# INACTIVATION OF γ-CYSTATHIONASE BY γ-FLUORINATED AMINO ACIDS

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## 1. Introduction

The discovery by Abeles and Walsh that γ-cystathionase and mechanistically similar enzymes are inactivated by the antibiotic β-ethynyl-L-alanine provided an elegant example of the phenomenon of suicide inactivation in which enzymes catalyze their own inactivation upon unmasking latent reactive functionalities of substrate analogs [1,2]. The analogous nitrile, the neurotoxin β-cyano-L-alanine, is a potent but freely reversible inhibitor of γ-cystathionase [3]. However, we have found that β-cyano-L-alanine is a suicide inactivator of porcine heart alanine aminotransferase, a pyridoxal enzyme which shares with γ-cystathionase the ability to abstract protons from both the β- and α-positions of its substrates (fig.1) [4,5].

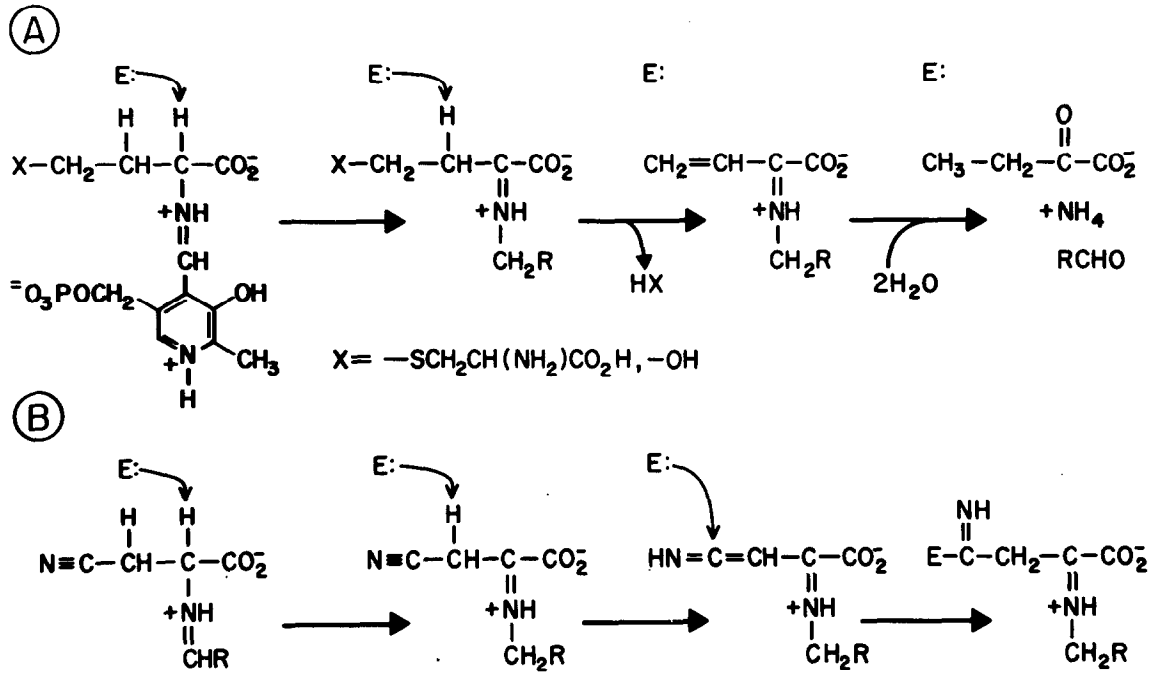


Fig.1. The γ-cystathionase reaction (A) and one putative suicide reaction of β-cyano-L-alanine (B). The enzyme ordinarily abstracts a β-proton from cystathionine or homoserine in a β,γ-elimination reaction that ultimately yields α-oxobutanoate. Enzymatic removal of a β-proton from cyanoalanine may generate a reactive ketenimine which transiently imino acylates an active-site nucleophilic group. The ketenimine is much more electrophilic than the ordinary olefinic intermediate. This scheme is analogous to that proposed in [1] for the suicide reaction of β-ethynyl-L-alanine (propargylglycine) with γ-cystathionase. Any covalent modification of the active site of the rat liver γ-cystathionase by cyanoalanine must be labile since the potent inhibition by cyanoalanine is rapidly reversible. However, the inhibition of a β-proton-abstracting alanine aminotransferase by cyanoalanine is not rapidly reversible ($t_{1/2} \simeq 30$ min) [5].

