Inactivation of Pyridoxal Phosphate Dependent Enzymes by Mono- and Polyhaloalanines†

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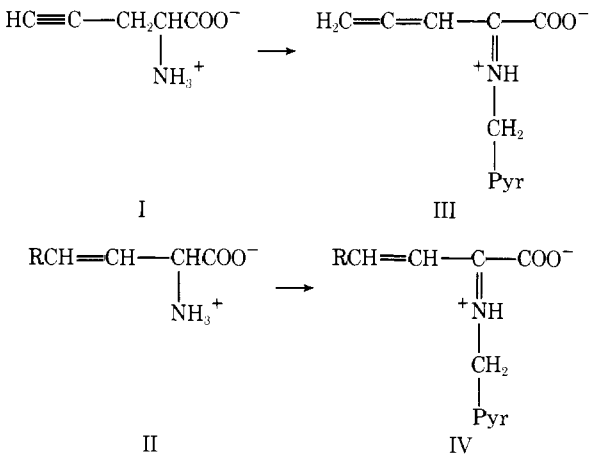
ABSTRACT: β,β-Dichloro- and β,β,β-trifluoroalanine irreversibly inactivate a number of pyridoxal phosphate dependent enzymes which catalyze β- or γ-elimination reactions. The inactivation is time dependent and the rate of inactivation is first order in enzyme concentration. This suggests that inactivation is due to covalent modification of the enzyme by a species generated at the active site from the polyhaloalanine (i.e., suicide inactivation). Monohaloalanines are substrates and do not inactivate. For γ-cystathionase, covalent and stoichiometric attachment of [1-^{14}C]β,β,β-trifluoroalanine was shown. It is proposed that the mechanism of inactivation involves Schiff base formation between inactivator and enzyme-bound pyridoxal and subsequent elimination of HCl from dichloroalanine or HF from trifluoroalanine. This results in the formation of a β-halo-α,β unsaturated imine, an activated Michael acceptor. Michael addition of a nucleophile at the active site leads to covalent labeling of the enzyme and inactivation. Alanine racemase is also inactivated by the two polyhaloalanines. Glutamate-pyruvate and glutamate-oxaloacetate transaminase are inactivated by monohaloalanines but not by polyhaloalanines.

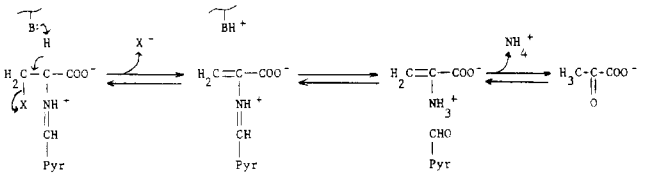
We have reported the inactivation of several enzymes by compounds that we call suicide inactivators (Abeles and Walsh, 1973; Walsh et al., 1972; Hevey et al., 1973, Maycock et al., 1975). Suicide inactivators are relatively unreactive molecules which bind to the active site where, as a result of the catalytic capabilities of the enzyme, they are converted to active species which irreversibly modify the active site. An important characteristic of suicide inhibitors is that the inactivating species are generated at the active site and are not molecules which react from solution. Similar inhibitors have also been prepared by others and some general principles of this type of inactivation have been reviewed (Rando, 1974a; Rando, 1975).

Several pyridoxal phosphate dependent enzymes have been inactivated by compounds (e.g., I and II of Scheme I) which, upon interaction with the enzyme-bound pyridoxal phosphate, give rise to adducts containing a double bond conjugated with an imino group (III and IV of Scheme I) (Abeles and Walsh, 1973; Rando, 1974b,c). These adducts can react with a nucleophile at the active site via a Michael addition, which leads to covalent labeling and inactivation of the enzyme. Nucleophilic attack, however, takes place at the γ-carbon of the adduct. In order to increase the probability of interaction between the inactivator and the base which abstracts the α proton from the substrate, it seemed desirable to design an inactivator which could interact at the β carbon, rather than at the γ position. Structure V represents an example of an adduct which is subject to nucleophilic attack at the β carbon. This type of intermediate (V, where R = H) is produced during the normal catalytic process involving β eliminations (Scheme II), yet its formation does not lead to enzyme inactivation. Therefore, it must not be very reactive toward Michael additions, because, if it were, the substrate would cause inactivation of the enzyme. However, an effective inactivator might be obtained if the adduct (V) were activated for Michael addition by attaching an electronegative substituent to the β carbon (Patai and Rappoport, 1964). This consideration led us to examine the effect of β,β-dichloroalanine and β,β,β-trifluoroalanine on

