Purification of an Alanine Racemase from *Streptococcus faecalis* and Analysis of Its Inactivation by (1-Aminoethyl)phosphonic Acid Enantiomers†

Bernard Badet‡ and Christopher Walsh*

Departments of Chemistry and Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139
Received August 10, 1984

ABSTRACT: An alanine racemase has been purified some 30 000-fold almost to homogeneity from Gram-positive *Streptococcus faecalis* NCIB 6459; the enzyme has been purified to the same extent (4000-fold) from an *O*-carbamyl-D-serine-resistant mutant with a 7-fold higher enzyme level in crude extract. The racemase has one pyridoxal phosphate molecule per 42-kDa subunit, has a V_{max} of 3570 units/mg and a K_m of 7.8 mM in the L to D direction, and has a V_{max} of 1210 units/mg and a K_m of 2.2 mM in the D to L direction. The K_{eq} is 0.8 and k_{cat}/K_m values are ca. 3×10^5 M^{-1} s^{-1}. The purified enzyme is inhibited in a time-dependent manner by both L- and D-(1-aminoethyl)phosphonates (Ala-P), confirming observations of Atherton et al. in crude extracts of this organism [Atherton, F. R., Hall, M. J., Hassal, C. H., Holmes, S. W., Lambert, R. W., Lloyd, W. J., & Ringrose, P. S. (1980) *Antimicrob. Agents Chemother. 18*, 897]. Studies with [1-^{2}H]-, [1-^{3}H]-, and [1,2-^{14}C]Ala-P rule out enzymic activation and processing as the basis for irreversible inhibition. Thus, enzyme after exposure to [^{14}C]Ala-P or [α-^{3}H]Ala-P and gel filtration contains stoichiometric amounts of radioactive label, but denaturation quantitatively releases intact Ala-P into solution as revealed by high-performance liquid chromatography and cocrystallization with authentic material. The Ala-P isomers are slow binding inhibitors of this racemase as is the α,α'-dimethyl analogue but not the D or L isomers of the corresponding phosphinate. The extrapolated limiting k_{inact} for L-Ala-P is 5.3 min^{-1}, and an extrapolated $t_{1/2}$ for regain of enzyme activity is about 19 days, suggesting a ratio of ca. 10^6 in favor of isomerization of an initial, rapidly formed E·I complex to an isomerized (E·I)* tight complex with very slow dissociation properties. No such slow-binding, time-dependent inhibition was detected with pure alanine racemases from Gram-negative bacteria.

Bacterial alanine racemases equilibrate the α-carbon configuration of alanine, thereby converting L-alanine used in normal metabolism to the D isomer used either as an energy source or as a building block in assembly of the peptidoglycan layer of bacterial cell walls. In the latter function, alanine racemases have been identified as targets for antibacterial agents, and both natural and synthetic inhibitors are known. Thus, *O*-carbamyl-D-serine and -D-cycloserine are naturally occurring inhibitors (Neuhaus, 1967; Roze & Strominger, 1966; Johnston et al., 1968; Lambert & Neuhaus, 1972) while β-fluoro-D-alanine (Kollonitsch & Barash, 1976) and L-(1-aminoethyl)phosphonic acid (L-Ala-P)[1] (Atherton et al., 1979) are synthetic racemase inhibitors.

We have shown that the β-substituted alanines are irreversible inhibitors of purified alanine racemases from Gram-negative organisms such as *Escherichia coli* (Wang & Walsh, 1978), *Salmonella typhimurium* (Badet et al., 1984), and *Pseudomonas striata* (Roise et al., 1984) and have recently determined that the time-dependent loss of activity stems from covalent attack of nascent aminoacrylate product on the PNP–lysyl protein aldimine in the racemases' active site.

Much less molecular information has been available about alanine racemases from Gram-positive bacteria. Perhaps the most intriguing observation is that Ala-P is, in crude extracts, a reversible inhibitor of Gram-negative alanine racemases (*E. coli* and *S. typhimurium*) but a time-dependent inhibitor of racemases in crude extracts of two Gram-positive organisms, *Staphylococcus aureus* and *Streptococcus faecalis* (Atherton et al., 1979).

The molecular basis of this distinction or of the mode of irreversible inactivation on Gram-positive organisms has been unknown. In this paper we report the 30 000-fold purification close to homogeneity of the *S. faecalis* NCIB 6459 alanine racemase and the initial studies on the mechanism of irreversible inhibition by D and L isomers of Ala-P.

Experimental Procedures

Materials

L- and D-alanine, D-serine, *Bacillus subtilis* L-alanine dehydrogenase (EC 1.4.1.1) (30 units/mg in 50% glycerol), bovine plasma monoamine oxidase (EC 1.4.3.4), porcine kidney D-amino acid oxidase (EC 1.4.3.3) (15 units/mg in 3.2 M ammonium sulfate), yeast alcohol dehydrogenase (EC 1.1.1.1) (200 units/mg, lyophilized), bovine pancreas DNase I (EC 3.1.21.1) (1350 Kunitz units/mg, lyophilized), CHES, HEPES, bis-Tris-propane, piperazine, pyridoxal phosphate, malachite green, and alumina (type 305) were purchased from Sigma. L-Serine was from Calbiochem. D- and L-β-chloro-

† Supported in part by National Science Foundation Grant GM 07287.
‡ Present address: ENSCP-CERCOA, 75231 Paris Cedex 05, France.

