Suicide Substrates for the Alanine Racemase of *Escherichia coli* B†

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ABSTRACT: Alanine racemase (EC 5.1.1.1) from *Escherichia coli* B has been purified about 9000-fold and found to be a dimer of 100 000 daltons, containing one molecule of pyridoxal phosphate per subunit. The molecular basis for irreversible active site-directed inactivation of the enzyme by a number of suicide substrates has been examined. Both the D and L isomers of β-fluoroalanine and β-chloroalanine partition between (a) α,β elimination to pyruvate, ammonia, and halide ion or (b) inactivation. No racemization is detectable. The V_{max} for pyruvate formation from L-chloroalanine is ca. 50-fold lower than from the D isomer of chloroalanine or either fluoroalanine. However, both enantiomeric pairs partition identically, ca. 830 turnovers per inactivating event. This invariant partition ratio suggests that a common intermediate, the eneamino acid–PLP complex, is the species responsible for inactivation, probably by Michael attack from a nucleophilic residue at the enzyme active site. In keeping with this idea, *O*-carbamoyl-D-serine and *O*-acetyl-D-serine also undergo enzyme-catalyzed elimination for 830 turnovers before causing irreversible inactivation, presumably from the same intermediate. In contrast, the L isomers of *O*-carbamoyl- or *O*-acetylserine do not eliminate nor do they induce inactivation, but serve merely as reversible, competitive inhibitors of the enzyme. This suggests asymmetric binding regions for bulky β substituents at the active site and suggests D isomers of substituted β-alanines would be preferentially effective enzyme inactivators. Finally, D-cycloserine also inactivates the alanine racemase in time-dependent fashion. Thus, both natural antibiotics *O*-carbamoyl-D-serine and D-cycloserine, previously reported as reversible alanine racemase inhibitors, are in fact suicide substrates along with the β-haloalanines.

The D-amino acids are important constituents of bacterial metabolism, especially D-alanine and D-glutamate which are components of the peptidoglycan layer of cell walls in many bacterial species. Since the L isomers of amino acids are generally produced in biosynthetic pathways, formation of D isomers can arise either from equilibration of configuration at the α-carbon starting from the L enantiomer (racemase action) or from appropriately chiral reduction of an imino acid (transaminase action). For example, in *Bacillus sphaericus* and *Bacillus subtilis* a D-amino acid transaminase converts α-ketoglutarate to D-glutamate (Martinez-Carrion & Jenkins, 1965). This may be the source of D-glutamate incorporated into the cell wall of those organisms. On the other hand, D-alanine appears to be formed directly from L-alanine by action of an alanine racemase in many species of bacteria (Adams, 1976).

Given the absence of corresponding racemases or D-amino acid-specific transaminases in animal cells, these bacterial enzymes are likely targets for antibiotics (Park, 1958). Indeed, alanine racemase appears to be a major site of action for two naturally elaborated antibiotics, D-cycloserine and *O*-carbamoyl-D-serine (Neuhaus, 1967), reported as competitive inhibitors of the racemase in *S. aureus* (Roze & Strominger, 1966), *E. coli* (Lambert & Neuhaus, 1972), and *B. subtilis* (Johnston et al., 1968). Recently, Manning and colleagues reported that the D isomer of β-chloroalanine is bacteriocidal (Manning et al., 1974) and noted in crude extracts from treated bacteria that alanine racemase and D-amino acid transaminase activities were no longer detectable. Recent reports have shown that homogeneous D-amino acid transaminases are inactivated by D-β-chloroalanine (Soper et al., 1977b) and D-vinylglycine (Soper et al., 1977a), but we restrict our attention to alanine racemase in this paper. In studies on active transport of solutes in *E. coli* B membrane vesicles, we have noted that both D- and L-chloroalanine could cause time-dependent, irreversible inactivation of alanine racemase associated with the isolated cytoplasmic membrane vesicles (Kaczorowski et al., 1975a,b). Additionally, Kollonitsch et al. (1973) have reported that D-β-fluoroalanine (Kollonitsch & Barash, 1976) is bacteriocidal and in combination with D-cycloserine showed promising activity in terminating infections in experimental animals (Kahan et al., 1975), presumably by inhibition of alanine racemase and D-alanyl-D-alanine ligase. They further noted the interesting point that D-[2-^{2}H]-3-fluoroalanine was more efficacious than the protio compound reflecting a kinetic isotope effect in some enzymatic processing of fluoroalanine (Kahan et al., 1975).

Although homogeneous alanine racemase has been prepared from *Pseudomonas putida* (Rosso et al., 1969) and considerably purified preparations reported from *B. subtilis* (Diven et al., 1964), and *E. coli* W (Lambert & Neuhaus, 1972), little if any mechanistic information has been reported in detail, especially on the molecular aspects of antibiotic action.

In continuation of our previous studies on membrane-associated alanine racemase from *E. coli* B (Kaczorowski et al., 1975a,b), we have now purified the alanine racemase from that organism to ca. 50% homogeneity and studied its mode of interaction with both β-chloroalanines and β-fluoroalanines. We report here that the D and L enantiomers of each haloalanine are suicide substrates (Bloch, 1971; Walsh, 1977; Maycock & Abeles, 1976; Rando, 1974), partitioning between (a) α,β elimination of HX to form pyruvate and ammonia (as noted by us earlier for chloroalanine [Kaczorowski et al., 1975b]) and (b) enzyme inactivation. We also show that the natural products D-cycloserine and *O*-carbamoyl-D-serine, long thought to be reversible competitive inhibitors, are also mechanism-based irreversible inactivators.

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Experimental Section

Materials

The following compounds were synthesized according to published procedures: chloropyruvate (Cragoe & Robb, 1960); D-*erythro*- and *threo*-chloroaminobutyrate (Walsh et al., 1973); *O*-acetyl-D-serine (Sakami & Toennies, 1942); *O*-sulfo-D-serine (Tudball, 1962); *S*-carbamoyl-D-serine (McCord & Skinner, 1963); *S*-methyl-D-cysteine (duVigneaud et al., 1934); and L-cycloserine (Plattner et al., 1957).

