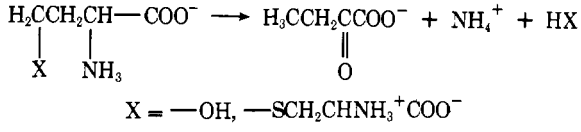
Mechanism of Inactivation of γ-Cystathionase by the Acetylenic Substrate Analogue Propargylglycine†

Wendy Washtien‡ and Robert H. Abeles*

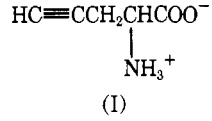
ABSTRACT: The acetylenic substrate analogue propargylglycine (2-amino-4-pentynoic acid) irreversibly inactivates rat liver γ-cystathionase. The inactivation is accompanied by covalent labeling of the enzyme (1 mol of ^{14}C-labeled inactivator per 80 000 daltons per two subunits). Catalytically active enzyme and the presence of the acetylenic functionality are required for covalent labeling. The inactivation is pseudo-first-order and shows saturation kinetics. A V_{max} deuterium isotope effect of 2.2 is observed with [α-^{2}H]propargylglycine. An almost identical isotope effect is observed when an α-deuterated substrate is used in the catalytic reaction. In addition, ^{3}H is released into the solvent during inactivation of γ-cystathionase by [α-^{3}H]propargylglycine. An inactivator-enzyme adduct can be isolated using D,L-[^{14}C]inactivator. Structure II, Scheme I, is proposed for the adduct structure. Consistent with this structure is the isolation of 2-amino-4-keto[2-^{14}C]pentanoic acid (only the L-isomer) from 1 N HCl hydrolysis of the ^{14}C-inactivated enzyme. Hydrolysis studies suggest a cysteine or tyrosine residue as the nucleophilic amino acid involved in covalent linkage with the inactivator. The mechanism proposed for inactivation (Scheme I) involves enzymatic conversion of the acetylenic inactivator at the active site to a reactive allene. The allene interacts with a nucleophile at the active site to form a covalent bond with the enzyme.

Since the original discovery by Bloch and co-workers (Endo et al., 1970) that acetylenic compounds can act as irreversible enzyme inhibitors, a number of additional examples of inactivation by acetylenic substrate analogues have been reported (Walsh et al., 1972; Holland et al., 1973; Hevey et al., 1973; Batzold and Robinson, 1975; Marcotte and Walsh, 1975).

Among the enzymes inactivated by acetylenic analogues is rat liver γ-cystathionase, which catalyzes the following reactions. Propargylglycine (I) acts as an irreversible inactivator



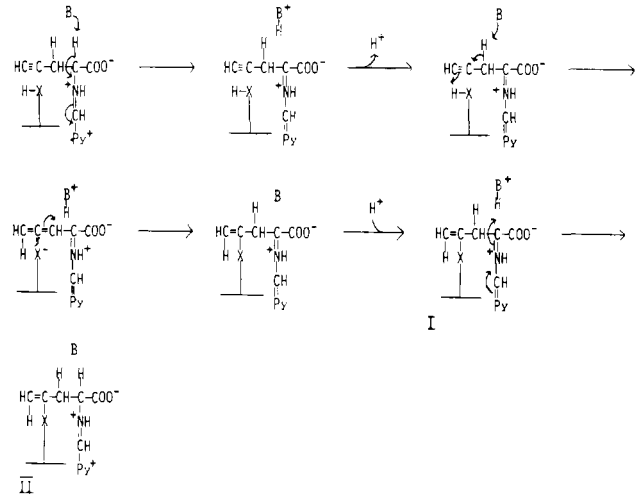
X = —OH, —SCH$_2$CHNH$_3^+$COO$^-$



(I)

of this enzyme. The rate of inactivation follows pseudo-first-order kinetics and 1 mol of ^{14}C-labeled inactivator is incorporated per 80 000 daltons (Abeles and Walsh, 1973). The mechanism shown in Scheme I is proposed for inactivation.[1] According to this mechanism, the enzyme catalyzes the rearrangement of the acetylenic inhibitor to an allene. The enzyme-bound allene then reacts with a nucleophile at the active site. This results in covalent labeling of the active site and inactivation of the enzyme. An important aspect of this type of inactivation, which has been referred to as suicide inactivation (Abeles and Maycock, 1976), is that the actual inactivator is generated enzymatically at the active site.

SCHEME I. Mechanism of Inactivation of γ-Cystathionase by Propargylglycine.[a]



[a] Py$_T^+$-CHO = enzyme-bound pyridoxal phosphate. B, X = bases at the active site. In the mechanism shown, two bases participate in the catalytic process. It is possible that more than two bases are involved.

Acetylene to allene rearrangements have been proposed as essential to the mechanism of inactivation of several enzymes by acetylenic substrate analogues (Endo et al., 1970; Hevey et al., 1973; Walsh et al., 1972; Marcotte and Walsh, 1975; Batzold and Robinson, 1975) but in no case has an enzyme-inactivator adduct been isolated to substantiate this mechanism.

Experimental Procedures

Materials

Enzymes and substrates were obtained from the following sources: D-amino acid oxidase, L-amino acid oxidase, D,L-

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‡ Present address: Department of Biochemistry and Biophysics, University of California Medical School, San Francisco, Calif.

