Egmond, M. R., Veldink, G. A., Vliegenthart, J. F. G., & Boldingh, J. (1973) *Biochem. Biophys. Res. Commun. 54*, 1178–1184.
Egmond, M. R., Brunori, M., & Fasella, P. M. (1976) *Eur. J. Biochem. 61*, 93–100.
Fukuzumi, S., & Kochi, J. K. (1982) *J. Am. Chem. Soc. 104*, 7599–7609.
Hanzlik, R. P., & Shearer, G. O. (1975) *J. Am. Chem. Soc. 97*, 5231–5233.
Hanzlik, R. P., & Shearer, G. O. (1978) *Biochem. Pharmacol. 27*, 1441–1444.
Hanzlik, R. P., & Westkaemper, R. B. (1980) *J. Am. Chem. Soc. 102*, 2464–2467.
Knier, B. L., & Jencks, W. P. (1980) *J. Am. Chem. Soc. 102*, 6789–6798.
Kühn, H., Schewe, T., & Rapoport, S. M. (1986) *Adv. Enzymol. Relat. Areas Mol. Biol. 58*, 273–311.
Lands, W. E. M. (1984) *Prostaglandins, Leukotrienes Med. 13*, 35–46.
Maas, R. L., Ingram, C. D., Taber, D. F., Oates, J. A., & Brash, A. R. (1982) *J. Biol. Chem. 257*, 13515–13529.
Martens, F. M., Verhoeven, J. W., & de Boer, T. J. (1979) *Tetrahedron Lett. 1979*, 2919–2920.
Melander, L., & Saunders, W. H., Jr. (1987) *Reaction Rates of Isotopic Molecules*, pp 95, 170–201, Krieger Publishing, Malabar, FL.
Miller, L. L., Nordblom, G. D., & Mayeda, E. A. (1972) *J. Org. Chem. 37*, 916–918.
Puustinen, T., Scheffer, M. M., & Samuelsson, B. (1987) *FEBS Lett. 217*, 265–268.
Rouzer, C. A., Matsumoto, T., & Samuelsson, B. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 857–861.
Schewe, T., Rapoport, S. M., & Kühn, H. (1986) *Adv. Enzymol. Relat. Areas Mol. Biol. 58*, 191–272.
Skoog, M. T., Nichols, J. S., & Wiseman, J. S. (1986) *Prostaglandins 31*, 561–576.
Veldink, G. A., & Vliegenthart, J. F. G. (1984) *Adv. Inorg. Biochem. 6*, 139–161.
Viswanathan, T. S., & Cushley, R. J. (1981) *J. Biol. Chem. 256*, 7155–7159.
Wiseman, J. S., Skoog, M. T., Nichols, M. S., & Harrison, B. L. (1987) *Biochemistry 26*, 5684–5689.

Mechanism-Based Inactivation of L-Methionine γ-Lyase by L-2-Amino-4-chloro-4-pentenoate†

Nobuyoshi Esaki,‡ Harumi Takada,‡ Mitsuaki Moriguchi,§ Shin-ichi Hatanaka,∥ Hidehiko Tanaka,‡,⊥ and Kenji Soda*,‡

Institute for Chemical Research, Kyoto University, Uji, Kyoto-Fu 611, Japan, Department of Environmental Chemistry and Engineering, Faculty of Engineering, Oita University, Oita 870-11, Japan, and Department of Biology, College of Arts and Sciences, The University of Tokyo, Meguro-Ku, Tokyo 153, Japan

Received July 19, 1988; Revised Manuscript Received November 7, 1988

ABSTRACT: L-2-Amino-4-chloro-4-pentenoic acid (L-ACP), an antibacterial amino acid produced by *Amanita pseudoporphyria* Hongo [Moriguchi, M., Hara, Y., & Hatanaka, S.-I. (1987) *J. Antibiot. 15*, 904–906], time dependently and irreversibly inactivates L-methionine γ-lyase. The inactivation obeys biphasic pseudo-first-order kinetics and is carried out completely with a minimum molar ratio ([L-ACP]/[enzyme tetramer]) of 5. During the incubation of enzyme, 4.4–5.0 mol of chloride ions is formed per mole of tetramer enzyme. The tetrameric enzyme is labeled with 4 mol of DL-[2-^{14}C]ACP/mol. We have isolated ^{14}C-labeled acetopyruvate and pyridoxamine 5′-phosphate from the [^{14}C]ACP-modified enzyme. The enzyme fully inactivated shows λ_{max} at 460 and 495 nm, which probably is derived from a conjugated pyridoximine paraquinoid. We have proposed a mechanism which involves enzymatic dehalogenation from C_4 of ACP to form a reactive allene. The allene is attacked by a nucleophilic amino acid residue at the active site. Analysis results of the thiol content of enzyme suggest that a cysteine residue is a possible nucleophilic residue covalently bound to the inactivator.

Mechanism-based enzyme inactivators are inherently inactive but are unmasked by the catalysis of the targeted enzyme itself to generate a reactive intermediate to inactivate the enzyme irreversibly. Thus, they show much higher selectivity toward the targeted enzyme than conventional affinity labeling agents (Walsh, 1982). Various functional groups, which can be activated by rearrangement or elimination, have been used for mechanism-based enzyme inactivators: e.g., acetylenes and olefins. The activated intermediates, generally electrophiles, react with a nucleophile at the active site as a Michael acceptor to inactivate the enzyme (Walsh, 1982).

