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Mechanism of Inactivation of γ-Cystathionase by β,β,β-Trifluoroalanine†

Richard B. Silverman‡ and Robert H. Abeles*

ABSTRACT: β,β,β-Trifluoroalanine irreversibly inactivates γ-cystathionase. The inactivator becomes covalently attached to the enzyme. Two moles of inactivator is incorporated per tetramer (Silverman, R. B., and Abeles, R. H. (1976), *Biochemistry 15,* 4718). The rate of inactivation, the rate of [^{14}C]trifluoroalanine incorporation into the protein, and the rate of release of F^- from trifluoroalanine are equal. F^-, 2.7 mol, is released per mol of inactivator which becomes covalently attached to the protein. In the course of the inactivation, the β carbon of trifluoroalanine is converted to an acyl group which is bonded to an amino group of the protein. (See Scheme I.) It is proposed that trifluoroalanine labels the functional group, probably a lysine ε-amino group, which protonates or deprotonates the α and β positions of the substrate in the normal catalytic process. Propargylglycine inactivates and covalently labels the native enzyme (Washtien, W., and Abeles, R. H. (1977), *Biochemistry 16* (in press)). Enzyme inactivated with trifluoroalanine does not react with propargylglycine. However, enzyme inactivated with 2 mol of propargylglycine per tetramer reacts with trifluoroalanine and 2 mol of trifluoroalanine become covalently bonded per mol of enzyme (tetramer). It is proposed that propargylglycine does not affect the functional group required for reaction with trifluoroalanine. Trifluoroalanine labels a group required for the activation of propargylglycine and thus prevents reaction of the enzyme with propargylglycine.

Recently, we reported our studies on the irreversible inactivation of several pyridoxal phosphate dependent enzymes, including γ-cystathionase, by polyhaloalanines (Silverman and Abeles, 1976). Some aspects of the mechanism of inactivation of γ-cystathionase were also described. When γ-cystathionase was inactivated with [1-^{14}C]trifluoroalanine and then subjected to gel filtration, ^{14}C was found with the protein fraction. Dialysis or gel filtration of the ^{14}C-labeled protein did not result in loss of ^{14}C. Two moles of inactivator was incorporated per mol of protein, which consists of 4 subunits and contains 4 mol of pyridoxal phosphate. Subsequent acid denaturation of this labeled protein released all of the radioactivity in the form of $^{14}CO_2$. Scheme I shows the mechanism which we proposed for this inactivation (Silverman and Abeles, 1976). In this paper, we report a detailed investigation of the mechanism of the inactivation of γ-cystathionase by trifluoroalanine.

Materials and Methods

Reagents. γ-Cystathionase was purified from rat liver by the procedure of Greenberg (1971). Enzyme activity is expressed in the same units as previously used (Greenberg, 1971), i.e., μmol of α-ketobutyrate produced in 60 min under standard assay conditions. Enzyme with specific activity of 350 showed a single band on acrylamide electrophoresis. β,β,β-Trifluoro-D,L-alanine and [1-^{14}C]trifluoro-D,L-alanine (specific activity 7.8×10^5 dpm/μmol) were synthesized by the procedure of Weygand et al. (1967). Potassium [^{14}C]cyanide was used to prepare the intermediate 2,2,2-trifluoro-1-[^{14}C]cyano-*N*-benzoylethylamine. Propargylglycine and [1-^{14}C]propargylglycine were generous gifts of Dr. W. Washtien. β-(*N*-Acetyl)aminoethyl thioacetate was kindly donated by Ms. S. Moore and glycine phenyl ester hydrobromide, by Mr. R. Suva. Pyridoxal 5′-phosphate (PLP)[1] and iodoacetic acid were purchased from Sigma Chemical Co. Urea (recrystallized from

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[1] Abbreviations used: PLP, pyridoxal 5′-phosphate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

95% ethanol), ferric chloride, sodium fluoride, and sodium borohydride were bought from Fisher Scientific Co. Guanidine hydrochloride (recrystallized from methanol), α-picoline (redistilled), and sodium dodecyl sufate (recrystallized from 95% ethanol) were obtained from Aldrich Chemical Co. ^3H_2O and [2-^{14}C]glycine were bought from New England Nuclear; glycine ethyl ester hydrochloride, ethyl cyanoacetate, and hydroxylamine hydrochloride were from Eastman Chemical Co.; benzamide (recrystallized from water) was from Matheson Coleman and Bell; glycylglycine was from Mann Research Laboratories; and trypsin-TPCK was from Worthington Biochemical Corp. Buffer A contains 0.2 M potassium phosphate, pH 7.2, 2×10^{-2} M ethylenediaminetetraacetic acid (EDTA), 7×10^{-2} M β-mercaptoethanol, and 5×10^{-4} M pyridoxal 5′-phosphate (PLP). 2 M hydroxylamine reagent was prepared by mixing equal volumes of 4 M hydroxylamine hydrochloride with 3.5 M sodium hydroxide. Ferric chloride reagent was prepared by mixing equal volumes of 5% ferric chloride in 0.1 N HCl with 3 N HCl.