Here, we report that γ-cystathionase is subject to suicide inactivation by β-trifluoromethyl-D,L-alanine (2-amino-4,4,4-trifluorobutanoic acid, fig.2A) and by β-trifluorovinyl-D,L-alanine (2-amino-4,5,5-trifluoro-4-pentenoic acid, fig.2B). The inactivation of this enzyme catalyzing β,γ-elimination reactions by a compound multiply halogenated at the γ-carbon supports the view inferred from studies of β-polyhalogenated amines [6,7] that this strategy of multiple halogenation, at the appropriate carbon, will prove generally applicable to the design of mechanism-based enzyme inactivators.

## 2. Materials and methods

Crystalline γ-cystathionase was isolated from the livers of adult male Sprague-Dawley rats [8]. Taking $\epsilon_{280}$ to be $1.3 \times 10^5$ $M^{-1}$ $cm^{-1}$ for the tetramer [8], the molecular turnover number at saturating D,L-homoserine was 32 $s^{-1}$ at 25°C and pH 7.4 in a solution containing 0.1 mM pyridoxal 5′-phosphate, 5.0 mM mercaptoethanol, 1.0 mM EDTA and 100 mM potassium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate (Hepes). β-Trifluoromethyl-D,L-alanine [9], β-trifluorovinyl-D,L-alanine [10], β-trichloromethyl-L-alanine [11], β-dichloromethyl-L-alanine [11], and β-acetyl-D,L-alanine [12] were synthesized by published procedures.

We found that β-chloroalanine serves as a substrate of the cystathionase. Since α-oxobutanoate (fig.1A) is a poor substrate of lactate dehydrogenase, in some of our experiments in which the activity of cystathionase was followed by the coupling of a cystathionase-catalyzed reaction to a dehydrogenation, β-chloroalanine was employed as a pyruvate-generating substrate of