L-2-Amino-4-chloro-4-pentenoic acid (L-ACP)[1] was isolated from fruit bodies of *Amanita pseudoporphilia* Hongo (Hatanaka et al., 1974), and it inhibits growth of various bacterial strains, e.g., *Bacillus subtilis*, *Escherichia coli*, and *Pseudo-*

† This work was supported in part by a Grant-in-aid for Research of Formulation and Management of Man-environment System from the Ministry of Education, Science and Culture of Japan.
* To whom correspondence should be addressed.
‡ Kyoto University.
§ Oita University.
∥ The University of Tokyo.
⊥ Present address: Faculty of Agriculture, Okayama University, Okayama 700, Japan.

[1] Abbreviations: ACP, 2-amino-4-chloro-4-pentenoate; pyridoxal-P, pyridoxal 5′-phosphate; pyridoxamine-P, pyridoxamine 5′-phosphate; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNB, 5-thio-2-nitrobenzoate.

0006-2960/89/0428-2111$01.50/0

monas aeruginosa (Moriguchi et al., 1987). It is a unique amino acid in which chlorine binds to the olefinic γ-carbon of L-allylglycine. As chloride is a good leaving group, L-ACP is most probably converted into 2-amino-3,4-pentadienoic acid when its β-hydrogen atom is removed enzymatically. 2-Amino-3,4-pentadienoic acid is reactive enough to react with an electrophilic amino acid residue at an active site of enzymes. Thus, L-ACP (and also D-ACP) most likely acts as a mechanism-based enzyme inactivator to the enzymes that catalyze the release of its β-hydrogen atom.

L-Methionine γ-lyase [L-methionine methanethiol-lyase (deaminating), EC 4.4.1.11] inherently catalyzes the α,γ-elimination of L-methionine to produce α-ketobutyrate, methanethiol, and ammonia and requires pyridoxal-P as a coenzyme. We have purified the enzyme from *Pseudomonas putida* to homogeneity and studied its physicochemical and enzymological properties (Nakayama et al., 1984). The enzyme is inactivated by L-propargylglycine (Johnston et al., 1979) and *S*-(*N*-methylthiocarbamoyl)-L-cysteine (Esaki et al., 1984). Johnston et al. (1980) also found its novel mechanism-based enzyme inactivator, 2-amino-4-chloro-5-[(*p*-nitrophenyl)sulfinyl]pentanoic acid, which is activated enzymatically by a 2,3-sigmatropic rearrangement. Removal of a β-hydrogen atom from the substrate amino acids is essential to the pridoxal-P-catalyzed α,γ-elimination (Davis & Metzler, 1972). L-Methionine γ-lyase removes β-hydrogen atoms of the substrate and nonsubstrate amino acids (Esaki et al., 1985). It is in fact inactivated by L-ACP as expected. We here show the inactivation of the enzyme with L-ACP and its mechanism.

Experimental Procedures

Materials. L- and D-ACP were prepared by enzymatic optical resolution of the racemate synthesized chemically (Hatanaka et al., 1974, 1985). *O*-Acetyl-L-homoseine was synthesized by the method of Nagai and Flavin (1971). L-Methionine γ-lyase was purified from the extract of *Pseudomonas putida* ICR 3460 as described previously (Nakayama et al., 1984). Leucine dehydrogenase (EC 1.4.1.9) of *Clostridium thermoaceticum* was purified according to the method of Shimoi et al. (1987).

Synthesis of DL-[2-^{14}C]*ACP.* [2-^{14}C]Diethyl acetamidomalonate (Amersham; 125 μCi/5 mg) was dissolved in 20 μL of ethanol containing 19 mg/mL sodium metal. 2,3-Dichloropropene (1.9 mg/7 μL of ethanol) was added to the mixture to be incubated in a sealed vial (inner volume, 0.2 mL) at 100 °C for 17 h. The mixture was then evaporated to dryness in a Sakuma vacuum centrifuge (Model EC-57C). The residue was extracted with 150 μL of chloroform and dried again in the same manner. The resiude was then dissolved in 120 μL of 2 N HCl and heated in a sealed vial at 130 °C for 4 h. The mixture was evaporated to dryness in the vacuum centrifuge, and the residue was dissolved in 50 μL of 40% trifluoroacetic acid in water. DL-[2-^{14}C]ACP was then isolated by high-performance liquid chromatography (HPLC): column, Ultron NC_{18} (Shinwa Kako Inc., Kyoto, Japan; 46 mm × 15 cm); mobile phase, 0.1% trifluoroacetic acid; flow rate, 1.0 mL/min; detection, A_{210}; retention time, 2.9 min. The product gave a single peak by amino acid analysis (Hitachi Model 835) and showed a specific radioactivity of 1.0×10^4 dpm/nmol, when measured with a Packard TRICARB 300C liquid scintillation system.

Kinetic Assay. The enzymatic α,γ-elimination of *O*-acetyl-L-homoserine was routinely followed by determination of α-ketobutyrate formed with 3-methyl-2-benzothiazolone hydrazone hydrochloride (Soda, 1968). The standard assay mixture contained 100 mM potassium phosphate buffer (pH 8.0), 25 mM *O*-acetyl-L-homoserine, and 0.01 mM pyridoxal 5′-phosphate (pyridoxal-P).