[1] The mechanism shown differs from that originally proposed (Abeles and Walsh, 1973) in final reprotonation at the α carbon. This step is implicated by the work presented in this paper.

homoserine, and pyridoxal 5′-phosphate were from Sigma Chemical Co.; Pronase was from Calbiochem; lactate dehydrogenase and TPCK trypsin were from Worthington Biochemicals; ^3H_2O (1 Ci/g) was from New England Nuclear; and diethyl [2-^{14}C]acetamidomalonate (4.8 μCi/μmol) was from Amersham Searle Co. All other materials used were of commercial analytical grade.

γ-Cystathionase was purified from rat liver (specific activity, 380–500 units/mg) by the procedure of Greenberg (1962), who reported a specific activity of 350 units/mg. Purified enzyme with specific activities ranging from 380–500 was subjected to sodium dodecyl sulfate gel electrophoresis (Weber and Osborn, 1969). Enzyme preparations within that specific activity range showed one protein band with a molecular weight of 40 000. No other protein bands were apparent.

D,L-Propargylglycine (2-amino-4-pentynoic acid) was synthesized from propargyl bromide and diethyl acetamidomalonate by the method of Jansen and co-workers (1969), mp 238–240 °C (lit. 235–240 °C). D,L-[2-^{14}C]Propargylglycine (specific activity, 3.5×10^5 cpm/μmol) was synthesized using diethyl [2-^{14}C]acetamidomalonate. The synthesis was performed on a 1 mmol scale. A single radioactive compound was detected upon chromatography of the synthetic material on Eastman Silica TLC[2] plates in solvent A and high-voltage electrophoresis at pH 8.9.

D,L-[α-^{3}H]Propargylglycine (specific activity, 1.68×10^5 cpm/μmol) was prepared by the method of Miles and McPhie (1974), as modified by Dr. R. Silverman of this laboratory. D,L-Propargylglycine (1.2 mmol) is dissolved in ^3H_2O (2.5 mL, 200 mCi/mL) and treated with aluminum sulfate and pyridoxal as described. Aluminum tritoxide was precipitated by the addition of 7 volumes of ethanol. The solution was filtered and the pH of the filtrate was adjusted to 6.5 with 5 N HI. Upon standing at room temperature, crystals began to form. When crystallization appeared complete, the crystals were collected and recrystallized from water–ethanol. Further purification was obtained by chromatography on a Dowex 50-X8 H^+ column with 1 N HCl as eluent. This procedure gave D,L-[α,δ-^{3}H]propargylglycine. ^{3}H in the δ position of this compound was then exchanged for ^{1}H by equilibration of the purified compound with H_2O at pH 11 for 24 h, giving D,L-[α-^{3}H]propargylglycine as the final product. D,L-[α-^{2}H]-Propargylglycine was prepared in an analogous manner with ^2H_2O replacing ^3H_2O in the initial incubation.

D,L-[α-^{2}H]Homoserine was prepared by methods analogous to those used for preparation of D,L-[α-^{2}H]propargylglycine. The material was purified on a column of Dowex 50-X8 H^+ with 2 N ammonia as the eluent.

2-Amino-4-ketopentanoic acid was synthesized from bromoacetone (redistilled) and diethyl acetamidomalonate by the method of Wiss and Fuchs (1952). The product was identified by NMR (Tsuda and Friedman, 1970).

Ethyl vinyl sulfide was prepared by the method of Price and Gillis (1953) for methyl vinyl sulfide; bp 86.5–88 °C; NMR: triplet, 1.35 δ(3); quartet, 2.75 δ(2); multiplet, 5.2 δ(2); quartet, 6.3 δ(1).

Methods

Analytical Procedures. γ-Cystathionase was assayed by coupling α-ketobutyrate production to the oxidation of NADH as catalyzed by lactate dehydrogenase (Flavin and Slaughter, 1969). The reaction mixture contained pyridoxal 5′-phosphate (36 μM), β-mercaptoethanol (5.4 mM), potassium phosphate buffer, pH 7.5 (75 mM), D,L-homoserine (35 mM), NADH (0.2 mM), and LDH (26 units) in a total volume of 1.4 mL. The assay is begun with the addition of γ-cystathionase (0.05–0.1 IU) and the decrease in absorbance at 340 nm followed. Enzymatic activity was recorded in units of μm/h at 37 °C. The protein concentration was determined by the method of Lowry et al. (1951).

Radioactivity was determined on a Nuclear Chicago scintillation counter. Chromatograms were scanned for radioactivity with a Tracerlab 4π scanner.

Paper chromatography was performed by the ascending method on Whatman No. 3 paper. Papers were equilibrated with the solvent atmosphere for 15 to 20 min in the chromatography jar before lowering them into the solvent. The solvent systems used were: (A) butanol/acetic acid/water (4:1:1), (B) 2-propanol/ammonia/water (8:1:1), (C) 80% aqueous phenol, (D) methanol/pyridine/water (20:5:1).

Electrophoresis was performed on Whatman No. 3 paper. The conditions used were: pH 1.9, formic acid/acetic acid/water (1:4:45), 4.2 kV, 26 min; pH 8.9, 1% $(NH_4)_2CO_3$ in water, 2.5 kV, 50 min.

Isolation of the [^{14}C]Propargylglycine–γ-Cystathionase Adduct. A 67 mM solution (0.05–0.1 mL) of D,L-[2-^{14}C]-propargylglycine (specific activity, 3.5×10^5 cpm/μmol) in potassium phosphate buffer, 0.2 M, pH 7.5, was added to 3–5 mL of a 4–8 mg/mL solution of γ-cystathionase in the same buffer. Aliquots were withdrawn and assayed to monitor the rate of inactivation. When inactivation was greater than 90% complete (20–40 min, depending on the concentration of the inactivator), the reaction mixture was applied to a Sephadex G-25 column (1.5 × 45 cm) and eluted with distilled water. Protein-containing fractions from Sephadex G-25 chromatography were pooled and the amount of radioactivity and protein in the pooled sample was determined.

