a reliable measure of changes in binding energy between the enzyme and substrate and the stabilization of one state relative to a previous one, there is an important exception. This is when groups in the ES complex shed their hydrogen bonds with water to form bonds with each other (Fersht, 1988). As emphasized in the accompanying paper (Fersht, 1988), deletion of a hydrogen-bond donor to a charged group will considerably overestimate the stabilization energy. This could well be the case here.

ACKNOWLEDGMENTS

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Specific Labeling of the Essential Cysteine Residue of L-Methionine γ-Lyase with a Cofactor Analogue, *N*-(Bromoacetyl)pyridoxamine Phosphate†

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ABSTRACT: L-Methionine γ-lyase from *Pseudomonas putida* is composed of four identical polypeptide chains and contains four cysteinyl residues per subunit. We have found one of them catalytically essential by its specific cyanylation with 2-nitro-5-thiocyanobenzoic acid. We have shown its essentiality also with *N*-(bromoacetyl)pyridoxamine 5′-phosphate (BAPMP), which is a cofactor analogue and also an affinity-labeling agent. The kinetic data show that the apoenzyme forms a binary complex with BAPMP prior to covalent binding. The stoichiometry of inactivation was 1 mol of BAPMP per subunit. We have shown that the cysteine residue modified with BAPMP is identical with that labeled specifically with [^{14}C]iodoacetic acid. The amino acid sequences of the peptides containing the essential cysteine residue and the lysine residue to which pyridoxal 5′-phosphate is bound were determined by automated Edman degradation.

L-Methionine γ-lyase (EC 4.4.1.11) is a pyridoxal-P[1] enzyme that catalyzes the α,γ-elimination of L-methionine to α-ketobutyrate, methanethiol, and NH_3. We have purified the enzyme from *Pseudomonas putida* and characterized it (Esaki et al. 1979, 1984, 1985; Nakayama et al., 1984). We have studied the mechanism of inactivation of the enzyme by a suicide substrate, L-propargylglycine, as well and shown that a nucleophilic amino acid residue located at the active site is modified with L-propargylglycine (Johnston et al., 1979). The presence of an essential cysteine residue of the enzyme was

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[1] Abbreviations: pyridoxal-P, pyridoxal 5′-phosphate; BAPM, *N*-(bromoacetyl)pyridoxamine; BAPMP, *N*-(bromoacetyl)pyridoxamine 5′-phosphate; NTCB, 2-nitro-5-thiocyanobenzoic acid; DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetate; HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoin; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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suggested on the basis of inactivation by a novel suicide substrate, 2-amino-4-chloro-5-[(*p*-nitrophenyl)sulfinyl]pentanoic acid (Johnston et al., 1980). The present study has been undertaken to show whether an essential cysteine residue is located near the pyridoxal-P binding region by affinity labeling with cofactor analogues *N*-(bromoacetyl)pyridoxamine (BAPM) and *N*-(bromoacetyl)pyridoxamine 5′-phosphate (BAPMP). BAPM was used to label specifically an active cysteine residue of tryptophan synthase (EC 4.2.1.20) (Higgins & Miles, 1978) and also an active lysine residue of cytosolic aspartate aminotransferase (EC 2.6.1.1) (Farach et al., 1983).

Experimental Procedures

Materials. L-Methionine γ-lyase was purified from an extract of *Pseudomonas putida* ICR 3460 as described previously (Nakayama et al., 1984). BAPMP and BAPM were synthesized by modification of the previous method (Higgins & Miles, 1978). 2-Nitro-5-thiocyanobenzoic acid (NTCB) was synthesized according to the method of Degani et al. (1970). [^{14}C]NTCB (1.4 mCi/mmol) was prepared with [^{14}C]KCN (New England Nuclear, 8.0 mCi/mmol). *O*-Acetyl-L-homoserine was prepared by the method of Nagai and Flavin (1971). N^{ϵ}-(Phosphopyridoxyl)lysine was a gift from Dr. Y. Morino, Kumamoto University. The other chemicals were of analytical grade.