Scheme I

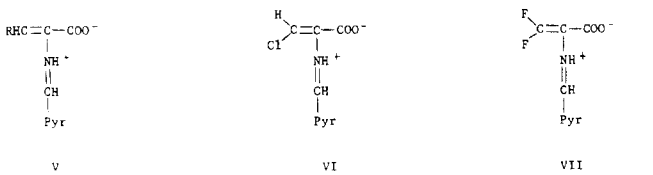


Scheme II



Pyr represents the pyridoxal ring system.

B is a base on the enzyme surface.



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several pyridoxal enzymes. These compounds can, after reacting with enzyme-bound pyridoxal phosphate, eliminate HCl or HF to form adducts VI and VII, which should be more reactive towards Michael additions than V (R = H). Precedence for the formation of a highly reactive difluoromethylene intermediate is provided by Santi's work on model studies for the inactivation of thymidylate synthetase by 5-trifluoromethyl-2'-deoxyuridylic acid (Santi and Sakai, 1971). In that reaction, elimination of fluoride from the trifluoromethyl group by a pair of electrons adjacent to it occurs. The adduct so formed subsequently reacts with a nucleophile on the enzyme. We found that β,β-dichloro- and β,β,β-trifluoroalanine are suicide inactivators for several pyridoxal phosphate dependent enzymes. Results of this investigation are reported here.

Materials and Methods

Chemicals. Pyridoxal 5'-phosphate, NADH,[1] 5,5'-dithiobis(2-nitrobenzoic acid) (NbS_2), dithiothreitol (DTT), glutathione, α-ketoglutarate, and all amino acids, except L-tryptophan, were purchased from Sigma Chemical Co. L-Tryptophan was a product of California Biochemical Research and indole was bought from Fisher Scientific Co. β-Chloro-D,L-alanine was obtained from Cyclo Chemicals and ^3H_2O (1 Ci/ml) and Liquifluor from New England Nuclear. β,β,β-Trifluoro-D,L-alanine was a generous gift of Dr. F. Weygand and β-fluoro-D,L-alanine was kindly donated by Dr. H. Gershon (Boyce Thompson Institute).

Enzymes. Lactic dehydrogenase (beef heart) was purchased from Worthington Biochemicals and Sigma Chemicals; malic dehydrogenase, glutamate-pyruvate transaminase, and glutamate-oxaloacetate transaminase (all from pig heart) were obtained from Boehringer. Except for cystathionine synthase, which was isolated from rat liver, all of the other enzymes used in this study were generous gifts: γ-cystathionase (rat liver)—Ms. Wendy Washtien; β-cystathionase—Professor J.-S. Hong; tryptophanase (*Escherichia coli*)—Professor E. E. Snell; D-serine dehydratase (*E. coli*)—Professor J. Shafer; tryptophan synthase (*E. coli*; β_2 and $\alpha_2\beta_2$)—Dr. E. W. Miles; alanine racemase (*Pseudomonas putida*)—Professor H. C. Dunathan; and threonine dehydrase (*E. coli*)—Professor C. P. Dunne.

Enzyme Assays

γ-Cystathionase. The standard assay mixture in a volume of 1.4 ml consisted of pyridoxal 5'-phosphate (36 μM), β-mercaptoethanol (5.4 mM), EDTA (5.0 mM), potassium phosphate, pH 7.5 (75 mM), D,L-homoserine (34 mM), NADH (0.2 mM), and lactic dehydrogenase (26 units) at 25 °C. The assay was begun with the addition of enzyme (0.05–0.1 IU) and the decrease in absorbance at 340 nm was followed.

β-Cystathionase. The enzyme was assayed by the procedure of Holloway et al. (1970).

Tryptophanase (Morino and Snell, 1970). Apo-tryptophanase was activated by incubation in 1.0 ml of 0.1 M potassium phosphate, pH 7.8, containing glycerin (10%), DTT (10 mM), EDTA (2 mM), and PLP (10^{-4} M) (activation buffer) at 37 °C for 40 min. Assay mixtures consisted of 1.3 ml of the activation buffer containing L-tryptophan (5 mM), NADH (0.05 mM), and lactic dehydrogenase (0.5 unit) at 37 °C. The assay was begun with the addition of enzyme (0.02 IU) and the decrease in absorbance at 340 nm followed.

