxal-P, 1.8 μ mol of DL-selenomethionine, and enzyme in a final volume of 1.0 mL. Water was substituted for selenomethionine in a blank. After incubation at 37 °C for 0.5, 1, and 3 h, the residual DL-selenomethionine was determined by paper chromatography as described above. Ammonia was assayed spectrophotometrically by determining NADH at 25 °C. The reaction mixture contained 60 μ mol of potassium phosphate buffer (pH 8.0), 25 μ mol of sodium α -ketoglutarate, 0.3 μ mol of NADH, a sample solution, and 30 units of glutamate dehydrogenase in a final volume of 1.0 mL. α -Ketobutyrate was determined spectrophotometrically with lactate dehydrogenase as well. The assay mixture in a final volume of 1.0 mL consisted of 40 μ mol of potassium

phosphate buffer (pH 8.0), 0.3 μmol of NADH, a sample solution, and 24 units of lactate dehydrogenase. The reaction was carried out at 25 °C for 30 min, and a change in A_{340} was read. Methaneselenol was determined with bis(5-carboxy-4-nitrophenyl)disulfide (DTNB) by use of a molar absorption coefficient of $13,600 \text{ M}^{-1} \text{ cm}^{-1}$ at 412 nm (Ellman, 1959; Nashef et al., 1977). The reaction was conducted at 30 °C for 3 h under anaerobic conditions in a Thunberg tube containing the reaction mixture in the main compartment and a mixed solution (2 mL) of 20 μmol of DTNB and 40 μmol of potassium phosphate buffer (pH 8.0) in the head compartment. After the DTNB solution was tipped into the enzyme reaction mixture, the absorbance at 412 nm was measured.

Selenols and thiols were determined with DTNB just before use, and the same amounts were added to the reaction mixture in the experiment where their γ -replacement reactions were compared. Concentrations of selenium amino acids other than selenomethionine were expressed in terms of selenomethionine concentration on the basis of its ninhydrin color yield. Specific activity of the enzyme was expressed as the amount of amino acid formed (μmol) per milligram of enzyme.

Gas chromatography-mass spectrometry. Gas chromatography-mass spectrometry was performed with a Shimadzu-LKB 9000 gas chromatograph-mass spectrometer. Ionization voltage, acceleration voltage, and trap current were 70 eV, 3.5 kV, and 60 μA , respectively. The ion source was kept at 290 °C. Gas chromatography was run on a coiled glass column (3 mm X 2 m) packed with 3% SE-52 on Chromosorb W (60 - 80 mesh; acid washed and silanized). Helium was used as a carrier gas at a flow rate of 30 mL/min. Injection port was kept at 280 °C. The column was programmed from 100 to 270 °C at a rate of 6 °C/ min.

Other instrumentation. Spectrophotometric measurements were made with a Carl-Zeiss PMQ II spectrophotometer with a 1.0-cm light path. ^1H -NMR spectra were taken with a JEOL JNM-FX 100

spectrometer operated at 99.65 MHz in the Fourier transform mode. Sodium 4,4-dimethyl-4-silapentane-5-sulfonate was used as an internal standard, and chemical shifts are reported in δ values (ppm).

RESULTS

α,γ -Elimination reaction of selenomethionine. When the enzyme reacted with DL-selenomethionine under the conditions described in CHAPTER II for L-methionine, I identified the products as α -ketobutyrate, ammonia, and methaneselenol as follows. Methaneselenol was identified by $^1\text{H-NMR}$ after oxidation with DTNB. The reaction was carried out in a Thunberg tube as described under Experimental Procedure except that all compounds were dissolved in D_2O . The enzyme solution in D_2O was prepared by repeated concentrations and dilutions with 10 mM potassium phosphate buffer in D_2O (pD 7.6) with an Amicon 202 ultrafiltration unit. After the tube was evacuated and flushed with N_2 three times, the reaction was started by tipping DL-selenomethionine into the main compartment and performed at 37°C for several hours. The DTNB solution in the head compartment was colored yellow (λ_{max} 412 nm), suggesting that volatile methaneselenol formed from selenomethionine reacted with DTNB to form 3-carboxylato-4-nitrothiophenolate anion (Ellman, 1959). The $^1\text{H-NMR}$ spectrum of the yellow solution showed a new singlet peak at δ 2.50, which probably arises from methyl proton, in addition to signals of HDO (δ 4.75) and phenyl protons (δ 7.50 - 8.12). When DL-selenomethionine was replaced by L-methionine, a singlet signal at δ 2.45 also appeared. The product from L-methionine is methanethiol as described in CHAPTER II, and methanethiol is certainly oxidized by DTNB to dimethyl disulfide or 3-carboxylato-4-nitrophenyl methyl disulfide under the conditions. Therefore, the singlet signal at δ 2.45 is assigned to the methyl proton of the oxidized product from methanethiol. The chemical shift of

the methyl proton of dimethyldiselenide is δ 2.548 as reported by Lardon (1970). This value is very consistent with that observed for the oxidized product from selenomethionine. Thus, the direct product from selenomethionine is methaneselenol. The formation of methaneselenol was confirmed also by the experiments in which solutions of mercuric acetate, lead acetate, silver nitrate, and cupric sulfate were used instead of the DTNB solution. Yellow precipitates were formed with gaseous methaneselenol in the head compartment of the Thunberg tubes. The precipitate was not formed under aerobic conditions. Baroni (1930) observed that the salts of methaneselenol formed with metal solutions are yellow.

α -Ketobutyrate was identified paper chromatographically and enzymatically as described in CHAPTER II. The formation of ammonia was confirmed by the glutamate dehydrogenase method (Kun & Kearney, 1974). Balance studies show that 1 mol of selenomethionine is converted to equimolar amounts of α -ketobutyrate, ammonia, and methaneselenol (Table I). The maximum reactivity was found at pH 8.0 for the α,γ -elimination reaction of selenomethionine. This value is closely similar to that observed for the α,γ -elimination of methionine.

TABLE I. Stoichiometry of Reaction.^a

Time (min)	Seleno- methionine disappeared (μ mol)	α -Keto- butyrate formed ^b (μ mol)	Ammonia formed (μ mol)	Methane- selenol formed (μ mol)
30	0.670	0.665	0.660	
60	0.755	0.750	0.756	
180	0.891	0.900	0.902	0.868

^aThe reactions and assays were carried out as described in the text. ^b α -Ketobutyrate was determined with MBTH and also with lactate dehydrogenase.

Kinetics. The kinetic parameters for DL-selenomethionine and L-methionine were determined under the standard assay conditions. K_m values were 0.51 mM for selenomethionine and 1.33 mM for methionine. I showed that D-methionine and D-cysteine are not substrates (CHAPTER II). Therefore, the value for selenomethionine was estimated on the assumption that only the L form is active. The V_{max} values for the α,γ -elimination reactions of selenomethionine and methionine were 2.27 and 1.88 ($\mu\text{mol/mg}$)/min, respectively.

γ -Replacement reactions of selenomethionine. I found that L-methionine γ -lyase catalyzes γ -replacement reaction between the selenomethyl group of selenomethionine and various thiols, e.g., ethanethiol, 1-propanethiol, and benzenethiol. The products were identified as the corresponding S-substituted homocysteines in the same manner as described in CHAPTER II. The enzyme showed an optimum reactivity at about pH 8.5 when examined in the DL-selenomethionine-1-propanethiol system. The rates of γ -replacement reactions of selenomethionine with ethanethiol, 1-propanethiol, and benzenethiol were approximately 24, 10, and 18%, respectively, lower than those of methionine.

Reactivity of selenols as substituent donors.

(1) *Formation of selenomethionine.* Gaseous methaneselenol was introduced into the reaction mixture (2.2 mL) containing 100 μmol of DL-methionine sulfone, 200 μmol of potassium phosphate buffer (pH 8.0), 0.04 μmol of pyridoxal-P, and 1.2 mg of enzyme. The reaction mixture was incubated at 30 °C for 2 h and heated at about 100 °C for 5 min, followed by centrifugation. The supernatant solution was chromatographed at 25 °C on Toyo No. 51 filter paper with 1-butanol-acetic acid-water (12:3:5, v/v/v) as a solvent. The area corresponding to the product (R_f 0.52) was cut off. The product was eluted with water and evaporated to dryness. To the residue was added 100 μL of a pyridine solution of *N,N*-dimethylformamide di-*n*-butyl acetal (2 mequi/mL), which reacts

with both carboxylic and amino groups of amino acids to form *N*-(*N*, *N*-dimethylaminomethylene)*n*-butyl ester (DMAM-BE) derivatives (Thenot & Horning, 1972). The reaction was carried out in a micro-bial with a Teflon cap liner at 60 °C for 20 min. The reaction mixture was subjected directly to gas chromatographic-mass spectrometric analysis. The derivative, whose retention time was 20 min under the conditions used, was identical by gas chromatography with the same derivative of authentic selenomethionine. The mass spectrum of DMAM-BE derivative of the product showed characteristic selenium isotope pattern at m/e 308 (M^+), 293 ($M - 15$), 264 ($M - 44$), and 251 ($M - 57$) (Agenäs, 1973). It coincided with that of authentic DL-selenomethionine. Thus, the product was identified as selenomethionine.

(2) *Formation of other Se-substituted selenohomocysteines.*

L-Methionine was incubated with the enzyme in the presence of benzeneselenol. The formation of a new amino acid, which reacts with a platinum reagent (Toennis & Kolb, 1951), was also observed by paper chromatography. To isolate and identify the amino acid, the reaction mixture on a four-times larger scale was incubated at 30 °C for 3 h under anaerobic conditions. After deproteinization the product was isolated and analyzed in the same manner as described for selenomethionine. The DMAM-BE derivative of the product gave a peak at 31.5 min by gas chromatography. The existence of Se atom in the molecule was suggested, on the basis of the relative abundance of each molecular ion peak and fragment, by mass spectrometry as described for selenomethionine. The spectrum was closely similar to that of the derivative of selenomethionine. The same fragment ion peaks were observed at m/e 44, 57, 84, 111, 112, 129, 170, 186, and 199 in both derivatives, and several other ion peaks of the derivative of product [e.g., molecular ion peak (m/e 370) and fragments at m/e 326 ($M - 44$) and m/e 269 ($M - 101$)] are higher than those of selenomethionine by 62 atomic mass units (amu). This difference is ascribed to the

difference between the methyl group (15 amu) of selenomethionine and the phenyl group (77 amu) of the product. The results show that the product is Se-phenyl selenohomocysteine.