Enzyme Assay. L-Methionine γ-lyase was assayed with *O*-acetyl-L-homoserine as a substrate (Esaki et al., 1984).

Resolution and Reconstitution of Enzyme. The holoenzyme was incubated with 1.25 M KSCN in 0.01 M potassium phosphate buffer (pH 7.2) containing 1 mM EDTA and 0.1 mM DTT at 37 °C for 15 min. Hydroxylamine hydrochloride was then added to a final concentration of 0.5 mM, and the mixture was incubated at 37 °C for 5–7 min. The enzyme, desalted with Sephadex G-25, exhibited no absorption peak at 420 nm and no activity but was reactivated more than 90% by addition of 10^{-5} M pyridoxal-P.

Reaction with 5,5′-Dithiobis(2-nitrobenzoic acid). Various amounts of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (1–16 nmol) were incubated with enzyme (2.4 nmol) in 10 mM potassium phosphate buffer (pH 7.2) containing 20 μM pyridoxal-P and 1 mM EDTA at 0 °C for 3 h (final volume, 1.0 mL). DTNB was replaced by water in a blank. After the reaction A_{412} was read, and an aliquot of the reaction mixture was removed to determine the remaining activity by measurement of the α,γ-elimination rate.

Reaction with NTCB. The 2–5 μM native enzyme solution was preincubated with 0.1 M potassium phosphate buffer (pH 7.2) containing 20 μM pyridoxal-P and 1 mM EDTA at 0 °C. After addition of the appropriate volume of 10 mM NTCB solution in 0.1 M potassium phosphate (pH 7.2), an increase in A_{412} was followed at 0 °C, and the amount of sulfhydryls reacted was determined by means of an absorption coefficient of 5-thio-2-nitrobenzoate (ϵ_{412} = 14 200 M^{-1} cm^{-1}) (Riddles et al., 1979). The surface of the cuvette was prevented from dewing by continuous flushing of dry air on the surface throughout the measurement. An aliquot of the above reaction mixture was removed at appropriate time intervals and added to the assay mixture to make a final concentration of NTCB lower than 0.2 μM. Protein was determined by the method of Bradford (1976).

Reaction with BAPM and BAPMP. Apo-L-methionine γ-lyase (2.03 μM in 0.2 M potassium phosphate buffer, pH 8.0) was incubated at 37 °C with various concentrations of BAPM or BAPMP. At time intervals, aliquots (10 μL) were withdrawn and mixed with 0.99 mL of the following buffer, which was chilled at 0 °C: 0.01 M potassium phosphate (pH 7.2) containing 0.1 mM DTT and 20 μM pyridoxal-P.[2] The remaining activity was determined as described above.

Carboxymethylation and $NaBH_4$ Reduction of Enzyme. L-Methionine γ-lyase [14 mg in 1.2 mL of 0.2 M potassium phosphate buffer (pH 7.2), 20 μM pyridoxal-P, and 0.1 mM EDTA] was incubated with 3.0 μmol of [2-^{14}C]iodoacetic acid in 30 μL of the above buffer at 37 °C for 1 h in the dark. Five microliters of 2-mercaptoethanol was then added to stop the reaction, and the mixture was dialyzed against 1.0 L of the above buffer at 4 °C. The radioactive enzyme was then reduced with $NaBH_4$ at room temperature for 1 h. The reaction mixture was dialyzed against 1.0 L of 0.1 M NH_4HCO_3 and lyophilized. The residue was dissolved in 1.0 mL of 0.1 M Tris-HCl buffer (pH 8.5) containing 10 mM EDTA and 6 M guanidine hydrochloride and incubated at 50 °C for 1 h. After addition of 2 μmol of DTT, the mixture was gently flushed with nitrogen gas and incubated at 37 °C for 1 h. Five micromoles of cold sodium iodoacetate was added to the mixture, which was incubated at 37 °C for 30 min. The reaction was terminated by addition of 5 μL of 2-mercaptoethanol. The carboxymethylated and $NaBH_4$-reduced enzyme was dialyzed against 0.1 M NH_4HCO_3 solution and deionized water successively and lyophilized.