D-Serine Dehydratase. Assays consisted of potassium phosphate, pH 7.8, containing 10^{-4} M EDTA (83 mM), D,L-serine (41.7 mM), NADH (0.2 mM), and lactic dehydrogenase (26 units) in a total volume of 1.2 ml at 25 °C. The assay was begun with the addition of enzyme (0.1 IU) and the decrease in absorbance at 340 nm was followed.

Tryptophan Synthase, $\alpha_2\beta_2$. The procedure of Smith and Yanofsky (1962), as modified by Miles (1970), was used.

Alanine Racemase. The procedure of Rosso et al. (1969) was followed.

Tryptophan synthase, β_2 subunit (Crawford and Ito, 1964). Apoenzyme was activated by dialysis against one change of 0.1 M potassium phosphate, pH 7.8, containing PLP (0.02 mM), DTT (0.2 mM), and EDTA (5 mM). Assay mixtures contained L-serine (40 mM), Tris-HCl, pH 7.8 (0.1 M), PLP (16 μM), DTT (1.0 mM), ammonium chloride (0.1 M), NADH (0.1 mM), and lactic dehydrogenase (26 units) in a total volume of 1.0 ml at 37 °C. The assay was begun with the addition of enzyme (0.05–0.1 IU) and the decrease in absorbance at 340 nm was followed.

Threonine Dehydrase. Standard assay mixtures in a total volume of 1.0 ml contained potassium phosphate, pH 7.8 (75 mM), AMP (5 mM), DTT (5 mM), L-threonine (20 mM), NADH (0.2 mM), LDH (5 units) at 25 °C. The assay was begun with the addition of enzyme (0.1 IU) and the decrease in absorbance at 340 nm was followed.

Cystathionine Synthase. Assayed by a modification of the procedure of Suda et al. (1971) and will be published separately.

Glutamate-Pyruvate Transaminase. The procedure of Segal and Matsuzawa (1970) was followed.

Glutamate-Oxaloacetate Transaminase (Schwartz, 1971). Assay mixtures contained sodium phosphate, pH 7.5 (0.13 M), L-aspartate (41.7 mM), α-ketoglutarate (8.3 mM), NADH (0.2 mM), and malic dehydrogenase (0.5 unit) in a total volume of 1.2 ml at 25 °C. The assay was begun with the addition of enzyme (10 μg) and the decrease in absorbance at 340 nm was followed.

β,β-Dichloro-D,L-alanine. β,β-Dichloro-D,L-alanine was synthesized by a modification of the procedure by Zaima et al. (1970). A solution of D,L-alanine (89 g, 1.0 mol) in concentrated H_2SO_4 (168 ml, 3 mol) at 70–75 °C was photolyzed with an Hanovia 450 W mercury arc lamp with a Pyrex filter while chlorine gas was bubbled in for ca. 10 h. The viscous solution, after cooling to room temperature, was neutralized with saturated barium hydroxide to pH 4. The mixture was centrifuged (10 000*g* for 15 min) and the supernatant was filtered through Celite and then concentrated by rotary evaporation. Acetone was used to precipitate the product as an off-white solid (95 g), which was shown by NMR to be a mixture of alanine, β-chloroalanine, and β,β-dichloroalanine in the ratio 3:2:3. The mixture[2] (6.0 g) was dissolved in concentrated hydrochloric acid (3 ml), then diluted with water to 10 ml, filtered, and placed on a column of Dowex 50 W X-8 (2.6 × 80 cm) which had been equilibrated with 0.2 N pyri-

[1] Abbreviations used are: NADH, nicotinamide adenine dinucleotide; DTT, dithiothreitol; AMP, adenosine monophosphate; EDTA, (ethylenedinitrilo)tetraacetic acid; NMR, nuclear magnetic resonance.

[2] An alternative procedure for the isolation of β,β-dichloroalanine was performed by Dr. Arthur Cooper and gives a better yield. The mixture (1.45 g) was dissolved in water (20 ml) and added to a column of Dowex AG 1 X-8, 100–200 mesh (2.5 × 18 cm) in the acetate form. The alanines were eluted with water. Alanine and β-chloroalanine came through in the first 150 ml; β,β-dichloroalanine was retarded somewhat. The fractions containing dichloroalanine were combined and concentrated at 20 °C and the product was precipitated as a white solid (200 mg) with acetone. A satisfactory elemental analysis for C,H,N,Cl was obtained.

