Characteristics of β,β-Difluoroalanine and β,β,β-Trifluoroalanine as Suicide Substrates for *Escherichia coli* B Alanine Racemase†

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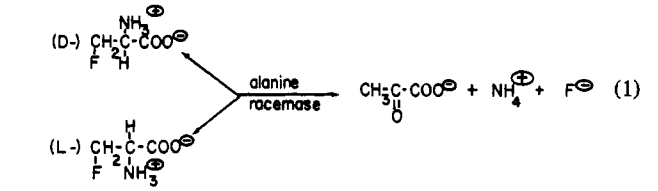
ABSTRACT: The alanine racemase from *Escherichia coli* B has been shown to process DL isomers of β-fluoroalanine as suicide substrates with an identical partitioning ratio for each enantiomer of 820 catalytic eliminations of HF per enzymatic inactivation event [Wang, E., & Walsh, C. T. (1978) *Biochemistry 17*, 1313], suggesting the aminoacrylate–PLP complex as a common, symmetrical partitioning species. In an attempt to vary the partition ratio, an index of killing efficiency, systematically the β,β-difluoroalanine and β,β,β-trifluoroalanine isomers have now been evaluated for substrate processing, suicidal inactivation kinetics and partitioning ratio, and stability of inactive, derivatized enzyme forms. Both difluoroalanine isomers show high K_m values (116 mM for D, 102 mM for L) in catalytic HF loss to form fluoropyruvate. The V_{max} for the D isomer is about 14-fold higher than that for the L isomer. Limiting inactivation rate constants, calculated from k_{cat} and observed partition ratios of 5000 and 2600, respectively, are 2.2 min^{-1} for D-difluoroalanine and 0.33 min^{-1} for L-difluoroalanine. For comparison, DL-trifluoroalanine turns over less than 10 times per enzyme molecule inactivated and so is a very efficient suicide substrate. The estimated inactivation rate constant is ≤1.0 min^{-1}. These data are analyzed in terms of partitioning behavior of the monofluoro- and difluoroaminoacrylate–PLP complexes as partitioning intermediates for turnover or for racemase inactivation. While mono- and trifluoroalanines yield stable inactive species, the difluoroalanine isomers produce labile enzyme derivatives, and regain of catalytic activity is analyzed in terms of the anticipated oxidation state at the β carbon of the substrate fragment adducted to the enzyme.

The D isomers of amino acids are key constituents of the peptidoglycan layer of bacterial cell walls but in general are not common metabolites in mammalian cells. Thus, bacterial enzymes which supply D-amino acids for cell wall biosynthesis are recognized targets for inhibition by synthetic and naturally occurring inhibitors (Park, 1958). There are two general enzymatic routes to D-α-amino acids, the first by equilibration of α-carbon configuration of L-amino acids via racemase (Adams, 1976) action and the second by appropriate chiral reductive amination of a keto acid to a D-α-amino acid isomer by D-specific transaminases. Both enzymatic types may be of wide bacterial distribution. We have previously reported studies with mechanism-based inactivators of homogeneous D-amino acid transaminases from *Bacillus* species (Soper et al., 1977a,b) and of alanine racemase from *Escherichia coli* (Wang & Walsh, 1978). Both are pyridoxal-P-dependent enzymes, accelerating substrate transformations by generation of stabilized α-carbanionic intermediates of bound substrate–PLP adducts, and this chemistry has been conscripted for rerouting intermediates to suicidal alkylation of the catalysts with D-vinylglycine and D-chloroalanine in the case of the *Bacillus* D-amino acid transaminases (Soper et al., 1977a,b) and with a variety of D- and L-β-substituted alanines, most notably D- and L-β-fluoroalanines, for *E. coli* B alanine racemase (Wang & Walsh, 1978). The β-fluoroalanines were first prepared by Kollonitsch and colleagues at Merck, and the D isomer was successfully validated to be a wide-spectrum experimental antibacterial agent (Kollonitsch et al., 1973; Kahan et al., 1975; Kollonitsch & Barash, 1976). This result has precipitated a systematic synthesis and evaluation of a series of β-fluoro amino acids, especially α-fluoromethyl α-amino acids, as pharmacologically useful inhibitors of PLP-dependent enzymes (Kollonitsch et al., 1978; Metcalf et al., 1978).

The *E. coli* alanine racemase processes either β-fluoroalanine isomer for catalytic elimination of HF to yield pyruvate (eq 1) in kinetic competition with the autoinactivation sequence. The partition ratio for turnover to inactivation measures the frequency with which an active site alkylation intervenes and inactivates an enzyme molecule before the electrophilic species uncovered in turnover can be deactivated chemically or physically (e.g., product release) at the active site. For both D- and L-β-fluoroalanines, an identical partition ratio of ~820/1 obtains (eq 2), indicating inter alia that each isomer yields a common intermediate, the aminoacrylate–PLP complex, I, which partitions between hydrolysis and release (the safe route) and Michael addition (and inactivation) (Wang & Walsh, 1978). The partition ratio observed is in general not predictable a priori with a given enzyme since one



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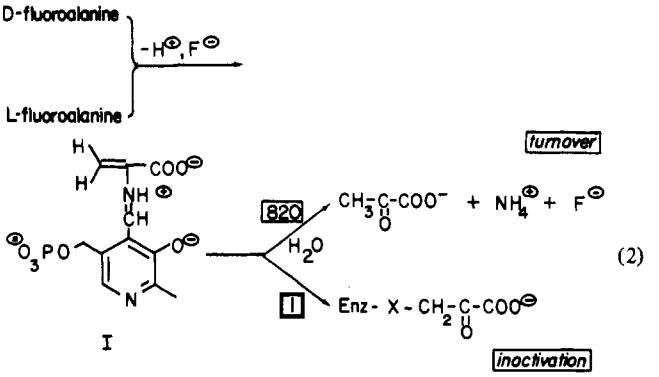

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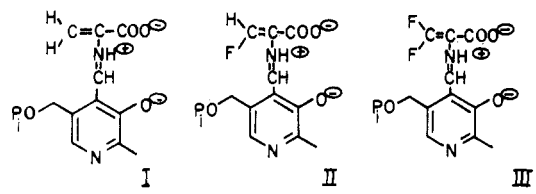
does not know the steric disposition of potential active-site nucleophiles in the absence of a high-resolution X-ray structure. Nonetheless, rational efforts to lower the partition ratio of a suicide substrate seem worthwhile since the more flux is rerouted to the inactivation pathway, the less compound will be required (less catalytic consumption) and the less the chance that uncovered electrophiles will diffuse as reactive products away from the active site of the target enzyme to other cellular constituents.