Reaction with ACP. (*a*) *Kinetics of Inactivation.* The rate of inactivation of L-methionine γ-lyase by ACP was determined as follows. The enzyme was added to a solution containing an appropriate concentration of ACP in 50 mM potassium phosphate buffer (pH 8.0) at 37 °C. Aliquots were removed at intervals and diluted 330 times with a standard reaction mixture for α,γ-elimination to determine the remaining activity. Various concentrations of ACP (0.2–1.0 mM) were used to determine the kinetic constants.

(*b*) *Determination of Ammonia and Chloride Ions Formed from* L-*ACP.* L-Methionine γ-lyase (10.8 mg) was incubated with 2 mM L-ACP in 0.2 mL of 20 mM Tricine/NaOH buffer (pH 8.0) at 37 °C. Enzyme or L-ACP was omitted in a blank. Aliquots were withdrawn and assayed to follow the enzyme inactivation. When the enzyme was inactivated completely (after about 3 h), ammonia and chloride ions formed in the reaction mixture were determined as follows. Ammonia was determined with glutamate dehydrogenase. The reaction mixture (0.1 mL) contained 10 mM α-ketoglutarate, 10 mM Tricine/NaOH buffer (pH 8.0), 0.13 mM NADH, 1 unit of glutamate dehydrogenase (Boehringer; bovine liver), and the sample solution (0.05 mL). The change in A_{340} was follwed with a Beckman DU 50 spectrophotometer, and the molar absorption coefficient of NADH, 6220 M^{-1} cm^{-1}, was used for the calculations. Chloride was determined by the method of Iwasaki et al. (1956); 0.15 mL of 0.1% (w/v) $Hg(SCN)_2$ in dioxane–ethanol (9:1 v/v) and 0.05 mL of 8% (w/v) $FeNH_4(SO_4)_2 \cdot 12H_2O$ in 6 N HNO_3 were added to a sample solution (0.05 mL) and A_{460} was measured with a Beckman DU 50 spectrophotometer after 10 min. Chloride ions were determined with a Shimadzu HIC-6A ion chromatograph equipped with a Shimadzu CDD-6A conductivity detector: column, Shim-pack IC-A1 (4.6 mm × 10 cm); mobile phase, 2.5 mM phthalic acid (pH 4.0); flow rate, 1.5 mL/min; temperature, 40 °C.

Labeling with DL-[2-^{14}C]*ACP.* L-Methionine γ-lyase (2.1 mg) was incubated with 5 mM DL-[2-^{14}C]ACP in 0.2 mL of 50 mM potassium phosphate buffer (pH 8.0) at 37 °C. Inactivation was allowed to proceed until no residual activity was detected (after about 4 h). The reaction mixture was applied to a Bio-Gel 6PDSG column (1.5 × 25 cm) and chromatographed with 10 mM potassium phosphate buffer (pH 7.2). The absorbance at 278 nm and the radioactivity of the fractions (0.7 mL) collected were measured.

Isolation of Acetopyruvate and Pyridoxamine-P from the [2-^{14}C]*ACP–*L-*Methionine* γ-*Lyase Adduct.* The solution (6 mM, 0.1 mL) of DL-[2-^{14}C]ACP in 160 mM Tricine/NaOH buffer (pH 8.0) was added to 0.15 mL of a 3.17 mg/mL solution of L-methionine γ-lyase in 20 mM Tricine/NaOH buffer (pH 8.0). Aliquots were withdrawn and assayed until more than 90% of the original activity was lost (3 h). The reaction mixture was applied to a Bio-Gel 6PDSG column (1.5 × 25 cm), and the radioactive enzyme was eluted with water. The enzyme fractions were combined and concentrated to about 50 μL with an Ultracent 30 ultrafiltration unit (Tosoh Manufacturing Co., Ltd., Tokyo, Japan). Trifluoroacetic acid was added to the enzyme solution at a concentration of 75%, and the mixture was incubated at 37 °C for 16 h. The mixture was then centrifuged, and the supernatant solution was evaporated to dryness in a vacuum centrifuge. The residue was dissolved in 55 μL of water and analyzed by HPLC: column, Ultron PS-80H (8 mm × 30 cm; Shinwa Kako Inc., Kyoto Japan); mobile phase, 0.18% $HClO_4$ in water (v/v); flow

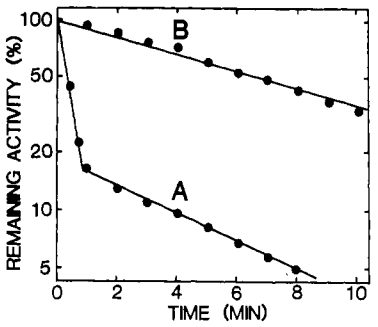


FIGURE 1: Kinetics of inactivation of L-methionine γ-lyase with 5.0 mM L-ACP in the absence (A) and presence (B) of 10 mM L-norleucine.

rate, 1.0 mL/min; detection, A_{210}. The ^{14}C-labeled compound was analyzed also after reductive amination with a reaction mixture (50 μL) containing 15 units of leucine dehydrogenase, 100 μmol of NH_4HCO_3, 10 nmol of NADH, and 20 μL of the above sample solution. The reaction was monitored by A_{340} change with a Beckman DU50 spectrophotometer. After 1 h, 450 μL of water was added to the reaction mixture, which was filtered through an Ultracent 30 to remove protein. The filtrate was evaporated to dryness in a vacuum centrifuge, and then the residue was dissolved in 40 μL of water. The amino acid produced was analyzed with a Beckman 7300 high-performance amino acid analyzer. Pridoxal-P and pyridoxamine 5′-phosphate (pyridoxamine-P) were also analyzed by HPLC according to the method of Tryfiates and Sattsangi (1982) with an Ultron NC_{18} column (4.6 × 200 mm).