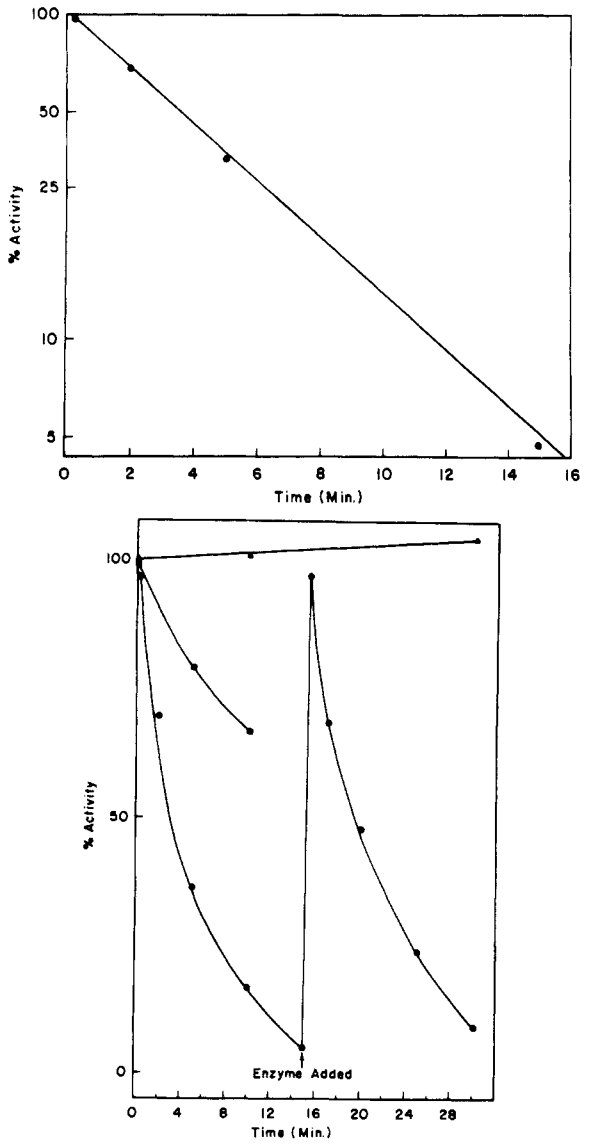


FIGURE 1: Kinetics of inactivation of γ-cystathionase by β,β-dichloro-D,L-alanine. (a, top) Enzyme (0.05–0.1 IU) was incubated in 130 μl of a solution containing KPO_4 buffer, pH 7.5 (75 mM), PLP (36 μM), β-mercaptoethanol (5.4 mM), EDTA (5 mM), and β,β-dichloro-D,L-alanine (20 mM). Periodically, 25-μl aliquots were removed and added to 1.4 ml of the assay mixture (see Methods). (b, bottom) (●—●) as in 1a; (■—■) as in 1a + 200 mM D,L-homoserine; (▲—▲) β,β′-dichloro-D,L-alanine omitted.

dine–acetic acid buffer, pH 3.1. The alanines were eluted with the same buffer at a rate of 0.5–0.7 ml/min, collecting 7-ml fractions. Amino acids were located by examining aliquots of the chromatographic fractions by high-voltage electrophoresis (pH 1.9) (45:1:4), water: 98% formic acid: glacial acetic acid; $R_{alanine}$ dichloroalanine, 0.22; chloroalanine, 0.47. Dichloroalanine was found in tubes 39–78. The tubes containing dichloroalanine were combined and concentrated by rotary evaporation at room temperature. When crystals began to form, the flask was removed and acetone was added to precipitate the remainder of the product (478 mg). The dichloroalanine was triturated in concentrated HCl (0.5 ml) and then filtered to give an off-white solid (400 mg). NMR (D_2O; relative to DSS) δ 4.75 (d, J = 2.5 Hz, 1 H), 6.63 (d, J = 2.5 Hz, 1 H). n-BuOH:HOAc:H_2O (120:30:50), R_f 0.43; pyridine:H_2O (65:35), R_f 0.72; n-PrOH:pyridine:H_2O (1:1:1), R_f 0.64.