D- and L-chloroalanine and D- and L-*O*-carbamoylserine were purchased from the Vega Fox Biochemical Co. L-Alanine dehydrogenase was obtained from Boehringer-Mannheim and was dialyzed against 20 mM Tris-HCl[1] pH 8.0, 0.1 mM EDTA before use. D-Amino acid oxidase was prepared from frozen hog kidneys obtained from the Pel-Freeze Co. by the method of Brumby & Massey (1968). [^{3}H]H_2O (100 mCi/g) was obtained from New England Nuclear; [^{14}C]proline (270 mCi/mmol) was purchased from Amersham/Searle. All fluoroalanines (Kollonitsch & Barash, 1976) were the generous gift of Dr. D. J. Kollonitsch of Merck, Sharp and Dohme. The [1,2-^{14}C,2-^{2}H]-D-fluoroalanine was labeled at carbons 1 and 2 (specific activity of 1.28 mCi/mmol). All other chemicals and biochemicals were purchased from Sigma.

All inhibitor solutions were prepared fresh daily. Cycloserine was stored desiccated at −20 °C and solutions prepared in 100 mM potassium phosphate buffer (pH 8.0) to minimize decomposition and dimerization (Khomutov et al., 1963).

Methods

Preparation of Alanine Racemase. E. coli B cells were grown at 37 °C on the mineral medium of Davis & Mingioli (1959) supplemented with 1.0% glycerol and 0.5% D,L-alanine and harvested at *late log* phase.

Alanine racemase was prepared essentially according to Lambert & Neuhaus (1972) except that 0.1% Triton X-100 was used in the initial bacterial cell suspension to replace the 1-butanol extraction. Typically the yield from 23 g of cells after the first DEAE column was fractionated on a 1.6 × 40 cm DEAE-Sephadex A-50 column equilibrated with 20 mM ethylenediamine, 1.0 mM mercaptoethanol, 0.1 mM EDTA, and 5% glycerol and eluted with a gradient of 0.04 to 0.28 M NaCl to provide another tenfold purification. A portion of this enzyme was then fractionated on a 1.5 × 60 Sephadex G-200 column in 20 mM Tris, 5% glycerol, 1 mM mercaptoethanol, and 0.1 mM EDTA. Enzyme emerged at a position corresponding to about 103 000 molecular weight. Active fractions were concentrated on an Amicon ultrafiltration apparatus and frozen in liquid nitrogen. The Sephadex G-200 column was calibrated using pyruvate kinase, yeast alcohol dehydrogenase, ovalbumin, and lysozyme as molecular weight standards.

Purity of the enzyme was estimated from Gilford gel densitometer scans of 7% polyacrylamide disc gels, prepared according to Ornstein (1964) and stained with 1% Amido Black. Active enzyme was eluted from unfixed gels by incubating 3-mm slices in 0.1 M potassium phosphate buffer (pH 8.0) at 4 °C overnight. This pure enzyme was analyzed on sodium dodecyl sulfate–polyacrylamide gels (Weber & Osborn, 1969).

Measurement of Enzymatic Activity. All assays were performed at 37 °C. A unit of enzyme is that amount which catalyzes the formation of 1 μmol of product per min at pH 8.0. Activity was measured in either direction (L → D or D → L) by monitoring pyruvate formation by a coupling enzyme from the product on a Gilford 220 spectrometer. In the L to D direction, the D-alanine initially produced was converted to pyruvate by a saturating amount of D-amino acid oxidase. Activity was followed by the oxidation of NADH at 340 nm as the pyruvate was then reduced by lactate dehydrogenase and NADH. A standard assay consisted of 0.05–0.30 unit of enzyme, 0.60 unit of D-amino acid oxidase, 80 units of lactate dehydrogenase, 20 μmol of L-alanine, 0.16 μmol of NADH, and 50 μmol of potassium phosphate at pH 8.0 in a 1-mL volume. In the D to L direction activity was monitored by the production of NADH as the L-alanine was converted to pyruvate and ammonia by saturating amounts of L-alanine dehydrogenase. A standard assay contained 0.02–0.10 units of enzyme, 0.45 unit of L-alanine dehydrogenase, 5 μmol of D-alanine, 100 μmol of NAD^+, and 50 μmol of potassium pyrophosphate at pH 9.0 in a 1-mL volume. Enzyme used in all assays was 30–50% pure.

Reaction of Enzyme and Inactivators. The products from alanine racemase action on various β-haloalanines were determined as the 2,4-dinitrophenylhydrazones spectrophotometrically by the method of Böhme & Winkler (1954) and Walsh et al. (1971). Authentic chloropyruvate, fluoropyruvate, and pyruvate served as standards.

The catalytic turnover of substrates which also caused irreversible inactivation was measured by following pyruvate formation (the sole product from the inactivators) coupled to lactate dehydrogenase and NADH. Racemization of the isomer was tested by using the standard L-alanine to D-alanine assay or the D-alanine to L-alanine assay.

Time-dependent inactivation was measured by incubating alanine racemase and inhibitor in pH 8.0 buffer (total volume 0.1–0.15 mL) at 20 °C and periodically removing 10–25-μL aliquots for dilution into a standard assay. Irreversibility was assayed by overnight dialysis against a 4000-fold volume of buffer with or without pyridoxal-P (1 mM), mercaptoethanol (10 mM), or pyruvate (10 mM).

Substrate α-proton exchange catalyzed by alanine racemase was measured by incubating [^{3}H]H_2O, alanine racemase, substrate, and buffer for 1 h at 25 °C. The solution was frozen and lyophilized repeatedly to remove the remaining [^{3}H]H_2O. The amino acids were isolated by elution from a Dowex 50 [H^+] column by 1 M NH_4OH or 1 M HCl. Recovery of the amino acids was estimated by the ninhydrin method of Rosen (1957).

Covalent, Irreversible Inactivation by [^{14}C]-D-*Fluoroalanine.* Alanine racemase, 0.59 mg, at a specific activity of 28.5 U/mg (~44% pure), was incubated with 0.02 mmol of [1,2-^{14}C,2-^{2}H]-D-fluoroalanine in a volume of 1.5 mL; the enzyme showed no remaining activity after 15 min. The solution was then applied to a 1.4 × 30 cm Sephadex G-25 column and eluted with 20 mM Tris-HCl (pH 8.0) at 4 °C. The protein fractions were combined, dialyzed against 50 mM potassium phosphate (pH 8.0), and then lyophilized.

Protein was estimated by the method of Lowry et al. (1951) and samples were counted for radioactivity.

The absorption spectra of alanine racemase (44% pure, 0.4 mg/mL) on a Hitachi Perkin-Elmer 200 spectrophotometer and the fluorescence spectra of the same enzyme solution on a Perkin-Elmer fluorescence spectrometer MPF-4 were recorded before and after racemase inactivation by [1,2-^{14}C,-2-^{2}H]-D-fluoroalanine.