Haloform Reaction of the Hydrolysis Products. The purified radioactive hydrolysis product (0.01 mL, 1000 cpm) was mixed with 0.05 mL of water and the solution was made basic by addition of 0.03 mL of 10% NaOH. To this solution was added 0.05 mL of haloform reagent (2 g of potassium iodide and 1 g of iodine in 10 mL of water; Pasto and Johnston, 1969). The solution was left at room temperature for 1 h, after which time 10% NaOH was added until the solution appeared colorless. The solution was washed twice with chloroform (0.1 mL) and the aqueous layer was taken to dryness by rotary evaporation.

Sodium Borohydride Reduction of the Hydrolysis Product. The purified radioactive hydrolysis product (0.01 mL, 1000 cpm) was placed in 0.03 mL of water. A 0.1 M $NaBH_4$ solution in 0.1 M NaOH (0.01 mL) was added to the radioactive solution. After incubation at room temperature for 30 min, the reaction was stopped with the addition of 0.05 mL of 1 N HCl to remove excess borohydride. Methanol was then added to the solution to produce methyl borate, which was removed by repeated concentration of the sample from methanol by rotary evaporation.

Chemical Reduction of the [^{14}C]Propargylglycine–γ-Cystathionase Adduct. The [^{14}C]propargylglycine–γ-cystathionase adduct isolated from Sephadex G-25 chromatography was reduced with $NaBH_4$ under various conditions: (1) as isolated, (2) in 6 M urea, (3) in 8 M guanidine. In each case, $NaBH_4$ (0.2 mL of a 0.1 M solution) was added to the enzyme solution (2 mL) and the reaction mixture was kept at room

[2] Abbreviations used are: NADH, reduced nicotinamide adenine dinucleotide; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride; TLC, thin-layer chromatography; NMR, nuclear magnetic resonance.

temperature for 3 h. At this time, the reaction was stopped by the addition of acetone. The reaction mixture was then dialyzed against 0.001 M HCl and placed in a hydrolysis vial, and the HCl concentration of the solution was brought to 6 N with the addition of concentrated HCl. The vial was sealed under vacuum and placed at 110 °C for 6 h. After hydrolysis, the sample was concentrated by rotary evaporation and the residue was taken up in water, applied to a Dowex 1-X2 Cl^- column (0.5 × 60 cm), and eluted by the method of Schroeder (1972).

Sodium cyanoborohydride reduction was performed in 8 M guanidine hydrochloride as described above. The pH of the adduct solution was adjusted to pH 5 prior to the addition of the sodium cyanoborohydride. Reduction by sodium cyanoborohydride was also carried out on lyophilized protein samples which were suspended in methanol prior to addition of the reducing agent.

H_2/Pt reduction of the [^{14}C]propargylglycine-γ-cystathionase adduct was performed in aqueous 1% sodium dodecyl sulfate.

Proteolytic Digestion of the [^{14}C]Propargylglycine-γ-Cystathionase Adduct. The [^{14}C]propargylglycine-γ-cystathionase adduct isolated from Sephadex G-25 chromatography was denatured in a 100 °C water bath until obvious coagulation of the protein had occurred. A solution of Pronase in 0.2 M piperazine buffer, pH 7.8, was added to the denatured protein solution. The final Pronase concentration was 1 mg/mL. The solution was made 0.1 M in Ca^{2+} and placed in a 37 °C water bath. After 3 h, the pH of the digestion was checked and readjusted, if necessary, to pH 8. Incubation at 37 °C was continued for another 4 h. At this time, the solution was lyophilized and the residue was resuspended in 0.5 mM picoline-acetate buffer, pH 6.5. The digested material was applied to a Sephadex G-10 column (0.8 × 111 cm) and eluted with the same buffer. Two peaks of radioactive material were found and were separately pooled. Each fraction was further purified on a Dowex 50-X8 H^+ column (0.3 × 30 cm), eluted with 0.1–4 N HCl (30 mL each of 0.1, 0.5, 1.0, 2.0, and 4.0 N HCl).

Tryptic digestion of the [^{14}C]propargylglycine-γ-cystathionase adduct was performed in an analogous manner.

Peptic digestion of the [^{14}C]propargylglycine-γ-cystathionase adduct was performed by the method of Smyth (1967), and the digestion products were purified as described above.

Hydrolysis of Ethyl Vinyl Sulfide. Kinetic measurements of the acid hydrolysis of ethyl vinyl sulfide were carried out in 1-cm quartz cells using a Cary 15 spectrophotometer. Ethyl vinyl sulfide absorbs between 200 and 250 nm (E_{230} = 3760 at pH 7; E_{230} = 3000 at pH 2). The reaction was followed by repeatedly scanning the region from 195 to 300 nm and recording the optical density values at 230 nm. A 10 mM solution of ethyl vinyl sulfide in ethanol (0.025 mL) was added to a cuvette containing 0.975 mL of H_2O. The reaction was initiated by adding to the reaction cell a small quantity of perchloric acid, sufficient to adjust the sample to the desired pH. Initial readings were made 1 min after the reaction was started with subsequent readings (10 to 20 per experiment) taken at convenient intervals. Infinity points were taken after 7 to 10 half-lives. pH measurements were made at the beginning and end of a kinetic experiment. Rate constants were estimated from plots of log ($abs_t - abs_\infty$) against time.