In this paper, we report the results of variation of the electronic distribution at the β carbon of the key partitioning intermediate, the aminoacrylate–PLP complex, I, in alanine racemase inactivation. This is the likely carbon site undergoing covalent attack by an active-site nucleophile. Increasing its electrophilicity might raise the frequency of alkylative capture relative to the frequency of H_2O addition to the pyridoxal aldimine carbon for hydrolytic release of the product fragment. We have used D- and L-β-difluoroalanines and DL-β-trifluoroalanine with the expectation that they would generate β-mono- and β-difluoroenamino-PLP adducts, II and III, respectively, as the partitioning species in the alanine racemase active site. A preliminary note on a portion of these results has appeared in a conference symposium volume (Walsh et al., 1978).



I II III

Experimental Procedures

Materials

β-Mono- and β,β-difluoroalanines, D and L isomers, were the generous gifts of Dr. J. Kollonitsch of Merck Sharp & Dohme (Kollonitsch et al., 1973; Kollonitsch & Barash, 1976). DL-β,β,β-Trifluoroalanine was purchased from E. Merck. ^3H_2O was obtained from New England Nuclear. *O*-Propionyl-D-serine was synthesized by a modification of *O*-acetylserine synthesis (Sakami & Toennies, 1942). All other chemicals and biochemicals were purchased from Sigma, Boehringer Mannheim, Calbiochem, or Pharmacia.

Methods

Preparation of O-Butyryl-D-serine. *N*-Carbobenzoxy-D-serine was synthesized as described by Moore (1954). To the *N*-carbobenzoxy-D-serine (1.0 g, 0.0042 mol), dissolved in 100 mL of freshly distilled benzene, was added 5 equiv (2.2 mL, 0.21 mol) of *n*-butyryl chloride dropwise with stirring. The solution was refluxed for 1 h, and the solvent was removed under vacuum. After hydrogenolysis by palladium on charcoal (Greenstein & Winitz, 1961), the material was recrystallized in 2-propanol, water, and ether. The white crystals gave a sharp melting point of 163.5–164 °C.

Preparation of Alanine Racemase. Alanine racemase was prepared as described (Wang & Walsh, 1978; Lambert & Neuhaus, 1972), except that the ammonium sulfate fraction (from 120 g wet weight of cells) was applied directly to a 3.4 × 40 cm phenyl-Sepharose column equilibrated in 0.8 M NaCl, 10 mM potassium phosphate, pH 8.0, 0.1 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM mercaptoethanol. After being extensively washed, the enzyme was eluted by the same buffer without NaCl. This step normally gave a 9-fold purification. Enzyme used in all experiments reported here was 30–50% pure and has specific activities of 18–30 units/mg.

Measurement of enzymatic activity, reaction of enzyme with inactivators, and fluoride ion measurement have been described (Wang & Walsh, 1978). Elimination rates were measured in 50 mM potassium phosphate, pH 8.0. Catalytic turnover was measured by following pyruvate (or fluoropyruvate) formation coupled to lactate dehydrogenase (80–800 units/mL) and NADH. All turnover numbers have been corrected with a factor derived from enzyme purity.

Incorporation of Solvent ^3H_2O into Fluorolactate. Alanine racemase (0.86 unit), L-difluoroalanine (20 μmol), NADH (5 μmol), and lactate dehydrogenase (400 units) were combined in 0.5 mL of 100 mM potassium phosphate, pH 8.0, and 0.15 mL of ^3H_2O (1 Ci/g) and incubated at 22 °C for 1 h. An additional 160 units of lactate dehydrogenase and 5 μmol of NADH were added, and the solution was incubated another 2 h (final specific activity of water was 82 mCi/g). The solution was lyophilized repeatedly to remove ^3H_2O and then applied to a 2-mL Dowex 50 column. The water eluate was lyophilized. This material was applied to a 2.2-mL Dowex 1 column. The column was washed extensively with water before development with a 40-mL gradient of 0.005–0.10 N HCl. The radioactive fractions were pooled and lyophilized. Lactate and fluorolactate were estimated by the lactate dehydrogenase hydrazine method (Gotmann & Wahlefeld, 1974). About 30% of the original material was isolated as fluoroacetate.

D-Fluoroalanine was also converted to fluorolactate by D-amino acid oxidase (0.5 unit) and lactate dehydrogenase as described above. The final specific activity of H_2O in the incubation was 81 mCi/g. About 33% of the original fluoroalanine was isolated as fluorolactate.

Results

Inactivation by Di- and Trifluoroalanines. We have previously shown that both D and L isomers of β-fluoroalanine are mechanism-based irreversible inactivators of *E. coli* alanine racemase (Wang & Walsh, 1978). The polyhalo analogues, D-β-difluoroalanine, L-β-difluoroalanine, and DL-β-trifluoroalanine, also show a pseudo-first-order time-dependent inactivation of *E. coli* alanine racemase. Representative inactivation curves are shown in Figure 1. Greater than 98% inactivation of initial enzyme activity was observed consistently. As evidenced in Table I, D-difluoroalanine at a given concentration is a much more potent inactivator than the L isomer. As seen in Figure 2, an increase in the concentration of DL-trifluoroalanine results in a decrease in observed inactivation rate. We tentatively attribute this result to protection effected by the L isomer in the racemic mixture (see below) but have not yet resolved the trifluoroalanine isomers.