[1-^{14}C]Trifluoro-D,L-alanine. The procedure of Weygand et al. (1967) for the synthesis of trifluoro-D,L-alanine was adapted using potassium [^{14}C]cyanide to prepare the intermediate 2,2,2-trifluoro-1-[^{14}C]cyano-N-benzoylethylamine.

Purification of Cystathionine Synthase. The procedure of Suda et al. (1971) was modified and will be published elsewhere.

Enzyme Inactivations. In general, 25 μl of an enzyme solution (0.25–0.5 IU) was preincubated at the desired temperature with 95 μl of the assay mixture excluding substrate. After 5 min, 10 μl of a buffered solution of the inactivator was added and 25-μl aliquots were removed at intervals, diluted 40–120 times into the assay mixture, and the remaining enzyme activity was assayed as described (vide supra). A control was done for each enzyme in which 10 μl of buffer was added in place of the buffered inactivator solution. When glutamate-oxaloacetate transaminase was studied, the preincubation mixture also contained 2.0 M sodium formate (Morino et al., 1974).

Trifluoroalanine Incubation with Apotryptophanase. Apotryptophanase (containing no PLP) was incubated at 37 °C for 80 min with 64 mM trifluoro-D,L-alanine in 0.1 M potassium phosphate, pH 7.8, containing 10% glycerin, 10 mM DTT, and 2 mM EDTA. An aliquot was removed and diluted 40-fold into the same buffer mixture but containing 10^{-4} M PLP. After incubation at 37 °C for 40 min, the enzyme activity was assayed. The control contained no trifluoroalanine in the initial incubation and 2 mM trifluoroalanine in the PLP incubation. Another control, in which holoenzyme was inactivated and diluted as above, showed no regeneration of enzyme activity.

Incubation of β-Fluoroalanine with Pyridoxamine Phosphate Form of Glutamate-Pyruvate Transaminase. The following mixture was incubated for 30 min prior to the addition of inactivator: potassium phosphate, pH 7.5 (62 mM), L-alanine (10 mM), lactate dehydrogenase (10 units), NADH (0.2 mM), glutamate-pyruvate transaminase (25 μg). Then, β-fluoro-D,L-alanine (to give 2.0 mM) was added and aliquots were removed at various times and assayed.

Sephadex Gel Filtration of Inactivated Enzymes. The enzymes were incubated in the usual assay mixtures (excluding substrate) containing sufficient β,β,β-trifluoroalanine to cause >95% inactivation in 15 min at 25 °C. The enzyme mixture was then filtered through a column of Sephadex G-25 (medium, 0.9 × 25 cm) which was equilibrated at 4 °C with the assay buffer. Fractions shown to contain enzyme activity in a prior control experiment which excluded β,β,β-trifluoroalanine were assayed.

Labeling of γ-Cystathionase by [1-^{14}C]Trifluoro-D,L-alanine. To a solution of γ-cystathionase (6 mg/ml; sp act. 300 units/mg) in 0.2 M potassium phosphate, pH 7.2, containing 7×10^{-2} M EDTA, 7×10^{-2} M β-mercaptoethanol, and 5×10^{-4} M PLP, was added a solution of [1-^{14}C]trifluoro-D,L-alanine in buffer to give a final concentration of 4 mM. After incubation at 25 °C for 45 min, the orange-pink solution was applied to a column of Sephadex G-25 (medium, 1.8 × 21 cm) which was equilibrated with 10 mM potassium phosphate, pH 7.2, at room temperature and 1-ml fractions were collected. The tubes containing radioactively labeled protein were pooled.

Trichloroacetic Acid Denaturation of Labeled γ-Cystathionase. An aliquot of ^{14}C-labeled γ-cystathionase, after Sephadex G-25 filtration (200 μl containing 1000 cpm ^{14}C), was placed in a counting vial which was capped with a rubber

TABLE I: Irreversible Inactivation of PLP-Dependent Enzymes which Catalyze Elimination Reactions.[a]