The formation of Se-*n*-hexyl selenohomocysteine was shown in the same way with the reaction system in which benzeneselenol was replaced by 1-hexaneselenol.

When the derivatives of methionine (e.g., homocysteine) which are substrates for the α,γ -elimination reaction were incubated with benzeneselenol or 1-hexaneselenol, the corresponding Se-substituted selenohomocysteines were produced.

(2) *Other properties.* The reactivity of selenols was compared with that of thiols in a reaction system in which L-methionine was used as a substrate. Specific activities of the enzyme in γ -replacement reaction with benzeneselenol and 1-hexaneselenol were 0.094 and 0.167, respectively, whereas those with benzene-thiol and 1-hexanethiol were 0.900 and 0.211, respectively. The pH optimum of the enzyme in γ -replacement reaction with selenols was between 8.5 and 9.0.

DISCUSSION

Certain enzymes that participate in the metabolism of methionine are capable of acting on selenium analogues. Selenomethionine serves as a better substrate than methionine for ATP: L-methionine Se-adenosyltransferase of *Saccharomyces cerevisiae*, *Escherichia coli*, rabbit liver, and rat liver (Mudd & Cantoni, 1957; Greene, 1969). Se-Adenosylselenomethionine formed by the yeast enzyme also was shown to be an efficient methyl donor in various methylation systems (Coch & Greene, 1971). Hoffman et al. (1970) showed that methionyl-tRNA synthetase of *E. coli* works on both methionine and selenomethionine with almost the same affinity. The evidence presented here shows that selenomethionine is a substrate of L-methionine γ -lyase with higher affinity and

reactivity than methionine in α, γ -elimination, although is a little less reactive in γ -replacement reaction.

Pyridoxal-P dependent enzymes which catalyze replacement reactions play important role in biosynthesis of amino acids such as homocysteine, cysteine, and tryptophan. The formation of cysteine from *O*-acetylserine and H_2S by *O*-acetylserine sulfhydrylase has been demonstrated in microorganisms (Kredich & Thomkins, 1966; Wiebers & Garner, 1967) and higher plants (Giovanelli & Mudd, 1967). Chen et al. (1970) reported that serine is used as an intermediate in the biosynthesis of selenocysteine and Se-methylselenocysteine in *Astragalus bisulcatus* and suggested that *O*-acetylserine sulfhydrylase reacts with H_2Se to produce selenocysteine through γ -replacement. No evidence, however, has been obtained for the incorporation of Se atom into the selenium amino acids. I showed here that L-methionine γ -lyase can replace the γ -substituent of methionine and its derivatives with selenoalkyl or selenoaryl groups to form the corresponding Se-substituted selenohomocysteines. This is the first evidence showing that selenols are incorporated into selenium-containing amino acids. This enzymatic replacement reaction facilitates the synthesis of various selenium amino acids labeled with ^{14}C , ^{75}Se , and 3H .

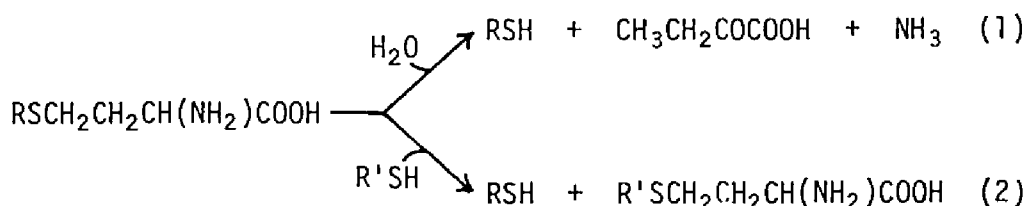
Snell & Di Mari (1970) and Davis & Metzler (1972) proposed that β - and γ -replacement reactions by pyridoxal-P enzymes proceed through nucleophilic addition of a substituent donor to the intermediate derived from the substrate amino acid. It is most likely that selenols are more reactive as substituent donors than thiols in the replacement reactions, since selenols are more nucleophilic (Klayman, 1973). I, however, found that selenols are less efficient substituent donors than thiols in the γ -replacement reaction. L-Methionine γ -lyase was slowly inactivated and the reaction rate decreased to 70% of the initial one when incubated at 25 °C for 1 h in the reaction mixture containing benzeneselenol. The low reactivity of selenols is not attributable to this partial inac-

tivation because the replacement reaction rate was determined as the initial velocity. Some other properties of selenols and thiols such as solubility and dissociation constant of ionization may affect their reactivity in the enzyme reaction, although further investigation is needed.

CHAPTER IV^d

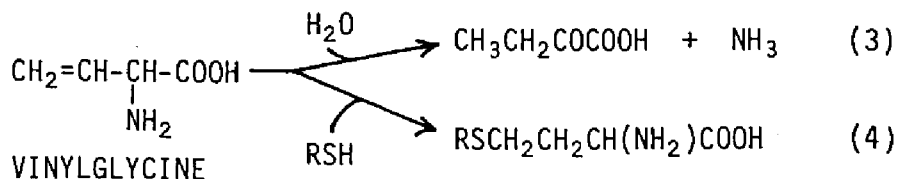
DEAMINATION AND γ -ADDITION REACTIONS OF VINYLGLYCINE BY L-METHIONINE γ -LYASE.

L-Methionine γ -lyase catalyzes α,γ -elimination (1) and γ -replacement (2) reactions of L-methionine and its derivatives, and also α,β -elimination and β -replacement reactions of S-substituted-L-cysteines. A Schiff base between pyridoxamine 5'-P and 2-keto-3-butenic acid is regarded as a key intermediate in the α,γ -elimination (1) and γ -replacement (2) reactions as reviewed by Snell & Di Mari (1970) and Davis & Metzler (1972).



The conversion of vinylglycine into α -ketobutyrate was demonstrated with serine-threonine dehydratase of sheep liver (Kapke & Davis, 1975) and tryptophan synthase of *Escherichia coli* (Miles, 1975). Cooper et al. (1976) reported the γ -addition reaction of vinylglycine by L-amino acid oxidase of snake venom.

The present investigation was undertaken to elucidate whether vinylglycine can be a substrate for deamination (3) and γ -addition (4) reactions by L-methionine γ -lyase.



EXPERIMENTAL PROCEDURE

Materials. DL-Vinylglycine was prepared as described previously (Rando, 1974). S-Substituted homocysteines were synthesized according to the method described in CHAPTER II. Crystalline lactate dehydrogenase of beef heart and crystalline glutamate dehydrogenase of beef liver were obtained from Sigma and Boehringer Mannheim GmbH, respectively. L-Methionine γ -lyase was purified to homogeneity from *Pseudomonas ovalis* (IFO 3738) (CHAPTER I).

Enzyme assay. The enzymatic α,γ -elimination reaction was followed by determining α -ketobutyrate by a method described in CHAPTER I with a quarter scale reaction system (final volume, 0.5 mL). The reaction mixture for the stoichiometric studies contained 4 μ mol of DL-vinylglycine as a substrate. The γ -replacement and γ -addition reactions were assayed as described in CHAPTER II except that 10 μ mol of DL-vinylglycine was used as a substrate. Ammonia was determined with indophenol (Russel, 1944) and with glutamate dehydrogenase (Kun & Kearney, 1974). DL-Vinylglycine was determined with ninhydrin after separation by paper chromatography (Soda et al., 1961).

Gas chromatography-mass spectrometry was performed with a Shimadzu LKB-9000 gas chromatograph-mass spectrometer. Gas chromatography was run on a coiled glass column (3 mm X 2 m) packed with 10% Silar 10 C on Gas Chrom Q 100 - 120 mesh. Helium was used as carrier gas, flow rate 30 mL/min. Injection port was kept at 200 °C. The column was programmed from 70 - 200 °C at a rate of 6 °C/min. Mass spectra were taken at 70 eV with 3.5 kV of accelerating voltage.

RESULTS

Deamination of vinylglycine. After the reaction mixture containing DL-vinylglycine was incubated at 30 °C for 30 min and 60 min, the residual substrate and the products were determined. Ammonia was identified and determined chemically and enzymatically as described above. α -Keto acid derived from vinylglycine reacted with lactate dehydrogenase and NADH to yield NAD. The product was identified as α -ketobutyrate by a comparison of the R_f values of its 2,4-dinitrophenylhydrazone with those of the authentic α -ketobutyrate by paper chromatography, carried out at 25 °C using several solvent systems, e.g., 1-butanol-water-ethanol (5:1:1, v/v/v) (R_f 0.6) and methanol-benzene-1-butanol-water (2:1:1:1, v/v/v/v) (R_f 0.8). An attempt also was made to identify the product by gas chromatography-mass spectrometry. After incubation for 2 h, the reaction mixture was deproteinized by addition of 0.1 mL 6 N HCl followed by centrifugation. The supernatant solution was passed through Dowex 50-H⁺. The eluate was evaporated to dryness under reduced pressure. The residue was dissolved in a small volume of ethylether, and esterified with diazomethane derived from *p*-tosyl-*N*-methyl-*N*-nitrosoamine (De Boer & Backer, 1963). The derivative, which showed a peak at 9.6 min by gas chromatography, was identical with the standard methyl α -ketobutyrate. The mass spectrum of the authentic methyl α -ketobutyrate was identical with that of the product; molecular ion peak at m/e 116, fragment peaks at 101 ($\text{CH}_3\text{CH}_2\text{CO-CO-O}^+$), 59 ($\text{CH}_3\text{O-C=O}$), 57 ($\text{CH}_3\text{CH}_2\text{C=O}^+$), 43 ($\text{CH}_3\text{C=O}^+$), and 29 (CH_3CH_2^+), among which those at m/e 29 and 57 were much more abundant than the others. Thus, the product was identified as α -ketobutyrate, which was determined with MBTH. The results in Table I indicate that the enzyme catalyzes reaction (3), and that the reaction proceeds stoichiometrically.

γ -Addition reaction of vinylglycine. When vinylglycine was

TABLE I: Stoichiometry of Reaction (3).