The stability of the variously inactivated enzymes was also examined. Alanine racemase that had been inactivated to greater than 95% by D- or L-β-difluoroalanine showed a 44–48% return of the control activity after overnight dialysis in 50 mM potassium phosphate, pH 8.0, 5 mM mercaptoethanol, 0.1 mM PLP, and 0.1 mM EDTA. In contrast, the DL-trifluoroalanine-inactivated alanine racemase regained no activity after dialysis under identical conditions. Also, for comparison, enzyme inactivated by either β-monofluoroalanine isomer was stable to dialysis (Wang & Walsh, 1978), so only the difluoro case (each enantiomer) generates a labile inactive enzyme.

Catalytic Turnover of Di- and Trifluoroalanines. Both D- and L-difluoroalanine isomers are also substrates for net HF elimination with alanine racemase. The products of en-

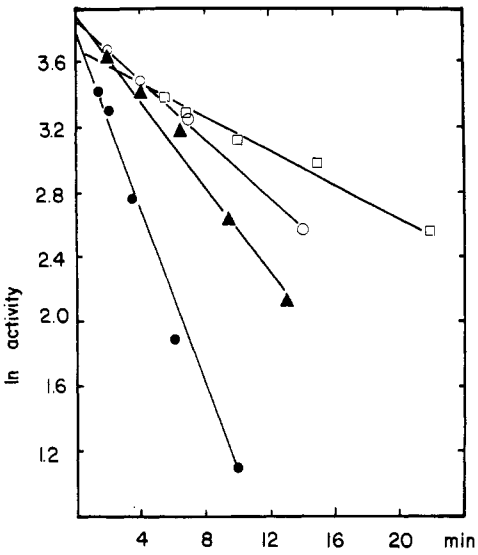


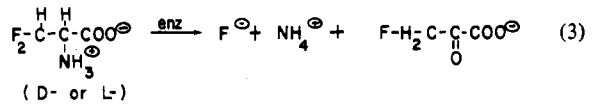
FIGURE 1: Time-dependent inactivation of alanine racemase by D-difluoroalanine: (□) 1.25 mM; (○) 1.67 mM; (▲) 2.5 mM; (●) 5.0 mM.

Table I: Inactivation of Alanine Racemase by Fluoroalanines[a]

compd	concn (mM)	half-time (min)
D-difluoroalanine	1.25	13.9
	1.67	6.1
	2.5	5.0
	5.0	1.63
L-difluoroalanine	8.33	12.4
	10.0	7.6
DL-trifluoroalanine	20.0	2.4
	25.0	14.5
	33.3	40.7
D-fluoroalanine	0.1	0.5[b]
L-fluoroalanine	0.1	7.0[b]

[a] Alanine racemase inactivations were conducted as described previously (Wang & Walsh, 1978). [b] Wang & Walsh (1978).

zyme-mediated elimination are fluoride ion, ammonium ion, and fluoropyruvate (eq 3). Fluoropyruvate was identified by



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the spectrum of its 2,4-dinitrophenylhydrazone derivative (Bohme & Winkler, 1954; Walsh et al., 1971). No pyruvate (5% would have been detectable) was found in the enzymatic incubations. The product was quantitated by coupling the reduction of fluoropyruvate to fluorolactate by lactate dehydrogenase and NADH and also by specifically measuring fluoride ion production with a fluoride ion electrode. The steady-state kinetic constants for the D- and L-difluoroalanine catalytic elimination reactions are shown in Table II.

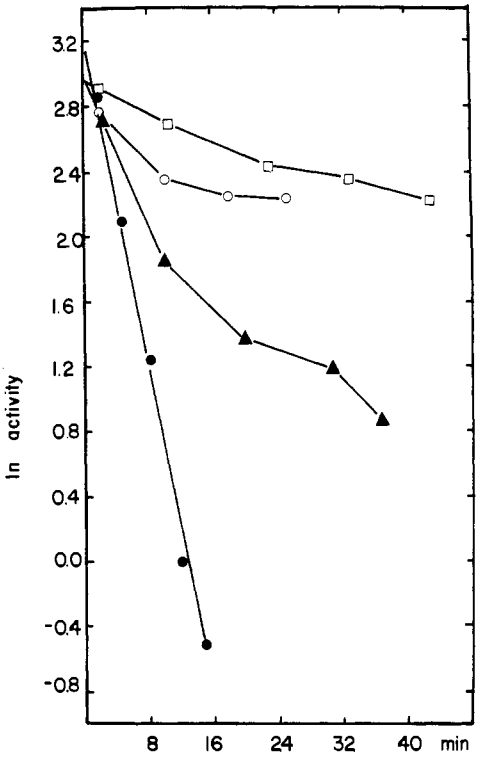
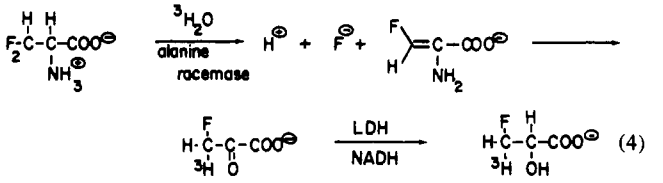


FIGURE 2: Time-dependent inactivation of alanine racemase by DL-trifluoroalanine: (□) 33 mM; (○) 50 mM; (▲) 25 mM; (●) 20 mM.