Determination of Cysteine Content. The half-cystine content of the enzyme was determined with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) by the method of Cavallini et al. (1966). The total cysteine content of the enzyme was measured in 20 mM Tricine/NaOH buffer (pH 8.0) containing 4.5 M guanidine hydrochloride with a 20-fold molar excess of DTNB over enzyme. The amount of 5-thio-2-nitrobenzoate (TNB) released was estimated with the published molar absorption coefficient in guanidine hydrochloride, ϵ_{412} = 13 600 M^{-1} cm^{-1} (Riddles et al., 1979).

Digestion and Purification of Peptides. L-Methionine γ-lyase was labeled with DL-[2-^{14}C]ACP as described above and dialyzed against 1.0 L of 0.1 M NH_4HCO_3, followed by lyophilization. The enzyme (2.5 nmol) was digested with trypsin (0.05 nmol) in 100 μL of 0.1 M NH_4HCO_3 (pH 7.8) at 37 °C for 5 h. Peptides were purified with a Jasco LC-800 HPLC system (Japan Spectroscopic Co., Ltd., Tokyo, Japan) equipped with an Asahipak ODP-50 column (6 × 150 mm). A linear gradient elution from 0.1% (v/v) TFA in water to 80% (v/v) acetonitrile in 0.1% TFA was carried out at a flow rate of 0.8 mL/min. Peptides were followed by measurement of A_{220}.

RESULTS

Kinetic Profiles of Inactivation by L-ACP. L-Methionine γ-lyase was inactivated effectively by L-ACP with time, but D-ACP was inert. This is consistent with the finding that only L-amino acids are the substrates (Nakayama et al., 1984; Esaki et al., 1985). Typical semilogarithmic plots of remaining enzyme activity against time are shown in Figure 1. The kinetics of inactivation was biphasic. A double-reciprocal plot of the initial first-order rate constants of inactivation versus L-ACP concentrations gave a straight line. The maximal inactivation rate constant and the binding constant for L-ACP were calculated to be 0.046 s^{-1} and 1.0 mM, respectively. L-Norleucine and some other straight-chain L-amino acids are

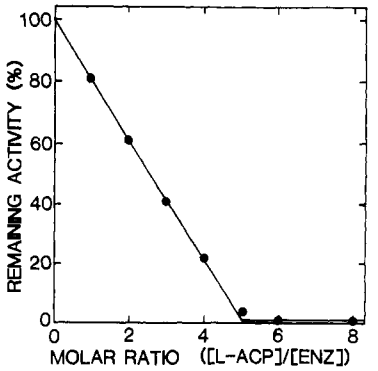


FIGURE 2: Inactivation of L-methionine γ-lyase as a function of the molar ratio of L-ACP to enzyme.

Table I: Determination of Chloride and Ammonia Formed during Inactivation of L-Methionine γ-Lyase with L-ACP

reaction system[a]	NH_4^+ (nmol)	Cl^- (nmol)	
		method 1[b]	method 2[c]
minus enzyme	1.6	0.4	0.8
minus substrate	21	0.8	0.8
complete	20	78	70

[a] Experimental conditions are described under Experimental Procedures, and 15.7 nmol of L-methonine γ-lyase was used. [b] Determined by ion chromatography. [c] Determined by colorimetric method (Iwasaki et al., 1956).

competitive inhibitors of the enzyme. The enzyme abstracts their α- and β-hydrogen atoms but cannot cleave the C–C bond at the β- or γ-position (Esaki et al., 1985). The rate of inactivation was decreased by the presence of L-norleucine under the conditions employed. The apoenzyme was not affected by L-ACP. These results suggest that the inactivation results from enzyme catalysis.

Turnover of L-ACP. We incubated L-methionine γ-lyase with various amounts of L-ACP. The extent of inactivation is linearly dependent on the L-ACP/enzyme molar ratio (Figure 2). Extrapolation of the initial linear part of the curve to the line of complete inactivation showed about 5 mol/mol of tetramer enzyme. These suggest that essentially none of the L-ACP molecules are turned over before every subunit of enzyme is inactivated. When L-ACP reacted with the enzyme, 4.4–5.0 mol of chloride ions was formed per mole of tetramer enzyme (Table I). Therefore, L-ACP is dechlorinated enzymatically to form an active intermediate, which probably reacts readily with an amino acid residue at the active site to inactivate the enzyme. Ammonia was produced as well in the same reaction mixture, but this is probably due to desorption from protein: the same amount of ammonia was produced in a control system in which L-ACP was omitted (Table I).

Spectral Analyses. Addition of L-ACP to L-methionine γ-lyase results in a decrease in the 420-nm absorbance and in gradual appearance of an absorption peak and a shoulder at 460 and 495 nm (Figure 3). The fully inactivated enzyme showed a shoulder of absorption at 435 nm as well. A similar spectral change was observed for the enzyme inactivated with L-propargylglycine (Johnston et al., 1979). These suggest that a common enzyme–inactivator adduct was produced with L-ACP and L-propargylglycine.