Enzyme	Trifluoro-D,L-alanine			Dichloro-D,L-alanine		
	Concn	$t_{1/2}$	Temp (°C)	Concn	$t_{1/2}$	Temp (°C)
β-Cystathionase[b]	1 mM	50 sec	25	Does not bind[c]		25
	250 μM	5 min	25			
	100 μM	7 min	25			
γ-Cystathionase[b]	20 mM	<1 min	25	20 mM	2 min	25
	4 mM	2 min	25	2 mM	19 min	25
Tryptophanase	80 mM	9 min	37	80 mM	47 min	37
	20 mM	20 min	37			
D-Serine dehydratase	Does not bind[c]		25	Does not bind[c]		25
Tryptophan synthase (β_2)	40 mM	9 min	37	Does not bind[c]		37
Tryptophan synthase ($\alpha_2\beta_2$)	40 mM	12 min	37	40 mM	10 min	37
Cystathionine synthase	Competitive inhibition		37	Competitive inhibition		37
Threonine dehydrase	40 mM	14 min	25	Does not bind[c]		25

[a] Rates of inactivation were measured by incubating inhibitor at the concentration indicated with the enzyme (0.05–0.1 IU) in a total volume of 130 μl. Periodically, 25-μl aliquots were removed and added to 1.0–1.4 ml of appropriate assay mixtures (see Methods). [b] Sephadex gel filtration after inactivation did not regenerate enzyme activity. [c] Does not competitively inhibit the substrate reaction at 40–50 times the substrate concentration.

septum through which a plastic center well (Kontes Glass Co., catalog no. K-882320-0000) was suspended. Sodium hydroxide (8% solution; 0.2 ml) was injected into the center well and then 20% trichloroacetic acid (0.2 ml) was injected into the counting vial. The vial was shaken at 37 °C for 2 h, and then the basic solution and the acidic solution were counted in 10 ml of Liquifluor counting mixture (1 l. of toluene, 500 ml of methanol, 278 ml of dioxane, and 68 ml of Liquifluor).

Results and Discussion

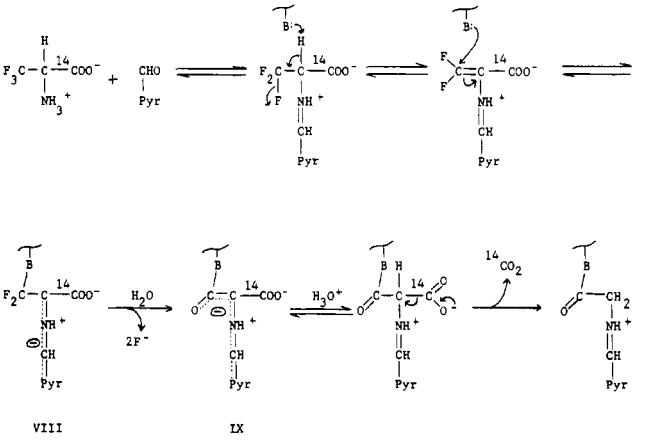
Figure 1a represents the time course of the inactivation of γ-cystathionase by β,β-dichloroalanine. The loss of enzyme activity is first order in enzyme concentration. Figure 1b shows that additional enzyme, added after completion of the inactivation, is inactivated at the same rate as originally observed. These data provide good evidence that inactivation occurs by a molecule generated at the active site, and not by a molecule from solution produced by the action of the enzyme upon the inactivator. Time dependence of the inactivation is good evidence for irreversible inactivation, probably due to covalent modification. Finally, reduction of the rate of inhibition (Figure 1b) by the addition of substrate suggests that the inactivator and substrate bind at the same site.

The effect of β,β-dichloroalanine and β,β,β-trifluoroalanine on a number of pyridoxal enzymes which can catalyze β elimination reactions was examined. The results are summarized in Table I. In all cases where inactivation was observed, the kinetics of the inactivation is qualitatively similar to that described for the inactivation of γ-cystathionase. In some cases, as indicated in Table I, additional evidence for irreversible inactivation was obtained by showing that Sephadex gel filtration does not regenerate catalytic activity. In the case of tryptophanase, further evidence for "suicide" inactivation was realized. The apoenzyme of tryptophanase was exposed to trifluoroalanine under conditions which inactivate holoenzyme and then the apoenzyme was converted to holoenzyme. Enzyme activity was found to be the same as a control containing no trifluoroalanine during preincubation. In another experiment using holoenzyme, it was shown that after inactivation by trifluoroalanine, enzyme activity does not return during conditions used to convert apoenzyme to holoenzyme. The presence of pyridoxal phosphate is, therefore, essential for inactivation.