Labeling of Propargylglycine Inactivated γ-Cystathionase by [1-^{14}C]Trifluoro-D,L-alanine. To 1.0 mL of a solution of γ-cystathionase (6 mg/mL; specific activity 300 units/mg) in buffer A was added a solution of propargylglycine in 0.2 M potassium phosphate, pH 7.2, to give a final concentration of 1 mM. After incubation at 25 °C for 30 min, the yellow protein solution was dialyzed overnight at 4 °C against two changes at 3-h intervals of 0.2 M potassium phosphate, pH 7.2. To the inactivated enzyme solution were added EDTA, β-mercaptoethanol, and PLP to give the concentrations found in buffer A. Then a solution of [1-^{14}C]trifluoro-D,L-alanine in buffer was added to give a final concentration of 4 mM. After incubation at 25 °C for 45 min, the bright pink solution was applied to a column of Sephadex G-25 (medium, 1.8 × 21 cm) equilibrated with 10 mM potassium phosphate, pH 7.2, at room temperature and 1-mL fractions were collected. The tubes containing radioactivity-labeled protein were pooled.

Sodium Borohydride Inactivation of γ-Cystathionase Followed by Treatment with [1-^{14}C]Trifluoroalanine. To 1.0 mL of γ-cystathionase (6 mg/mL; specific activity 250 units/mg) in 0.2 M potassium phosphate, pH 7.2, at 4 °C was added solid sodium borohydride (1 mg). The foaming, nearly colorless protein solution was allowed to stand in the cold for 30 min, and then it was dialyzed overnight against two changes at 3-h intervals of 0.2 M potassium phosphate–EDTA, pH 7.2. β-Mercaptoethanol and PLP were added to give final concentrations as in buffer A. [1-^{14}C]Trifluoroalanine was then added to this solution to bring the final concentration of the inactivator to 4 mM. After standing at room temperature for 45 min, the nearly colorless protein solution was applied to a column of Sephadex G-25 (medium, 1.8 × 21 cm) equilibrated with 10 mM potassium phosphate, pH 7.2. A parallel control experiment was done in which sodium borohydride was omitted.

Release of Fluoride Ion from Trifluoroalanine Catalyzed by γ-Cystathionase. Fluoride was determined with a fluoride electrode (Model 96-09 Orion Research Inc.) and was used in conjunction with an Orion Model 801A digital ionalyzer.

Method A: Continuous Assay. A solution containing 1 M potassium phosphate, pH 7.2, 1 M potassium chloride, 10^{-2} M EDTA, and 10^{-6} M NaF in 1.0 mL was added to 1.0 mL of γ-cystathionase in buffer A (specific activity 300, 9 mg/mL) and, when the fluoride electrode gave a steady potential reading, a solution of trifluoroalanine was added to make the final concentration 2 mM. The decrease in potential was recorded as a function of time and compared with a calibration curve using known concentrations of fluoride ions.

Method B: Aliquot Assay. To γ-cystathionase (3 mL, specific activity 265, 6 mg/mL) in buffer A was added a solution of trifluoroalanine to give a final concentration of 2 mM. At various times, aliquots (0.5 mL) were removed and added to 0.5 mL of 1.0 M sodium acetate buffer containing 1 M NaCl and 10^{-2} M EDTA, pH 5.0. Potential readings were taken 2 min after immersion of the fluoride electrode and compared with a calibration curve using known concentrations.

Labeling of γ-Cystathionase and Denaturation in the Presence of ^3H_2O. To 4 mL of γ-cystathionase (specific activity 200 units/mg, 8 mg/mL) in buffer A was added a solution of unlabeled trifluoroalanine in 0.2 M potassium phosphate, pH 7.2, to give a final concentration of 4 mM. The enzyme solution was kept at 25 °C for 90 min and then dialyzed against two changes of 2 L of cold 10 mM potassium phosphate, pH 7.2. After lyophilization, the residue was taken up in 400 μL of ^3H_2O)1.0 Ci/g) and then guanidine hydrochloride (230 mg) was added to give a final concentration of 6 M. This solution was allowed to stand at room temperature overnight, then the ^3H_2O was removed by bulb-to-bulb distillation, and the residue taken up in 400 μL of water, and applied to a column of Sephadex G-25 (1.2 × 30 cm) equilibrated with 6 M urea in 50 mM α-picoline acetate, pH 7.0. The protein fractions were combined ("tritiated γ-cystathionase").