Results

Inactivation of γ-Cystathionase by Propargylglycine. Incubation of γ-cystathionase with D,L-propargylglycine at 25 °C causes a time-dependent irreversible inactivation of the enzyme (Figure 1); when D,L-propargylglycine is present at a concentration of 6 × 10^{-4} M, the half-time for inactivation is 30 s. The presence of substrate (below saturation) in the inactivation mixture slows, but does not prevent the eventual inactivation of the enzyme. The inactivation is irreversible, since no activity can be recovered upon dialysis of inactivated enzyme for 48 h against 0.2 M potassium phosphate buffer, pH 7.2. The rate of inactivation is saturable with a K_{inact} of 0.7 mM. Similar measurements made with D,L-[α-^{2}H]propargylglycine show that the inactivation rate displays a deuterium isotope effect of 2.2 ± 0.1. This value is similar to the deuterium isotope effect (V_H/V_D = 2) observed in the normal enzymatic reaction with homoserine. The similarity of the isotope effects for these two reactions lends support to the idea that the initial steps in normal catalysis and in inactivation are analogous.

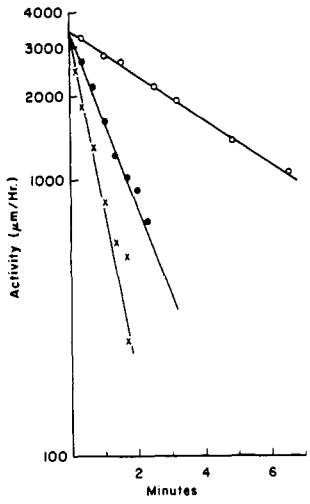


FIGURE 1: Rate of inactivation of γ-cystathionase by D,L-propargylglycine. γ-Cystathionase (0.5 mg) was incubated with D,L-propargylglycine (6 × 10^{-4} M) in phosphate buffer, pH 7.5, 0.2 M. Aliquots were withdrawn at time intervals and diluted into 1.4 mL of the standard assay mixture. The reaction was followed by the disappearance of absorbance at 340 nm (x). Two reaction mixtures also contained L-homoserine: (●) 2.4 × 10^{-4} M; (○) 9.6 × 10^{-4} M.

Incubation of γ-cystathionase with D,L-[2-^{14}C]propargylglycine results in the incorporation of 1 mol of ^{14}C-labeled inactivator per 80 000 daltons (experimental values ranged from 0.89 to 1.1). Enzyme which has been labeled with D,L-[2-^{14}C]propargylglycine and passed through Sephadex G-25 can be dialyzed against 0.001 M HCl or 6 M urea. Neither procedure releases radioactivity from the protein, suggesting that a covalent linkage exists between the enzyme and propargylglycine.

To determine whether the labeling of γ-cystathionase by propargylglycine requires active enzyme, γ-cystathionase was inactivated with sodium borohydride (Churchich and Bieler, 1971) prior to incubation with D,L-[2-^{14}C]propargylglycine. Gel filtration of the incubation mixture demonstrated that no radioactivity was associated with the protein. These results indicate that inactivation and labeling of γ-cystathionase by propargylglycine requires active enzyme and probably involves the pyridoxal 5′-phosphate cofactor.

Inactivation of γ-cystathionase by propargylglycine is pictured as initially proceeding through reactions analogous to those of the normal enzymatic reaction; therefore, labilization of the α proton of propargylglycine should occur during inactivation. γ-Cystathionase was inactivated with a mixture of

TABLE I: Inactivation of γ-Cystathionase by a Mixture of D,L-[2-^{14}C]Propargylglycine and D,L-[2-^{3}H]Propargylglycine.[a]

	$^{3}H/^{14}C$ (cpm)	μmol of radioactivity[b] ^{3}H	^{14}C
Initial inactivator	2.37		
Protein isolated from Sephadex G-25 column	0.13	0.003	0.157[c]
Unreacted inactivator isolated from Dowex 50	2.5		
^{3}H found in H_2O isolated from bulb-to-bulb distillation	0.088		

[a] γ-Cystathionase (13 mg) was inactivated with a mixture of D,L-[2-^{14}C]propargylglycine (specific activity 0.54×10^5 cpm/μmol). Separation of inactivated enzyme from excess inactivator was performed on a Sephadex G-25 column, with distilled water as the eluent. The small molecule fraction was bulb-to-bulb distilled, and the amount of ^{3}H present as $^{3}H_2O$ was quantitated. The unreacted propargylglycine was purified on a Dowex 50-X8 column and the $^{3}H/^{14}C$ ratio of this material determined. [b] Based on original specific activity of the inactivator. [c] This represents incorporation of 1 μmol of [^{14}C]-propargylglycine per μmol of γ-cystathionase (1 μmol = 80 mg).