Attempts were made to determine whether formation of the activated Michael intermediates generated from dichloro- or trifluoroalanine are ever released into solution to give chloropyruvate or difluoropyruvate. The inactivators were incubated with γ-cystathionase and tryptophanase in the presence of lactic dehydrogenase and NADH. No decrease in absorbance at 340 nm was observed. In the case of γ-cystathionase with trifluoroalanine, no carbonyl compounds were detected with 2,4-dinitrophenylhydrazine reagent. Furthermore, when γ-cystathionase was incubated with [1-^{14}C]trifluoroalanine and the mixture was applied to a column of Dowex 50 (H^+ form), no radioactivity was found in the non-amine fraction. Thus, it appears that any product formation is small relative to inactivation.

Covalent attachment of inactivator to protein was demonstrated with γ-cystathionase. Incubation of γ-cystathionase with [1-^{14}C]trifluoroalanine, followed by Sephadex gel filtration, leads to incorporation of 2 mol of ^{14}C label/160 000 g of protein.[3] This represents the same amount of radioactive incorporation as previously reported for γ-cystathionase with [2-^{14}C]propargylglycine (Abeles and Walsh, 1973). After denaturation of the [1-^{14}C]trifluoroalanine-labeled protein by trichloroacetic acid, all of the radioactivity associated with

Scheme III



[3] 1 mol of enzyme contains 4 subunits of identical molecular weight and 4 pyridoxal residues/160 000 g (Churchich et al., 1975).

the protein can be accounted for as $^{14}CO_2$. Under the same conditions [1-^{14}C]trifluoroalanine produces no $^{14}CO_2$. Concomitant with inactivation by trifluoroalanine is an increase in the pyridoxal absorbances at 490 and 518 nm. The absorbance at 490 nm is associated with the quinoid form of pyridoxal (John and Fasella, 1969). These results suggest that interaction with γ-cystathionase causes a chemical modification in the structure of the inactivator molecule. A possbile mechanism for labeling of γ-cystathionase and for the formation of $^{14}CO_2$ upon acid denaturation is shown in Scheme III.

The formation of enzyme-bound VIII or IX is reminiscent of the mechanism proposed for trifluoromethyldeoxyuridylate inactivation of thymidylate synthetase (Santi and Sakai, 1971). The detailed mechanism of trifluoroalanine inactivation of pyridoxal phosphate enzymes is under investigation.

All of the enzymes listed in Table I, except cystathionine synthase, catalyze elimination reactions from serine, chloroalanine, and fluoroalanine. (In general, the rate of formation of pyruvate from fluoroalanine was slower than that from chloroalanine.) This establishes that a simple elimination reaction from a substituted alanine does not lead to inactivation and, therefore, provides support for the hypothesis that electronegative substituents on the β carbon of the inactivator facilitate inactivation, presumably by activating the unsaturated intermediate adduct towards Michael addition.

An interesting change in susceptibility to inhibitors is seen between the β_2 subunit and the $\alpha_2\beta_2$ complex of tryptophan synthase. Both the $\alpha_2\beta_2$ complex and β_2 subunits are inactivated at approximately the same rate by trifluoroalanine. Dichloroalanine inactivates only the $\alpha_2\beta_2$ complex and does not bind appreciably to the β_2 subunit. The steric requirements for the β_2 subunit must be fairly strict, since L-threonine is deaminated at only 3% the rate of the substrate L-serine (Crawford and Ito, 1964). However, combination of the $\alpha_2\beta_2$ subunits may create a binding site which can accommodate larger substrate molecules. Thus, the steric requirements are diminished and the bulky dichloroalanine can bind at the active site.

Cystathionine β-synthase, a β replacement pyridoxal phosphate dependent enzyme, is competitively inhibited by both trifluoro- and dichloroalanine, but not inactivated. Braunstein and Goryachenkova (1976) have suggested that the mechanism for PLP-dependent enzymes, which catalyze exclusive β-replacement reactions, may not involve an α,β-elimination step. Incubation of cystathionine synthase with chloro- or fluoroalanine produced no pyruvate. Furthermore, incubation with [1-^{14}C]trifluoroalanine did not give rise to any new radioactive metabolites. These data are in accord with a mechanism which does not involve a simple β elimination and, therefore, the polyhaloalanines are not expected to cause irreversible inactivation.