Fluoride Ion Measurement. Fluoride ion was measured with

[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate; NADH, reduced form of nicotinamide adenine dinucleotide; NAD^+, nicotinamide adenine dinucleotide; DEAE, diethylaminoethyl.

an Orion Model 94-09A fluoride electrode and a Model 90-01 reference electrode on a Beckman Model 3500 pH meter. Measurements were taken using an equal volume of Orion total ionic strength activity buffer. The instrument was calibrated using Orion fluoride standard.

Membrane Preparation and Transport Assays. Membrane vesicles were prepared and assayed as in Kaczorowski et al. (1975a). Vesicles (4 mg of protein) were incubated at 25 °C with 5 mM fluoroalanine (either isomer), 10 mM $MgSO_4$, and 100 mM potassium phosphate, pH 7.5 (final volume 1 mL), for 15 min. Then 9 mL of cold buffer was added and the mixture centrifuged. The pellet was resuspended again and recentrifuged before assaying the vesicles for active transport.

Results

Purification and Properties of E. coli B Alanine Racemase. Lynch & Neuhaus (1972) reported the partial purification of alanine racemase from *E. coli* W, grown either on L-alanine or D,L-alanine as carbon source to increase levels of enzyme activity. At specific activities of ca. 1700 μmol of amino acid racemized per h per mg of protein, they reported two bands on disc gel electrophoresis. By gel filtration analysis, active alanine racemase migrated as a species of 98 000 molecular weight. K_m values of 0.97 mM for L-alanine and of 0.42 mM for D-alanine were obtained in discontinuous assays at pH 8.0.

We have followed the general outlines of the Lynch & Neuhaus procedure for the *E. coli* B cells except that the low temperature butanol step was troublesomely irreproducible. Since we believe, on the basis of our experiments in *E. coli* B membrane vesicles (Kaczorowski et al., 1975a,b), that alanine racemase is a peripheral enzyme of the *E. coli* cytoplasmic membrane, 0.1% Triton X-100 was substituted for butanol as a solubilizing agent. The best specific activities obtained to date have been ca. 28.5 U/mg, by continuous assay in the L-alanine → D-alanine direction at pH 8.0. By disc gel analysis under nondenaturing conditions two protein bands are detectable; only one of these showed activity on elution from the gel. When this eluted enzyme band was subsequently subjected to sodium dodecyl sulfate-polyacrylamide disc gel electrophoresis, a single band with a mobility corresponding to molecular weight of 49 000 was observed. We thus suspected that active enzyme is dimeric, in view of the data of Lynch & Neuhaus (1972) for *E. coli* W and have confirmed this by gel filtration chromatography on Sephadex G-200, where active enzyme eluted in a single peak with Stokes radius corresponding to a 103 000 molecular weight.[2]

It is worth passing mention that we have used continuous spectrophotometric assays in each direction: measuring O_2 uptake (or NADH production) in the presence of homogeneous hog kidney D-amino acid oxidase for the L-alanine → D-alanine direction, and measuring NADH production in the presence of NAD and *B. subtilis* L-alanine dehydrogenase for the D-alanine → L-alanine direction. The D- to L-alanine assays were performed at pH 9.0 rather than pH 8.0 as in the L to D assay because the pH optimum of the coupling enzyme L-alanine dehydrogenase is pH 10.0 (Yoshida & Freese, 1965). These assays have facilitated enormously our ability to detect the irreversible inactivations discussed later in this manuscript.

In their 1972 paper Lynch & Neuhaus had not shown directly that the *E. coli* W enzyme contained pyridoxal-P. They did note, as do we with the *E. coli* B enzyme, that there is no dependence on or stimulation by added pyridoxal phosphate, in contrast to other alanine racemases (Adams, 1976). They also demonstrated, as have we previously (Kaczorowski et al., 1975a), that hydroxylamines are reversible inhibitors. We have now recorded both the absorption and fluorescence spectra of the *E. coli* B enzyme. Enzyme at a specific activity of ca. 28 U/mg has a characteristic enzyme-bound pyridoxal phosphate absorbance peak at 420 nm. Assuming ~44% purity and one pyridoxal phosphate per 50 000 dalton subunit, we calculate an extinction coefficient of $\epsilon_{420} = 4.4 \times 10^4$ within the range expected (Snell and DiMari, 1972) for enzyme-bound pyridoxal phosphate chromophore. The enzyme also shows a fluorescence excitation maximum at 425 nm and an emission maximum at 510 nm typical of the vitamin B-6 fluorophore.

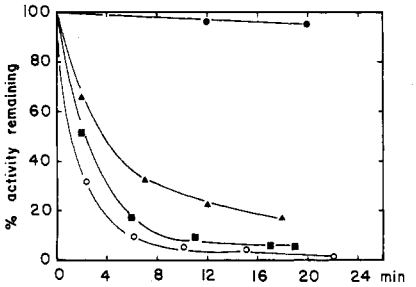


FIGURE 1: Time-dependent inactivation of alanine racemase by fluoroalanine. (●—●) Control; (▲—▲) 0.1 mL L-fluoroalanine; (■—■) 0.4 mM L-fluoroalanine; (○—○) 0.1 mM D-fluoroalanine.

For the chemically symmetrical reaction D-alanine ⇌ L-alanine, the equilibrium constant must equal unity and this has been validated with other racemases (Adams, 1975). The Haldane equation (Briggs & Haldane, 1925) allows one to compute K_{eq} from measured V_{max} and K_m values. With enzyme of specific activity 6.83 the $V_{max_{L\to D}}$ was 1124 s^{-1} and $V_{max_{D\to L}}$ was 974 s^{-1}, under identical conditions at pH 9.0. The K_m for L-Ala was 1.3 mM and for D-Ala was 1.2 mM.

$$K_{eq} = \frac{K_m^{\text{D-Ala}} V_{max}^{\text{L-Ala}}}{K_m^{\text{L-Ala}} V_{max}^{\text{D-Ala}}} = 1.07$$

Enzymatic Behavior with β-Chloro- and β-Fluoroalanines Inactivation. When the purified alanine racemase was exposed to either the D isomer or the L isomer of β-fluoroalanine (Figure 1), a time-dependent loss of activity ensued as shown. The activation displayed pseudo-first-order kinetics in remaining enzyme activity as expected for a process which occurs only from some preformed complex of amino acid and enzyme (Shaw, 1970; Walsh, 1977). The inactivation was irreversible as judged by failure to regain catalytic activity after gel filtration to remove excess fluoroalanine or dialysis against mercaptoethanol (10 mM), pyridoxal phosphate (1 mM), or pyruvate (10 mM). Similar kinetic patterns of irreversible inactivation were observed with the D and L isomers of β-chloroalanine. The resultant enzyme was inactive whether assayed in the L → D direction or in the D → L direction with alanine as substrate.