TABLE II: Specific Activity of 2-Amino-4-ketopentanoic Acid during Purification Procedures.[a]

Step	Total counts (cpm)	μmol of 2-amino-4-ketopentanoate	Sp act. (cpm/μmol)
Initial	57 000	90	620
Following 6 N HCl hydrolysis	59 400	84	660
Following Dowex 1 column	39 600	62.4	640
Following Dowex 50 column	29 900	46.8	638
	52% recovery	51% recovery	

[a] γ-Cystathionase (24 mg) was inactivated with a fivefold molar excess of D,L-[2-^{14}C]propargylglycine (specific activity 3×10^5 cpm/μmol) and the inactivated enzyme (96% activity lost) was isolated on a Sephadex G-25 column. The protein peak was pooled, its content of radioactivity was determined, and a known amount of synthetic D,L-2-amino-4-ketopentanoic acid added to the solution. The sample was then hydrolyzed in 6 N HCl and purified on columns of Dowex 50 and Dowex 1. Radioactivity was determined at each step and the number of micromoles of 2-amino-4-ketopentanoate was quantitated.

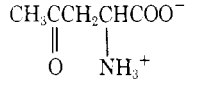
D,L-[2-^{14}C]-propargylglycine and D,L-[α-^{3}H]propargylglycine. After inactivation was complete, the amount of radioactivity associated with protein, solvent, and unreacted propargylglycine was determined. The results of this experiment are summarized in Table I.

The formation of $^{3}H_2O$ after inactivation has occurred indicates that the α hydrogen of D,L-[α-^{3}H]propargylglycine is labilized during the inactivation, as required by the proposed mechanism. The amount of ^{3}H released (0.088 μmol), however, represents only a little over half the number of micromoles of ^{14}C (0.157 μmol) incorporated into the protein (propargylglycine reacts stoichiometrically with the enzyme: i.e., no turnover occurs). No substantial intramolecular transfer of ^{3}H from the α position to another position on the inactivator–enzyme adduct has occurred, since only 0.003 μmol of ^{3}H is found associated with the protein fraction. This value is 20 times less than would be necessary to explain the reduced amount of ^{3}H released into the solvent. These data indicate, therefore, that a discrimination against propargylglycine molecules containing ^{3}H in the α position occurs prior to inactivation. Such discrimination is consistent with the deuterium isotope effect seen for the inactivation. Also consistent with discrimination against [α-^{3}H]propargylglycine is the small increase observed in the $^{3}H/^{14}C$ ratio of the reisolated D,L-propargylglycine.

Acid Hydrolysis of the [^{14}C]Propargylglycine–γ-Cystathionase Adduct. Acid hydrolysis of γ-cystathionase which has been labeled with [^{14}C]propargylglycine was performed to investigate the structure of the inactive adduct. γ-Cystathionase was inactivated with D,L-[2-^{14}C]propargylglycine and the radioactively labeled protein was separated from excess inactivator on a Sephadex G-25 column. The labeled protein was subjected to 1 N HCl hydrolysis (under vacuum) at 110 °C for 4–6 h and the hydrolysate was purified on Sephadex G-10. One peak of radioactive material was obtained from this column. Further purification of the radioactive hydrolysis product was effected on columns of Dowex 50-X8 H^+ (Schroeder, 1972) and Dowex 1-X2 Cl^- (1 N HCl eluent). One product, representing about 45% of the original radioactivity, was isolated and shown to be homogeneous by chromatography in solvents A and B and high-voltage electrophoresis at pH 1.9. Hydrolysis of the adduct in 6 N HCl gave identical results.

A control experiment was performed to ensure that the product obtained upon 1 or 6 N HCl hydrolysis of the [^{14}C]propargylglycine–γ-cystathionase adduct did not merely represent a hydrolytic breakdown product of propargylglycine itself. While 6 N HCl hydrolysis converts approximately half of the [^{14}C]propargylglycine to a compound with chromatographic behavior identical to that of the isolated hydrolysis product, 1 N HCl hydrolysis leaves propargylglycine unchanged. It can be concluded, therefore, that the radioactive product obtained from acid hydrolysis of the ^{14}C-labeled inactivated enzyme does not represent an artifact produced by breakdown of propargylglycine, but is derived from an enzyme–inactivator adduct in which propargylglycine has undergone chemical modification.

The hydrolysis product was subsequently identified as 2-amino-4-ketopentanoic acid (II). Identification of this compound was achieved through the following experiments. (1) The electrophoretic and chromatographic behavior of the radioactive hydrolysis product indicated that it was an amino acid. (2) A sample of the radioactive hydrolysis product was exposed to sodium borohydride under conditions which reduce pyruvate to lactate. A clear change in the mobility of the sample in electrophoresis at pH 8.9 was observed after exposure to $NaBH_4$. In addition, the ninhydrin-positive spot associated with the radioactivity is purple, whereas prior to reduction a yellow color was observed. These results suggested the presence of a ketone or aldehyde function which was reduced to the corresponding alcohol upon exposure to sodium borohydride. (3) Identification of the borohydride-sensitive group as a ketone located on the C-4 carbon of a five-carbon chain, i.e., a methyl ketone, came from reaction of the radioactive hydrolysis product with iodine in a haloform reaction. This reaction converts a methyl ketone to the corresponding acid. Treatment of the radioactive hydrolysis product with the



(II)

TABLE III: Stereochemistry of the α Hydrogen of 2-Amino-4-keto[2-^{14}C]pentanoic Acid Isolated from Hydrolysis of the [^{14}C]Propargylglycine-γ-Cystathionase Adduct.[a]

Enzyme used	Length of Incubation	Radioact. in Acetopyruvate (cpm)	Radioact. in 2-amino-4-ketopentanoate (cpm)
L-amino acid oxidase	0 min	25	7100
L-amino acid oxidase	7 h	3579	3309
D-amino acid oxidase	0 min	24	7000
D-amino acid oxidase	4 h	53	7120