Reaction of Hydroxylamine with Tritiated γ-Cystathionase. To 200 μL of a solution of tritiated γ-cystathionase (13 000 cpm) was added 100 μL of 2 M hydroxylamine reagent. After incubating at 24 °C for 30 min, the solution was applied to Sephadex G-25 (1.2 × 30 cm) equilibrated with 50 mM α-picoline–acetate, pH 7.0. A control experiment under identical conditions but with hydroxylamine omitted was carried out.

Reaction of Base with Tritiated γ-Cystathionase. To 150 μL of a solution of tritiated γ-cystathionase in 50 mM α-picoline–acetate, pH 7.0, containing 6 M urea was added 100 μL of 0.1 M sodium phosphate buffer, pH 11.8, containing 6 M urea. The pH was raised to ca. 12 (pH paper) by the addition of 5 μL of 1 M NaOH and allowed to incubate at 23 °C for 80 min. This solution was applied to Sephadex G-25 (1.2 × 30 cm) equilibrated with 25 mM α-picoline–acetate, pH 7.0, containing 1% sodium dodecyl sulfate. The amount of radioactivity in small molecules was compared with a control which was not subjected to an elevated pH.

Nonenzymatic Reactions of Hydroxylamine with Carbonyl Compounds. To a solution of β-(*N*-acetyl)aminoethyl thioacetate (8 mM), glycine phenyl ester hydrobromide (50 mM), glycine ethyl ester hydrochloride (8 mM), or ethyl cyanoacetate (100 mM) in water at 23 °C was added an equal volume of 1 M hydroxylamine reagent. At various times, aliquots were removed and added to an equal volume of ferric chloride reagent. The concentration of ferric hydroxamate complex formed was read on a Klett colorimeter with a 54 filter or at 500 nm.

Nonenzymatic Base Hydrolysis of Esters and Amides. An ester (ethyl acetate) or amides (benzamide; glycylglycine) (0.01 M) were added to 0.01 M NaOH (pH 12) at 23 °C. At various times, aliquots were removed and added to excess 0.01 M HCl. The remaining HCl was back tritated with 0.001 M NaOH to a phenol-phthalein end point.

Tryptic Digestion of Tritiated γ-Cystathionase. The pooled tritiated γ-cystathionase was dialyzed overnight against two changes at 3-h intervals of 25 mM α-picoline–acetate, pH 7.0 (2 L), and then lyophilized. The residue was dissolved in 10 mL of a solution containing 8 M urea, 5.4 mM EDTA, 0.43 M Tris-HCl, pH 8.6, and 0.146 M β-mercaptoethanol. The so-

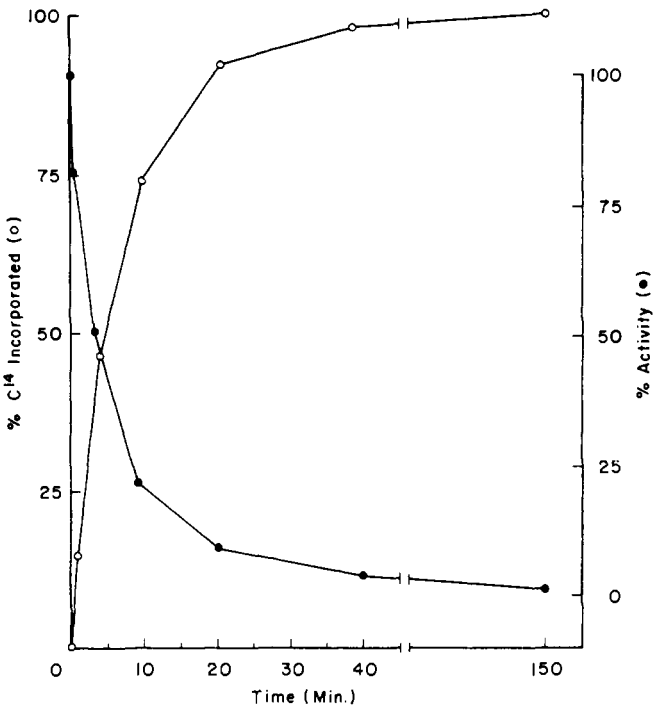


FIGURE 1: Rate of incorporation of [1-^{14}C]trifluoroalanine into γ-cystathionase vs. loss of enzyme activity. γ-Cystathionase (1.2 mL, 6 mg/mL, specific activity 350) in buffer A at 25 °C was incubated with [1-^{14}C]-β,β,β-trifluoro-DL-alanine (4 mM). Periodically, 5-μL aliquots were removed and added to 1.4 mL of the assay mixture; (●—●) 200-μL aliquots were also removed and applied to Sephadex G-25 (1.2 × 9 cm) equilibrated with 10 mM potassium phosphate, pH 7.2, and the protein fraction was collected. These fractions were used to determine the amount of ^{14}C incorporated into protein (O—O).