There are kinetic differences observed in the first-order rate constants for inactivation by the D or L enantiomer of each β-haloalanine. As noted in Table I, the half-time for L-fluoroalanine-induced loss of activity at 0.1 mM concentration is 7 min. An identical concentration of D-fluoroalanine gives a half-time of ≤0.5 min,[3] some 14-fold more efficient in time. An analogous pattern holds with the chloroalanine isomers,

[2] We estimate that homogeneous *E. coli* B alanine racemase has a specific activity of ca. 64 U/mg corresponding to an active site turnover number of ~53 catalytic events per s at 37 °C, pH 8.0, 20 mM L-alanine.

[3] This is an upper limit. Rapid kinetic experiments may be required for obtaining good precision of k_{inact}.

TABLE I: *E. coli* Alanine Racemase Suicide Substrates.

Substrate	Product	$\frac{V_{max}\text{ (elimination)}}{\text{alanine racemization}}$[a]	K_m (elimination)	Partition ratio[b]	Half-time for inactivation (min)[c]
D-Fluoroalanine	Pyruvate	0.9	0.05 mM	790	0.5 (0.1)
L-Fluoroalanine	Pyruvate	1.4	2.34 mM	810	7.0 (0.1)
D-Chloroalanine	Pyruvate	1.00	0.15	920	3.0 (0.5)
L-Chloroalanine	Pyruvate	0.1	25.3	790	7.0 (20)

[a] L to D direction, at 20 mM L-alanine, pH 8.0. [b] Per mole of dimer. [c] Concentration is in parentheses in units of mM.

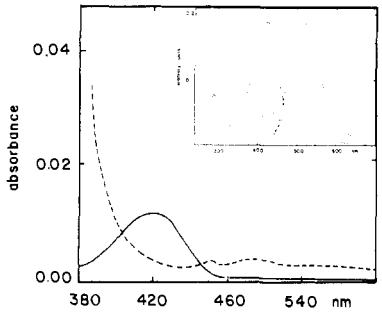


FIGURE 2: Absorption spectra of alanine racemase. (—) Native enzyme; (- - -) enzyme treated with [1,2-^{14}C, 2-^{2}H]-D-fluoroalanine (see text). (Insert) Fluorescence spectra of alanine racemase. (—) Native enzyme; (- - -) enzyme treated with [1,2-^{14}C, 2-^{2}H]-D-fluoroalanine. Excitation spectrum, λ_{em} = 500 nm; emission spectrum, λ_{ex} = 420 nm.

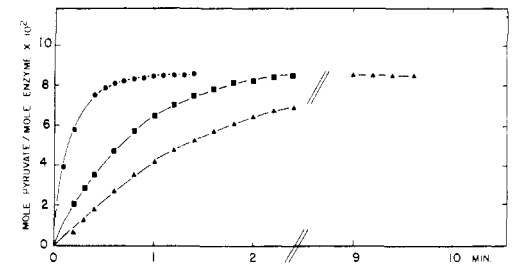
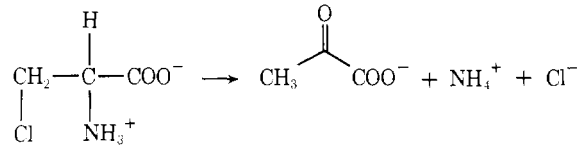


FIGURE 3: Pyruvate production from L-fluoroalanine catalyzed by alanine racemase at (●—●) 3 mM, (■—■) 0.5 mM, (▲—▲) 0.2 mM.

the D much more efficient than the L isomer by this kinetic criterion. At 40-fold higher concentrations than the D isomer, the L isomer of chloroalanine still exhibits a half-time for inactivation about twofold longer (3 vs. 7 min), a 100-fold sensitivity difference.[4]

Covalent Labeing. To test for covalent labeling of alanine racemase by some moiety of the haloalanine inactivators [1,2-^{14}C]-D-fluoroalanine, kindly provided by Dr. Janos Kollonitsch and his colleagues of Merck, Sharp and Dohme, was used. The radioactive sample was deuterated at carbon 2 but it was determined separately that there was no kinetic isotope effect on inactivation rate. The difference in UV–visible spectrum on inactivation after incubation of alanine racemase and [^{2}H, ^{14}C]-D-fluoroalanine is shown in Figure 2. The pyridoxal chromophore was drastically modified on inactivation. The absorbance below 380 nm dramatically increased but this was due to pyruvate production before inactivation (vide infra). Separation of the inactive enzyme from unreacted [^{14}C]fluoroalanine and product molecules on a column of Sephadex G-25 yielded a protein peak containing radioactivity corresponding to 1 to 1.3 labels per subunit. After extensive dialysis of the pooled protein fractions 1.8 nmol of fluoroalanine-derived label remained per nmol of enzyme dimer.

Catalytic Turnover before Inactivation. In our previous studies on *E. coli* B cytoplasmic membrane vesicles which contain alanine racemase and a D-alanine dehydrogenase (but no L-alanine dehydrogenase) (Kaczorowski et al., 1975a), we noted that, while the membranes racemized L-alanine to D-alanine, they did not racemize L-chloroalanine to D-chloroalanine; rather they converted L-β-chloroalanine to pyruvate, NH_4^+ and Cl^-, suggesting the racemase could carry out an



α,β elimination of HCl, as noted for some other pyridoxal enzymes and some flavoenzymes (e.g., Tate et al., 1969; Morino & Okamoto, 1973; Soper et al., 1977b; Walsh et al., 1971, 1973; Cook & Wedding, 1976).

We have now analyzed the ability of the purified enzyme to carry out catalytic turnover of D- and L-β-chloroalanine and D- and L-β-fluoroalanine, to α-keto acid, by a coupled spectrophotometric assay with NADH and lactate dehydrogenase. As shown in Figure 3, pyruvate is produced initially but its rate of production falls off and ceases with time, exactly paralleling the time-dependent loss of activity of alanine racemase. Aliquots were assayed for identity of α-keto acid by analysis of the spectra of the 2,4-dinitrophenylhydrazone derivatives (Böhme & Winkler, 1954; Walsh et al., 1971) and shown to be pyruvate, not chloropyruvate or fluoropyruvate, proving that the halogen had been eliminated. Equimolar amounts of fluoride ion, determined by a fluoride selective electrode, and pyruvate, determined by its reduction to lactate by NADH and lactate dehydrogenase, were found upon inactivation of alanine racemase by D-fluoroalanine. Data for the two enantiomeric pairs of the β-haloalanines are collected in Table I and emphasize two points. First, the initial rates of pyruvate production, at comparable concentrations, normalized to L-alanine racemization, are faster from the D isomers of the haloalanines than from the L isomers, paralleling the half-times for enzyme inactivation noted above and also shown in this table. These data are consistent with processing of the β-haloalanine suicide substrates to an intermediate which can partition either to form a product molecule of pyruvate or to cause enzyme inactivation. It is interesting that the smaller β-fluorine substituent permits faster enzymic processing than the β-chlorine substituent, despite the chemical expectation that chloride ion will be a better leaving group than fluoride ion.