[a] 2-Amino-4-keto[2-^{14}C]pentanoic acid isolated from hydrolysis of the ^{14}C-labeled inactivator-enzyme adduct, was mixed with synthetic D,L-2-amino-4-ketopentanoate to give material with a final specific activity of 533 cpm/μmol. The reaction with L-amino acid oxidase contained Tris-HCl buffer, pH 7.8 (0.1 M), KCl (16 mM), catalase (2×10^5 Sigma units), 0.3 unit of L-amino acid oxidase and 13 μmol of 2-amino-4-ketopentanoate in a total volume of 1 mL. The reaction with D-amino acid oxidase contained sodium pyrophosphate buffer, pH 8.3 (0.01 M), catalase (2×10^5 Sigma units), 4.4 units of D-amino acid oxidase, and 13 μmol of 2-amino-4-ketopentanoate in a total volume of 1 mL. At the end of the incubation period, each reaction was acidified and placed on a column of Dowex 50-X8 H^+ (0.5 × 5 cm). The column was washed with water to elute any keto acid product. Acetopyruvate (product) formation was indicated by the characteristic UV spectrum. The column is then eluted with 2 N NH_4OH, which elutes any remaining 2-amino-4-ketopentanoate. The radioactivity in each eluate was determined. Acetopyruvate and 2-amino-4-ketopentanoate were identified by paper electrophoresis at pH 3.5.

haloform reagent (see Methods) results in conversion of the hydrolysis product to a compound which cochromatographed with authentic aspartic acid in solvents A and B and high-voltage electrophoresis at pH 1.9 and 8.9. (4) Finally, the properties of the hydrolysis product were compared directly with those of synthetic II. The radioactive product isolated from acid hydrolysis of ^{14}C-labeled inactivated enzyme cochromatographed with synthetic 2-amino-4-ketopentanoic acid in systems A, B, and C and in high-voltage electrophoresis at pH 1.9 and 8.9. Synthetic 2-amino-4-ketopentanoic acid behaved identically to the hydrolysis product when reduced with $NaBH_4$ or oxidized in the haloform reaction.

To determine whether the 50% or greater loss of radioactivity which accompanied purification of the hydrolysis product was due to technical losses, or, in fact, represented the destruction of some other major product, the hydrolysis reaction was repeated in the presence of synthetic D,L-2-amino-4-ketopentanoic acid and the hydrolysis products were purified as previously described. At each step in the purification, the specific activity of the radioactive material was determined. The results, shown in Table II, demonstrate that 2-amino-4-ketopentanoic acid is the major product of acid hydrolysis of the [^{14}C]propargylglycine-γ-cystathionase adduct.

Stereochemistry of the α Hydrogen of 2-Amino-4-keto-[2-^{14}C]pentanoic Acid. It has been shown that the α hydrogen of D,L-[α-^{3}H]propargylglycine is labilized during the initial steps of inactivation. The isolation of 2-amino-4-keto[2-^{14}C]pentanoic acid from acid hydrolysis of ^{14}C-labeled inactivated enzyme therefore requires that the α proton be replaced sometime subsequent to its initial labilization. Reprotonation of the α position is probably stereospecific if it occurs while the enzyme is still in its native state. If it occurs after denaturation of the protein, protonation is most likely nonstereospecific. Determination of the stereochemistry of the α hydrogen of the 2-amino-4-keto[2-^{14}C]pentanoate isolated from hydrolysis of ^{14}C-labeled inactivated enzyme was therefore undertaken. 2-Amino-4-keto[2-^{14}C]pentanoic acid, isolated from hydrolysis of ^{14}C-labeled inactivated enzyme, was combined with carrier D,L-2-amino-4-ketopentanoic acid. This mixture was then incubated with either L-amino acid oxidase or D-amino acid oxidase and the reaction products were isolated. (Previous experiments had demonstrated that synthetic D,L-2-amino-4-ketopentanoate was an adequate substrate for both enzymes.) Control experiments were performed in which the reaction was terminated immediately after the addition of enzyme, to ensure that keto acid production did not occur nonenzymatically. The results of this experiment are shown in Table III and demonstrate that the L isomer of 2-amino-4-keto[2-^{14}C]pentanoic acid is isolated as a hydrolysis product, since radioactive acetopyruvate is produced only in the reaction of the 2-amino-4-keto[2-^{14}C]pentanoic acid mixture with L-amino acid oxidase. Therefore, reprotonation of the α position is stereospecific.

pH Stability and Rate of Hydrolysis of the Linkage Present in the [^{14}C]Propargylglycine-γ-Cystathionase Adduct. The stability of the enzyme-inactivator adduct was investigated to further characterize the covalent linkage present in the adduct. Since preliminary experiments had indicated that extremes in pH caused precipitation of the protein adduct from solution, all studies were performed in 1% sodium dodecyl sulfate, which maintained protein solubility. ^{14}C-labeled inactivated enzyme in 0.01 M sodium phosphate, pH 7.0, was made 1% in sodium dodecyl sulfate and heated at 65 °C for 10 min. Subsequent passage through a Sephadex column equilibrated with 1% sodium dodecyl sulfate indicated that 93% of the radioactivity remained associated with the protein during this treatment. The pyridoxal cofactor, however, was released from the enzyme during this process. The fact that pyridoxal 5′-phosphate can be released from the protein without concomitant loss of radioactivity indicates that the linkage between the inactivator and pyridoxal (presumably an imine linkage) is more labile than the other point of contact between propargylglycine and γ-cystathionase. The pH stability of the enzyme-inactivator linkage was examined and the results, summarized in Figure 2, show the presence of a linkage whose hydrolysis is acid, but not base, catalyzed. The radioactive molecule released from the protein was isolated and purified on a Dowex 50-X8 H^+ column, in the presence of synthetic 2-amino-4-ketopentanoic acid. One peak of radioactivity was found which cochromatographed with the synthetic carrier.

