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Properties of L-Methionine γ-Lyase from *Pseudomonas ovalis*†

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ABSTRACT: The distribution of bacterial L-methionine γ-lyase (L-methionine methanethiollyase (deaminating) (EC 4.4.1.11)) 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 5′-phosphate per mol of the enzyme. The formyl group of pyridoxal 5′-phosphate is bound in an aldimine linkage to the ε-amino group of lysine residues of the protein. The holoenzyme is resolved to the apoenzyme by incubation with hydroxylamine, and reconstituted by addition of pyridoxal 5′-phosphate. 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.

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 dethiomethylated with the release of methanethiol as reported for various aerobic bacteria and soil fungi (Segal and Starkey, 1969; Ruiz-Herrera and Starkey, 1969a). Alternatively, methionine is deaminated-dethiomethylated simultaneously to form methanethiol. The first evidence for the occurrence of this pathway was obtained by Onitake (1938) using the dried cells

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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 and Matsuo, 1950), *Pseudomonas* sp. (Miwatani et al., 1954; Kallio and Larson, 1955), *Clostridium sporogenes* (Wiesendanger and Nisman, 1953), rumen bacteria (Merricks and Salsbury, 1974), *Ps. ovalis* (Tanaka et al., 1974), and *Ps. putida* (Ito et al., 1975). Little effort, however, has been devoted to the purification and the characterization of the enzyme. 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. Recently we have purified the enzyme to homogeneity from the cell-free extract of *Ps. ovalis* (Esaki et al., 1975), and reported briefly (Tanaka et al., 1976) that the enzyme catalyzes α,β elimination (eq 1) as well as α,γ elimination (eq 2).

$$S\text{-methyl-L-cysteine} + H_2O \rightarrow \text{pyruvate} + NH_3 + CH_3SH \quad (1)$$

$$\text{L-methionine} + H_2O \rightarrow \alpha\text{-ketobutyrate} + NH_3 + CH_3SH \quad (2)$$

In the present paper, more detailed studies on enzymological and physicochemical characteristics of L-methionine γ-lyase are described.

Experimental Procedure

Materials. *S*-Methyl-L-methionine (Toennis, 1940), *S*-(β-aminoethyl)-L-cysteine (Cavallini et al., 1955; Rothfus and Crow, 1968), *S*-(β-aminoethyl)-L-homocysteine (Hope and Horncastle, 1966), α-keto-γ-methiolbutyrate (Meister, 1952), lead methylmercaptide (Sliwinski and Doty, 1958), and N^{ϵ}-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 crystallized.[1] Thiols were obtained from Tokyo Chemical Industry Co., Tokyo, *S*-methyl-L-cysteine and *S*-ethyl-L-cysteine from Fluka AG, Buchs, pyridoxal-P[2] was from Dainippon Seiyaku Co., Osaka, D-cycloserine from Shionogi Seiyaku Co., Osaka, D- and L-penicillamine were from Calbiochem, Calif., 3-methyl-2-benzothiazolinone hydrazone hydrochloride was from Aldrich Chemical Co., Inc. Pyridoxal-P was chromatographically purified by the method of Peterson and Sober (1954). Sodium lauryl sulfate was a specially prepared reagent for protein research (Nakarai Chemicals, Kyoto). The other chemicals were analytical grade reagents.

Microorganisms and Conditions of Culture. Pseudomonas ovalis IFO 3738 and other organisms were grown in a medium containing 0.25% L-methionine as described previously (Tanaka et al., 1976). The harvested cells were washed twice with 0.85% NaCl solution and subsequently with 0.01 M potassium phosphate buffer (pH 7.2) containing 10^{-5} M pyridoxal-P and 0.01% 2-mercaptoethanol. The washed cells were stored frozen at −20 °C until used.

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

[2] Abbreviations used: pyridoxal-P, pyridoxal 5′-phosphate; Tris, tris(hydroxymethyl)aminomethane; NADH, reduced nicotinamide adenine dinucleotide.

Enzyme Preparation. The enzyme was purified to homogeneity from a cell-free extract of *Ps. ovalis* as described previously (Tanaka et al., 1976).

Enzyme Assay. Method A. The enzymatic α,γ-elimination reaction was routinely followed by determining α-ketobutyrate formed. The standard assay system contained 100 μmol of potassium phosphate buffer (pH 8.0), 100 μmol of L-methionine, 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 in the supernatant solution was determined with 3-methyl-2-benzothiazolinone hydrazone as described previously (Soda, 1968).