[4] At high concentrations of D-alanine, substrate inhibition (Lynch & Neuhaus, 1972) is observed in the D → L direction, producing upward curvature in double-reciprocal plots. Similar curvature is observed in plots of $1/k_{obsd}$ for inactivation vs. 1/[inactivator] with all the D isomers of inactivators tested: K_I values for these compounds are thus not easily computed.

TABLE II: *E. coli* Alanine Racemase Suicide Substrates.

Substrate	Inhibition	Product	$\frac{V_{max}\text{ elimination}}{\text{alanine racemization}}$[a]	K_m elimination (mM)	Partition ratio[b]	Half-time (min) for inactivation[c]
O-Carbamoyl-D-serine	Irreversible	Pyruvate	0.22	1.6	800	4.5 min (1 mM)
O-Carbamoyl-L-serine	Competetive reversible					K_i = 4 mM
O-Acetyl-D-serine	Irreversible	Pyruvate	0.23	1.9	860	9.0 min (1 mM)
O-Acetyl-L-serine	Competitive reversible					K_i = 58 mM

[a] L to D direction, at 20 mM L-alanine, pH 8.0. [b] Per mole of dimer. [c] Concentration in parentheses.

The second point of interest is the calculation of the partition ratio (Walsh, 1977; Walsh et al., 1978)—the number of times a haloalanine substrate will provide a product molecule for each time an enzyme molecule is inactivated, since both the amounts of enzyme and pyruvate are known. Table I shows that this ratio is between 790 and 870, essentially identical within experimental error, for all four forms of the two haloalanine suicide substrates. Thus, independent of how fast a D or L isomer of chloro- or fluoroalanine turns over with the enzyme, the fate of the partitioning intermediate is constant, inactivating the enzyme on the average once in about every 830 catalytic cycles.

The aggregate data of Table I strongly imply that these four suicide substrates produce a common inactivator since the partitioning ratio is independent of the nature of -X, the initial chirality of the substrate, and the V_{max} of processing. The most likely candidate for this common intermediate is the eneamino acid-pyridoxal-P complex shown as the partitioning intermediate in Scheme I. It is symmetric and of identical geometry whether formed from a D- or L-amino acid.

Behavior of Alanine Racemase with o-Carbamoylserine and Cycloserine. At this juncture two naturally occurring inhibitors of bacterial alanine racemase noted in the introductory section, *O*-carbamoylserine and cycloserine (Neuhaus, 1967), were investigated; in each case it is the D enantiomer which is the natural product.

O-Carbamoyl-D-serine was studied by Lynch & Neuhaus with the *E. coli* W racemase (1972). In their discontinuous assays the compound behaved as a competitive reversible inhibitor with a K_i of 0.75 mM. However, it appears that the inhibitor and either enantiomer of alanine were preincubated together with enzyme and then an aliquot was removed and assayed (Lambert & Neuhaus, 1972). Such a protocol would minimize detection of irreversible inactivation since substrate would have a protectant effect. We suspected that *O*-carbamoyl-D-serine might be, in fact, an irreversible inactivator since the carbamoyl substituent in the β position is a potential leaving group, either as carbamate, or perhaps collapsing to CO_2 and ammonia with general acid catalysis. Such a β elimination would lead to the eneamine suggested above to be the inactivator of alanine racemase. Figure 4 shows that indeed *O*-carbamoyl-D-serine induces irreversible first-order loss of enzyme activity. Further, Table II shows that *O*-carbamoyl-D-serine qualifies as a suicide substrate, turning over an average of 800 times to pyruvate for every enzyme inactivation event. This is precisely the same partition ratio observed with the haloalanines and strongly corroborates the idea that the bound eneamino acid is the partitioning intermediate.

When *O*-carbamoyl-L-serine was tested with the purified alanine racemase, neither irreversible inactivation nor catalytic β elimination of the *O*-carbamoyl group could be detected. The L isomer is a reversible inhibitor with $K_i \sim 58$ mM; it can bind

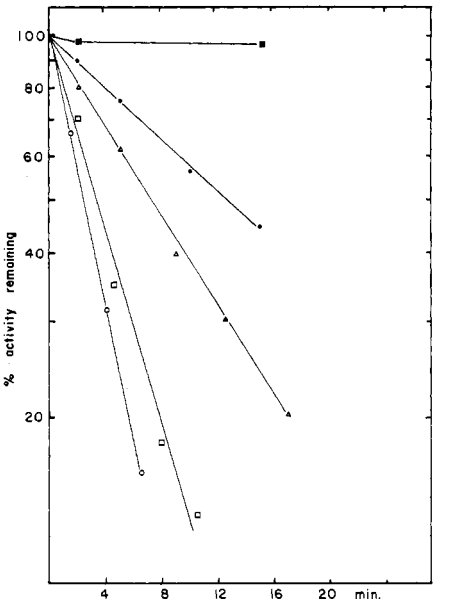


FIGURE 4: Inactivation of alanine racemase by *O*-carbamoyl-D-serine. (■—■) Control; (●—●) 0.5 mM; (△—△) 0.57 mM; (□—□) 1.0 mM; (○—○) 1.33 mM.

to the active site but apparently cannot undergo catalysis (see next section also).