To demonstrate that the difluoroalanine processing by racemase for HF elimination does indeed involve an enamine–PLP intermediate and then the enamino acid, 3-fluoro-2-aminoacrylate as initial product, incorporation of a solvent-derived hydrogen at C-3 of fluoropyruvate was examined (eq 4). L-Difluoroalanine was incubated with alanine



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racemase in the presence of ^3H_2O, and the fluoropyruvate product reduced immediately to fluorolactate by lactate dehydrogenase (LDH) and NADH. The purified β-[β-^{3}H]-fluorolactate had a specific activity of 2.3×10^5 dpm/μmol, which was 11.2% of the maximal theoretical incorporation (i.e., with no allowance for isotope effect). A control experiment, enzymatic production of fluoropyruvate through an imine, rather than enamine, intermediate from D-monofluoroalanine catalyzed by D-amino acid oxidase (Dang et al., 1976) with analogous reduction to fluorolactate, showed one-tenth the specific activity, 2.32×10^4 dpm/μmol, which was 1% of the

Table II: Kinetic Constants for Catalytic Turnover and Inactivation of Fluoroalanines[a]

compd	V_{max} (min^{-1})[b]	K_m (mM)	source	calcd k_{inact}[c] (min^{-1})	partition ratio[d]
D-fluoroalanine	2860	0.05	Wang & Walsh (1978)	3.6	790
L-fluoroalanine	4475	2.4	Wang & Walsh (1978)	5.6	810
D-difluoroalanine	11730	116	this work	2.2	5000 ± 1000[e]
L-difluoroalanine	820	102	this work	0.33	2600 ± 500[e]
DL-trifluoroalanine	<0.1	ND[f]	this work	≤1.0	10 ± 2
L-alanine racemization	6360	1.3	Wang & Walsh (1978)	none	none

[a] Assays for keto acid production or F^- production were conducted at pH 8.0 as described under Experimental Procedures. [b] Per subunit. [c] From V_{max} and partition ratios (Table III). [d] Turnovers/inactivation event. [e] Error limits are quite large due to kinetic lability of the enzyme adduct formed from the difluoroalanines. [f] ND, not determined.

Table III: Kinetic Data for *O*-Acyl-D-serines

compd	V_{max} elimination (min^{-1})	K_m (mM)	partition ratio	competitive K_I (mM)
O-propionyl-D-serine	3900	4.4	860 (±100)	2.8
O-butyryl-D-serine	3910	10.9	820 (±100)	10.5

theoretical incorporation from water of the same specific radioactivity as above. Thus, the racemase product picks up 10-fold more tritium from ^3H_2O than the oxidase product.

Similarly, the expected products of alanine racemase catalyzed HF elimination from DL-β,β,β-trifluoroalanine would be difluoropyruvate, NH_4^+, and F^-. No lactate dehydrogenase reducible product was observed even with extremely high concentrations of alanine racemase and lactate dehydrogenase. However, production of fluoride ion was measured, and at 0.3% enzymatic activity remaining after exposure to trifluoroalanine, approximately 10 equiv of fluoride ion were produced per mol of enzyme dimer. Given that difluoropyruvate is likely to exist essentially entirely as the *gem*-diol rather than the ketone (Goldstein et al., 1978), it may not be a substrate for the LDH couple, especially at low product concentrations.

The partition ratio for a suicide substrate is defined as noted in the introduction as the ratio of the number of times a substrate turns over to each enzyme inactivation event (Walsh, 1977; Wang & Walsh, 1978). That partition ratio can be calculated either as the ratio of product formed per mole of enzyme inactivated or as the ratio of the turnover rate to the inactivation rate. The partition ratio was calculated in the first way with DL-trifluoroalanine since kinetic production of the product fluoride ion is low; because of the partial reversibility of D- or L-difluoroalanine-induced inactivation, the partition ratio in that case was estimated in the second manner, measuring the initial rates of both fluoride and fluoropyruvate production. The partition ratios for di- and trifluoroalanines are shown in Table II and are compared with our previous values obtained with the monofluoroalanine enantiomers.

Other Substrates for Elimination. The β-substituted compounds, *O*-propionyl-D-serine, and *O*-butyryl-D-serine were also found to be suicide substrates of alanine racemase. At 1 mM final concentration, the compounds induced a pseudo-first-order, time-dependent inactivation of the racemase with half-times of inactivation of 2.1 and 8.1 min, respectively. Inactivation of alanine racemase by *O*-butyryl-D-serine is shown in Figure 3. Inactivation by both suicide substrates was irreversible as judged by failure to regain catalytic activity after dialysis against buffer containing 5 mM mercaptoethanol and 0.1 mM PLP.

As expected, alanine racemase catalyzed a β-elimination sequence on *O*-propionyl-D-serine and *O*-butyryl-D-serine to form pyruvate, ammonium ion, and the corresponding carboxylic acid. Pyruvate was identified and quantitated as described above. The kinetic constants and the partition ratios are listed in Table III. The partition ratios are identical with those obtained with the D- and L-fluoroalanines, the D- and L-chloroalanines, *O*-acetyl-D-serine, and *O*-carbamoyl-D-serine (Wang & Walsh, 1978) and confirm our postulation of a common intermediate, the enamino–PLP complex, which is formed after β elimination and which is the inactivating species. The L isomers of these amino acids do not inactivate and are not detectably processed by the *E. coli* B enzyme (Wang & Walsh, 1978).

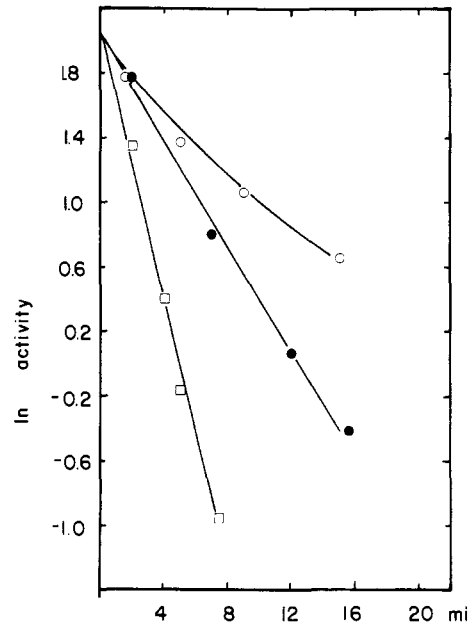


FIGURE 3: Time-dependent inactivation of alanine racemase by *O*-butyryl-D-serine: (○) 0.67 mM; (●) 1.0 mM; (□) 2.0 mM.