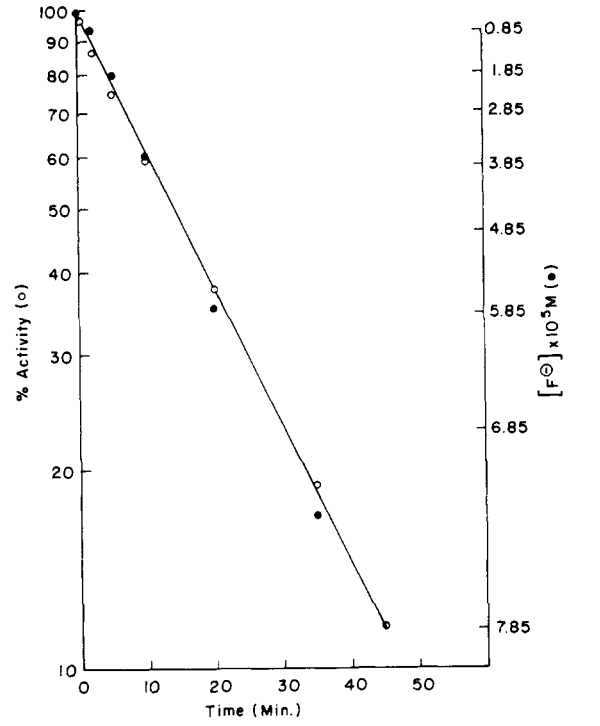


FIGURE 2: Loss of γ-cystathionase activity and release of fluoride ions from trifluoroalanine. γ-Cystathionase (9 mg/mL, specific activity 300) was incubated at 24 °C in 2.0 mL of a solution containing potassium phosphate, pH 7.2 (1 M), potassium chloride (1 M), EDTA (0.01 M), sodium fluoride (10^{-6} M), and trifluoroalanine (2 mM). Fluoride ion concentration (●—●) was determined as described under Materials and Methods. Under identical conditions, 5-μL aliquots were removed periodically and added to 1.4 mL of the assay mixture (O—O), to determine enzyme activity.

lution was kept at room temperature under nitrogen for 5 h. Iodoacetic acid (268 mg) in 1.0 mL of 1.0 M NaOH was added under anaerobic conditions. After standing at room temperature for 30 min, the solution was dialyzed in the cold against two changes of 0.1 M ammonium formate containing 0.01 M $CaCl_2$, pH 8.0 (1 L). To the dialyzed solution was added 50 μL of trypsin-TPCK (40 mg/mL) in 1 mM HCl. This slightly cloudy solution was kept at 37 °C for 4 h, and then another 50 μL of the trypsin-TPCK solution was added. After incubation at 37 °C for an additional 12 h, the solution was lyophilized. The residue was taken up in 0.3 mL of 25 mM α-picoline-acetate, pH 7.0, and applied to Sephadex G-25 (1.2 × 30 cm). The peptide fractions were pooled ("tritiated γ-cystathionase peptides").

Reaction of Hydroxylamine with Tritiated γ-Cystathionase Peptides. To 100 μL of tritiated γ-cystathionase peptides (19 000 cpm) in 50 mM α-picoline-acetate, pH 7.0, was added 100 μL of 1 M hydroxylamine reagent. The solution was kept at 25 °C for 30 min, then was applied to Sephadex G-25 (1.2 × 30 cm) equilibrated with 50 mM α-picoline-acetate, pH 7.0, and compared with a control to which buffer had been added.