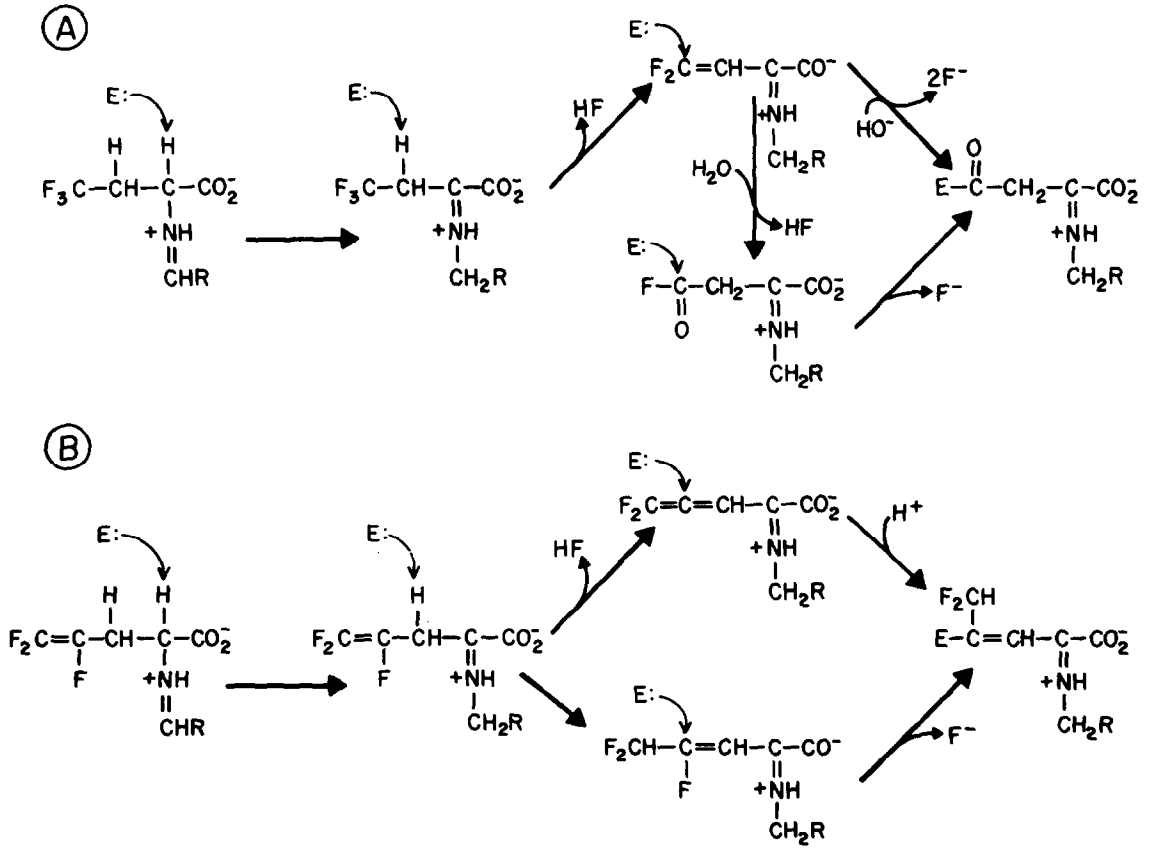


Fig.2. Probable suicide reaction mechanisms of β-trifluoromethyl-L-alanine (A) and of β-trifluorovinyl-L-alanine (B). After β-proton abstraction, the former compound may acylate γ-cystathionase via either a fluorine-activated olefin or an acyl fluoride. The latter compound may trap the enzyme via either an allene or a fluorine-activated olefin. Tautomeric structures are also plausible, and the ∂-carbon of the latter compound is potentially reactive.

cystathionase. At 25°C and pH 7.4 in a solution containing 0.1 mM pyridoxal 5′-phosphate, 5.0 mM mercaptoethanol, 1.0 mM EDTA, 0.2 mM NADH, excess lactate dehydrogenase and 100 mM Hepes, the $V_{max}$ value for β-chloro-D,L-alanine is 1.7-fold greater than that of D,L-homoserine whereas the $K_m$-value (10 mM) is 1/3rd that (33 mM) observed for D,L-homoserine.

## 3. Results

Progressive inactivation of γ-cystathionase by γ-fluorinated amino acids is sensitively monitored by means of continuous recordings of NADH absorbance following the addition of cystathionase to dehydrogenase-coupled assay solutions containing the suicide inactivator and a sub-saturating level of homoserine or β-chloroalanine (fig.3). We have confirmed the irreversibility of the inactivation by these fluoro compounds in experiments in which the cystathionase was incubated with the inactivators at 25°C and pH 7.4. Progressive inactivation is seen when small aliquots (10 μl) of the incubated solutions are withdrawn at intervals and then diluted 100-fold into assay solutions containing saturating (200 mM) levels of D,L-homoserine. Inactivation is also not reversed when the inactivated enzyme is washed by dialysis against 10 mM Hepes buffer containing 0.1 mM pyridoxal 5′-phosphate at pH 7.4 and 4°C for 48 h. The enzyme treated with either no amino acid or, interestingly, with β-cyano-L-alanine retains full activity during dialysis.

We have also examined β-trichloromethyl-L-alanine and β-dichloromethyl-L-alanine for activity as suicide substrates. The γ-dichloro compound, armentomycin, is a toxic antibiotic elaborated by a strain of *Streptomyces armentosus* [13]. Upon incubation of γ-cysta-