Stoichiometry of Labeling with DL-[2-^{14}C]ACP. When the enzyme incubated with DL-[2-^{14}C]ACP was chromatographed on a Bio-Gel 6PDSG column, a peak of radioactivity was coeluted with the protein. This suggests that the inactivation is caused by covalent and irreversible modification of the enzyme with ACP. The recovery of radioactivity bound to the enzyme shows incorporation of 4.0 mol of [^{14}C]ACP into

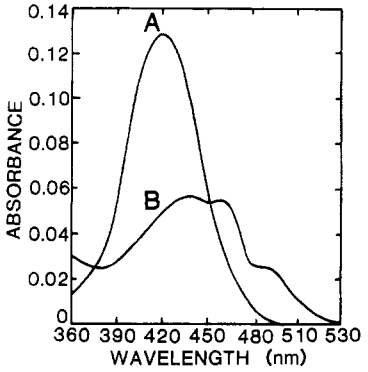


FIGURE 3: UV–visible spectra of the native L-methionine γ-lyase (A) and the enzyme inactivated by L-ACP (B).

Table II: Ultrafiltration of [2-^{14}C]ACP-Modified Enzyme

conditions[a]	radioactivity (dpm) residue[b]	filtrate
none	7800	0
dimethyl sulfoxide (75%)	7300	200
acetone (75%)	7500	200
1-propanol (75%)	7800	100
dimethylformamide (75%)	3500	3800
trifluoroacetic acid (75%)	1100	6400
formic acid (75%)	2500	5600
NaOH (50 mM)	7000	500
urea (8 M)	7200	200

[a] The [^{14}C]ACP-modified enzyme (0.196 nmol; 7800 dpm) was incubated with the compounds indicated at 37 °C for 16 h and ultrafiltered with an Ultracent 30 unit. [b] The residue was dissolved in 0.2 mL of 8 M urea, and the activity was counted.

1 mol of tetramer enzyme: this is consistent with the pyridoxal-P content (4 mol/mol) of the enzyme (Nakayama et al., 1984). The ACP-treated enzyme eluted from the Bio-Gel 6PDSG column did not act on L-methionine, *O*-acetyl-L-homoserine, or *S*-methyl-L-cysteine. This shows that the inactive enzyme–ACP complex is stable under the conditions. However, when the radioactive enzyme was treated with 75% trifluoroacetic acid or formic acid, most of the original radioactivity was released from the enzyme (Table II). Therefore, the complex of the enzyme with ACP is susceptible to solvolysis under acidic conditions.

Isolation of Acetopyruvate and Pyridoxamine-P from the Enzyme Complex with DL-[2-^{14}C]*ACP.* L-Methionine γ-lyase was inactivated with DL-[2-^{14}C]ACP, and the radioactively labeled protein was separated from the excess inactivator by gel filtration with a Bio-Gel 6PDSG column. The labeled protein was incubated with 75% trifluoroacetic acid, and the 2-^{14}C-labeled compound was isolated as described under Experimental Procedures. The isolated product gave a single peak upon Ultron PS-80H HPLC and was cochromatographed with authentic acetopyruvate (Sigma). Most of the radioactivity injected into an Ultron PS-80H column was recovered in the fraction collected for the peak (Table III). The product was identified as well by amino acid analysis after reductive amination with leucine dehydrogenase as described under Experimental Procedures. A single major amino acid appeared at the same retention time as the amino acid produced from authentic acetopyruvate (Table III). We have also attempted to analyze the cofactor released from the ^{14}C-labeled enzyme by ordinary-phase ion pair chromatography (Tryfiates & Sattsangi, 1982). Production of pyridoxamine-P was shown, but pyridoxal-P was not detected (Table III).

Content of Thiols in the Inactivated Enzyme. L-Methionine γ-lyase contains 16 mol of cysteine residues/mol of enzyme,

Table III: Chromatography of the Compounds Isolated from ACP-Modified Enzyme

	acetopyruvate isolated from ACP-modified enzyme[a]	amino acid produced from acetopyruvate[b]	pyridoxamine-P[c]
retention time (min)	17.9	7.9	8.3
amount injected			
dpm	32 300	29 400	
nmol	3.23[d]	2.94[d] (3.15)[e]	
amount recovered			
dpm	29 100	28 000	
nmol	2.91[d] (3.26)[f]	2.80[d] (2.97)[g]	1.23[h]
recovery (%)	90[i]	95[i]	76[j]