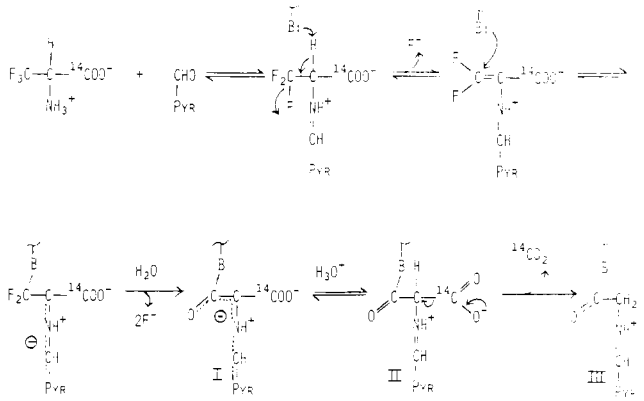
Results

To obtain evidence for the involvement of pyridoxal phosphate in the inactivation process, the enzyme-bound pyridoxal phosphate was reduced with $NaBH_4$. After dialysis, the reduced enzyme was exposed to [1-^{14}C]trifluoroalanine under conditions which would have resulted in incorporation of ^{14}C into unreduced enzyme. No radioactivity was incorporated into the enzyme. These results provide support for the participation of pyridoxal phosphate in the reaction of γ-cystathionase with trifluoroalanine.

Experiments were carried out to establish the relative rates of enzyme inactivation, covalent attachment of [1-^{14}C]trifluoroalanine and F^- release. The results of these experiments are shown in Figures 1 and 2. When γ-cystathionase is inactivated by [1-^{14}C]trifluoroalanine, the loss of enzyme activity is proportional to the amount of radioactivity incorporated into the protein. When the enzyme is completely inactivated, 1 mol of inactivator is incorporated per 80 000 daltons (2 subunits). The rate of loss of enzyme activity and the rate of release of fluoride ions (Figure 2) are the same. Furthermore, complete inactivation of the enzyme corresponds to the release of 2.7 equiv of fluoride ions per mol of inactivator incorporated (average of seven experiments). The same result was obtained whether the release of fluoride ions was followed continously or with aliquots removed at various time points. According to the proposed mechanism, 3 mol of F^- should be released per mol of trifluoroalanine which becomes covalently attached to the enzyme. This, then, in addition to the lack of formation of difluoropyruvate during inactivation (Silverman and Abeles, 1976) is further evidence that every turnover of inactivator produces inactivation of the enzyme and that none of the intermediate is released into solution.

We have previously reported that denaturation of the inactive enzyme with Cl_3CCOOH leads to loss of the carboxyl group of the inactivator, (Silverman and Abeles, 1976). We have now found that this loss of CO_2 occurs under all conditions which denature the protein, e.g., 6 M urea or guanidine hydrochloride, 1% sodium dodecyl sulfate, heat, acid, and organic solvents. The occurrence of this decarboxylation is not surprising and is consistent with the structure proposed for the

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



[a] B = a base at the active site; Pyr-CHO = enzyme-bound pyridoxal phosphate.

enzyme inhibitor adduct (structure II, Scheme I). Structure II is an adduct between a derivative of aminomalonic acid and pyridoxal. Such compounds are known to decarboxylate readily (Thanassi, 1970). It may, at first, be surprising that the adduct does not decarboxylate prior to denaturation. We attribute this failure to decarboxylate to the following: (1) A large fraction, but probably not all of the inhibitor, is present as the carbanion (structure I, Scheme I) (see discussion of spectrum below). This carbanion would not be expected to decarboxylate. (2) The geometry to which the carboxyl group is restricted may not be favorable for decarboxylation (Dunathan, 1971). Upon denaturation, the carbanion is protonated and the geometrical restraint on the carboxyl group is released so that decarboxylation can occur.

Loss of label after decarboxylation makes identification of the bound inactivator difficult. However, denaturation of the protein and, therefore, decarboxylation of the bound inactivator in the presence of ^3H_2O should lead to incorporation of ^3H into the inactivated protein. We, therefore, inactivated the enzyme with unlabeled trifluoroalanine and carried out the decarboxylation in ^3H_2O. This resulted in the incorporation of 0.4 atom of ^3H per mol of inactivator bound to the enzyme. According to the proposed mechanism of inactivation, 2 atoms of ^3H could be incorporated per mol of inactivator. The lower incorporation is partially due to the occurrence of decarboxylation during the lyophilization, prior to exposure to ^3H_2O. It was determined in separate experiments with [^{14}C]trifluoroalanine that about 35% decarboxylation occurs during lyophilization. Isotope selection can also be a contributing factor.

The fact that nonexchangeable ^3H is incorporated into the inactivated enzyme allowed us to further pursue the structure of the inactivated enzyme. The tritiated denatured protein (80 000 cpm) was lyophilized and then hydrolyzed with 1 mL of 6 N HCl at 110 °C for 13 h. The hydrolysate was lyophilized, redissolved in H_2O, and chromatographed by ascending paper chromatography (Whatman 3MM) in 1-butanol:acetic acid:water (120:30:50) and in pyridine:water (130:70). In both solvent systems, a single broad radioactive peak was detected which cochromatographed with glycine. These results suggest that glycine, derived from trifluoroalanine, is covalently attached to the enzyme. Formation of glycine is consistent with the proposed mechanism (structure III, Scheme I).