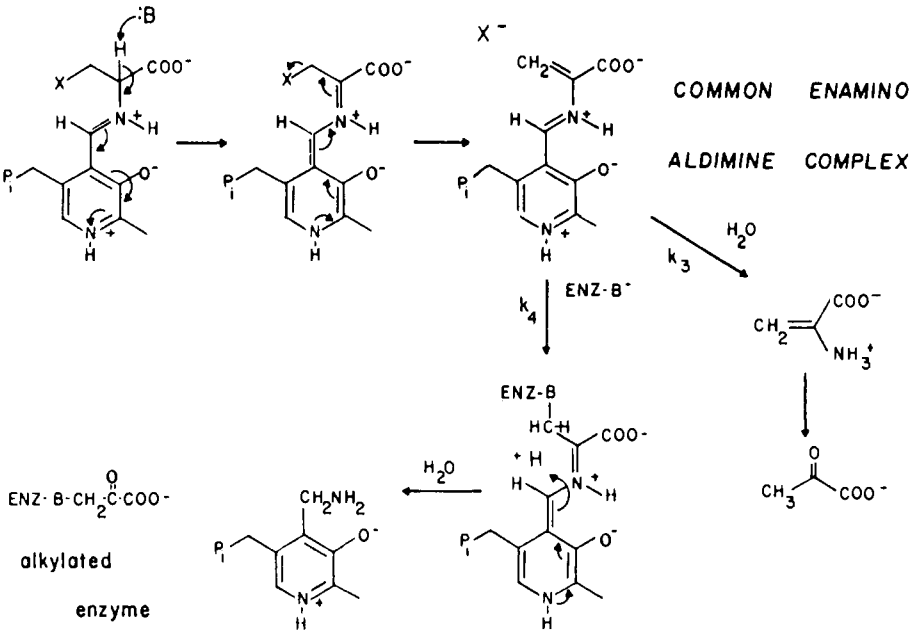
Discussion

Since the initial reports by Kollonitsch, Kahan, and coworkers (1973, 1975) that β-fluoro-D-alanine was a potent antibacterial candidate by way of alanine racemase inhibition, a large number of β-fluoro α-amino acids and related α-fluoromethyl α-amino acids have been prepared and found effective as inhibitors of pyridoxal-P-dependent enzymes in vitro and in vivo. The major common feature of these fluoro amino acids is that they function as mechanism-based inactivators of the target enzymes, partitioning between catalytic loss of HF [for compounds such as β-fluoroalanine (Wang & Walsh, 1978)] or of CO_2, F^- (for α-fluoromethyl amino acids) and enzyme suicidal inactivation (Kollonitsch et al., 1978; Maycock et al., 1980; Metcalf et al., 1978).

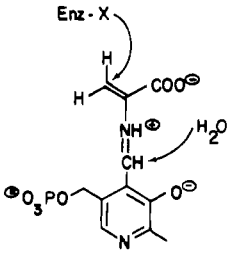
These suicide substrates have not been limited to monofluoro analogues. For example, difluoromethylornithine appears to be a particularly effective suicide substrate for ornithine decarboxylase (Metcalf et al., 1978; Bey, 1978), and its consequent antitrypanosomal activities are recently reported (Bacchi et al., 1980). The trifluoromethyl case has been examined for β,β,β-trifluoroalanine by Silverman & Abeles (1976), who surveyed several PLP-linked enzymes and found trifluoroalanine to be a much more potent inactivator than monofluoroalanine. Two facts were particularly notable in the context of this work. First, they reported, without details, that a *Pseudomonas* alanine racemase (Adams, 1976) was inactivated by trifluoroalanine and second, in a careful and elegant study, observed that the inactivation of rat liver γ-cystathionase occurred with loss of all three fluorines from C-3 of trifluoroalanine and led to an amide linkage in the covalent adduct between the nucleophilic amino group of the attacking enzyme and carbon 3 of the inactivator (now at the acyl oxidation state) (Silverman & Abeles, 1977).

In this paper, we have attempted a systematic comparison of di- and trifluoroalanines with our previous work on monofluoroalanines (Wang & Walsh, 1978) as suicide substrates for the alanine racemase from *E. coli* B, a target enzyme for antibiotics, in an effort to understand how partition ratios,

Scheme I



turnovers/inactivation event, vary with electronic effects on the key partitioning intermediate, the aminoacrylate–PLP complex, which develops after enzyme-catalyzed HF elimination. We have previously adduced evidence (Wang & Walsh, 1978), reinforced here with the *O*-propionyl- and *O*-butyryl-D-serine data, that compound I is the inactivating



species which can either undergo hydrolysis by H_2O attack at the imine carbon, a safe, product-forming route, or be captured by covalent conjugate addition at the olefinic terminus of the electron-deficient enamine. Since the electrophilicity of the enamine should affect the frequency of covalent conjugate addition by nucleophiles, we used isomeric D- and L-difluoroalanines, which should yield monofluoroenamine–PLP (II), and DL-trifluoroalanine, which should yield the corresponding disubstituted fluoroenamine–PLP complex III, as probes for how the electrophilicity of the partitioning species would control reaction flux between turnover and inactivation.