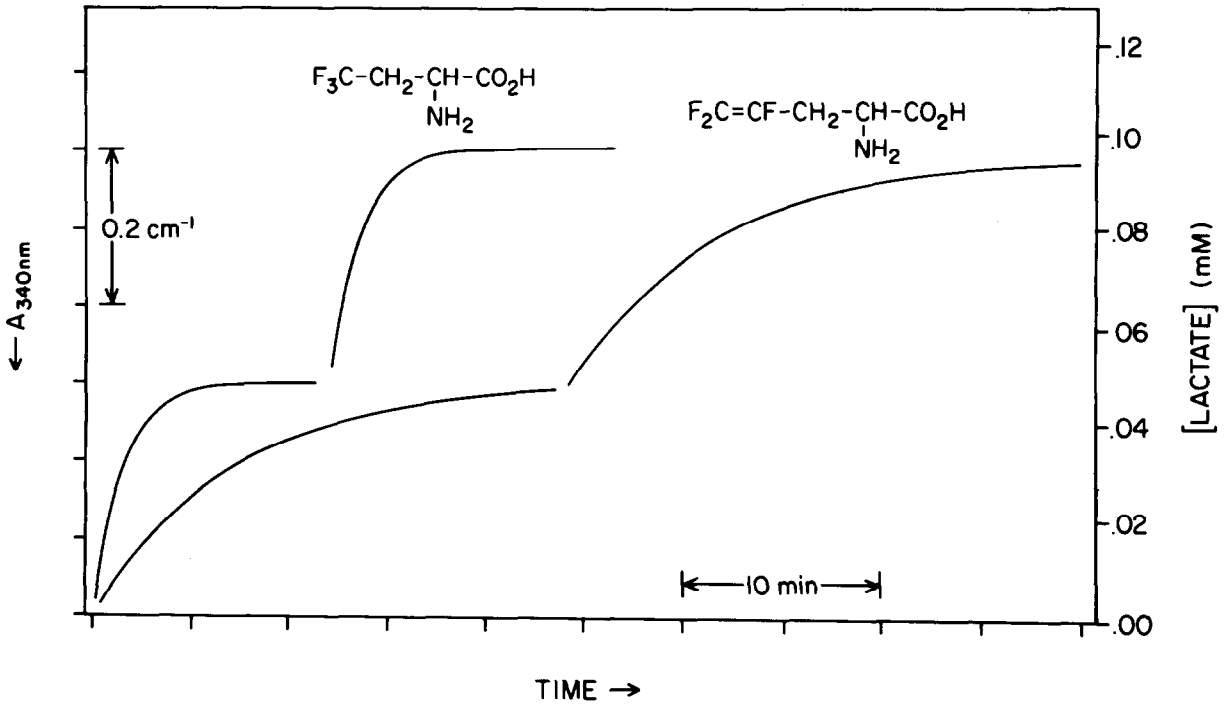


Fig.3. Time-dependent inhibition of γ-cystathionase by γ-fluoro compounds. The production of pyruvate from β-chloroalanine is followed by the coupling of that cystathionase-catalyzed reaction to the lactate dehydrogenase reaction. The decrease in absorbance of NADH is given as a function of time. The reaction solutions initially contain either 9.6 mM β-trifluoromethyl-D,L-alanine or else 120 mM β-trifluorovinyl-L-alanine as well as 6.0 mM β-chloro-D,L-alanine, 5.0 mM mercaptoethanol, 1.0 mM EDTA, 0.2 mM NADH, 0.1 mM pyridoxal 5′-phosphate, lactate dehydrogenase (70 μg/ml), and 100 mM Hepes/KOH at pH 7.4 and 25°C. The reactions are initiated by the addition of cystathionase (4.6 or 2.3 μg/ml). The absorbance is read against a slight non-enzymatic rate of pyruvate production from β-chloro-D,L-alanine. The first-order exponential decay of the velocity is secondary to inactivation of the cystathionase rather than to accumulation of an inhibitory product because successive additions of enzyme produce identical bursts of pyruvate.