Method B. For the replacement reaction, the reaction system consisted of 40 μmol of potassium phosphate buffer (pH 8.0), 20 μmol of L-methionine or other sulfur amino acids, 20 μmol of alkanethiols or substituted thiols, 0.02 μmol 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).

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 ($A_{1cm}^{1\%}$ = 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 mg of protein.

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 6166 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 Methods. 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 and Kolb, 1951). Thiols such as methanethiol and ethanethiol were identified

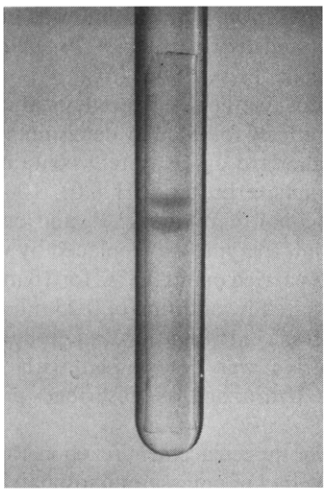

FIGURE 1: Sodium lauryl sulfate disc gel electrophoresis of carboxymethylated L-methionine γ-lyase. The conditions of carboxymethylation and sodium lauryl sulfate disc gel electrophoresis are given in the text. The direction of migration is from the cathode (top) to the anode.

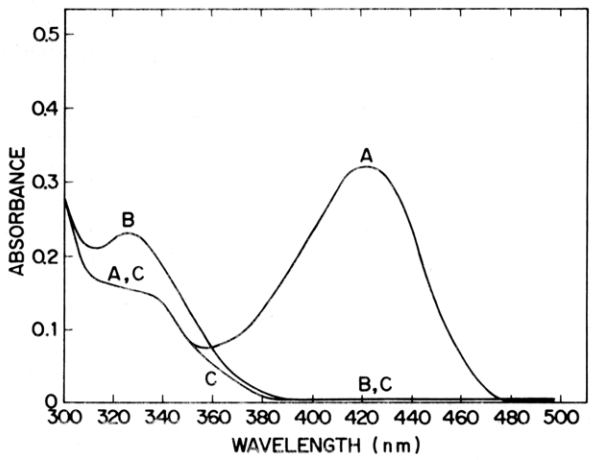

FIGURE 2: Absorption spectra of L-methionine γ-lyase. (Curve A) Holoenzyme in 0.01 M potassium phosphate buffer (pH 7.2); (curve B) holoenzyme reduced with $NaBH_4$ and dialyzed against the same buffer; (curve C) apoenzyme.

and determined with a Shimadzu Gas Chromatograph GC-4 BM-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/cm^2, 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 proton magnetic resonance spectra with a Varian Associates recording spectrometer (A60) 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 (parts per million).

Results

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 Thumberg 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.

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; Tris-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 K_2HPO_4–KOH, pH 11.5.

Purity and Molecular Weight. The purified enzyme was shown to be homogeneous upon ultracentrifugation and disc gel electrophoresis as reported in a previous paper (Tanaka et al., 1976). The sedimentation coefficient ($s_{20,w}^0$) of the enzyme is 8.5 S. The molecular weight of the enzyme was determined to be about 180 000 by the Sephadex G-200 gel filtration method (Tanaka et al., 1976). A molecular weight of 173 000 ± 2000 was also obtained by the sedimentation equilibrium method, assuming a partial specific volume of 0.74.

Structure of Subunit. The subunit structure of the enzyme was examined by disc gel electrophoresis. The enzyme was treated with 6.5 M guanidine hydrochloride 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 preparations (10 μg) were subjected to electrophoresis in the presence of 0.1% sodium lauryl sulfate (Weber and Osborn, 1969). There were two bands of stained protein (Figure 1). To determine the molecular weight of the polypeptides in these bands, we ran a series of marker proteins treated in the same manner: chymotrypsinogen A (mol wt 25 000), carboxypeptidase A (mol wt 34 600), D-amino acid oxidase (mol wt 37 000), ovalbumin (mol wt 45 000), glutamate dehydrogenase of bovine liver (mol wt 50 000), pyruvate kinase (mol wt 57 000), catalase (mol wt 60 000), and L-amino acid oxidase of snake venom (mol wt 63 000). The molecular weights were calculated to be approximately 40 000 and 48 000, respectively, from a semilogarithmic 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 shifts 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.

Reduction of the enzyme with sodium borohydride by the dialysis method of Matsuo and Greenberg (1959) affects both

the absorption spectrum (Figure 2B) and the activity. Reduced enzyme was catalytically 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 and Snell, 1961) and the 3-methyl-2-benzothiazolinone 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.