Digestion and Purification of Peptides. The carboxymethylated and $NaBH_4$-reduced enzyme (75 nmol in 2.5 mL of 70% formic acid) was treated with a 100-fold molar excess of cyanogen bromide at 25 °C for 24 h in the dark. Peptides obtained were digested further with trypsin (0.15 nmol) or chymotrypsin (0.15 nmol) in 100 μL of 0.1 M NH_4HCO_3 (pH 7.8) at 37 °C for 5 h. Peptides were purified with an LKB HPLC system equipped with a Bakerbond Widepore C_{18} column (4.6 × 25 cm). A linear gradient elution from 0.1% (v/v) trifluoroacetic acid in water to 80% (v/v) acetonitrile in 0.1% trifluoroacetic acid was carried out at a flow rate of 0.8 mL/min. Peptides isolated were purified further with a TSK-TMS250 column under the same conditions as described above except that trifluoroacetic acid was replaced by heptafluorobutyric acid. Peptides were detected by measurement of A_{220}. Phosphopyridoxyl peptide was analyzed by its characteristic absorption at 330 nm. ^{14}C-Carboxymethylated peptides were monitored by their radioactivity with a Packard TRICARB 300C liquid scintilation system.

Amino Acid Analysis and Sequencing of Peptides. Peptides were hydrolyzed in 5.7 N HCl under reduced pressure and analyzed with a Hitachi high-performance amino acid analyzer (Model 835). The amino acid sequence of the peptides was determined by automated Edman degradation with an Applied Biosystems gas-phase sequencer (Model 470A) equipped online with a PTH-amino acid analyzer (Model 120A). Peptides analyzed were as follows: CBR1, 2 nmol; CBP, 2 nmol; T2, 100 pmol; C1, 1 nmol.

Results

Subunit Structure. We have shown that L-methionine γ-lyase has a molecular weight of about 172 000 and consists of four subunits identical in molecular weight (Nakayama et al., 1984). When the enzyme was subjected to two-dimensional gel electrophoresis according to the method of O'Farrell (1975), it showed only a single protein band that had a mobility corresponding to a M_r of 42 000 ± 1000 and a p*I* value of 6.75. The four polypeptide chains are identical also in

[2] When the apoenzyme was incubated with 0.1 mM BAPMP (or 1 mM BAPM) in the same buffer at 0 °C for 30 min, it was not inactivated significantly. Thus, we confirmed that the inactivation can be stopped under the conditions.

Table I: Amino Acid Composition of L-Methionine γ-Lyase and Peptides Derived from It

amino acid	native enzyme (mol %)[a]	CBR1[b]	CBR2[b]	CBP[b]
Asp	7.2	2.9 (3)	3.1 (3)	5.9 (6)
Thr	5.4[c]	5.0 (5)	5.0 (5)	4.3 (5)
Ser	5.2[c]	1.3 (1)	0.4 (1)	3.3 (4)
Glu	9.2	2.2 (2)	1.7 (2)	2.8 (3)
Pro	5.0	1.1 (1)	1.1 (1)	2.1 (2)
Gly	10.2	7.5 (7)	6.5 (7)	6.5 (7)
Ala	12.9	2.4 (2)	2.0 (2)	8.4 (9)
Cys	1.0[d]	1.0 (1)[e]	0.9 (1)[e]	
Val	5.8	3.3 (3)	3.4 (3)	6.0 (6)
Met	3.5	0.0 (0)	0.0 (0)	0.0 (0)
Ile	4.1	2.3 (2)	2.0 (2)	3.2 (3)
Leu	11.6	9.8 (8)	7.6 (8)	8.1 (8)
Tyr	3.0	1.0 (1)	0.6 (1)	1.8 (3)
Phe	3.5	2.9 (3)	3.1 (3)	0.0 (0)
His	4.4	2.6 (3)	2.6 (3)	2.4 (2)
Lys	2.4	0.9 (1)	1.1 (1)	2.0 (2)
Arg	5.1	2.2 (2)	2.3 (2)	3.1 (3)
Hse		1.2 (1)	0.9 (1)	0.8 (1)
Trp	0.5[f]	nd[g]	nd[g]	nd[g]
total	100	46	46	63