D-Cycloserine (4-amino-3-isoxazolidinone) was investigated for several reasons. First, Lynch & Neuhaus (1972) had noted it was also a competitive reversible inhibitor of the *E. coli* W alanine racemase with a K_i of 0.65 mM. Yet, it is known that cycloserine can cause time-dependent inactivation of other pyridoxal-P enzymes. For instance, L-cycloserine inactivates L-aspartate transaminase (Braunstein, 1973). Third, D-fluoroalanine and D-cycloserine were used in combination against infections in experimental animals (Kahn et al., 1975), and the synergism observed might derive in part, on a molecular level, from both serving as irreversible inactivators of alanine racemase. (Neuhaus (1967) has pointed out that D-cycloserine competitively inhibits the next enzyme, D-alanine-D-alanine ligase, in peptidoglycan biosynthesis.) Indeed, D-cycloserine does inactivate the *E. coli* B alanine racemase in a time-dependent manner. It has not yet been determined whether this isoxazolidinone undergoes catalytic turnover before inactivation. L-Cycloserine (Plattner et al., 1957) has been examined for irreversible inactivation in preliminary experiments; Lynch & Neuhaus (1972) noted it was as good a reversible inhibitor of the *E. coli* W racemase as D-cycloserine with a K_i of 2.1 mM. Our preparations of L-cycloserine inactivated the purified *E. coli* B racemase much more slowly than the D-cycloserine. As a comparison the half-time for loss of enzymatic activity is 4 min at 1.0 mM D-cycloserine but is 10 min at 16.7 mM

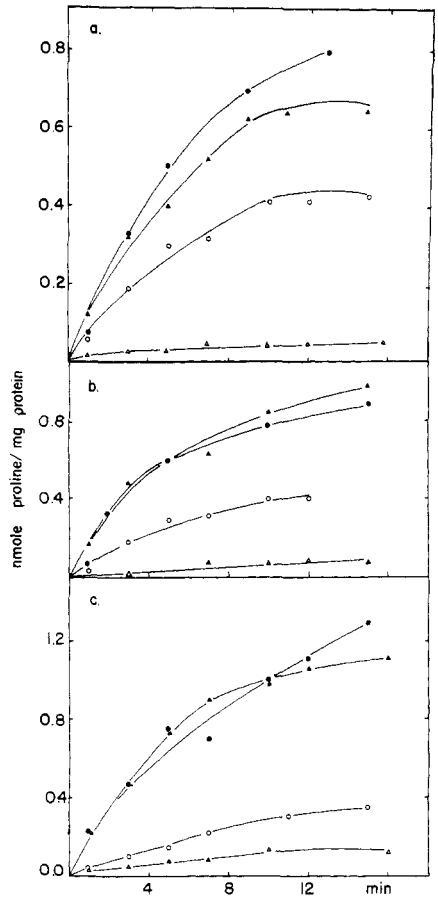


FIGURE 5: Effect of fluoroalanine and D-cycloserine on active transport. (a) D-fluoroalanine: (●—●) D-alanine energized, control; (▲—▲) D-alanine energized, 5 mM D-fluoroalanine; (○—○) L-alanine energized, control; (△—△) L-alanine energized, 5 mM D-fluoroalanine. (b) L-Fluoroalanine: (●—●) D-alanine energized, control; (▲-▲) D-alanine energized, 5 mM D-fluoroalanine; (○—○) L-alanine energized, control; (△—△) L-alanine energized, 5 mM L-fluoroalanine. (c) D-cycloserine: (●-●) D-alanine energized, control; (▲-▲) D-alanine energized, 5 mM D-cycloserine; (○-○) L-alanine energized, control; (△—△) L-alanine energized, 5 mM D-cycloserine.

L-cycloserine. Thus, while both enantiomers inactivate the *E. coli* B alanine racemase, the D isomer is ca. 40-fold more effective.[5]

Catalytic Behavior with Other Amino Acids. At this point features which condition the specificity of the *E. coli* B alanine racemase for elimination and subsequent irreversible inactivation were probed. The parent compound for *O*-carbamoylserine, serine, either as the D or L isomer, does not undergo catalytic elimination and does not cause time-dependent inactivation. Each enantiomer of serine is slowly racemized by the enzyme at rates ca. 1% that of the corresponding alanine enantiomers. This lack of elimination and inactivation may be due to the fact that the β-OH of serine would yield a much more basic anion, OH^-, than *O*-carbamoylserine, $NH_3{}^+COO^-$. *O*-Acetylserine (which would eliminate acetate, conjugate acid $pK_a = 4.76$) might be a racemase inactivator even though serine is not. In fact, the data in Table II are consistent with the *O*-carbamoylserine behavior. The D isomer of *O*-acetylserine does eliminate, giving the same ~830/1 partitioning ratio before causing irreversible inactivation. By contrast the L isomer of *O*-acetylserine *does not undergo elimination,* does not cause inactivation, and serves only as a *reversible,* competitive inhibitor of the racemase. No racemization of either *O*-carbamoyl-L- or *O*-acetyl-L-serine to their respective D isomers was detected in coupled assays containing D-amino acid oxidase; as indeed, formation of D isomer should lead irreversibly to time-dependent inactivation.

To determine whether the L isomers of *O*-carbamoyl- and *O*-acetylserine do not inactivate because the enzyme cannot achieve a favorable geometry for elimination once the α-H has been abstracted or because the enzyme does not initiate α-hydrogen abstraction with these isomers, experiments were conducted with 10 mCi of $[^3H]H_2O$, mixing enzyme (0.9 unit) separately with 20 μmol of *O*-acetyl-L-serine, *O*-carbamoyl-L-serine, and L-alanine (as control). The isolated alanine sample (which had been completely racemized to the D,L mixture as analyzed by D-amino acid oxidase) contained 3×10^5 dpm/μmol; half of this tritium was volatilizable by D-amino acid oxidase action. The L-*O*-acetylserine (19 μmol recovered) and L-*O*-carbamoylserine (20 μmol recovered) contained less than 10^3 dpm/μmol. Therefore, the racemase does not appear to initiate catalysis (as monitored by α-H abstraction, exchange into solvent, and reincorporation with the stabilized α-carbanion) with these two L-amino acids.

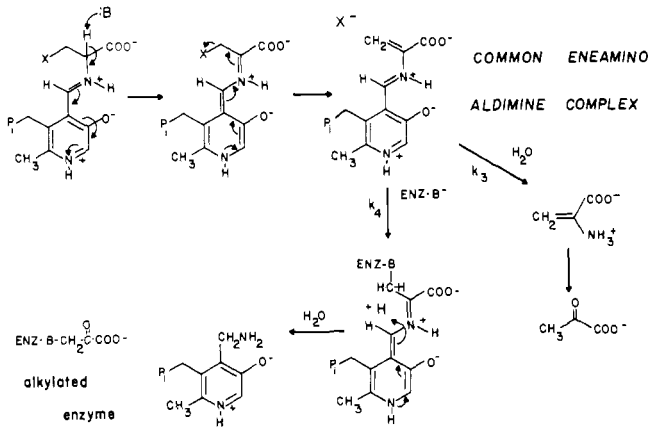
Other potential amino acid substrates for the *E. coli* B racemase were then examined. As with serine, both D and L isomers of cysteine are racemized very slowly (<0.2% V_{max} for alanine) and also only slowly processed for elimination (<0.2%); HS^- would appear not to be a good enough leaving group. Similarly *S*-methyl-D- and *S*-methyl-L-cysteine do not cause noticeable enzyme inactivation or pyruvate formation. The pK_a of 10.7 of methanethiol may likewise slow elimination relative to racemization. In both cases the β-sulfur substituted amino acids are very slow substrates for racemization (0.2-0.4% the alanine rates). *O*-Phospho-D,L-serine and *O*-sulfo-D-serine which have very good potential leaving groups do not inactivate alanine racemase detectably in short incubations (ca. 15-30 min), quite possibly because the anionic charges on the β-substituents provide electrostatic barriers to reaction or binding.