Incubation time (min)	DL-Vinylglycine disappeared (μmol)	α -Ketobutyrate formed (μmol)	Ammonia formed (μmol)
0	0	0	0
30	1.80	1.75	1.80
60	2.00	2.07	2.03

incubated with the enzyme in the presence of l-propanethiol, the formation of a sulfur-containing amino acid was observed by paper chromatography. To isolate and identify the amino acid, incubation was carried out at 30 °C for 2 h with a ten-times larger scale reaction mixture. After deproteinization the reaction mixture was chromatographed on Toyo filter paper No. 51 with 1-butanol-acetic acid-water (12:3:5, v/v/v) as a solvent. The area corresponding to the position of the product was cut off and eluted with water. The product was crystallized from the eluate as described in CHAPTER II.

The infrared spectra of the product and the authentic S-n-propyl homocysteine were identical. On elemental analysis of the enzymatic product, the following result was obtained. Anal. Calcd for S-n-propyl homocysteine ($\text{C}_7\text{H}_{15}\text{NO}_2\text{S}$): C, 47.5; H, 8.5; N, 7.9%. Found: C, 47.6; H, 8.3; N, 8.0%. These results reveal that the compound is S-n-propyl homocysteine. When l-propanethiol was replaced by several other thiols (e.g., ethanethiol, 1-butanethiol, toluenethiol, benzenethiol), the corresponding sulfur amino acids were formed. They were identified paper-chromatographically by a comparison of their R_f values with those of the authentic S-substituted homocysteines using two solvent systems, 1-butanol-acetic acid-water (12:3:5, v/v/v) and phenol-water (4:1, w/v).

Vinylglycine also served as a substrate in γ -addition reaction

with benzeneselenol and 1-hexaneselenol. The products were isolated and identified as Se-phenyl selenohomocysteine and Se-n-hexyl selenohomocysteine, respectively, in the same manner as described in CHAPTER III.

Kinetics. The kinetic parameters, K_m and V_{max} for DL-vinylglycine and L-methionine in reactions (3), (1), (4), and (2) in which 1-propanethiol was used as a substituent donor, are listed in Table II. The K_m value was calculated on the assumption that only the L-form is active as a substrate since D-methionine and D-cysteine are not substrates (CHAPTER II).

Vinylglycine has been shown to act as a suicide substrate upon L-aspartate aminotransferase (Rando, 1974; Rando et al., 1976), D-amino acid aminotransferase (Soper et al., 1977), and L-amino acid oxidase (Marcotte & Walsh, 1976). No inactivation of L-methionine γ -lyase occurred on incubation with 100 mM DL-vinylglycine at 25 °C for several hours.

TABLE II: Kinetic Constants for Reaction (1) - (4).

Reactions	K_m (mM)	V_{max} ($\mu\text{mol/mg/min}$)
Vinylglycine		
Reaction (3)	15	1.81
Reaction (4)	12	1.39
Methionine		
Reaction (1)	1.3	1.88
Reaction (2)	11	1.80

The enzyme shows a high affinity for L-methionine in α, γ -elimination reaction. The K_m and V_{max} values for methionine and vinylglycine in reactions (2), (3), (4) are closely similar. Relative activity of the enzyme for various alkanethiol in γ -addition reaction of vinylglycine is very close to that in

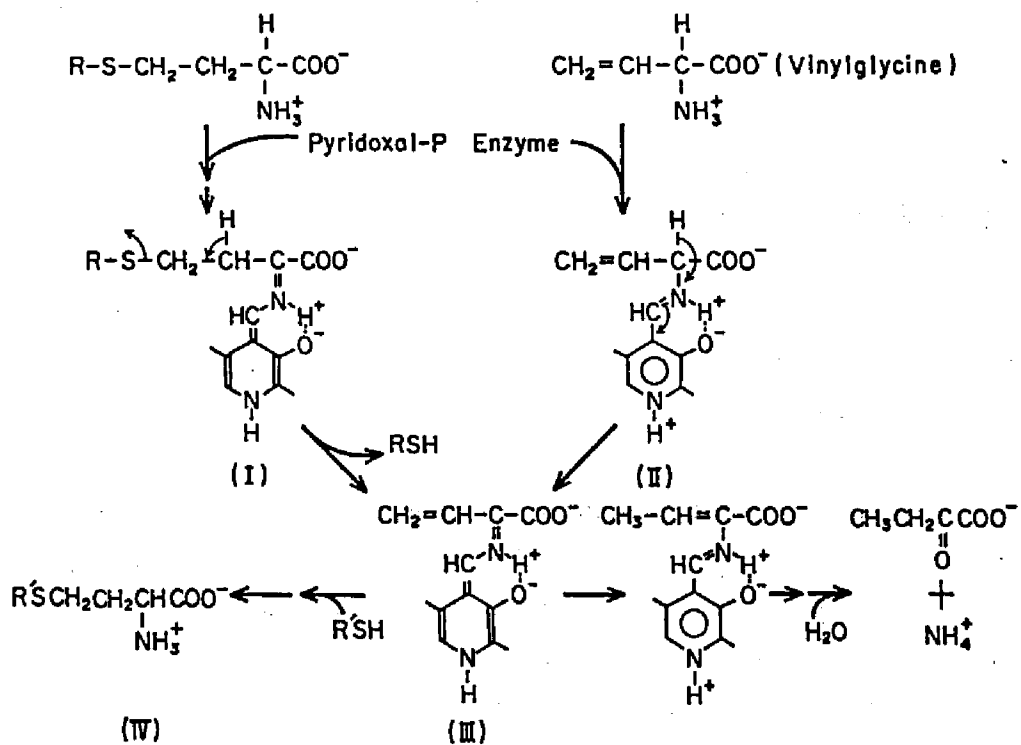
TABLE III: Relative Activity of Alkanethiols.

RSH R =	Vinylglycine (%)	Methionine (%)
Ethyl-	100	100
<i>n</i> -Propyl-	82	81
<i>n</i> -Hexyl-	23	17
Benzyl-	72	71

γ -replacement reaction of methionine (Table III).

DISCUSSION

The results obtained here support the proposed reaction mechanism (Davis & Metzler, 1972; Snell & Di Mari, 1970; Guggenheim & Flavin, 1969) for the α,γ -elimination and γ -replacement reactions through a pyridoxal-P Schiff base of vinylglycine as illustrated in SCHEME I. The formation of a ketimine quinoide intermediate of *S*-substituted homocysteine (I) is followed by loss of a β -hydrogen and γ -substituent. The resulting β,γ -unsaturated compound (III) is derived also from a pyridoxal-P aldimine intermediate of vinylglycine (II). The intermediate III can undergo γ -addition of R'SH to form a product IV, or tautomerization followed by hydrolysis to form α -ketobutyrate and NH_3 .



SCHEME I

CHAPTER V

REACTION MECHANISM OF L-METHIONINE γ -LYASE

The generally accepted mechanism for α,γ -elimination catalyzed by pyridoxal-P enzymes involves initial labilization of α and β -hydrogen atoms of the substrate amino acid, with subsequent elimination at the γ -position (Davis & Metzler, 1972). Flavin et al. proposed that a β,γ -unsaturated compound, a vinylglycine intermediate, is the common key intermediate of α,γ -elimination and γ -replacement reactions of cystathionine γ -synthase. Their proposal is based on the studies from the hydrogen exchange reaction of substrate amino acids in $^2\text{H}_2\text{O}$ and the analysis of deuterium distribution in α -ketobutyrate molecule formed in α,γ -elimination reaction (Guggenheim & Flavin, 1969; Posner & Flavin, 1972a, 1972b). However, no critical investigation has been done on the reactivity of the enzyme toward the postulated intermediate; vinylglycine.

L-Methionine γ -lyase purified to homogeneity from *Pseudomonas ovalis* catalyzes deamination and γ -addition reactions of vinylglycine. The products and kinetic properties of these reactions are consistent with the proposal of Flavin et al.

In this chapter, I describe the results of experiments undertaken to determine (1) α and β -hydrogen atoms of substrate amino acids are labilized; (2) the distribution of deuterium atom in α -ketobutyrate formed from methionine in $^2\text{H}_2\text{O}$ is the same as that from vinylglycine in $^2\text{H}_2\text{O}$; and (3) the spectrophotometric properties of the reaction intermediate from methionine is similar to those of vinylglycine.

EXPERIMENTAL PROCEDURE

Materials. L-Methionine γ -lyase was purified to homogeneity from a cell-free extract of *Pseudomonas ovalis* (IFO 3738) as described in CHAPTER I. $^2\text{H}_2\text{O}$ (99.75%) was purchased from Merck, *N,O*-bis-(trimethylsilyl)acetamide from Pierce, crystalline lactate dehydrogenase of bovine heart from Sigma, L-methionine from Ajinomoto, and other amino acids from Nakarai. The other chemicals are analytical grade reagents.

Methods. The enzymatic elimination reactions were followed by determining α -keto acids formed with MBTH (Soda, 1968). The standard assay systems and conditions were described in CHAPTER I.

The exchange reaction of the substrate proton with the solvent deuteron was conducted in an NMR tube containing 100 μmol of potassium phosphate buffer (pD 8.3) and 100 μmol of the substrate in 0.45 mL of $^2\text{H}_2\text{O}$. The enzyme solution in $^2\text{H}_2\text{O}$ was prepared as described in CHAPTER III. The reaction was initiated by addition of 0.05 mL of the enzyme (0.1 - 1.0 mg/mL) and performed at 28 °C. At appropriate time intervals, the ^1H -NMR spectra and peak integrals were taken with a JEOL JNM MH 100 spectrometer.

Gas chromatography-mass spectrometry was performed in the same manner as described in CHAPTER III except that the column was programmed from 70 - 90 °C at a rate of 3 °C/min.

Spectrophotometric measurements were made with a Carl-Zeiss PMQ II spectrometer or a Union SM 401 spectrometer with a 1.0-cm light path. The integrated amount of ΔA_{480} which corresponds to the area under the experimental trace of ΔA_{480} during the entire course of the reaction was determined by the method of Chance (1957).