The effect of β,β-dichloroalanine and β,β,β-trifluoroalanine on glutamate-pyruvate transaminase and glutamate-oxaloacetate transaminase was also tested. These enzymes, in contrast to the enzymes listed in Table I, do not catalyze elimination reactions as part of their normal catalytic process. Dichloroalanine does not bind to either enzyme and, consequently, cannot be expected to inactivate them. Trifluoroalanine binds, as demonstrated by its inhibition of the catalytic reaction, but does not inactivate. The α-proton exchange with solvent of trifluoroalanine was followed by NMR and shown to be glutamate-pyruvate transaminase catalyzed, but to occur at an extremely slow rate. This slow rate of α-proton removal may explain why it is not an effective inactivator.

However, β-chloroalanine and β-fluoroalanine irreversibly inactivate both transaminases, as shown in Table II. The inactivation by the monohaloamino acids is first order in enzyme concentration over at least three half-lives. Dilution of the inactivated enzyme does not restore catalytic activity, nor does Sephadex gel filtration. When glutamate-pyruvate transaminase was converted to the pyridoxamine phosphate form, no inactivation was observed. This indicates that the aldehyde form of the cofactor is necessary for inactivation and that Schiff base formation with the substrate is a prerequisite for inactivation. It is, therefore, probable that the monohaloalanines function as "suicide" inactivators. The inactivation of glutamate-oxaloacetate transaminase by chloroalanine (Morino et al., 1974) and serine *O*-sulfate (John and Fasella, 1969) has been reported. Inactivation by monohaloalanines may occur by covalent attachment of the enzyme to the reactive α-haloimine formed after enzyme-catalyzed tautomerization of the Schiff base intermediate, as proposed for chloroalanine inactivation of glutamate-oxaloacetate transaminase (Morino et al., 1974) and aspartate decarboxylase (Tate et al., 1969). If this were the mechanism, than an α-fluoroimine would be generated from β-fluoroalanine. We are unaware of an appropriate model reaction for this fluoride displacement. Alternatively, inactivation by monohaloalanines may occur by attachment of the enzyme to a pyridoxal-bound aminoacrylic intermediate generated from the elimination of HX (Scheme II). This mechanism parallels that proposed for serine *O*-sulfate inactivation of glutamate-oxaloacetate transaminase (John and Fasella, 1969).

TABLE II: Inactivation of PLP-Dependent Transaminases.

Transaminase	β-Chloro-D,L-alanine Concn	β-Chloro-D,L-alanine $t_{1/2}$[a] (min)	β-Fluoro-D,L-alanine Concn	β-Fluoro-D,L-alanine $t_{1/2}$[a] (min)
Glutamate-Pyruvate[b]	100 μM	7.6	100 μM	2.8
	5 mM	1.2	500 μM	2
Glutamate-Oxaloacetate[c]	40 mM	8.0	40 mM	53
	60 mM	6.3	250 mM	16

[a] Rates of inactivation were measured by incubating inhibitor at the concentration indicated with the enzyme (0.05–0.1 IU) in a total volume of 130 μl at 25 °C. Periodically, 25-μl aliquots were removed and added to 1.2 ml of the appropriate assay mixtures (see Methods). Substrate protected the enzymes against inactivation. [b] Sephadex gel filtration after inactivation by β-fluoro-D,L-alanine did not regenerate activity. [c] Preincubation mixtures contained 2 M sodium formate.

The effect of DL-trifluoroalanine on alanine racemase was also examined. At 40 and 20 mM β,β,β-trifluoroalanine (Tris-HCl, pH 7.8, buffer), $t_{1/2}$ for inactivation is 6 and 15 min, respectively. Inactivation was first order in enzyme concentration for at least three half-lives. No activity was restored upon dilution. β,β-Dichloroalanine (40 mM) gave a very slow inactivation (41% in 18 min). The mechanism of this inactivation could involve elimination of HX, initiated by α-proton abstraction which occurs during the normal catalytic process. Inactivation can then occur through nucleophilic addition to the activated double bond as discussed above.

The results reported here establish that β,β-dichloroalanine and β,β,β-trifluoroalanine are active site directed inactivators. Our data strongly suggest that the activity of the compounds is the result of their conversion at the active site to activated Michael acceptors. Although the experiments available so far

are consistent with this proposal, a more detailed investigation of the inactivation is required to establish the mechanism.

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