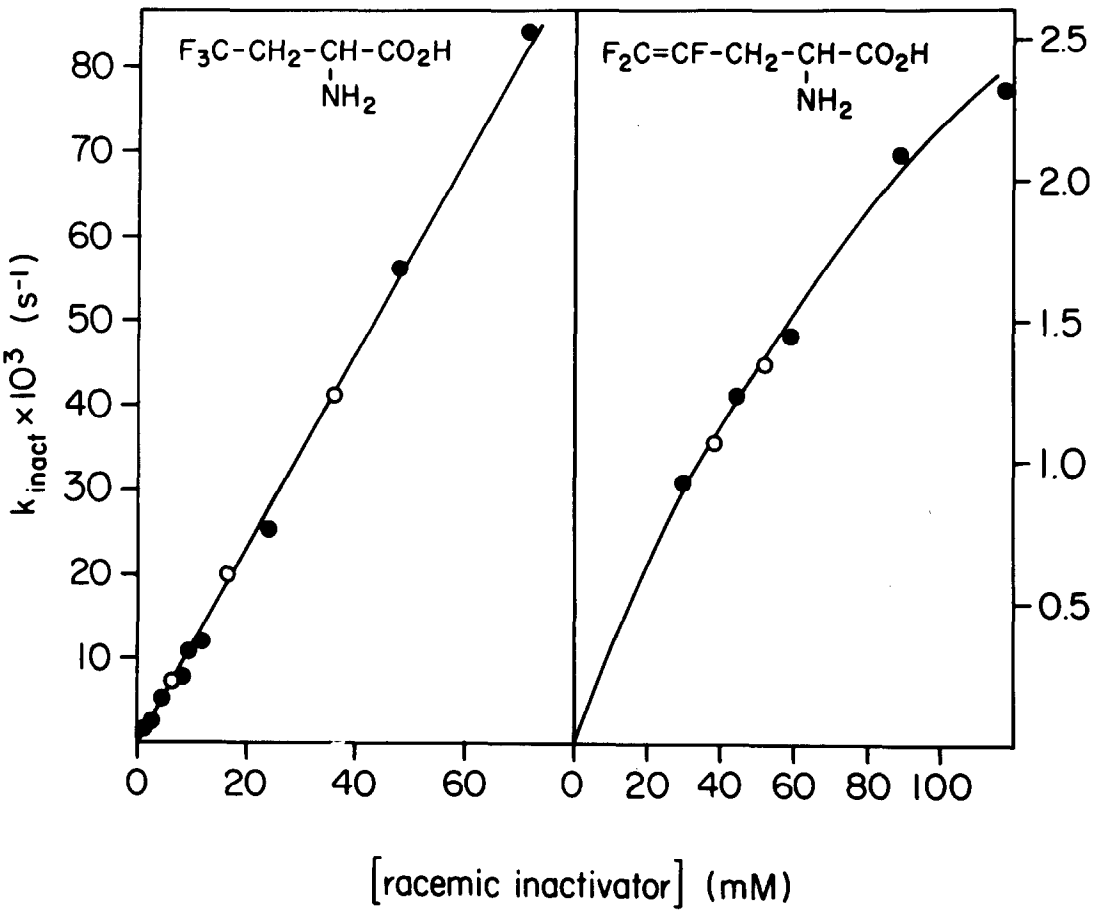


Fig.4. Observed first-order rate constants for the inactivation of γ-cystathionase by γ-fluoro α-amino acids at pH 7.4 and 25°C. These data were obtained from experiments such as those shown in fig.3. The reaction mixtures contained either 5.0 mM (•) or 50 mM mercaptoethanol (○). The inhibitor concentration varied as indicated, and the enzyme varied from 140–1.1 μg/ml. Because of the low solubility of the fluoro compounds and their low affinity for the enzyme, it was not possible to accurately measure their kinetic binding constants nor to extrapolate to a maximal rate of inactivation. However, the insensitivity of the rate of inactivation toward the nucleophilic agent mercaptoethanol indicates that an electrophilic species which inactivates the enzyme would have to be generated within a space sheltered from nucleophiles in free solution (as in a Michaelis complex at the active site).

thionase with the chloro compounds (25 mM) in 100 mM potassium phosphate buffer (pH 7.4) also containing 0.1 mM pyridoxal 5′-phosphate at 25°C for 3.0 h, aliquots of the solution containing the trichloro compound were fully active, and the enzyme treated with armentomycin retained 85% of the initial activity. Furthermore, the porcine heart alanine aminotransferase which is subject to inactivation by β-ethynyl-L-alanine [2] and by β-cyano-L-alanine [5] is not inactivated by the γ-fluoro nor γ-chloro compounds. No inactivation is detected after its incubation with the compounds (25 mM) for 2.0 h at 25°C in 100 mM potassium phosphate buffer (pH 7.4) also containing 10 mM α-oxoglutarate. The γ-halo compounds were not effective competitive inhibitors of either enzyme, the $K_i$-values all exceeding 25 mM.

## 4. Discussion

Since inactivation of γ-cystathionase by β-trifluoromethyl-D,L-alanine is irreversible, the potent but reversible inhibition of the enzyme by β-cyano-L-alanine may not involve an acylation reaction such as

that depicted in fig.1B. Alternatively, removal of the β-proton from β-cyano-L-alanine may result in tautomerization to a cyano enamine rather than to the ketenimine shown. Such an enamine may be metastable in view of the stability of ketoenamines [5,14]. However, the $K_i$-value of β-acetyl-D,L-alanine, 50 mM, is orders of magnitude greater than that of β-cyano-L-alanine, 0.1 μM [3].

β-Polyhalogenated amines such as β,β-dichloro- and β,β,β-trifluoroalanine are suicide substrates for a wide range of pyridoxal enzymes, including γ-cystathionase [6,7]. In general, γ-halogenated amines ought to prove more selective than β-halogenated compounds as inactivators of pyridoxal 5′-phosphate-dependent and similar enzymes since all pyridoxal enzymes generate α-carbanionic species whereas only a small subset also abstract β-protons from their substrates.

## Acknowledgements

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