RESULTS

^1H -NMR Studies of L-methionine γ -lyase-catalyzed incorporation of deuterium into substrates. (1). L-Methionine. Figure 1 shows

the ^1H -NMR spectral change of L-methionine observed during incubation with L-methionine γ -lyase in $^2\text{H}_2\text{O}$. The multiplet of β protons, which overlaps with the singlet of S-methyl protons at pD 8.3, can be shifted upfield from it in alkaline region. Therefore, ^1H -NMR spectra were recorded immediately after addition of 0.05 mL of 2% NaOD to the reaction mixture (0.5 mL) at appropriate time intervals. Peaks of the α and β protons disappeared almost completely after 57 min as these protons were replaced by deuterium, while those of the γ and the S-methyl protons did not disappear. After 183 min, the signal of the γ protons was transformed into a singlet, indicating that both of the β protons had exchanged. After 10 h, a broad singlet peak due to γ protons of α -ketobutyrate and a sharp singlet peak of methanethiol appeared in addition to two singlet peaks of γ and S-methyl protons of the deuterated methionine. In order to

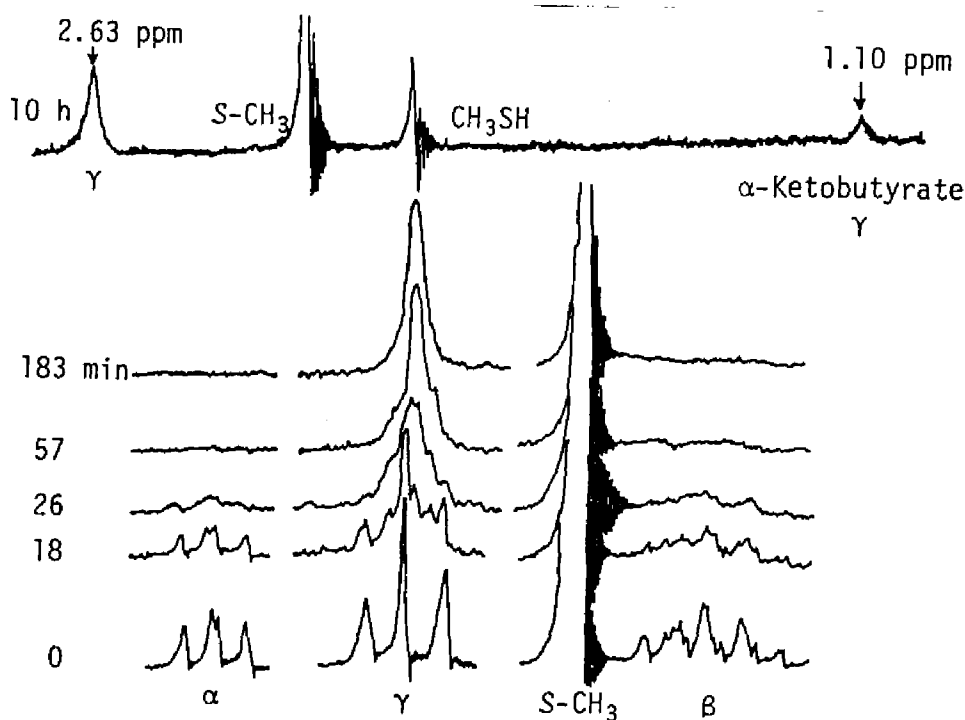


FIGURE 1: L-Methionine γ -lyase-catalyzed removal of α and β protons of L-methionine. The spectrum of L-methionine was recorded at zero time, and 56 μg of the enzyme was added.

compare the exchange rates of each protons, the log of peak integrals was plotted against time as shown in Figure 2A. A linearity was observed until more than 90% of the α proton was exchanged, whereas the time course of β proton exchange was bi-phasic; a faster process was followed by a slower one after about 65% of the β protons was deuterated. The peak integrals of the γ protons and the *S*-methyl protons decreased in the same rate of about 2.5% of the α proton loss. This decrease is certainly ascribed to the α,γ -elimination of methionine, since α -ketobutyrate was formed at the same velocity when aliquots of the reaction mixture were withdrawn and determined with MBTH.

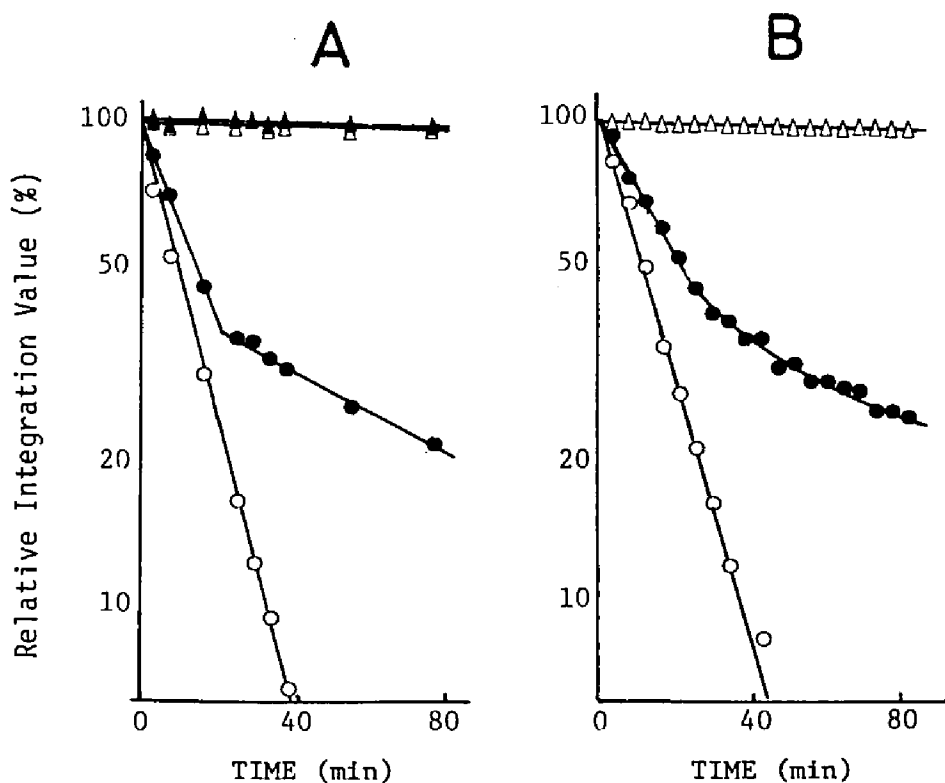


FIGURE 2: Rate of disappearance of proton signals of L-methionine (A) and *S*-methyl-L-cysteine (B): (○) α protons, (●) β protons, (▲) γ protons, and (Δ) *S*-methyl protons. The experimental conditions are described in EXPERIMENTAL PROCEDURE; 56 μ g of the enzyme was used.

(2). *S-Methyl-L-cysteine*. When *S*-methyl-L-cysteine was used as a substrate, a complete loss of the α and β protons was observed after 65 min. In semilog plots of each peak integrals against time, a similar relationship was found for a loss of the α proton (linear) and the β protons (biphasic) (Figure 2B). The rate of decrease of the *S* methyl protons was the same as that of the pyruvate formation due to α, β -elimination. Thus, the substrate proton exchange was found to proceed much faster than the net elimination reaction for both methionine and *S*-methylcysteine. The rate of net reaction in $^2\text{H}_2\text{O}$ was about 45% lower than that in $^1\text{H}_2\text{O}$ in both cases.

(3). *Amino acids with alkyl side chains*. Table I summarizes the results of the enzyme-catalyzed exchange of the α and β protons with solvent deuterons for several amino acids. All the amino acids listed except L-methionine and *S*-methyl-L-cysteine are non substrates for L-methionine γ -lyase in both elimination and replacement reactions. However, they competitively inhibited α, β -elimination of *S*-methyl-L-cysteine and α, γ -elimination of L-methionine, and did act as substrates in the hydrogen-deuterium exchange reaction. In every case, a loss of the α proton was of first order through more than four half-lives. For only L-alanine and L- α -aminobutyrate, the β proton exchange was also of first order through at least three half-lives. This is in contrast to the other cases; the exchange rate of the β protons decreased in a biphasic fashion when about a half of the β protons were lost as observed for methionine and *S*-methylcysteine cases. The exchange rates of β protons calculated for the faster processes are listed in Table I. The exchange rate in the slower process was one-fourth to one-third slower than the faster process in every case. However, all the β protons were eventually deuterated on prolonged incubation. Of non-substrate amino acids tested, L-norleucine showed the highest binding affinity (K_i), and was followed by L-norvaline, L-alanine, and L- α -aminobutyrate

TABLE I: Rates of Exchange of α and β Protons.

Compound RCHNH ₂ COOH R =	K _i or K _m (mM)	Exchange Rate (μ mol/min)		Number of β -H Exchanging
		α -H	β -H	
CH ₃ - Alanine	5.1	0.2	0.1	3
CH ₃ CH ₂ - α -Aminobutyrate	8.4	2.7	1.7	2
CH ₃ CH ₂ CH ₂ - Norvaline	3.0	14,8	5.2	2
CH ₃ CH ₂ CH ₂ CH ₂ - Norleucine	0.5	18.8	6.0	2
CH ₃ SCH ₂ - S-Methylcysteine	0.8 (K _m)	28.6	15.0	2
CH ₃ SCH ₂ CH ₂ - Methionine	1.3 (K _m)	31.6	15.8	2

in this order. The exchange rate of α and β protons increased with a increase in length of the alkyl side chain of amino acids.

Deuterium distribution in α -ketobutyrate formed from methionine in $^2\text{H}_2\text{O}$. α -Ketobutyrate formed from methionine by α,γ -elimination after 10 h incubation was found to contain hydrogen atom only at its γ -position when examined by ^1H -NMR as described above. Since the enzyme catalyzes the rapider incorporation of deuterium into methionine itself than the net α,γ -elimination reaction, the product, α -ketobutyrate should be derived from non-deuterated and also from deuterated methionine. Therefore, it is important to analyze the deuterium distribution in α -ketobutyrate formed at different stages during incubation when methionine remains almost intact or is deuterated to a considerable extent. I analyzed the product by (1) the gas chromatography-mass spectrometry of the α -hydroxy derivative, and (2) the ^1H -NMR spectra of the

2,4-dinitrophenylhydrazone derivative.