The acid-catalyzed rate of hydrolysis of the enzyme-inactivator adduct was determined in order to help identify the nature of the covalent bond formed between propargylglycine and γ-cystathionase. ^{14}C-labeled inactivated enzyme was first denatured with 1% sodium dodecyl sulfate at 65 °C and cooled to 25 °C, and any radioactivity which had been released by this procedure was removed by passage through Sephadex G-25. A sample of the denatured inactivated protein so isolated was then adjusted to the desired pH by the addition of perchloric acid and aliquots were withdrawn at time intervals. These al-

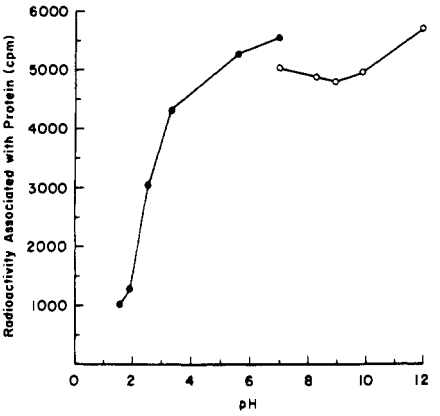
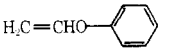
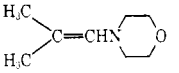
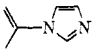


FIGURE 2: pH stability of the [^{14}C]propargylglycine-γ-cystathionase adduct. Samples of the ^{14}C-labeled inactivator-enzyme adduct in 1% sodium dodecyl sulfate were adjusted to a particular pH with the addition of perchloric acid (●) or NaOH (○) and heated at 65 °C for 10 min. The amount of radioactivity associated with the protein was then determined by passage of the sample through Sephadex G-25. Protein-bound radioactivity is plotted as a function of pH.

TABLE IV: Acid-Catalyzed Rate of Hydrolysis for Several Compounds Containing a Vinyl Linkage.

Compound	k_{H^+} (s^{-1} M^{-1}) 25 °C
$H_2C{=}CHOCH_2CH_3$ Ethyl vinyl ether	2.3 ± 0.01[a]
$H_2C{=}CHSCH_2CH_3$ Ethyl vinyl sulfide	4×10^{-3} [b]
 Phenyl vinyl ether	2.9×10^{-3} [c]
 1-N-Morpholino-1-isobutene	3.1×10^{2} [d]
 *N*-Vinylimidazole	Stable to refluxing in 5 M HCl for 21 h[e]

[a] Kresge and Chiang (1967). [b] Experimentally determined (see Methods). [c] Estimated from the value for ethyl vinyl ether, based on data which indicate a rate 750 × slower for the phenyl compound relative to the ethyl compound (Matsumura et al., 1968; Okuyama et al., 1967). [d] Maas et al. (1967). [e] Hupe et al. (1972).

iquots were passed through a Sephadex G-25 column and the amount of radioactivity associated with the protein was determined. The rates of hydrolysis at several phs were determined from first-order plots and these values were replotted against the hydrogen ion concentration. From such a plot, one can obtain a second-order rate constant, $k_{H^+} = 2.2 \times 10^{-3}$ s^{-1} M^{-1} for the acid-catalyzed hydrolysis of the [^{14}C]propargylglycine-γ-cystathionase adduct.

Table IV contains the rates of acid-catalyzed hydrolysis for several vinyl compounds. A comparison of the second-order rate constants of these compounds with the constant determined for hydrolysis of the [^{14}C]propargylglycine-γ-cystathionase adduct indicates that reaction of either a sufhydryl group (cysteine) or a phenolic oxygen (tyrosine) with the proposed allene intermediate (structure I, Scheme I), would produce an adduct whose rate of hydrolysis is comparable to the one observed for the enzyme-inactivator adduct.

Attempted Isolation of a Labeled Peptide from [^{14}C]Propargylglycine-Inactivated γ-Cystathionase. The studies on the pH stability of the linkage present in the [^{14}C]propargylglycine-γ-cystathionase adduct indicated that proteolytic digestion under neutral conditions might enable us to isolate a labeled peptide from the inactivated enzyme. Pronase and trypsin were employed to digest the ^{14}C-labeled inactivator enzyme adduct under nonacidic conditions. The major digestive fragment isolated in both experiments was a compound which was stable to acid hydrolysis and thus no longer contains the original enzyme-inactivator linkage. The compound is distinct from 2-amino-4-ketopentanoic acid, but has not been further characterized. It is thought to represent secondary reaction of the original adduct with nucleophiles or pyridoxal released during digestion.

Since digestion with Pronase and trypsin was unsuccessful, possibly due to the chemical reactivity of the protein-inactivator adduct, we thought that it would be useful to reduce the lability of the adduct by chemical modification. We attempted to reduce the acid-labile vinyl linkage which exists between propargylglycine and γ-cystathionase with sodium borohydride, sodium cyanoborohydride, and H_2/Pt. ^{14}C-labeled inactivated enzyme was exposed to the chosen reagent, and the treated sample was hydrolyzed in 6 N HCl. In all cases, 2-amino-4-ketopentanoic acid was the major hydrolysis product, indicating that no reduction of the vinyl linkage to an acid stable form had occurred.