[a] Average of values obtained by hydrolysis for 24, 48, and 72 h. [b] Ratio of amino acids recovered after hydrolysis for 24 h. [c] Extraporated to zero time of hydrolysis. [d] Determined as cysteic acid by the method of Hirs (1967). [e] Determined as *S*-(carboxymethyl)-cysteine. [f] Determined spectrophotometrically (Edelhoch, 1967). [g] Not determined.

charge. The enzyme was subjected to manual Edman degradation to determine the amino-terminal amino acid sequence through 11 cycles: NH_2-Met-His-Gly-Ser-Asn-Lys-Leu-Pro-Gly-Phe-Ala-. Only one major PTH-amino acid was detected in each cycle. The yield of the PTH derivative of the amino-terminal methionine was determined to be 64% by HPLC. These results show that L-methionine γ-lyase consists of four identical polypeptide chains.

Amino Acid Composition and Cysteine Content of Enzyme. The enzyme oxidized with performic acid was found to contain 16.0 mol of cysteic acid per mole of enzyme of amino acid analysis (Table I). When the enzyme reacted with DTNB in the presence of 4.5 M guanidine hydrochloride, 16.1 mol of 5-thio-2-nitrobenzoate was released per mole of enzyme. Thus, each subunit has four cysteine residues, but no disulfide bonds.

Reactivity with DTNB and NTCB. The native enzyme reacted with DTNB very rapidly (within 10 s) even at 0 °C. The enzyme was fully inactivated when 4 mol of DTNB reacted with 1 mol of enzyme. The enzyme was inactivated also with NTCB. About 70% of the activity was lost by cyanylation of 2.0 mol of reactive residues per mole of enzyme, and more than 95% was lost after 4–5 mol of reactive residues was cyanylated. When the enzyme was treated with a 20–40 molar excess of [^{14}C]NTCB and then chromatographed with Sephadex G-25, it was labeled with 4.9 mol of [^{14}C]CN/mol. The radioactive enzyme reacted further with 11.1 mol of DTNB/mol in the presence of 4.5 M guanidine hydrochloride. The sum of the amount of reactive residue was 16.0 mol/mol and coincided with the value obtained with DTNB alone (see above). This indicates that the same functional group (i.e., cysteine) reacted with NTCB and DTNB.

Inactivation by BAPM and BAPMP. Incubation of apo-L-methionine γ-lyase with excess BAPMP resulted in an irreversible loss of activity (Figure 1A). When half-times of inactivation ($t_{1/2}$) were plotted against reciprocal concentrations of the inactivator, a straight line, which intersects on the ordinate, was obtained (Figure 1B). This shows that the

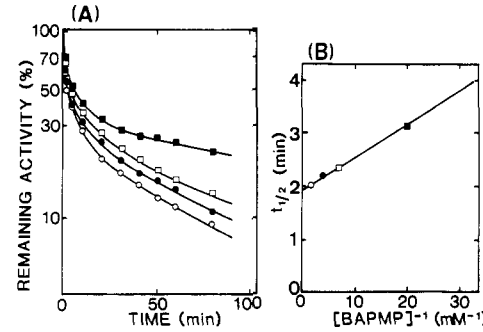

FIGURE 1: (A) Inactivation of apo-L-methionine γ-lyase by BAPMP. The apoenzyme was incubated with 0.05 (■), 0.14 (□), 0.25 (●), and 0.5 mM (○) BAPMP, and the residual activity was determined after quenching the inactivation by addition of pyridoxal-P and DTT as described under Experimental Procedures. (B) The $t_{1/2}$ of inactivation was plotted against reciprocal concentrations of BAPMP.