(1). *Gas chromatographic-mass spectrometric analysis.*

In order to avoid the deuterium incorporation into the β -position of α -ketobutyrate by enolization, α -ketobutyrate formed was transformed into its α -hydroxy derivative by the action of lactate dehydrogenase and NADH as follows. The reaction mixture was made on a 3.2-times larger scale (a final volume, 1.6 mL in $^2\text{H}_2\text{O}$) than that described in EXPERIMENTAL PROCEDURE, and contained L-methionine as a substrate, 2 μmol of NADH and 64 units of lactate dehydrogenase. After incubation at 28 °C for 10 and 120 min, the reaction was stopped by addition of 0.05 mL of concentrated HCl followed by centrifugation. The supernatant solution was passed through Dowex 50- H^+ , and the eluate was evaporated to dryness under reduced pressure. The residue was further dried over P_2O_5 and dissolved in 50 μL of *N,O*-bis-(trimethylsilyl)acetamide. The TMS derivative thus obtained, whose retention time was 6.2 min under the conditions used, was identical with the same derivative of authentic α -hydroxy-*n*-butyric acid ($(\text{TMS})_2$ - α -hydroxy-*n*-butyric acid). The mass spectrum of the derivative of enzyme reaction product showed peaks of $M - 15$, $M - 43$, and $M - 117$ which were higher than those of the authentic compound by 2 or 3 atomic mass units (Figure 3). This indicates that α -ketobutyrate molecule containing 2 and 3 deuterium atoms were formed in the enzyme reaction. When calculated from the relative abundance of each of $M - 117$ (m/e 133 and 134), the ratio of $^2\text{H}_2$ - α -ketobutyrate to $^2\text{H}_3$ - α -ketobutyrate was 0.55 in the 10-min reaction, whereas in the 120-min reaction was 0.04. Almost the same results were obtained from peaks of $M - 15$ and $M - 43$. These results suggest that $^2\text{H}_2$ - α -ketobutyrate initially present was increasingly diluted with $^2\text{H}_3$ - α -ketobutyrate as the reaction proceeded. The formation of non-deuterated or mono-deuterated α -ketobutyrate must be negligible if any, since fragment peaks derivable from them were not included in the spectrum.

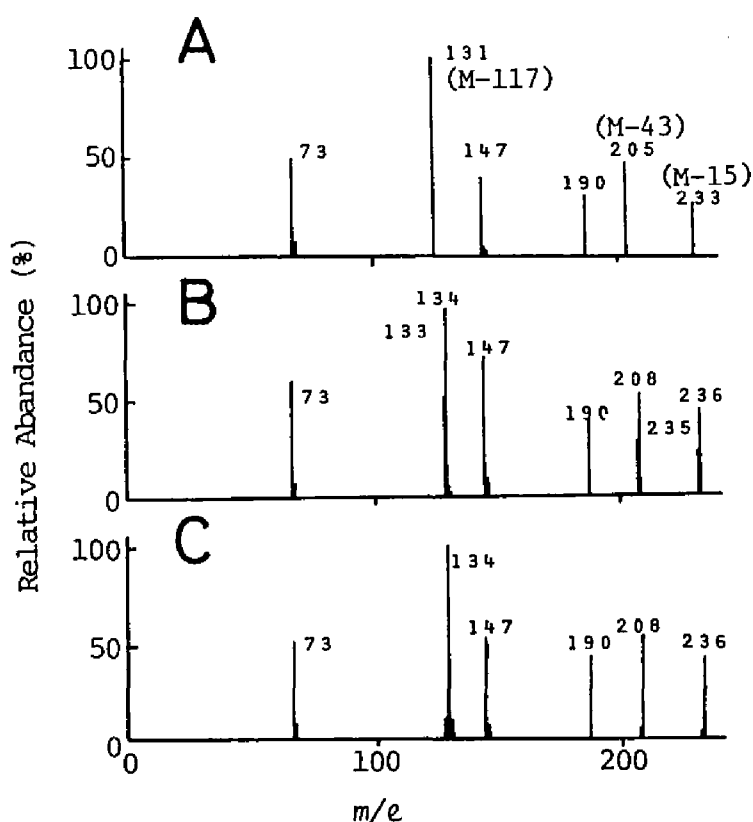


FIGURE 3: Mass spectra of TMS-derivative of α -hydroxybutyrate which was derived from (A) authentic α -ketobutyrate, (B) the product of the 10-min reaction, and (C) the product of the 120-min reaction.

(2). $^1\text{H-NMR}$ analysis. I determined how many deuterium atoms were incorporated into α -ketobutyrate at β and γ -positions. The reaction mixture containing L-methionine as a substrate was made on a 40-times larger scale (a final volume, 20 mL in $^2\text{H}_2\text{O}$) than that shown in EXPERIMENTAL PROCEDURE. After incubation at 28 °C for 10 and 120 min, α -ketobutyrate was isolated as its 2,4-dinitro-phenylhydrazone essentially by the method of Posner & Flavin (1972). The number of hydrogen atoms at β and γ -positions of α -ketobutyrate was expressed as the relative value of peak

integrals against the phenyl proton at C₃ carbon taken as 1. The β and γ protons measured by deuterium decoupling suggested the presence of (β -²H₁, γ -²H₁)- α -ketobutyrate and (β -²H₂, γ -²H₁)- α -ketobutyrate (Figure 4). The results of mass spectrometry indicate that α -ketobutyrate containing less than one deuterium atom must be negligible. Thus, α -ketobutyrate formed in the 10-min reaction contained 36% of (β -²H₁, γ -²H₁)-type and 64% of (β -²H₂, γ -²H₁)-type, and that in the 120-min reaction contained 4% of the former and 96% of the latter. The values are in good agreement with the results from mass spectrometry. However, it is important to ascertain whether some deuterium incorporation into the β -position occurred by enolization. There was less than 5% loss of β protons after 3 h in a control experiment where L-methionine was replaced by α -ketobutyrate. Therefore, α -ketobutyrate formed in the enzyme reaction must retain its β -hydrogen almost completely even after 120 min.

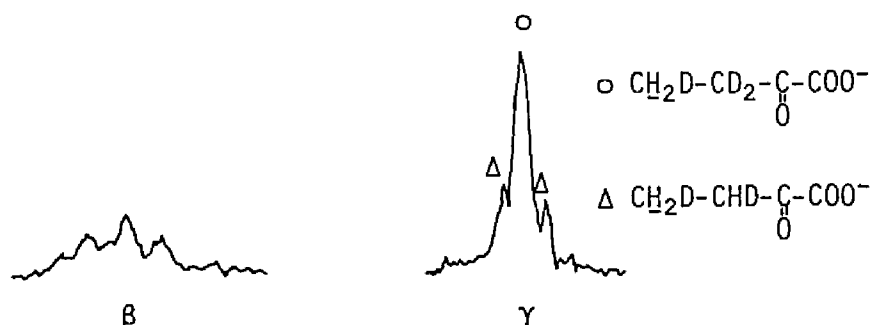


FIGURE 4: ¹H-NMR spectra of β and γ -hydrogens of 2,4-dinitrophenylhydrazone of α -ketobutyrate formed from L-methionine in ²H₂O.

Deamination reaction of vinylglycine in ²H₂O. L-Methionine γ -lyase catalyzes deamination and γ -addition reactions of vinylglycine. Pyridoxal-P Schiff base of it is proposed to be an intermediate of α,γ -elimination. Therefore, I monitored the deamination reaction of L-vinylglycine by ¹H-NMR. Figure 5 demonstrates the time course of disappearance of α , β , and γ protons of vinylglycine and appearance of β and γ protons of α -ketobutyrate.

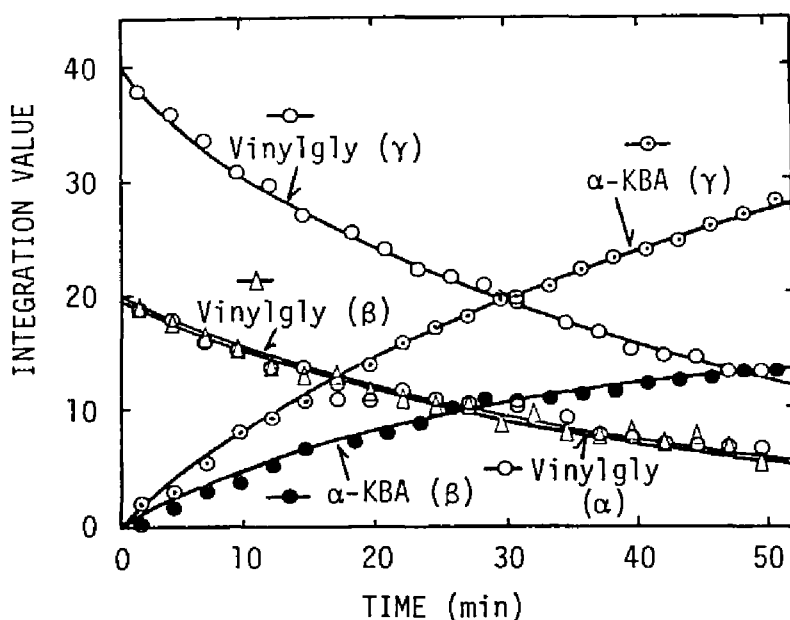


FIGURE 5: Disappearance of α , β , and γ protons of L-vinylglycine, and appearance of β and γ protons of α -ketobutyrate. The conditions are given in the text. The notations Vinylgly and α -KBA refer to vinylglycine and α -ketobutyrate, respectively.

A change in signal integration of each protons of vinylglycine and α -ketobutyrate was of first order. Disappearance of each protons of vinylglycine and appearance of each protons of α -ketobutyrate occurred in the same rate. This indicates that the enzyme catalyzes only deamination, but not proton exchange reaction.

The $^1\text{H-NMR}$ spectra after 196 min showed that α -ketobutyrate formed was only the (β - $^2\text{H}_1$, γ - $^2\text{H}_1$)-type (Figure 6). This was further confirmed by the experiments where α -ketobutyrate was analyzed as its 2,4-dinitrophenylhydrazone as in the case of methionine.

Absorption spectrum of L-methionine γ -lyase during α,γ -elimination. L-Methionine γ -lyase exhibits an absorption maximum at 420 nm as shown in Figure 7A. When L-methionine was

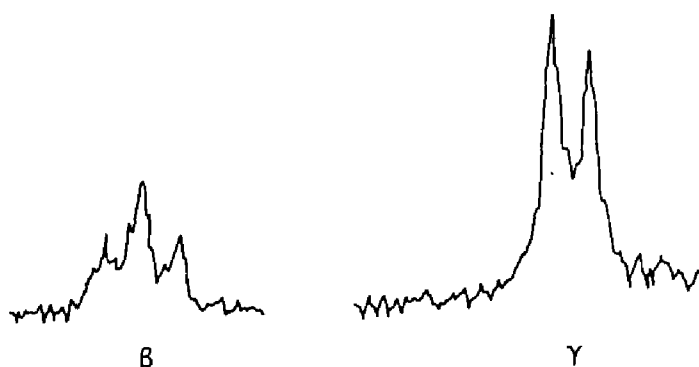


FIGURE 6: ^1H -NMR spectra of β and γ -hydrogens of α -ketobutyrate produced from L-vinylglycine.