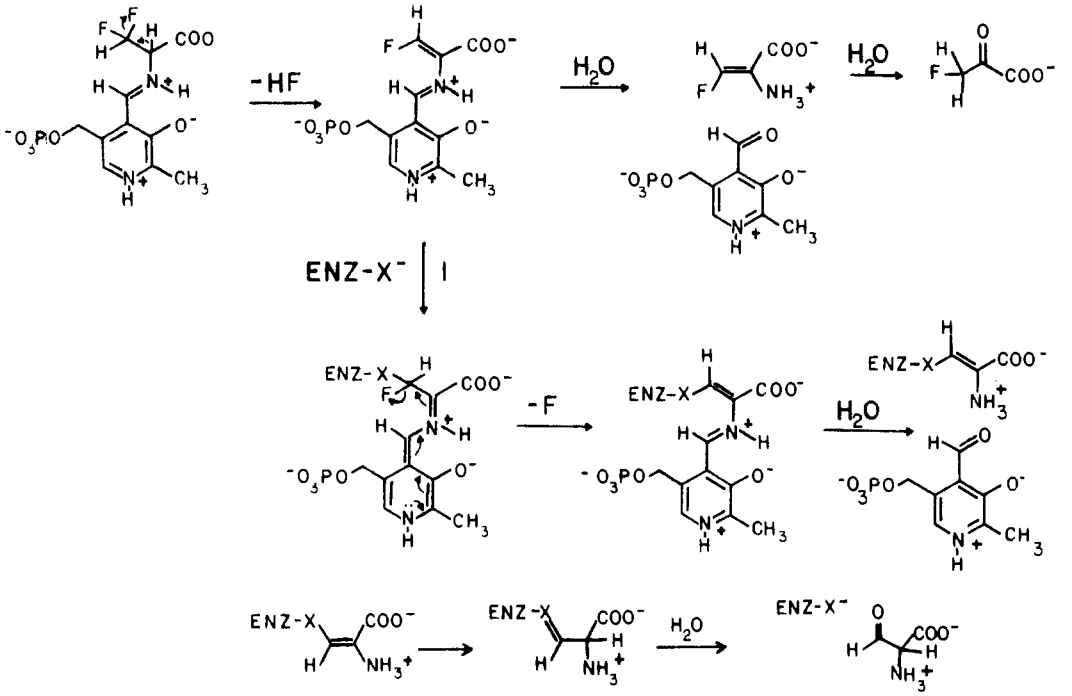
In fact, all three haloalanines, mono, di, and tri, are substrates for HX elimination and suicidal autoinactivation by alanine racemase from *E. coli* B, but there are clear distinctions in elimination rates (V_{max} for turnover), partition ratios, and also the lability of inactivated enzyme species within this haloalanine series. There is the additional constraint that this racemase shows markedly different tolerance for β substituents in D isomers vs. L isomers of the same amino acid, a reflection, no doubt, of the chiral structure of the enzymatic catalyst.

With the natural enantiomeric D- and L-alanine substrates, the K_m and V_{max} values are approximately equal (Lambert & Neuhaus, 1972; Wang & Walsh, 1978). With monofluoro substitution at the β carbon, V_{max} for proton abstraction and β-fluoro group elimination is effectively the same for both isomers and equal to the V_{max} for alanine racemization. But, as we have noted previously (Wang & Walsh, 1978), the K_m for the L-fluoroalanine isomer is up almost 50-fold. In the D- and L-β,β-difluoroalanine cases, the K_m is elevated another 50-fold for both isomers. The substitution of a second fluorine atom at the β carbon has thus elevated the K_m of the D-difluoro isomer by $\sim(2.5 \times 10^3)$-fold compared to D-fluoroalanine. This enormous difference is unlikely to be simply a steric problem in the D-isomer case since *O*-propionyl- and *O*-butyryl-D-serine have K_m values for the equivalent catalytic α,β-elimination sequence of $\sim$4 and $\sim$10 mM, respectively. It is very likely that one needs to measure substrate K_D values separately to factor out rate constant contributions to K_m values, but this must await substrate quantities of enzyme. One possible explanation for the dramatic K_m change with D-difluoroalanine could be the pK_a change of the NH_3^+ group induced by adjacent fluorine substitution. A pK_a of 9.75 for the amino group of alanine is lowered to $\sim$8.35 for monofluoroalanine and to 7.25 for difluoroalanine. The pH of the incubation medium was 8.0, and we have not yet determined K_m and V_{max} as a function of pH to analyze this point. In β,β,β-trifluoroalanine, the pK_a of the α-amino group is down to 5.85, a 10^4-fold decrease in acidity from alanine.[1] Since turnover is so low with the trifluoro amino acid, we have been unable to determine K_m values for the DL mixture nor have we succeeded in resolving them yet. An extrapolation of V_{max} for DL-trifluoroalanine, from half-time for inactivation and the partition ratio (Table II), of $\sim$1 min^{-1} compares with the value of 12 000 min^{-1} for D-difluoroalanine and $\sim$4000 min^{-1} for L-difluoroalanine. The trifluoro V_{max} number may also be low if the L isomer binds but does not turnover and so functions as inhibitor as suggested by Figure 2. The apparent 10^4-fold decrease in V_{max} on moving from di- to trifluoro substitution is probably due to a combination of features, among them possibly the low pK_a of the α-NH_2 group and an electronic effect on α-H abstraction to initiate catalysis.

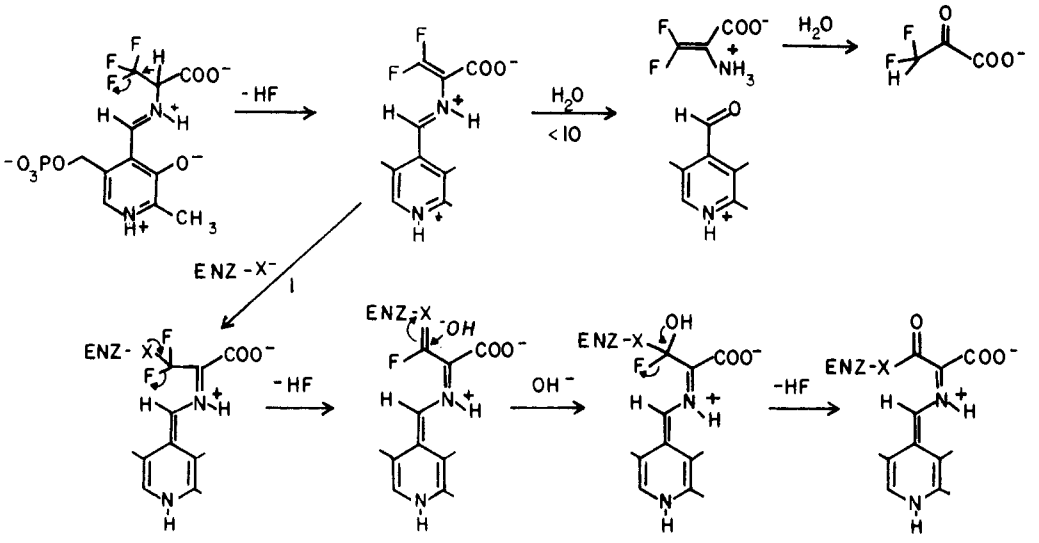
Despite its very low V_{max} value for catalytic processing, DL-trifluoroalanine is, *by criterion of partition ratio* (Table II), the most effective of the fluorinated alanines at inactivation