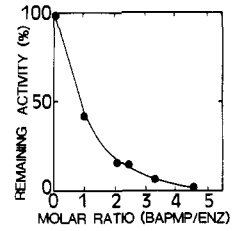

FIGURE 2: Stoichiometry of inactivation of L-methionine γ-lyase with BAPMP. The apoenzyme (0.5 mM) was incubated with BAPMP as described under Experimental Procedures. Aliquots were taken from the reaction mixture and mixed with DTT at a final concentration of 10 mM, followed by dialysis to remove unreacted BAPMP. The enzyme-bound BAPMP was determined with a reported absorption coefficient of 7750 M^{-1} cm^{-1} at 325 nm and pH 7.0.

apoenzyme forms a reversible binary complex with the inhibitor.[3] BAPM also inactivated the enzyme with a maximum inactivation rate (k_{inact}) of 0.28 min^{-1}, which is similar to the value for BAPMP (0.35 min^{-1}). This indicates that the same functional group reacts with the bromoacetyl group. However, the enzyme showed a higher affinity for BAPMP (K_i = 0.03 mM) than for BAPM (0.25 mM). This is consistent with the functional essentiality of the phosphate group of the coenzyme.

The BAPMP-modified enzyme showed a characterized absorption peak at 325 nm. The enzyme activity decreased with an increase in the amount of chromophore. Higgins and Miles (1978) and Farach et al. (1983) showed that the incorporation can be estimated with the absorption coefficient of free BAPMP. Thus, we have determined the stoichiometry of inactivation with respect to the amount of chromophore incorporated and shown that the tetrameric enzyme is fully inactivated by labeling with 4 mol of BAPMP/mol of enzyme (Figure 2); this is consistent with the number of pyridoxal-P binding sites in the enzyme (Nakayama et al., 1984). However, the extent of enzyme inactivaion was not proportional to the moles of BAPMP incorporated per mole of enzyme. This is also the case for the modification with NTCB (see above) and propargylglycine (Johnston et al., 1979) and probably due to a negative cooperativity among subunits. When the apoenzyme fully inactivated with BAPMP was treated with NTCB, no 5-thio-2-nitrobenzoate was released.

[3] We have found that BAPMP inactivates also the holoenzyme. Its inactivation rate, however, was much lower than that of the apoenzyme: relative rates were about 3 and 2% with 0.5 and 0.25 mM BAPMP, respectively. Plots of $t_{1/2}$ against reciprocal concentrations of BAPMP gave a straight line which passes through the origin of the coordinate axis; the holoenzyme is inactivated by second-order kinetics in contrast with the apoenzyme.