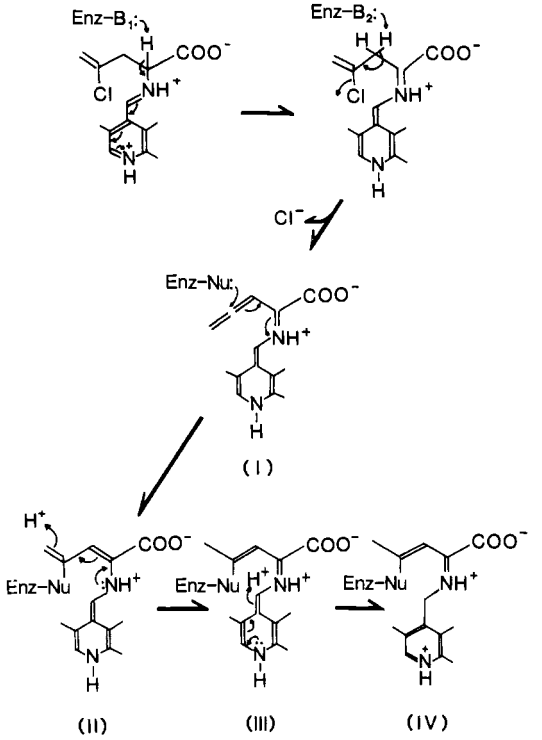
[a] The ^{14}C-labeled compound was isolated from the ACP-modified enzyme as described under Experimental Procedures. Twenty microliters of the final solution (55 μL) was analyzed by HPLC with an Ultron PS-80H column. The fraction collected at the peak appearing at 17.9 min was counted. [b] The ^{14}C-labeled compound was reductively aminated with leucine dehydrogenase as described under Experimental Procedures. The fraction collected at the peak appearing at 17.9 min was counted. [c] The ^{14}C-labeled enzyme was denatured and ultrafiltered as described under Experimental Procedures. Ten microliters of the final solution (55 μL) was analyzed by ordinary-phase ion pair HPLC (Tryfiates & Sattsangi, 1982). [d] Calculated by the specific radioactivity of DL-[2-^{14}C]ACP (1×10^4 dpm/nmol) used. [e] Determined by measurement of A_{340} change. [f] Determined by measurement of A_{210} with authentic acetopyruvate as a standard. [g] Determined with ninhydrin on the assumption that the product gives the same color yield as valine. [h] Determined by measurement of A_{254} with authentic pyridoxamine-P. [i] Calculated from the radioactivity. [j] Calculated on the assumption that the amount of pyridoxamine-P equals that of the ^{14}C-labeled compound.

and 4 mol of them is essential for the enzyme activity (Nakayama et al., 1987ab). Johnston et al. (1979, 1980) have suggested that the essential cysteine residues are most probably modified with suicide substrates, L-propargylglycine and 2-amino-4-chloro-5-[(*p*-ntirophenyl)sulfinyl]pentanoic acid. Therefore, we determined the content of thiols in the inactivated enzyme with DTNB. The ACP-modified enzyme showed 10.9 mol of thiols/mol of enzyme in the presence of 4.5 M guanidine hydrochloride: about 5 mol of cysteine residue/mol of enzyme was lost by modification with L-ACP. The [2-^{14}C]ACP-labeled enzyme was digested with trypsin, and the resulting peptides were separated by reverse-phase HPLC as described under Experimental Procedures. However, no radioactivity was recovered from the peptide fractions: the peptide radioactively labeled underwent acid hydrolysis under the conditions used.

Discussion

L-Methionine γ-lyase is inactivated irreversibly by L-ACP. The inactivation is accompanied by covalent attachment of 4 mol of ACP to 1 mol of the enzyme. The inactivation obeys pseudo-first-order kinetics. The rate of inactivation is zero order with respect to ACP at high ACP concentrations and is reduced in the presence of the competitive inhibitor L-norleucine. These meet criteria for the suicide inactivation (Walsh, 1982). However, the inactivation does not follow a simple process: a fast inactivation process is followed by a slow one. This is probably due to a kinetic cooperativity among subunits: modification of one subunit affects the reactivity of an adjacent subunit. The enzyme shows negative cooperativity with regard to inactivation by L-propargylglycine (Johnston et al., 1979) and modification with various thiol reagents (Nakayama et al., 1988ab). Such half-of-the-sites reactivity has been reported for suicide inactivation of other oligomeric enzymes: cystathionine γ-lyase (EC 4.4.1.1)

Scheme I: Proposed Inactivation Mechanism of L-Methionine γ-Lyase by L-ACP and L-Propargylglycine



(Washtien & Abeles, 1977; Silverman & Abeles, 1977) and cystathionine γ-synthase (EC 4.2.99.9) (Johnston et al., 1979).

A structure of the enzyme–inactivator complex is proposed as II (Scheme I). Isolation of acetopyruvate and pyridoxamine-P from the complex provides strong support for the proposed structure. Enzymatic generation of a carbanion at the β-position, adjacent to the vinyl linkage, allows dechlorination from the vinyl function to an allene. In fact, chloride was produced enzymatically from L-ACP, and the amount was almost equivalent to that of enzyme monomer used. The allene thus produced is then attacked by a nucleophilic active site residue, which results in covalent modification and inactivation. Although we have no direct evidence for the conjugated allene formation, this is one of the most reasonable intermediates in the formation of the complex. The involvement of an allenic intermediate in the inactivation is compatible with the observed inactivation of the enzyme by L-propargylglycine (Johnston et al., 1979). The same conjugated allene can be produced by rearrangement of the acetylenic functionality from the compound as proposed previously (Johnston et al., 1979). We suggest that the partition ratio of turnover vs inactivation is almost 1 for L-ACP. This is also the case for L-propargylglycine (Johnston et al., 1979) and probably attributable to a high reactivity of the conjugated allene. Our results of thiol determination of the enzyme modified with L-ACP suggest that a cysteine residue is bound to the conjugated allene. We have attempted to isolate the ACP-modified peptide without success.

We have proposed a two-base mechanism for the substrate hydrogen exchange reaction catalyzed by L-methionine γ-lyase: the base which abstracts an α-hydrogen from the substrate is different from that abstracting the β-hydrogen (Esaki et al., 1985). The inactivation probably follows the action of the functional groups participating in the normal catalytic cycle. Thus, the proposed inactivation mechanism involves several intermediary steps which are similar to those involved in the normal reactions. In the α,γ-elimination, abstraction of the β-hydrogen is followed by elimination of the γ-substituent. L-ACP is most likely dechlorinated in the same manner.