[1] The carboxyl group pK_as could also contribute, but they change less in the mono- to di- to trifluoro series: pK_a (COOH) for alanine, 2.50; for monofluoroalanine, 3.70; for difluoroalanine, 3.60; for trifluoroalanine, 3.50 (D. Roise, unpublished results).

Scheme II



Scheme III



of alanine racemase in vitro, presumably because nucleophilic capture on difluoroaminoacrylate–PLP complex III at the electron-deficient β carbon kinetically outcompetes Schiff-base hydrolysis. In the difluoroalanine isomers, the monofluoroaminoacrylate–PLP complex II has a β carbon more electrophilic than in I but less so than in III. By this effect, one anticipates a turnover/inactivation ratio somewhere between 800 and 10. The observed values of 2500–5000 suggest that the single fluorine in II has two effects: (1) enhancing reactivity toward Michael addition at the β carbon and (2) enhanced electrophilicity at the α-carbon (Chambers, 1973) which would speed H_2O attack on this Schiff-base carbon. Presumably effect 2 is differentially accelerated by a single β-fluorine, while effect 1 is dominant with two fluorines at the β carbon.

The probably distinct partition ratios for the difluoroalanine isomers are different from the results with monofluoroalanine isomers where identical partition ratios occur (Table II). Enzyme-mediated HF elimination from either isomeric pair produces the same chemical species, II or I, respectively. Until more is known about the binding domain for each substrate isomer and the planar carbanionic intermediate, one must be cautious at speculation, but our previous work on D- and L-*O*-acetyl- and *O*-carbamoylserines (Wang & Walsh, 1978) suggests dramatically different side-chain orientations at the active site or those D vs. L isomers. Under the assumption that D- and L-β,β-difluoroalanines produce the monofluoroenamine-PLP adduct II, it is possible that one isomer yields a geometrical isomer with fluorine cis to the amino group while the other difluoroalanine isomer yields the trans olefinic intermediate geometry. These geometric isomers of II could have distinct pK_as, and a ΔpK_a of $\sim$0.3 unit could produce a 2-fold difference in hydrolysis rates, explaining distinct partition ratios.

The question of differential adduct stability, or rather the unusual lability toward reactivation of the difluoroalanine-treated enzyme, is as yet unsolved, but there is precedent from the work of Sakai & Santi (1973) and Silverman & Abeles (1977) which is highly suggestive. Sakai & Santi used the vinyl-CF_3 analogue of dTMP to inactivate thymidylate

synthetase and observed the enzymatically attacked carbon to be at an acyl oxidation state from loss of all three fluorine atoms. Similarly, Silverman & Abeles (1977) have shown release of all three F^- atoms for each mole of [1-^{14}C]trifluoroalanine bound to γ-cystathionase on inactivation as the β-CF_3 group ends up at an acyl oxidation state after initial attack by an enzyme nucleophile which could be the ubiquitous ϵ-NH_2 of the lysine residue in the PLP–enzyme active site.

If an analogous process were indeed involved with alanine racemase, the structures in Scheme I show a mechanistic proposal essentially identical with that hypothesized by Silverman & Abeles. If enz-X is the lysine ϵ-NH_2 group (stoichiometry of labeling and adduct identification await larger quantities of pure alanine racemase), then the adduct is at the amide oxidation state and will be hydrolytically stable. Assuming the same nucleophile attacks species I from either monofluoroalanine isomer, we would anticipate the β carbon of the inactivator to be at the oxidation state of a secondary amine, also hydrolytically stable in line with our observations.

With the same reasoning for difluoroalanine, the initial adduct from II could readily lose the remaining fluorine as noted in Scheme II to yield a vinyl-X adduct. This is in a formal sense at the aldehyde oxidation state and, depending on the nature of the enzyme-X group, may be hydrolytically labile. The likely attacking nucleophiles are O, S, or N atoms, yielding vinyl ethers, vinyl sulfides, or vinyl amines (enamines), respectively. Of these three, the enamines are likely to be the most susceptible to facile isomerization to iminium ion followed by very rapid hydrolysis to the regenerated enz-NH_2 and aminomalonic semialdehyde. This is consistent with the observed regain of ~50% of initial activity on dilution and dialysis of racemase inactivated with either difluoroalanine isomer, but the idea will require studies with [^{14}C]difluoroalanine for validation. Whether some other enzyme nucleophile accounts for the remainder of adducted, inactive enzyme can also be solved with radioactive label.

In contrast to the lability of difluoroalanine-inactivated alanine racemase toward regain of catalytic activity, α-difluoromethylornithine yields stable inactive adducts with rat liver ornithine decarboxylase and appears curative in trypanosomal or coccidial infections in animals by mechanism-based inactivation of that enzyme in parasites (Scheme III) (Metcalf et al., 1978; Bacchi et al., 1980). Whether the second fluorine atom is eliminated from the initial Michael adduct to the ornithine decarboxylase enzyme to yield a now stable vinyl-X enzyme species (e.g., vinyl ether and vinyl sulfide) remains to be elucidated but suggests that difluoromethyl groups may not always yield unstable adducts.