To further verify that a ^3H-labeled glycine residue is covalently attached to the protein, the tritiated γ-cystathionase peptides were prepared (see Materials and Methods). The peptides (3×10^4 cpm) were dissolved in 0.3 mL of 25 mM α-picoline–acetate buffer (pH 7.0), and [^{14}C]glycine (3×10^4 cpm) was added to the solution. The solution was applied to Sephadex G-25 (1.2 × 30 cm). The tritiated peptides emerged near the void volume and were completely separated from the peak containing [^{14}C]glycine. It is therefore established that the radioactivity associated with the protein is not due to free glycine. The peptides obtained from the Sephadex column were lyophilized, redissolved in 1.0 mL of 6 N HCl, and then hydrolyzed under vacuum at 110 °C for 19 h. The hydrolysate, after removal of HCl, was subjected to paper chromatography in two solvent systems (1-butanol:acetic acid:water and pyridine:water) as well as high voltage paper electrophoresis at pH 1.9 and 8.9. In all cases, a single sharp peak of radioactivity was observed which cochromatographed with glycine. These results established that the inactivated enzyme, after decarboxylation in ^3H_2O, contains covalently bound [^{3}H]glycine, thus supporting the formation of structure II (Scheme I) and its conversion to III.

Experiments were then carried out to identify the nature of the residue to which glycine is attached. In these experiments, the chemical stability of the linkage between protein and labeled glycine toward OH^- and NH_2OH was examined. The tryptic peptides, as well as the labeled denatured protein, were exposed to NH_2OH under conditions described in Materials and Methods. No radioactivity (<5%) was released from either the tritiated peptides or the protein under these conditions. In another set of experiments, the tryptic peptides, as well as the denatured protein, were maintained at pH 12 for 80 min. Only 5% of radioactive material was released from the protein or the peptides. Nonenzymatic model reactions were performed to test the reactivity of various carbonyl compounds with 0.5 M hydroxylamine as well as their stability at pH 12. It was found that the reaction of NH_2OH with the thioester, β-(*N*-acetyl)-aminoethyl thioacetate, was 70% completed after 1 min and with glycine phenyl ester 95% completed in 1 min at 23 °C. There was no reaction in 30 min with glycine ethyl ester or ethyl cyanoacetate. It was found that, in 1 equiv of hydroxide ion at pH 12 (0.01 N NaOH) at 23 °C, ethyl acetate was saponified with a half-life of ca. 15 min. No hydrolysis of the amides, glycylglycine and benzamide, was observed in 2 h under the same conditions. These results then argue against the presence of an ester or thioester linkage but are consistent with the presence of an amide linkage between the carboxyl group of glycine (derived from trifluoroalanine) and the protein. The nitrogen which becomes labeled by trifluoroalanine is most probably an ε-amino group of lysine.

Trifluoroalanine as well as propargylglycine, another suicide inactivator which we previously described (Washtien and Abeles, 1977), label 2 of the 4 subunits of γ-cystathionase. We wanted to establish whether inactivation of γ-cystathionase by either of these inactivator prevents reaction with the other inactivator. γ-Cystathionase was inactivated with trifluoroalanine. After removal of unreacted trifluoroalanine by dialysis, the enzyme was exposed to [^{14}C]propargylglycine. No radioactivity was incorporated into the protein. This experiment was carried out under conditions where 2 mol of propargylglycine would be covalently bound to the active enzyme. Trifluoroalanine, therefore, prevents reaction between γ-cystathionase and propargylglycine. A surprising result was obtained when γ-cystathionase, completely inactivated by propargylglycine, as determined by the normal assay with homoserine as substrate, was exposed to [^{14}C]trifluoroalanine. It was found that [^{14}C]trifluoroalanine is incorporated into the protein to the same extent (i.e., 2 mol per mol of enzyme) as when the enzyme was not initially inactivated by propargylglycine. The rate of incorporation of [1-^{14}C]trifluoroalanine into γ-cystathionase which had been inactivated with pro-

pargylglycine was determined under the conditions described in Figure 1. The rate was found to be approximately one-third as fast as the rate observed with native enzyme. Treatment of propargylglycine-inactivated γ-cystathionase with trifluoroalanine causes a release of 2.6 equiv of fluoride ions into solution at a rate corresponding to the rate of incorporation of trifluoroalanine into the protein. Thus, when the enzyme is treated first with propargylglycine and then with trifluoroalanine, a total of 4 mol of inactivators is incorporated per mol of enzyme. However, reaction of the enzyme with either one of the inactivators alone leads to the incorporation of 2 mol of inactivator per mol of enzyme.