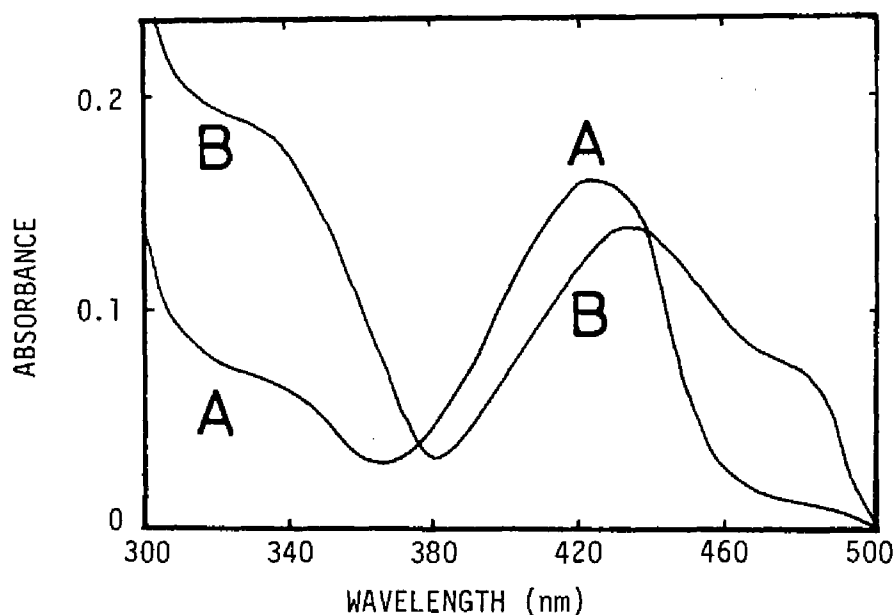


FIGURE 7: Spectral change of L-methionine γ -lyase with L-methionine. The reaction mixture contained, in a final volume of 0.7 mL, 1.03 mg of L-methionine γ -lyase and 140 μmol of potassium phosphate buffer (pH 8.0). The reaction was started by addition of 70 μmol of the substrate and performed at 25 $^{\circ}\text{C}$. Curve A, control, water was substituted for the substrate; curve B, absorption spectrum immediately after addition of the substrate.

added to the enzyme solution at 10 mM, the absorption maximum shifted to 428 nm (Figure 7B) and a new absorption shoulder appeared at about 480 nm. The spectrum gradually returned toward its initial form as the substrate became depleted. Changes occurring at lower wavelength were obscured by the absorption of α -ketobutyrate which was formed as the reaction progressed. However, the subtraction of the absorbance due to α -ketobutyrate ($\epsilon = 25$) formed in the reaction mixture revealed that this increase is due not only to the formation of α -ketobutyrate, but also to the transient appearance of a new spectral species during the incubation. In the experiment shown in Figure 7, an aliquot of the reaction mixture was removed from the reaction mixture after the scan was finished, and α -ketobutyrate was determined with MBTH. The absorbance at 320 nm due to the formation of α -ketobutyrate was about 70% less than the absorbance observed. The apoenzyme which was prepared by treatment with 5 mM phenylhydrazine caused no spectral change on addition of L-methionine. When pyridoxal-P was added to the apoenzyme in the presence of L-methionine, the absorption peak at 428 nm and the absorption shoulder at about 480 nm appeared. Pyridoxal-P is thus necessary for the formation of the species absorbing approximately at 480 nm.

The time course of α -ketobutyrate formation from L-methionine at 25 °C and the absorbance at 320 nm, 420 nm, and 480 nm of the enzyme are shown in Figure 8A. All the absorbance change at 320 nm, 420 nm, and 480 nm occurred immediately after addition of L-methionine. When most of the substrate had been consumed, the absorbance at 420 nm and 480 nm started to return to the original value simultaneously. After attaining to the maximum value, the absorbance at 320 nm began to decrease and reached to the stationary value. This indicates that a spectral species having the absorption maximum at about 320 nm appeared during the reaction.

The same spectral changes were observed during the deamination reaction of vinylglycine. After the addition of L-vinylglycine

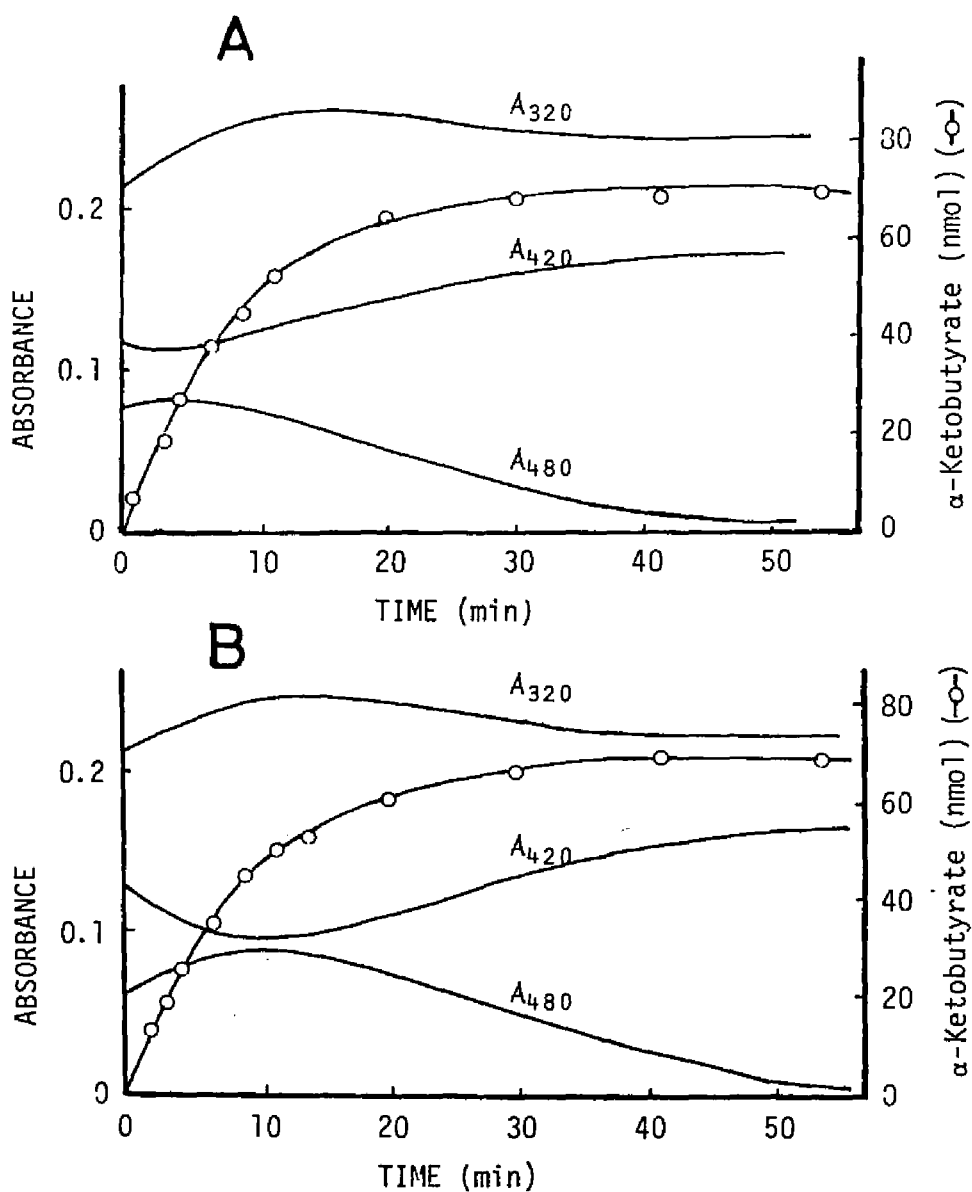


FIGURE 8: Time course of the accumulation of α -ketobutyrate and changes in the absorbance at 320 nm, 420 nm, and 480 nm during α,γ -elimination of L-methionine (A) and deamination of L-vinylglycine (B). The conditions are the same as those for Figure 7, curve B.

there was a quenching and a shift of the absorption band from 420 nm to 428 nm, and the appearance of a new absorption shoulder at about 480 nm. The appearance of a spectral species absorbing in the region of 320 nm also was shown by simultaneous investigation of time courses of α -ketobutyrate formation and changes in absorbance at 320 nm, 420 nm, and 480 nm (Figure 8B).

On addition of the other substrates which undergo α,γ -elimination reaction (e.g., L-ethionine, DL-selenomethionine, DL-methionine sulfone), the enzyme exhibited the same absorption shift as in the cases of L-methionine and vinylglycine.

Spectral change with other amino acids. No appreciable spectral changes of the enzyme were observed in the region of 480 nm during α,β -elimination of S-methyl-L-cysteine and S-ethyl-L-cysteine in contrast to the substrates for α,γ -elimination, although the absorption maximum shifted from 420 nm to 428 nm and the absorption was quenched a little (Figure 9A). Changes in the spectra below 360 nm were obscured by absorption of pyruvate. However, the final absorption was lower than the preceding one in the range of 310 to 360 nm. This suggests that other transient absorption bands absorbing at 320 nm appeared during the reaction. As the substrate was consumed the spectrum gradually returned to its initial form.

Non-substrate amino acids which undergo the hydrogen exchange reaction (e.g., L-alanine, L-norleucine) caused quenching of the 420 nm band of L-methionine γ -lyase but did no change at longer wavelength (Figure 9B). The spectrum was not affected by D-methionine, α -methyl-L-methionine, N-acetyl-L-methionine, and L-methionine methyl ester at concentrations of 20 mM.

Spectrum of L-methionine γ -lyase during replacement reaction.

Addition of L-methionine to the enzyme gave rise to the expected spectral shift described above. No appreciable changes were observed on the subsequent addition of ethanethiol. Under these conditions the enzyme catalyzes γ -replacement reaction.