Finally, given the observation that monofluoroalanine is a suicide substrate that yields stable adducts with alanine racemase and is an effective antibacterial in terminating infections in experimental animals (Kahan et al., 1975), one would expect (and finds) difluoroalanine to be ineffective as an antibiotic target against the racemase, probably due to the double drawbacks of high K_m and ready reversibility of inactivation. In contrast, one would have expected from these studies with the isolated *E. coli* alanine racemase that D-trifluoroalanine should be an effective antibiotic on the basis of its efficiency as a suicide substrate. However, it is not active as an antibacterial agent; the molecular basis of its failure must lie elsewhere than racemase susceptibility. Trifluoroalanine, with a pK_a of the α-amino group of 5.8, will not be zwitterionic at neutral pH and may for that reason fail to be recognized as a transport substrate for the D-alanine and L-alanine carrier proteins in the *E. coli* cytoplasmic membrane; failure to be internalized would prevent access to the intramolecular but otherwise susceptible alanine racemase.[2]

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[2] Preliminary studies suggest that DL-β,β,β-trifluoroalanine at 8 μM concentrations shows inhibition (75%) of transport of L-[^{14}C]alanine by isolated cytoplasmic membrane vesicles of *E. coli* (K. Haldar, unpublished results), so this issue is still complex.

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Solution Properties of Synthetic Chlorophyllide- and Bacteriochlorophyllide–Apomyoglobin Complexes†

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ABSTRACT: Well-defined 1:1 complexes have been formed between apomyoglobin (apoMb) and a number of chlorophyllide derivatives. The chlorophyllides substitute for heme in the pocket of myoglobin. These include magnesium chlorophyllide *a*, magnesium and zinc pyrochlorophyllide *a*, zinc pyrochlorophyllide *b*, zinc pyrochlorophyllide *d*, zinc pyromesochlorophyllide *a*, zinc 2-acetyl-2-devinylpyrochlorophyllide *a*, zinc protopyrochlorophyllide *a*, and zinc bacteriopyrochlorophyllide *a*. The effects of the protein on the electronic absorption, circular dichroism (CD), magnetic circular dichroism, and triplet state electron spin resonance spectra and fluorescence lifetimes in solution are compared with appropriate models in organic solvents. With the exception of the CD spectra, the protein causes shifts and intensity changes which are within the range observed for solvent effects. The CD spectra change substantially: the signs of several transitions are entirely reversed in the chlorins, and 3–6-fold intensity increases are observed with zinc bacteriochlorophyllide *a*. High-field ^{1}H NMR spectra of ring current shifted Val-E11 methyl protons for the series porphyrin–, chlorin–, and bacteriochlorin–apoMb are used to establish the probable absolute orientation of the chromophore in the heme pocket. Doubled peaks in the NMR spectra of certain complexes are shown to arise from interconvertible species. The temperature dependence of the peak intensities and saturation transfer studies show that the species giving rise to the doubled peaks exchange on the time scale of about 1–60 s. Arguments are presented against inversion of the macrocycle in the heme pocket by either an inter- or an intramolecular mechanism as the origin of doubled peaks, and simple two-site exchange is ruled out by the NMR data. We suggest that the data are consistent with the idea that at least two slowly interconverting conformational substates of the protein are populated, depending sensitively on small changes in rings I and II of the macrocycle and temperature.

Although it is widely believed that most chlorophyll in photosynthetic organisms is associated with proteins, little information is available on the consequences of this association. The spectroscopic and photochemical properties of chlorophyll in a large number of different reaction center and antenna complexes have been described (Thornber et al., 1979). Obvious features, such as the electronic absorption maxima and band shapes, and more subtle properties, such as oxidation and reduction potentials, excited state lifetimes, circular dichroism (CD)[1] intensities, and ESR properties of π radicals and excited triplet states, have been shown to differ substantially between various natural chlorophyll–protein complexes and chlorophylls in organic solvents (Sauer, 1978; Thornber et al., 1978; Norris & Katz, 1978). "Chlorophyll–protein interactions" of an undefined nature are commonly invoked to explain the variability of chlorophyll properties in vivo, yet we have little insight into the mechanism by which the protein exerts its influence. By contrast, a substantial literature has developed which suggests the basis for the diversity of properties of other important prosthetic groups in proteins, such as hemes (Collman et al., 1980), iron–sulfur clusters (Holm, 1977), retinal (Nakanishi et al., 1980), and flavins (McCormick, 1977).

An alternative hypothesis is that spectroscopic and functional variability are primarily a consequence of chlorophyll–chlorophyll interactions. Extensive studies of chlorophyll aggregates in organic solvents (Katz et al., 1976), and singly (Boxer & Closs, 1976) and doubly covalently linked chlorophylls (Wasielewski et al., 1978; Bucks & Boxer, 1981), do indeed show a considerable diversity of absorption and chemical properties. It is quite likely that both chlorophyll–protein and chlorophyll–chlorophyll interactions play significant roles in determining the properties of photosynthetic pigments in vivo, and extensive research is directed toward a deeper understanding of both types of interactions [see, for example, Rafferty et al. (1979)].

Our approach to understanding the role of chlorophyll–protein interactions is to prepare *synthetic chlorophyll–protein complexes of defined structure*. An example of this type of

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[1] Abbreviations: 2-Ac-PChl*a*, 2-acetyl-2-devinylpyrochlorophyllide *a*; ANS, 8-anilino-1-naphthalenesulfonate; BPChl*a*, bacteriopyrochlorophyllide *a*; CD, circular dichroism; DMF, dimethylformamide; DSS, 4,4-dimethyl-4-silapentanesulfonate; ESR, electron spin resonance; Hb, hemoglobin; Mb, myoglobin; MCD, magnetic circular dichroism; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; PChl*a*, pyrochlorophyllide *a*; PChl*b*, pyrochlorophyllide *b*; PChl*d*, pyrochlorophyllide *d*; PMesoChl*a*, pyromesochlorophyllide *a*; ProtoPChl*a*, protopyrochlorophyllide *a*; ZFS, zero-field splitting parameters.

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