Experiments with Alanine Racemase-Containing Membrane Vesicles of E. coli B. One of the potential values of suicide substrates with high specificity for target enzymes is the possibility of moving experiments from the purified enzyme to a more complex biological system to evaluate the role of the protein in that milieu by observation of the functional consequences from its in situ inactivation by the suicide substrate. We have previously studied the role of alanine racemase in isolated cytoplasmic membranes of *E. coli* B functional for solute active transport (Kaczorowski et al., 1975b, 1977). Addition of D-alanine drives radioactive amino acid (or sugar) concentrative uptake by oxidation via a membrane bound D-amino acid dehydrogenase. Addition of L-alanine also powers active transport because the membrane-associated alanine racemase can isomerize it to D-alanine which can then be oxidized.

As representative tests for irreversible inactivation in the membrane vesicle system, we tested both isomers of β-fluoroalanine and D-cycloserine for their effects on active transport of [^{14}C]proline stimulated by D-alanine (requiring a functional D-amino acid dehydrogenase) or stimulated by L-alanine (requiring both a functional alanine racemase and D-amino acid dehydrogenase). The data of Figures 5a-c clearly point out that preincubations with these racemase suicide substrates selectively block racemase-coupled transport but have no effect on D-amino acid dehydrogenase coupled transport. In fact, we could show separately that the dehydrogenase in the mem-

[5] Curiously concentrations of L-cycloserine above 20 mM gave slower rates of inactivation than at 1 mM. This protectant effect is as yet unexplained.

SCHEME I



branes does oxidize D-β-fluoroalanine to fluoropyruvate without inactivation. [D-Chloroalanine oxidation produces chloropyruvate which, as a more reactive electrophile than fluoropyruvate towards S_N2 displacements, does cause damage to active transport (Kaczorowski & Walsh, 1975).]

Discussion

Adams, in recent review articles (1972, 1976) on amino acid racemases, has noted that reports on well-characterized purified racemases for primary amino acids are few. He and his colleagues showed conclusively that alanine racemase from *Pseudomonas putida* contained pyridoxal-P while Soda and colleagues proved the same for both an arginine/lysine racemase from *P. graveolens* (1969) and a low specificity racemase from *P. striata* (1969). Other alanine racemases from *B. subtilis, S. aureus,* and *E. coli* W have been shown to be sensitive to pyridoxal phosphate enzyme inhibitors but not directly to contain pyridoxal phosphate (Adams, 1976). Racemases for the secondary amino acids proline (Cardinale & Abeles, 1968) and hydroxyproline (Finlay & Adams, 1970) are just as clearly pyridoxal-P independent. In this report we have shown that the *E. coli* B alanine racemase is a dimer of 100 000 molecular weight with one pyridoxal phosphate and one active site per 49 000 molecular weight subunit. Although K_{eq}, the equilibrium constant for racemase action, equals unity, it has been recognized (e.g., Adams, 1976) that this need not imply a symmetric active site for binding and processing of D and L enantiomers and the *E. coli* B enzyme can show a functional asymmetry, not so obvious in processing alanine, but most visible in its differential tolerance for the suicide substrates discussed in this paper.

We have previously elaborated some kinetic and chemical criteria, including partition ratios which should be applied to candidates for enzymatic suicide substrates (Walsh et al., 1978). All the kinetic data (and the preliminary stoichiometry data) summarized for the haloalanines, *O*-carbamoyl-D-serine and *O*-acetyl-D-serine in Tables I and II strongly support the catalytic mechanism shown in Scheme I to explain the turnover data, the nature of the product, and the partitioning of a symmetric, common eneamino acid-PLP aldimine intermediate. The partitioning ratio of ~830/1 reflects the ratio of k_3/k_4 in that scheme and is a comparison of the rate at which hydrolysis to free dehydroalanine (aminoacrylate) occurs to the rate at which some enzyme nucleophile, enz-ÿ, adds adventitiously in a Michael sense to the conjugated eneamine before release. Such eneamino acid intermediates are thought to be normal catalytic intermediates in many pyridoxal-P enzymes carrying out β-eliminations or β-replacements as their physiological function (e.g., D-serine dehydrase, L-serine dehydrase, threonine deaminase, *O*-acetylserine sulfhydrylase [Davis & Metzler, 1972]). Those enzymes must have active sites where the diposition of basic (nucleophilic) groups is such that inactivating Michael additions do not occur at any kinetically significant frequency. An exception is threonine deaminase while processing serine: that enzyme is inactivated by its physiological substrate; the partitioning ratio is ca. 10^4:1 (McLemore & Davis, 1968). In contrast, alanine racemase has not had the necessity to evolve to leave such conjugated adducts unattacked; under normal physiological conditions the racemase will never generate such an intermediate at its active site since its physiological substrates do not have good enough leaving groups at the β carbon. Other pyridoxal-P enzymes which also do not normally produce conjugated intermediates can be similarly inactivated by the xenobiotic β-chloroalanines, among them L-aspartate transaminase from heart muscle (Morino & Okamoto, 1973), bacterial D-amino acid transaminases (Soper et al., 1977b), and *A. faecalis* β-aspartate decarboxylase (Tate et al., 1969). The partition ratios have been reported only for one D-amino acid transaminase (1500 turnovers to inactivation).