In an attempt to gain further understanding of this phenomenon, the spectral changes accompanying the reaction with various inactivators were examined. Inactivation of γ-cystathionase by trifluoroalanine is accompanied by a color change from bright yellow to orange-pink. This corresponds to a concomitant change in the visible spectrum of the enzyme-bound pyridoxal phosphate (Figure 3). The strong absorption at 419 nm decreases slightly, but the shoulder at 495 nm increases markedly and a new absorption at 519 nm appears. When γ-cystathionase is inactivated with propargylglycine, no change in color and little change in the visible spectrum occur. However, when propargylglycine-inactivated enzyme is treated with trifluoroalanine, a remarkable change in color to bright pink occurs and the visible spectrum is converted to that shown in Figure 3. We attribute the absorption at 519 nm to the highly delocalized carbanion of the inhibitor–pyridoxal Schiff base (structure I, Scheme I). A quinoid intermediate is frequently observed in reactions involving pyridoxal, but the absorption maximum is usually between 490 and 505 nm (Davis and Metzler, 1972). However, in this case, there is additional resonance with the newly formed carbonyl group created by hydrolysis of the carbon–fluoride bonds. Thus, a highly conjugated carbanion (structure I, Scheme I) could account for the long wavelength absorption at 519 nm. Other instances have been reported in which the carbanion is more extensively conjugated and the absorption maximum is therefore shifted to larger wavelengths. Brown, et al. (1969) found that the addition to γ-cystathionase of either DL-homocysteine thiolactone or α-aminobutyro-γ-seleno lactone produced spectra very similar to that observed when trifluoroalanine is added to γ-cystathionase.

The intensity of the long wavelength absorption in the spectrum of the enzyme labeled with propargylglycine and trifluoroalanine is greatly increased over that observed when the enzyme is labeled only with trifluoroalanine. The presence of propargylglycine thus greatly increases the fraction of inactivator in the quinoid form. Upon denaturation with guanidine hydrochloride (6 M), the long wavelength absorption of the trifluoroalanine-treated γ-cystathionase, either with or without prior propargylglycine inactivation, gradually decreases and the spectrum changes to that observed when native enzyme is denatured with guanidine hydrochloride. We attribute this spectral change to protonation of the α-position of the adduct (I → II, Scheme I).

Discussion

Scheme I represents the mechanism which we have previously proposed for the inactivation of γ-cystathionase by trifluoroalanine. In support of this mechanism, we have now shown that pyridoxal phosphate is required, and that three fluoride ions are released per mol of inactivator which becomes covalently bonded to the enzyme. Our data also support the structure which we proposed for the inactivator–enzyme adduct (structure I and II, Scheme I). The nucleophile (B in Scheme I) through which the inactivator is bonded to the enzyme is an amino group, probably the ε-amino group of lysine. Although the overall course of the inactivation is now established, the detailed steps by which fluoride is eliminated are speculative. The proposed reaction sequence for fluoride release is consistent with the known ability of the enzyme to catalyze α,β elimination reactions.

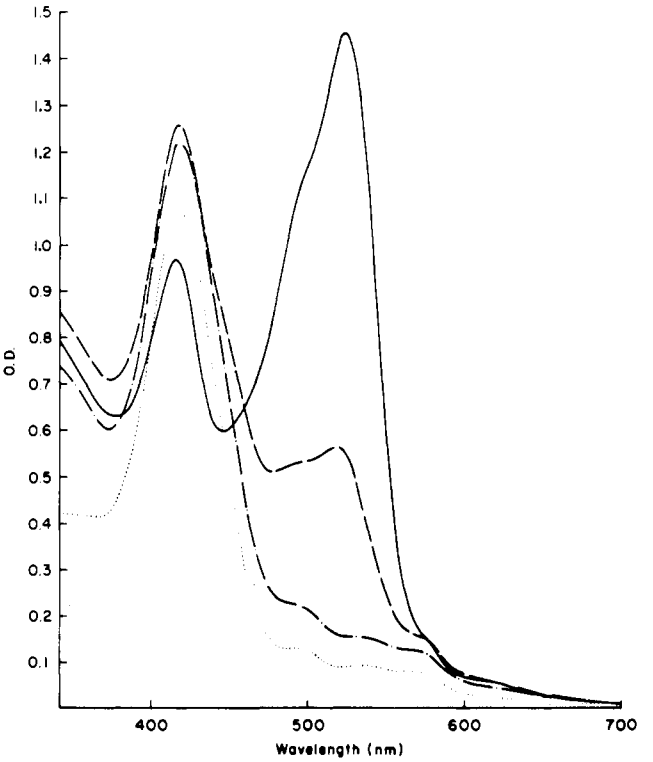


FIGURE 3: Visible spectrum of γ-cystathionase after reaction with inactivators. γ-Cystathionase (6 mg/mL, specific activity 350) in buffer A (····); γ-cystathionase inactivated with propargylglycine (· —); γ-cystathionase inactivated with trifluoroalanine (- - - -); γ-cystathionase first inactivated with propargylglycine and then, after dialysis, exposed to 4 mM trifluoroalanine at 25 °C for 1 h (—).