III is a pyridoximine quinoid with the extended β,γ-conjugation and expected to absorb at a high wavelength (e.g., 550 nm), because Karube and Matsushima (1977) have shown that an adduct of the β,γ-unsaturated ketimine of 2-ketobutenoate and the aluminum chelate of pyridoxal methochloride paraquinoid absorbs at 550 nm. L-Methionine γ-lyase actually shows chromophore absorbing at a high wavelength (λ_{max} = 550 nm) upon addition of L-2-amino-*cis*-3-pentenoate: a β,γ-unsaturated pyridoxal paraquinoid is accumulated (Johnston et al., 1981). We could not observe such a chromophore in the inactivated enzyme. IV shows a possible structure of the inactivated species. Rando et al. (1976) have studied the inactivation mechanism of aspartate aminotransferase (EC 2.6.1.1) by L-2-amino-4-methoxy-*trans*-3-butenoate and proposed that a structure similar to IV shows a triplet absorption band in the range of 350 nm. However, the wavelength is far away from those of absorption bands for L-methionine γ-lyase, even though we take the inherent difference between the two enzymes into consideration: the enzyme-bound pyridoxal-P of active aspartate aminotransferase absorbs at a much lower wavelength (360 nm) than that of L-methionine γ-lyase (420 nm). Probably, II gives rise to an absorption peak around 495 nm. On denaturation of the ACP-modified enzyme and subsequent hydrolysis of the enzyme–ACP complex, II probably is converted to acetopyruvate and pyridoxamine-P. It is not inconceivable that the ACP-modified enzyme has lost the ability to give a proton to C_5 of II. Thus, II shows the most probable structure of the final enzyme–inactivator complex.

Registry No. L-ACP, 55528-30-8; DL-[2-^{14}C]ACP, 118725-07-8; EC 4.4.1.11, 42616-25-1; [2-^{14}C]diethyl acetamidomalonate, 14109-61-6; 2,3-dichloropropene, 78-88-6; L-cysteine, 52-90-4.

References

Cavallini, D., Granziani, M. T., & Dupre, S. (1966) *Nature 212*, 294.

Davis, L., & Metzler, D. E. (1972) *Enzyme (3rd Ed.) 7*, 33–74.

Esaki, N., Kimura, T., Goto, J., Nakayama, T., Tanaka, H., & Soda, K. (1984) *Biochim. Biophys. Acta 785*, 54–60.

Esaki, N., Nakayama, T., Sawada, S., Tanaka, H., & Soda, K. (1985) *Biochemistry 24*, 3857–3862.

Hatanaka, S.-I., Kaneko, S., Niimura, Y., Kinoshita, F., & Soma, G. (1974) *Tetrahedron. Lett.*, 3931–3932.

Hatanaka, S.-I., Niimura, Y., & Takishima, K. (1985) *Trans. Mycol. Soc. Jpn. 26*, 61–68.

Iwasaki, I., Utsumi, S., Hagino, K., & Ozawa, T. (1956) *Bull. Chem. Soc. Jpn. 29*, 860–864.

Johnston, M., Jankoowski, D., Marcotte, P., Tanaka, H., Esaki, N., Soda, K., & Walsh, C. (1979) *Biochemistry 18*, 4690–4701.

Johnston, M., Raines, R., Walsh, C., & Firestone, R. A. (1980) *J. Am. Chem. Soc. 102*, 4241–4250.

Johnston, M., Raines, R., Chang, M., Esaki, N., Soda, K., & Walsh, C. (1981) *Biochemistry 20*, 4325–4333.

Karube, Y., & Matsushima, Y. (1977) *J. Am. Chem. Soc. 99*, 7356.

Moriguchi, M., Hara, Y., & Hatanaka, S.-I. (1987) *J. Antibiot. 15*, 904–906.

Nagai, S., & Flavin, M. (1971) *Methods Enzymol. 17B*, 423–424.

Nakayama, T., Esaki, N., Sugie, K., Beresov, T. T., Tanaka, H., & Soda, K. (1984) *Anal. Biochem. 138*, 421–424.

Nakayama, T., Esaki, N., Tanaka, H., & Soda, K. (1988a) *Agric. Biol. Chem. 52*, 177–183.

Nakayama, T., Esaki, N., Tanaka, H., & Soda, K. (1988b) *Biochemistry 27*, 1587–1591.
Rando, R. R., Relyea, N., & Cheng, L. (1976) *J. Biol. Chem. 251*, 3306–3312.
Riddles, P. W., Blakely, R. L., & Zerner, B. (1979) *Anal. Biochem. 94*, 75–81.
Shimoi, H., Nagata, S., Esaki, N., Tanaka, H., & Soda, K. (1987) *Agric. Biol. Chem. 51*, 3375–3381.
Silverman, R. B., & Abeles, R. H. (1977) *Biochemistry 16*, 5515–5520.
Soda, K. (1967) *Agric. Biol. Chem. 31*, 1054–1060.
Tryfiates, G. P., & Sattsangi, S. (1982) *J. Chromatogr. 227*, 181–186.
Walsh, C. (1982) *Tetrahedron 38*, 871–909.
Washtien, W., & Abeles, R. H. (1977) *Biochemistry 16*, 2485–2491.