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-dinitrophenylhydrazone 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 μmol of potassium phosphate buffer (pH 8.0), 100 μmol of L-methionine, 0.02 μmol 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 μmol of NH_4Cl–NH_4OH buffer (pH 9.0), 0.1 μmol 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 μmol. Formation of 6.30 μmol 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.

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

[a] The concentration of the amino acid: 25 mM; [b]12.5 mM; [c]5 mM. The enzyme activity was determined by method A.

Ammonia was identified and determined to be 6.25 μmol with Nessler reagent (Thompson and 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 and 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.[a]

System	L-Methionine Disappeared (μmol)	α-Ketobutyrate Formed (μmol)	Ammonia Formed (μmol)	Methanethiol 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

[a] Reactions and assays were carried out as described in the text.

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 by 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, $HgCl_2$, 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 and 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 μmol), 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 × 24 cm) of Dowex 50-X8 (H^+), washed thoroughly with water, and eluted with 0.3 N NH_4OH. 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 performed in the same way. The product was identified as ethionine based on the physicochemical analyses as follows. The proton magnetic resonance spectrum of the product in deuterium oxide 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 ($C_6H_{13}NO_2S$): 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 (C_3–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 alkanethiols (C_8–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 proton magnetic resonance 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 (2 H, triplet, >C-3-H_2), at 2.31–2.78 (4 H, multiplet, -CH_2-S-CH_2-), at 3.60 (2 H, triplet, OD-CH_2-), and 4.23 (1 H, triplet, >C-2-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 the absorption bands at 3200 and 1060 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 ($C_6H_{13}O_3NS$): C, 40.22; H, 7.31; N, 7.82%. Found: C, 40.83; H, 7.28; N, 7.89%. These results reveal that the compound 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 product was quantitatively deaminated with L-amino acid oxidase. Methanethiol 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 and Metzler (1972). Although the mechanism of α,β-elimi-

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

RSH, R =	Homocysteine Protons		*S*-Substituent Protons	Solvents
	α	β and γ		
Ethyl	3.35 t	1.63–2.86 m	*S*(α), 2.42–2.88 m *S*(β), 1.23 t	D_2O
n-Propyl	4.22 t	1.78–2.73 m	*S*(α), 2.30–2.69 m *S*(β), 0.83–1.52 m *S*(γ), 0.62 t	CF_3COOD
n-Butyl	4.67 t	2.34–3.11 m	*S*(α), 2.80–3.09 m *S*(β), 1.21–1.81 m *S*(γ), 0.75–1.13 m *S*(δ), 0.46 t	CF_3COOD
Benzyl	4.79 t	2.24–3.29 m	*S*(α), 4.09 s Phenyl, 7.74 s	CF_3COOD
Phenyl	4.90 t	2.34–2.89 m 3.42 t	Phenyl, 7.73 s	CF_3COOD

[a] The notations m, s, and t refer to multiplets, singlets, and triplets, respectively.

nation and β-replacement reactions of amino acids catalyzed by pyridoxal-P-dependent enzymes have been extensively studied and well established (Snell and Di Mari, 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 and Flavin, 1966; Kerr and Flavin, 1970) and γ-cystathionase (Matsuo and Greenberg, 1959; Flavin and Segal, 1964). L-Methionine γ-lyase is unique in catalyzing α,γ and α,β eliminations to produce alkanethiols.

The studies described here deal with the microbial distribution of L-methionine γ-lyase and the enzymological and physicochemical characterization of the enzyme. The high activity was chiefly found in *Pseudomonas* species. The enzyme has been purified to homogeneity from *Ps. ovalis*, in which L-methionine γ-lyase occurs most abundantly. The absorption spectrum of the enzyme (λ_{max} 420 nm with a shoulder at about 330 nm) closely resembles those of some other pyridoxal-P enzymes; e.g., L-leucine aminotransferase (λ_{max} 326 and 414 nm, Taylor and Jenkins, 1966), L-ornithine aminotransferase (λ_{max} 330 and 420 nm, Peraino et al., 1969), tyrosine phenol-lyase (λ_{max} 340 and 430 nm, Kumagai et al., 1970), and kynureninase (λ_{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 and 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 and 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 and 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 alkanethiols (C_2–C_7, probably also C_1), arylthio alcohols, and the derivatives of ethanethiol.

Some multifunctional lyases have been demonstrated. Guggenheim and 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 β-substituted amino acids. They showed as well that γ-cystathionase of *Neurospora* (Flavin and 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's base as an intermediate (Guggenheim and Flavin, 1969) as reviewed by Snell and Di Mari (1970), and Davis and Metzler (1972). More detailed investigations on the replacement reactions are currently in progress with particular emphasis on the mechanism.

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