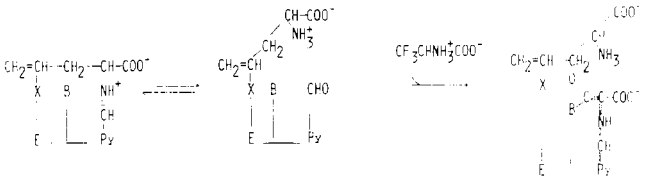
Further support for the reaction sequence (Scheme I) is provided by model studies (Sakai and Santi, 1973; Santi and Sakai, 1971; Heidelberger et al., 1964). In these studies, it was shown that, when a negative charge develops on a carbon bonded to a trifluoromethyl group, fluoride is eliminated. The resulting difluoroolefin in the compounds studied readily reacts with water or nucleophiles. The surprising fact brought out by these models studies is the relative ease with which the carbon–fluorine bond dissociates in elimination reactions, in which a negative charge can be stabilized on the carbon next to a CF_3 group.

A mechanism very similar to that shown in Scheme I has been proposed by Santi for the inactivation of thymidylate synthetase by 5-trifluoromethyl-2′-deoxyuridylate (Santi and Sakai, 1971).

The mechanism which has been proposed for the reaction catalyzed by γ-cystathionase (Davis and Metzler, 1972), as well as other experimental evidence, suggests that the active site of this enzyme contains at least two functional groups: one of these groups is involved in the protonation and deprotonation of the α, β position.[2] The other group interacts with the γ substituent and facilitates its elimination. In subsequent dis-

[2] Possibly more than one group is involved. At this point there is no experimental evidence which requires the involvement of more than one group.

SCHEME II: Reaction of Trifluoroalanine with γ-Cystathionase Inactivated by Propargylglycine.[a]



[a] X, B = functional groups at the active site. Py-CHO = enzyme-bound pyridoxal phosphate.

cussions, we will refer to these functional groups as B and X, respectively. Two different functional groups on the enzyme have been labeled with the suicide inactivators, trifluoroalanine (Silverman and Abeles, 1976) and propargylglycine (Washtien and Abeles, 1977). It is of interest to establish what the role of these functional groups is in the normal catalytic process. Since trifluoroalanine interacts with the enzyme through its β carbon, we suggest that it labels a group which normally interacts with that position, i.e., group B. We propose that propargylglycine, which interacts with the enzyme through its γ carbon, interacts with X. This group has been tentatively identified as either a phenolic OH group or a SH group (Washtein and Abeles, 1977).

When γ-cystathionase is inactivated by propargylglycine and then exposed to trifluoroalanine, 2 mol of trifluoroalanine become covalently bonded to the enzyme. A total of 4 mol of inactivator are then bonded per mol of enzyme. These experiments raise the question as to whether, in the enzyme labeled with 4 mol of inactivator, 2, or all 4 subunits, (Deme et al., 1971; Churchich et al., 1975) are labeled. In the event that only 2 subunits are labeled, 1 mol of trifluoroalanine and 1 mol of propargylglycine would have reacted with a single subunit. This question cannot be answered with certainty. The simplest model which will explain the experimental data is one in which both inactivators occupy the same subunit, i.e., 2 of the 4 subunits are occupied by both propargylglycine and trifluoroalanine. Scheme II schematically illustrates the reaction of γ-cystathionase with propargylglycine and trifluoroalanine. We proposed that propargylglycine is covalently attached to group X relatively far removed from pyridoxal phosphate and does not irreversibly combine with pyridoxal phosphate or with the base B involved in the abstraction of the α and β protons of the substrate or substrate analogues. Hence, the catalytic functions required, i.e., group B and the -CHO group of pyridoxal, to bring about the covalent attachment of trifluoroalanine are available. On the other hand, when trifluoroalanine reacts with the enzyme pyridoxal phosphate, group B becomes covalently labeled. Propargylglycine cannot react with trifluoroalanine-labeled enzyme since group B and pyridoxal phosphate which are required for the reaction between enzyme and propargylglycine are no longer available.

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