Staphylococcal Nuclease Active-Site Amino Acids: pH Dependence of Tyrosines and Arginines by ^{13}C NMR and Correlation with Kinetic Studies†

Charles B. Grissom‡ and John L. Markley*
Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin—Madison, 420 Henry Mall, Madison, Wisconsin 53706

Received January 20, 1988; Revised Manuscript Received October 21, 1988

ABSTRACT: The pH and temperature dependence of the kinetic parameters of staphylococcal nuclease (EC 3.1.4.7) have been examined with three *p*-nitrophenyl phosphate containing DNA analogues that vary as to 3′-substituent. With wild-type (Foggi variant) nuclease (nuclease wt) and the substrates thymidine 3′-phosphate 5′-(*p*-nitrophenyl phosphate) (PNPdTp), thymidine 3′-methylphosphonate 5′-(*p*-nitrophenyl phosphate) (PNPdTp*Me), and thymidine 5′-(*p*-nitrophenyl phosphate) (PNPdT), k_{cat} remains nearly constant at 13 min^{-1}. However, k_{cat}/K_m with nuclease wt varies considerably: 413, 13, and 0.52 mM^{-1} min^{-1} with PNPdTp, PNPdTp*Me, and PNPdT, respectively. When tyrosine-85 is changed to phenylalanine (nuclease Y85F) by site-directed mutagenesis, k_{cat} is unchanged at about 13 min^{-1}, except with PNPdTp where it drops to 1 min^{-1}. With nuclease Y85F, k_{cat}/K_m is 19.5 and 25 mM^{-1} min^{-1} with PNPdTp and PNPdTp*Me, respectively. With PNPdTp as the substrate, a bell-shaped k_{cat}/K_m vs pH profile is seen with pK_a values at 8.94 and 9.67 in 0.3 M KCl and H_2O. The pK_a at 9.67 disappears, and a new pK_a appears at 10.1 when tyrosine-85 is changed to phenylalanine (nuclease Y85F) or when the substrate 3′-phosphomonoester is changed to a 3′-methylphosphonate (PNPdTp*Me). This suggests that the inflection in k_{cat}/K_m with pK_a at 9.67 arises from ionization of tyrosine-85, which hydrogen bonds to the divalent 3′-phosphomonoester of substrates with this substituent. The enthalpy of ionization of both deprotonation steps in the k_{cat}/K_m versus pH profile is 5 kcal/mol. ^{13}C NMR has been used to determine the pK_a values of the arginine and tyrosine residues. The protein was enriched uniformly with 20% ^{13}C at all carbons and specifically with 90% ^{13}C at the guanidino carbon of the arginine residues. All five arginines in the protein have pK_a values greater than 11.6 in H_2O and 0.3 M KCl. This eliminates arginine as a candidate for the basic catalyst that deprotonates H_2O to facilitate nucleophilic attack on phosphorus. The results do not rule out arginine as a candidate for the acidic catalyst that protonates the 5′-ribose alkoxide prior to product release. The phenolic hydroxyl carbon of tyrosine-85 has been assigned by comparing the ^{13}C NMR spectrum of nuclease wt and nuclease Y85F. The structure of nuclease Y85F is not perturbed significantly by this substitution. Tyrosine-85 has a spectroscopically observed pK_a of 9.53 ± 0.05 in H_2O and 0.3 M KCl, which is similar to the basic-side pK_a of 9.67 seen in the k_{cat}/K_m pH profile. This correlation between pK_a values along with the absence of other candidates indicates that the ionization of tyrosine-85 is the pK_a seen in the k_{cat}/K_m vs pH profile for substrates with a divalent 3′-phosphomonoester. This conclusion is consistent with the proposed role of tyrosine-85 as a hydrogen-bond donor to the 3′-phosphomonoester of substrates poised for exonucleolytic hydrolysis.

Staphylococcal nuclease [ribonucleate (2′-deoxyribonucleate) 3′-nucleotidohydrolase, EC 3.1.4.7] catalyzes the hydrolytic cleavage of ribo- and 2′-deoxyribonucleotides between the 5′-phosphate and the 5′-oxygen of the ribose ring. The enzyme has been studied thoroughly and is a prototype for elucidation of the relationship between protein structure and catalytic activity (Hazen & Cotton, 1978; Tucker et al., 1979a–c). A detailed understanding of the catalytic role of active-site amino acids consistent with the pH dependence of the kinetic parameters (Dunn et al., 1973) has eluded researchers despite the availability of a 1.5-Å resolution X-ray crystal structure with the competitive inhibitor thymidine 3′,5′-diphosphate (pdTp)[1] and the required metal ion, Ca^{2+},

†Supported by NIH Fellowship GM10903 to C.B.G., NIH Grant GM35976, and NSF Grant DMB-8410222. A preliminary report of this work was presented at the American Chemical Society Meeting, New Orleans, LA, Sept 1, 1987.
‡NIH Postdoctoral Fellow. Present address: Department of Chemistry, University of California, Berkeley, CA 94720.

[1] Abbreviations: pdTp, thymidine 3′,5′-diphosphate; PNPdTp, thymidine 3′-phosphate 5′-(*p*-nitrophenyl phosphate); PNPdT, thymidine 5′-(*p*-nitrophenyl phosphate); PNPdTp*Me, thymidine 3′-(methylphosphonate) 5′-(*p*-nitrophenyl phosphate); Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Ches 2-(cyclohexylamino)ethanesulfonic acid; Caps, 3-(cyclohexylamino)-1-propanesulfonic acid; ΔH_{ion}, enthalpy of ionization; Mops, *N*-morpholinopropanesulfonic acid; TSP, (trimethylsilyl)propionic acid.

0006-2960/89/0428-2116$01.50/0