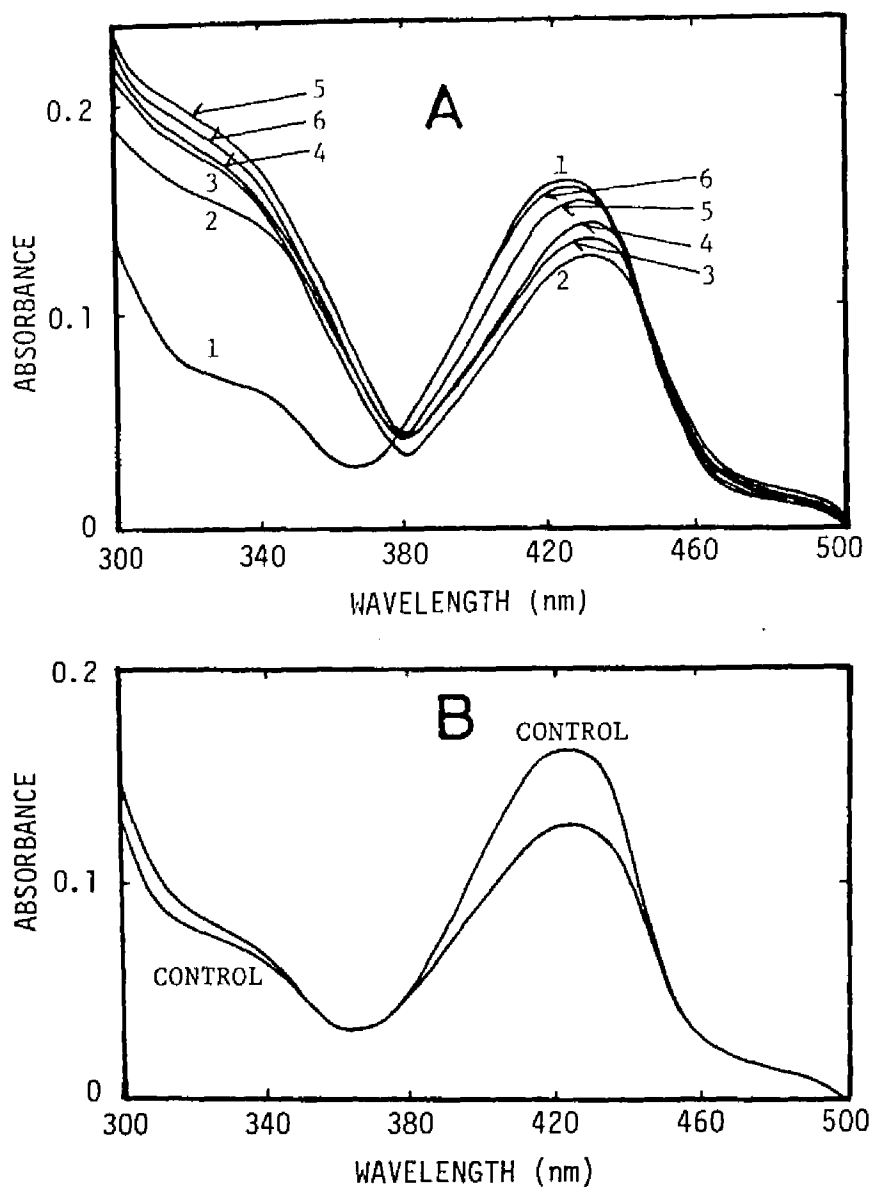


FIGURE 9: (A) Spectral change of L-methionine γ -lyase during α,β -elimination of S-methyl-L-cysteine. The conditions are described in the legend to Figure 7: spectrum 1, water was substituted for the substrate; spectrum 2, after 0.5 min; spectrum 3, after 3 min; spectrum 4, after 15 min; spectrum 5, after 57 min; spectrum 6, after 120 min. (B) Spectral change of L-methionine γ -lyase with L-alanine. Water was substituted for L-alanine in Control. The conditions are the same as those for Figure 7.

Ethanethiol, at concentrations of 10 mM, caused no changes in the shape of the spectrum. The subsequent addition of L-methionine caused the characteristic changes observed during α,γ -elimination. The spectra observed during α,γ -elimination of other substrates (homocysteine, methionine sulfone, and selenomethionine), deamination of vinylglycine, and α,β -elimination of S-methylcysteine were not influenced markedly by the subsequent addition of alkanethiols such as ethanethiol and 1-propanethiol.

Relationship between reaction and spectral change. In order to investigate the relationship between the reaction and the spectral change during the reaction, I examined the effect of substrate concentration on the change in the difference absorption at 480 nm (ΔA_{480}). Figure 10 shows the results of a typical experiment in which ΔA_{480} was determined as function of time at varying amounts of L-methionine. The area under each curve increased with an increase of methionine concentration. The integrated amount of ΔA_{480} during the entire course of the reaction is proportional to methionine concentration as shown in Figure 11A. The figure shows that the maximum change in the difference absorbance after addition of L-methionine ($\Delta A_{480} \cdot \max$) increased as methionine concentration increased. A reciprocal plot of $\Delta A_{480} \cdot \max$ values against methionine concentration gave a straight line as shown in Figure 12A. From this figure, an apparent affinity of the enzyme for L-methionine was estimated to be 0.5 mM.

When L-vinylglycine was used as a substrate, a similar relationship between the substrate concentration and ΔA_{480} was observed. The integrated value of ΔA_{480} was proportional to a vinylglycine concentration (Figure 11B). A reciprocal plot of $\Delta A_{480} \cdot \max$ values against vinylglycine concentration gave also a straight line (Figure 12B). An apparent affinity for vinylglycine was estimated to be 1.0 mM from the results. The value is much smaller than the K_m (15 mM) for vinylglycine in deamination reaction. This suggests that the spectral species at 480 nm does not

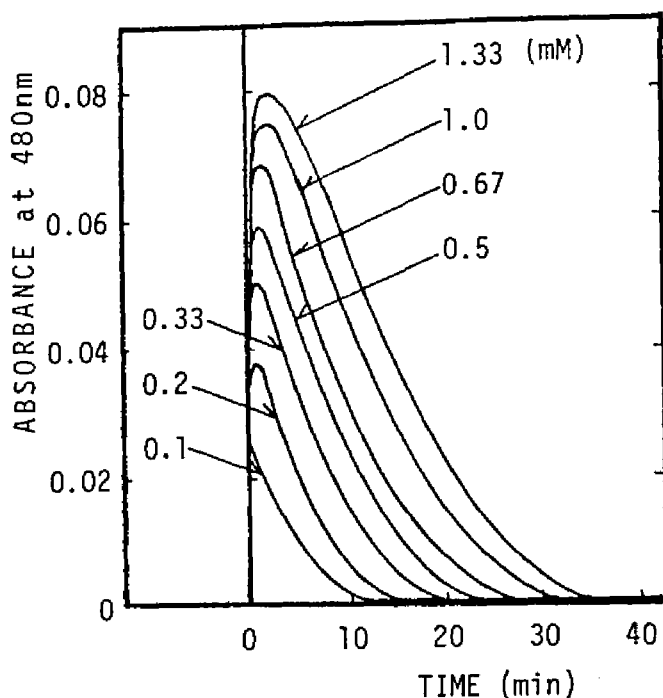


FIGURE 10: Time course of ΔA_{480} at different concentrations of L-methionine. The reaction mixture, in a final volume of 0.7 mL, 1.06 mg of L-methionine γ -lyase and 140 μ mol of potassium phosphate buffer (pH 8.0). The reaction was started by addition of various amounts of L-methionine (0.07, 0.14, 0.23, 0.35, 0.47, 0.70 and 0.93 mmol) and performed at 25 °C. The reference cuvette contained the same components except for the omission of L-methionine.

reflect the accumulation of an obligatory intermediate to form reaction products, because in that case an apparent affinity for vinylglycine determined from ΔA_{480} should agree with the K_m value (Chance, 1957). However, the integrated value of ΔA_{480} was proportional to the substrate concentration in both methionine and vinylglycine cases. In addition, $\Delta A_{480} \cdot \text{max}$ was decreased in the presence of L-norleucine in a competitive fashion (Figure 13). Thus, the 480-nm species is probably a pyridoxal-P Schiff base of a compound which can be derived from either methionine or vinylglycine, although this may not be an obligatory intermediate to produce the reaction products.

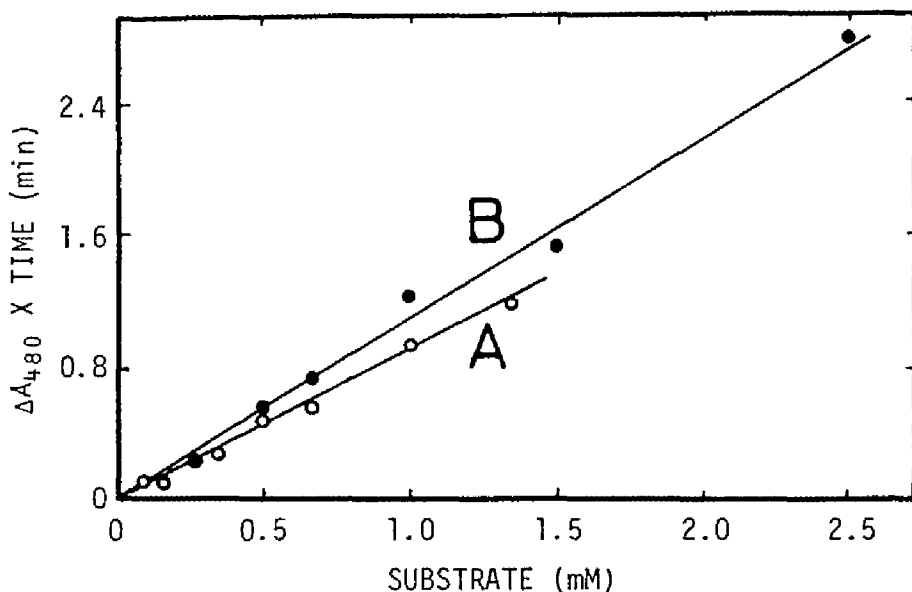


FIGURE 11: Effect of substrate concentration on the integrated amount of ΔA_{480} during the entire course of α, γ -elimination of L-methionine (A) and deamination of L-vinylglycine (B). The conditions for A are described in the legend to Figure 10. In the case of B, various amounts of L-vinylglycine (0.18, 0.35, 0.47, 0.70, 1.05, and 1.75 mmol) were added to the reaction mixture containing 1.06 mg of the enzyme and 140 μ mol of potassium phosphate buffer (pH 8.0) (a final volume, 0.7 mL). The reaction was performed at 25 °C.