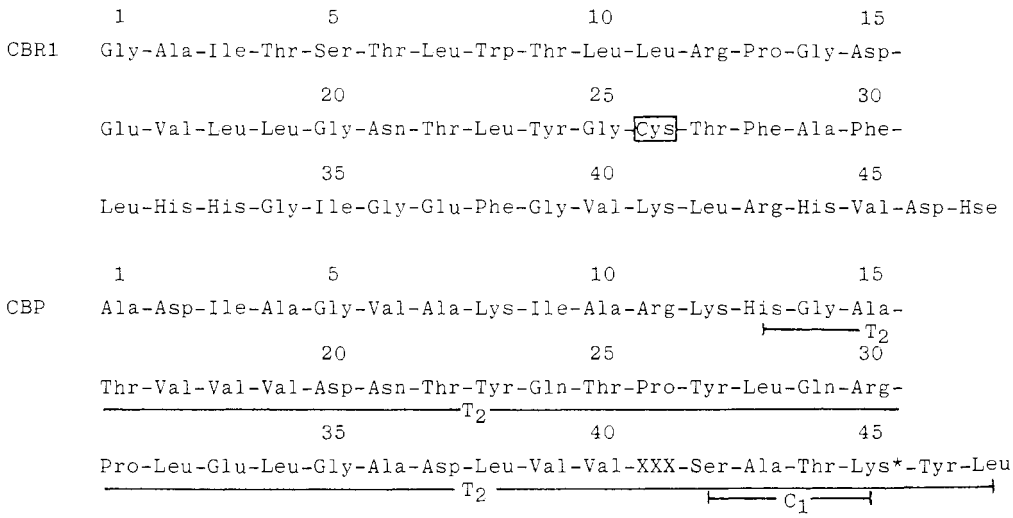

FIGURE 3: Amino acid sequence of the peptides derived from L-methionine γ-lyase. CBR1, CBP, T2, and C1 are the names of the peptides indicated in the text. PTH derivatives of the amino acids were identified by HPLC after automated sequencing. The essential cysteine residue in CBR1 is enclosed. Lys* indicates the lysine residue to which pyridoxal-P is bound. No PTH-amino acid could unambiguously be assigned at the position marked XXX.

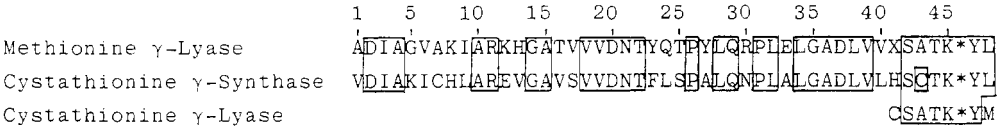

FIGURE 4: Linear alignment of the amino acid sequences around the active site lysine residue of L-methionine γ-lyase, cystathionine γ-synthase of *E. coli* (Duchange et al., 1983), and cystathionine γ-lyase of rat liver (Fearon et al., 1982). K* indicates the lysine residue to which pyridoxal-P is bound.

This suggests that the same cysteine residue was modified with NTCB and BAPMP.

Structure of Peptides Containing the Essential Cysteine and Active Site Lysine. The ^{14}C-carboxymethylated and $NaBH_4$-reduced enzyme was treated with cyanogen bromide, and the resulting fragments were separated by HPLC. A single radioactive peak, CBR1, and a peak absorbing at 330 nm, CBP, were observed, and fractions of both peaks were collected. They were further purified to homogeneity by HPLC. CBP was digested further with trypsin and chymotrypsin, and the peptides containing N^{ϵ}-pyridoxyllysine [Lys(Pxy)] were isolated and termed T2 and C1, respectively. The amino acid sequences of these peptides were determined by automated Edman degradation as summarzied in Figure 3. CBR1 also was sequenced, and most of radioactivity was found in the fraction of cycle 26, in which the PTH derivative of *S*-(carboxymethyl)cysteine was found. The BAPMP-labeled apoenzyme also was treated with cyanogen bromide, and the peptide modified with BAPMP (CBR2) was isolated in the same manner. CBR2 contains *S*-(carboxymethyl)cysteine, and its amino acid composition coincided with that of CBR1 (Table I). These results show that the cysteine residue carboxymethylated in CBR1 is the same one as is labeled with BAPMP and catalytically essential.

Discussion

Here we have presented evidence for the occurrence of four identical subunits of L-methionine γ-lyase; the identity has been shown in their molecular weight, charge, and amino-terminal amino acid sequence. One of four cysteine residues contained in each subunit is catalytically essential.

We have shown that the apoenzyme is rapidly inactivated with BAPMP, and that the inactivation obeys saturation kinetics. Stoichiometric studies of inactivation showed the incorporation of 1 mol of BAPMP per subunit; this corresponds to the content of the bound pyridoxal-P (Nakayama et al., 1984). These show that BAPMP acts as an affinity labeling agent on the pyridoxal-P binding site of the enzyme. We have also shown that BAPMP reacts with the essential cysteine residue. Thus, the essential cysteine most probably lies near the active site, although it is not close to the cofactor-binding lysine residue in the primary amino acid sequence as shown by isolation of the two separate peptides: CBP and CBR2.