Three other features implied by Scheme I are worthy of note. First, with none of the enantiomeric fluoroalanines is there any evidence that racemization is competitive with β elimination of halide. Assays were conducted with coupling enzymes, D-amino acid oxidase or L-alanine dehydrogenase, respectively, which would have detected racemization as an incremental increase in pyruvate formation; none was detected. This suggests that, although the racemase has evolved as a catalyst to deprotonate alanine isomers and then randomly reprotonate them at the α carbon, the energy barrier for elimination of either Cl^- or F^- from either D or L isomer is much lower than for reprotonation at the α carbon to produce the enantiomeric haloalanine. This is so despite the fact that L-chloroalanine eliminates as much as 140-fold more slowly than the D isomer.[6]

Secondly, inactivation with D-fluoroalanine dissipates the electronic and fluorescence maxima of the pyridoxal phosphate chromophore. While structural investigation remains, the stable form of inactive product cannot be either paraquinoid adduct of Scheme I. They would absorb in the 485–505-nm region (Snell & DiMari, 1972), but it may be a pyridoxamine-phosphate form of the coenzyme along with a pyruvylated enzyme residue, as postulated in Scheme I. If this is so, the alkylation must have disturbed active site geometry such that protonation at the benzylic position, essentially a transamination sequence and never observed with native racemase, is kinetically favored over reprotonation at the α carbon.

Thirdly, the partition ratio of 830/1 for catalytic events per inactivation event might be considered high for efficient in vivo specificity (Walsh, 1977). We have previously noted high partition ratios with hydroxybutynoate as flavin hydroxy acid oxidase suicide substrates (Cromartie & Walsh, 1975; Ghisla et al., 1976), with D-vinylglycine as D-amino acid transaminase suicide substrate (Soper et al., 1977a), and with L-vinylglycine as L-amino acid oxidase inactivator (Marcotte & Walsh, 1976). However, unlike the latter three instances where the products released are reactive electrophiles capable of reaction with cellular nucleophiles, the 830 product molecules released by alanine racemase are unreactive and not even xenobiotic,

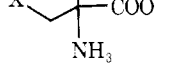
[6] The trapping experiments show that no *free* racemized species is formed. They cannot distinguish whether L-β-haloalanines are first racemized to D-β-haloalanines which stay *bound* to the PLP at the active site and, *before release,* there specifically undergo the elimination to the electrophilic eneamine.

but rather the physiological metabolite pyruvate. The common partitioning ratios in Tables I and II are powerful indices that covalent alkylation arises from Michael attack on bound amino acrylate rather than S_N2 displacement of a leaving group from the β carbon of the stabilized α carbanion, a distinction which could not be made with the other pyridoxal phosphate enzymes previously inactivated with β-chloroalanine.

The degree of active site asymmetry of an alanine racemase toward substrate binding and processing was first raised by Roze & Strominger (1966) for the *S. aureus* enzyme to explain their observations that D-cycloserine was a much better competitive inhibitor than L-cycloserine (it may well be worth reexamination for any irreversible effects of either isomer). The haloalanine data with the *E. coli* B alanine racemase support a functionally asymmetric active site. While D-fluoroalanine, L-fluoroalanine, and D-chloroalanine react at similar initial rates for elimination (and so produce proximal inactivator rapidly), the L-chloroalanine isomer eliminates 50–100-fold more slowly. The steric bulk of the β-chloro group in the L-isomer specifically must slow down reaction either in initial binding or subsequent chemistry. It may be that α-H removal from L-chloroalanine is now rate determining; this will be tested with L-[2-^{2}H]chloroalanine. There is no kinetic isotope effect with D-[2-^{2}H] fluoroalanine on either inactivation rates or partition ratios.

One possibility is that D and L enantiomers of amino acids must bind to pyridoxal phosphate at the active site with the amino and carboxylate groups in the same orientation (an unproved speculation); then the R groups would extend in different directions and bulk tolerance may differ in those subsites. The *O*-carbamoylserine and *O*-acetylserine data strongly support the idea that the R group subsite in D enantiomers could be larger. With the two *O*-acyl-L-serines examined, the steric bulk of the R group apparently affects positioning of the α carbon so that the C_α–H bond is not broken by the enzyme.

There is a preliminary correlation, requiring more experimentation, that pK_a of the conjugate acid of the leaving group (e.g., pK_a HX)



is related to observed elimination and consequent inactivation. The halide ions are nonbasic anions (pK_a(HCl) = −6.1; pK_a(HF) = 3.45) and can depart in a low energy transition state without acid catalysis; apparently, so can carbamoyl and acetyl substituents. But at pK_a values for HX of 8 and above, facile X-group expulsion may not occur.

It may be that *O*-carbamoyl-D-serine and D-cycloserine will be general irreversible inactivators (i.e., suicide substrates) of bacterial alanine racemases rather than mere competitive reversible inhibitors. This will bear examination as will general testing of the other suicide substrates reported in this paper. Henderson & Johnston (1976) reported that D- and L-β-chloroalanine are reversible inhibitors of alanine racemase from *B. subtilis*, yet Rando (1975) has suggested this same *B. subtilis* racemase is irreversibily blocked by D-cycloserine. The susceptibility of bacterial alanine racemases at a molecular level will probably be a function of whether a basic group in the active site can act in a kinetically adventitious and deleterious fashion as a nucleophile on a bound aminoacrylate species.

Although D-fluoroalanine is processed without isotope effect by the *E. coli* B alanine racemase, D-[2-^{2}H]fluoroalanine is a more potent antibiotic than D-[2-^{1}H]fluoroalanine (Kahan et al., 1975). An explanation for the increased efficacy of the D-[2-^{2}H]- vs. D-[2-^{1}H]fluoroalanine in experimental infections may reflect a kinetic isotope effect in removal of the hydrogen species by host enzymes. Slower catabolism of fluoroalanine would contribute to its antibacterial lifetime (Kollonitsch & Barash, 1976). Consistent with this idea is our report (Dang et al., 1976) that kidney D-amino acid oxidase, possibly the only mammalian enzyme that will oxidize D-fluoroalanine, shows a V_{max} ^{1}H/^{2}H isotope effect of 1.8 with this substrate. The product from that D-amino acid oxidase action is exclusively β-fluoropyruvate. It is likely that fluoropyruvate per se is a less reactive alkylating agent than chloropyruvate so that D-fluoroalanine may be a more specific antibacterial agent than D-β-chloroalanine on that basis. Whether fluoropyruvate would be significantly decarboxylated to toxic fluoroacetate may be an ancillary consideration.

Future antibiotic candidates targeted at alanine racemase might balance the features of being a D-amino acid isomer with a small good leaving group (yet kinetically stable in the molecule free in solution) at the β carbon, but one which when oxidized by an animal cell D-amino acid oxidase will yield a β-substituted α-keto acid of low reactivity (and toxicity). Further studies on the molecular aspects of alanine racemase catalysis are warranted not only for insights into the enzyme mechanism but also for prospective rational antibiotic evaluation and design.

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