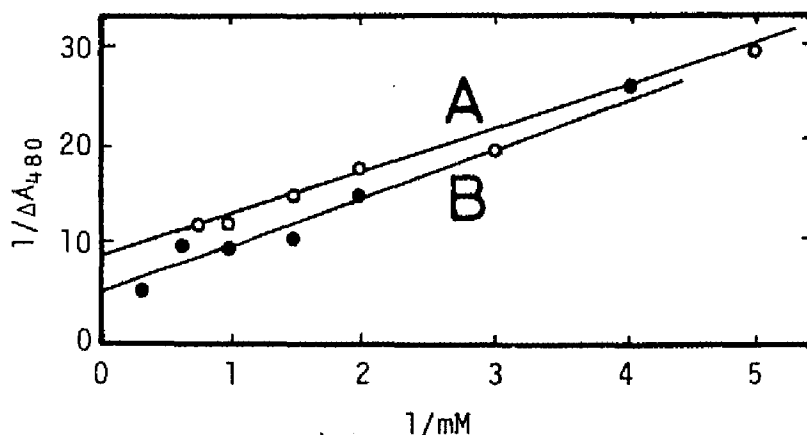


FIGURE 12: Reciprocal plots of $\Delta A_{480} \cdot \max$ values against substrate concentrations: A, L-methionine; B, L-vinylglycine. $\Delta A_{480} \cdot \max$ values are obtained from the data in Figure 10 for A. Although the results are not shown, the values for vinylglycine was obtained in the same manner. The conditions are shown in the legend to Figure 11.

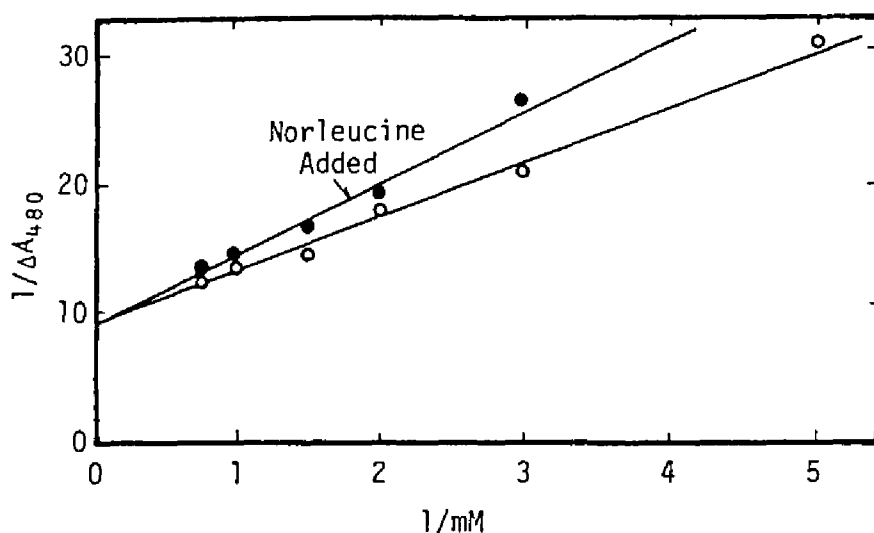


FIGURE 13: Reciprocal plots of $\Delta A_{480} \cdot \max$ values against L-methionine concentrations. $\Delta A_{480} \cdot \max$ values are obtained from the results of time course of ΔA_{480} at different concentrations of L-methionine in the absence or presence of 2.5 mM L-norleucine. The conditions are the same as those for Figure 10.

DISCUSSION

Labilization of the α -hydrogen of amino acids is a characteristic feature of most reactions catalyzed by pyridoxal-P enzymes such as transamination, racemization, α, β -elimination, β -replacement, α, γ -elimination, and γ -replacement (Davis & Metzler, 1972). Enzyme-catalyzed exchange of the β -hydrogen atoms has been also reported for aspartate aminotransferase (Babu & Johnston, 1976; Cooper, 1976), alanine aminotransferase (Babu & Johnston, 1976; Golichowski et al., 1977), cystathionine γ -lyase (Washtien et al., 1977) and cystathionine γ -synthase (Guggenheim & Flavin, 1969).

In this chapter I describe the rapid exchange reaction of α as well as of β hydrogen atoms in amino acids catalyzed by L-methionine γ -lyase. These exchange reactions were observed with substrate amino acids and also other amino acids which do not undergo enzymatic elimination or replacement reactions. In both

cases, the rate of β -proton exchange did not exceed the rate of α -proton exchange. This suggests that (1) β -proton exchange occurs only after α -proton removal and that (2) the α -proton is not conserved in the enzyme after initial abstraction. The β -proton exchange should occur via a reversible enzyme-catalyzed tautomerization of the α -keto acid-pyridoxamine-P Schiff base (ketimine) to the enamine as postulated by Babu & Johnston (1976). The finding that β -protons were exchanged in the same rate for alanine and α -aminobutyrate is distinct from other cases. Apparently, the methyl group of alanine and ethyl group of α -aminobutyrate which are attached to α -carbon are sufficiently free to rotate in the active site of enzyme. Thus, the β -protons become equivalent and exhibit identical exchange rates. However, for amino acids with longer carbon skeleton, the β -proton exchange reaction proceeded in a biphasic manner. Presumably, extension of the carbon chain allows sufficient interaction between the enzyme and substrate to prevent rotation of the β carbon.

Addition of substrates for α,γ -elimination to the enzyme causes appearance of a transient absorption shoulder in the region of 480 nm. The occurrence of a similar spectral species in the deamination reaction of vinylglycine is consistent with the proposed reaction mechanism that a pyridoxal-P Schiff base of vinylglycine is a key intermediate of α,γ -elimination. I have shown that the spectral change does not always indicate the formation of an obligatory intermediate in α,γ -elimination of L-methionine γ -lyase. However, the spectral species absorbing at 480 nm is probably a pyridoxal-P Schiff base of a compound which can be derived from either vinylglycine or substrates for α,γ -elimination. α -Ketobutyrate formed from methionine in the early stage of the reaction in $^2\text{H}_2\text{O}$ has the same structure as that from vinylglycine. The result is consistent with the mechanism that α,γ -elimination reaction proceeds through a vinylglycine-intermediate.

CONCLUSION

The distribution of L-methionine γ -lyase was investigated, and *Pseudomonas ovalis* (IFO 3738) was found to have the highest activity of enzyme, which was inducibly formed by addition of L-methionine to the medium. L-Methionine γ -lyase, purified to homogeneity from *Ps. ovalis*, has a molecular weight of about 173,000 and consists of nonidentical subunits (mol wt: 40,000 and 48,000). The enzyme exhibits absorption maxima at 278 and 420 nm, and a shoulder around 330 nm, which are independent of the pH (6.0 to 10.0), and contains 4 mol of pyridoxal-P per mol of the enzyme. The formyl group of pyridoxal-P is bound in an aldimine linkage to the ϵ -amino group of lysine residues of the protein. The holo-enzyme is resolved to the apoenzyme by incubation with hydroxylamine, and reconstituted by addition of pyridoxal-P. The enzyme activity is significantly affected by both carbonyl and sulfhydryl reagents. L-Methionine γ -lyase catalyzes α,γ - and α,β -elimination reactions of, in addition to L-methionine, several derivatives of L-methionine and L-cysteine, e.g., L-ethionine, DL-methionine sulfone, L-homocysteine, and S-methyl-L-cysteine. The enzyme catalyzes also γ -replacement reactions of the thiomethyl group of methionine with various alkanethiols ($C_2 - C_7$), arylthio alcohols (benzenethiol and β -naphthalenethiol) and the derivatives of ethanethiol (2-mercaptoethanol and cysteamine) to yield the corresponding S-substituted homocysteine. The thiomethyl group of S-methyl-L-cysteine also is replaced by ethanethiol to form S-ethyl-L-cysteine.

The enzyme catalyzes α,γ -elimination of selenomethionine to yield α -ketobutyrate, ammonia and methaneselenol, and also its γ -replacement reaction with various thiols to produce S-substituted homocysteines. Selenomethionine is an even better substrate than methionine in α,γ -elimination, but is less effective in γ -replacement. In addition, L-methionine γ -lyase catalyzes γ -replacement reaction of methionine and its derivatives with selenols to form

the corresponding Se-substituted selenohomocysteines, although selenols are less efficient substrate than thiols. This is the first proven mechanism for the incorporation of selenium atom into amino acids.

L-Methionine γ -lyase catalyzes deamination reaction of vinylglycine to produce α -ketobutyrate and ammonia. The enzyme catalyzes also γ -addition reaction of various alkanethiols to vinylglycine to yield the corresponding S-substituted homocysteines.

The enzyme catalyzes a rapid exchange reaction of α and β -hydrogen atoms of substrate amino acids (methionine and S-methylcysteine) and also other non-substrate amino acids with alkyl side chains (e.g., L-alanine and L-norleucine).

Vinylglycine gives [β - $^2\text{H}_1$, γ - $^2\text{H}_1$]- α -ketobutyrate in deamination reaction, when the reaction is performed in $^2\text{H}_2\text{O}$. α -Ketobutyrate formed from methionine in the early stage of α, γ -elimination in $^2\text{H}_2\text{O}$ has the same structure as that from vinylglycine.

On addition of the substrate which undergo α, γ -elimination reaction (e.g., L-methionine and DL-selenomethionine), the enzyme exhibits a new absorption shoulder in the region of 480 nm. This shoulder decreases as the substrates are converted into α -ketobutyrate, ammonia and RSH (or RSeH). Incubation with vinylglycine also gives rise to the same absorption shift. The D and L-amino acids which are not substrates for α, γ -elimination are ineffective. The 480-nm shoulder is probably attributable to a pyridoxal-P Schiff base of a compound which can be derived from either vinylglycine or substrates for α, γ -elimination. These results are consistent with the proposed mechanism that α, γ -elimination reaction proceeds through a vinylglycine intermediate (Davis & Metzler, 1972).

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