Discussion

γ-Cystathionase is irreversibly inactivated by the acetylenic substrate analogue propargylglycine (2-amino-4-pentynoic acid). The inactivation is accompanied by covalent attachment of the inactivator to the enzyme, with a stoichiometry of 1 mol of ^{14}C-labeled inactivator per 80 000 daltons. No covalent labeling by [^{14}C]propargylglycine is observed if the enzyme is first inactivated by exposure to sodium borohydride. This reagent reduces the essential pyridoxal cofactor and renders the enzyme inactive toward both substrate and inactivator. The kinetics of the inactivation shows the following properties. (1) It is pseudo-first-order for 3–4 half-lives. (2) At high inactivator concentration, the rate of inactivation becomes zero-order with respect to inactivator (K_{inact} = 0.7 mM). (3) The rate of inactivation is reduced in the presence of the substrate L-homoserine. These results suggest that the inactivation occurs at the active site and involves enzymatic catalysis. An alternative inactivation mechanism might involve enzyme-catalyzed conversion of propargylglycine to a reactive product which is released into solution and then inactivates the enzyme. This inactivation mechanism is unlikely in view of the first-order kinetics of the inactivation process. Inactivation by an enzyme-generated compound, i.e., a molecule from solution, would be expected to show a lag period followed by a continuously increasing inactivation rate.

A structure for the enzyme-inactivator adduct is shown in Scheme I, structure II. Salient features are (1) the presence of a vinyl linkage in the γ-δ position and (2) a Schiff base between the amino group of the inactivator and the pyridoxal cofactor. Isolation of L-2-amino-4-ketopentanoic acid as sole product from acid hydrolysis of the inactivated enzyme provides strong support for the proposed structure. The identity of the amino acid residue, X, is still under investigation. Hydrolysis data (Table IV) suggest that a cysteine or tyrosine residue is a reasonable possibility.

A mechanism for the formation of the covalent enzyme in-

activator adduct is shown in Scheme I. Enzymatic generation of a carbanion at the β position, adjacent to the acetylenic linkage, allows rearrangement of the acetylenic function to a reactive allene. The allene then undergoes nucleophilic attack by an active-site residue, resulting in the observed inactivation and covalent attachment. Although no direct evidence exists for allene formation, we believe this is the most reasonable intermediate in the formation of the enzyme inhibitor adduct. Further support for the involvement of an allene is the inability of 2-amino-4-pentenoic acid to inactivate γ-cystathionase (Abeles and Walsh, 1973). The olefinic linkage present in this molecule is not capable of rearrangement to an allene. Also consistent with the involvement of an allene intermediate in the inactivation is the observed inactivation of γ-cystathionase by 2-amino-4-chloro-4-pentenoic acid (Abeles and Brodsky, unpublished results). The same reactive allene intermediate can be generated by elimination of HCl from this compound as can be produced from propargylglycine through acetylenic rearrangement.

The inactivation mechanism we have proposed requires at least two bases at the active site. Two bases are required, since it is very likely that protonation of the α position of the inactivator occurs after formation of the covalent bond with the enzyme. Therefore, the base which protonated the α position cannot be the same base which adds to the allene. Involvement of two bases has been proposed for the γ-elimination reaction catalyzed by the pyridoxal enzyme, cystathionine-γ-synthase (Posner and Flavin, 1972).

The proposed mechanism of inactivation involves several intermediate steps which are very similar to those involved in the normal catalytic process, and, therefore, presumably involves the same functional groups. The elimination reaction which occurs as part of the catalytic process probably involves the following reaction sequence (Davis and Metzler, 1972): (1) Schiff-base formation between substrate and pyridoxal phosphate; (2) abstraction of the α-hydrogen and tautomerization of the Schiff base; (3) abstraction of the β proton; (4) elimination of the leaving group. All of these reactions, with the exception of reaction 4, occur in the proposed inactivation process (Scheme I). The similarity of the inactivation reaction and the catalytic process is further illustrated by the identity of the isotope effect observed when 2-^{2}H-labeled substrate or inhibitor is used. In the catalytic process, abstraction of the β proton is followed by elimination of the γ substituent. It is very likely that an acidic group at the active site facilitates the elimination by protonating the leaving group. We suggest that the same acidic group facilitates allene formation in the inactivation process by protonating the δ carbon of the inactivator. It is likely that, after deprotonation, this group adds to the allene to form the covalent bond between enzyme and inactivator.

γ-Cystathionase has a molecular weight of 160 000 and consists of four subunits (presumed identical) with a molecular weight of 40 000 each (Deme et al., 1972). The enzyme contains four pyridoxal 5′-phosphate molecules per 160 000 daltons (Churchich et al., 1975). The stoichiometry of inactivation indicates that only two of the four pyridoxal 5′-phosphate active sites are available for interaction with propargylglycine, indicating an essential nonequivalence of the sites or an alteration of their initial equivalence upon reaction, i.e., alternate-site reactivity. Differential reactivity of the pyridoxal 5′-phosphate residues of γ-cystathionase toward reagents such as cycloserine and sodium borohydride has been reported (Churchich and Bieler, 1971). In addition, recent studies (Churchich et al., 1975) indicate a differential binding of pyridoxal to γ-cystathionase ($K_m = 7.5 \times 10^5$ M^{-1}; 8.3×10^4 M^{-1}) and the presence of different environments for the two pairs of cofactors.

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