We have determined the amino acid sequences around the essential cysteine residue and the lysine residue to which pyridoxal-P is bound at the active site. L-Methionine γ-lyase shares a common sequence at the active site with several pyridoxal-P enzymes, -Ser-X-X-Lys(Pxy)- (Tanase et al., 1979). The serine residue probably is important in binding of the 5′-phosphate of pyridoxal-P as found for that of mitochondrial aspartate aminotransferase of pig heart (Jansonius et al., 1985).

By means of the National Biomedical Research Foundation (NBRF) protein sequence data bank with the SEQHP algorithm of the Integrated Database and Extended Analysis System (IDEAS) (Wilber & Lipman, 1983), we have found that the peptide containing the active site lysine residue isolated from L-methionine γ-lyase (CBP) has high sequence homology with a part of cystathionine γ-synthase (residues 154–200) of *Escherichia coli* (Duchange et al., 1983). ^{198}Lys of the *E. coli* enzyme was recently determined to be the active coenzyme-binding residue (Martel et al., 1987). Linear alignment of the sequence of CBP and residues 154–200 of the *E. coli* enzyme revealed a 61% identity (Figure 4), although no significant sequence homology was found between the peptide containing the essential cysteine residue (CBR1) and any part of the enzyme. We have found also that the sequence of five amino acid residues around the active site lysine of rat liver cystathionine γ-lyase (Fearon et al., 1982) coincides with that of L-methionine γ-lyase. Thus, the active site sequence is highly conserved in pyridoxal-P enzymes that catalyze α,γ-elimination and γ-replacement reactions. These suggest a

strong evolutionary relationship (divergent or convergent) in the origin of these enzymes.

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Registry No. BAPM, 54522-09-7; BAPMP, 67731-58-2; EC 4.4.1.11, 42616-25-1; L-Cys, 52-90-4; pyridoxal-P, 54-47-7.

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Mechanism and Stereochemical Course at Phosphorus of the Reaction Catalyzed by a Bacterial Phosphotriesterase†

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ABSTRACT: The reaction mechanism for the phosphotriesterase from *Pseudomonas diminuta* has been examined. When paraoxon (diethyl 4-nitrophenyl phosphate) is hydrolyzed by this enzyme in oxygen-18-labeled water, the oxygen-18 label is found exclusively in the diethyl phosphate product. The absolute configurations for the (+) and (−) enantiomers of *O*-ethyl phenylphosphonothioic acid have been determined by X-ray diffraction structural determination of the individual crystalline 1-phenylethylamine salts. The (+) enantiomer of the free acid corresponds to the R_P configuration. The R_P enantiomer of *O*-ethyl phenylphosphonothioic acid has been converted to the S_P enantiomer of EPN [*O*-ethyl *O*-(4-nitrophenyl) phenylphosphonothioate]. (S_P)-EPN is hydrolyzed by the phosphotriesterase to the S_P enantiomer of *O*-ethyl phenylphosphonothioic acid. The enzymatic reaction therefore proceeds with inversion of configuration. These results have been interpreted as an indication of a single in-line displacement by an activated water molecule directly at the phosphorus center of the phosphotriester substrate. (R_P)-EPN is not hydrolyzed by the enzyme at an appreciable rate.

A broad variety of organophosphate triesters have been used as pesticides, insecticides, and other biological control agents. First discovered in Germany in the 1930s (Schrader, 1963), some modern organophosphates are considered to be among the safest of all chemical pesticides. However, many compounds known to be mammalian neurotoxins (McEwen & Stephenson, 1979) are also included in this class of compounds. In general, these pesticides are considered to be chemically reactive materials that do not persist in the soil for long periods of time. The lack of persistence in the soil has been attributed to their susceptibility to microbial hydrolysis (Munnecke, 1981). The microbial degradation of organophosphates such as paraoxon (diethyl 4-nitrophenyl phosphate) or the related phosphorothioate (e.g., parathion) has been defined in several

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