[1] Abbreviations: Ala-P, (1-aminoethyl)phosphonic acid; PNP, pyridoxyl; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine; L-ADH, L-alanine dehydrogenase; D-AAO, D-amino acid oxidase; LDH, lactate dehydrogenase; GDH, glutamate dehydrogenase; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; bis-Tris-propane, 1,3-bis[[tris(hydroxymethyl)methyl]amino]-propane; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; FPLC, fast protein liquid chromatography; Cbz, benzyloxycarbonyl; d, doublet; t, triplet; m, multiplet; ppm, parts per million.

0006-2960/85/0424-1333$01.50/0

alanines were from Vega Biochemicals. D- and L-β-fluoroalanines were the gift of Dr. J. Kollonitsch (Merck Sharp & Dohme). [U-^{14}C]-DL-serine (50 Ci/mol) was from ICN. Hog muscle lactate dehydrogenase (EC 1.1.1.27) (550 units/mg, glycerol solution) and glutamate dehydrogenase (EC 1.4.1.3) (120 units/mg, glycerol solution) were from Boehringer Mannheim. Tritiated water was purchased from Amersham.

Streptococcus faecalis NCIB 6459, D- and L-(1-aminoethyl)phosphonic acids, [α-^{2}H]-D- and [α-^{2}H]-L-(1-aminoethyl)phosphonic acids, and [U-^{14}C]-L-(1-aminoethyl)-phosphonic acid were kindly provided by Dr. C. Hassal (Roche Products Ltd., U.K.). We are also indebted to Dr. J. Dingwall (Ciba-Geigy, U.K.) for providing us D- and L-(1-aminoethyl)phosphinic acids and (1-amino-1-methylethyl)phosphonic acid (α,α′-dimethyl-Ala-P).

Methods

(N-Phosphopyridoxyl)-L-Ala-P. (*N*-Phosphopyridoxyl)-L-Ala-P was synthesized following a procedure similar to that described by Forrey et al. (1971) on a 5-mmol scale. The compound was purified successively by anion exchange (AG 1-X8, formate, water elution), cation exchange (AG 50W-X4, H^+, 0–5 N HCl gradient) and filtration on Bio-Gel P-2 giving 700 mg (40%) of amorphous powder. The compound was pure as judged by TLC (cellulose; *n*-BuOH–AcOH–H_2O, 6:2:2) as visualized with Gibb's reagent (Waldi, 1965): ^{1}H NMR (D_2O) δ 8.07 (1 H, s), 4.91 (2 H, d, J_{HP} = 8.1 Hz), 4.43 (2 H, s), 3.32 (1 H, dq), and 1.35 (3 H, dd, J_{HH} = 7.2 Hz, J_{HP} = 14.6 Hz); ^{31}P NMR (D_2O–H_3PO_4) δ 12.46 and 14.81.

O-Acetylserine. Cold *O*-acetyl-D- and *O*-acetyl-L-serine, [U-^{14}C]-*O*-acetyl-D- and [U-^{14}C]-*O*-acetyl-L-serine were synthesized as previously described (Roise et al., 1984).

O-Carbamyl-D-serine. To 4.2 g (26 mmol) of 1,1′-carbonyldiimidazole in anhydrous benzene (200 mL) was added 7 g (21.3 mmol) of *N*-Cbz-D-serine benzyl ester (Skinner et al., 1956). After 1-h stirring at room temperature, ammonia was bubbled through the solution for 1 h. The solvent was removed, and the solid residue was washed with water and dried over P_2O_5, giving 9.3 g of crude *O*-carbamyl-*n*-Cbz-D-serine benzyl ester. The crude ester was reduced in ethanol (150 mL) and cyclohexene (50 mL) with 10% palladium on carbon (4.6 g) (Anantharamaiah & Sivanandaiah, 1977) under argon at 80 °C for 1 h. The reaction mixture was filtered, the catalyst was washed with hot water, and the combined filtrates were evaporated to dryness under reduced pressure. The solid was recrystallized from water–alcohol to give 1.85 g (59%) of compound pure by TLC (silica; *n*-PrOH–H_2O, 7:3): ^{1}H NMR (D_2O) δ 4.25 (2 H, d, J = 5 Hz) and 3.84 (1 H, t, J = 5 Hz).

[1-^{3}H]-DL-Ala-P. DL-Ala-P was prepared according to Kudzin & Stec (1979). *N*-Cbz-DL-Ala-P (Huber et al., 1975) was esterified with trimethylorthoformate at 105 °C according to Nicholson et al. (1970). The crude *N*-Cbz-DL-Ala-P dimethyl ester (10.4 g, 38 mmol) was transhydrogenated with cyclohexene and palladium (Anantharamaiah & Sivanandaiah, 1977), and the residue after evaporation under vacuum was redissolved in chloroform and filtered from nonsoluble material. After evaporation of the solvent, we obtained 4.77 g (26 mmol, 65%) of dimethyl (1-aminoethyl)phosphonate as an oil that exhibited one spot on TLC (cellulose; *n*-BuOH–AcOH–H_2O, 6:2:2; ninhydrin visualization): ^{1}H NMR ($CDCl_3$–Me_3Si) δ 3.73 (6 H, d, J_{HP} = 10 Hz), 3.15 (1 H, m), and 1.28 (3 H, dd, J_{HP} = 17 Hz, J_{HH} = 7 Hz). Benzaldehyde (2.6 g, 24.5 mmol) was added to the product isolated above; after 30 min, water was removed azeotropically with benzene under reduced pressure and the resulting oil dried over KOH under high vacuum.

The resulting imine (1.35 g, 5 mmol) was dissolved in 25 mL of dry tetrahydrofuran and the solution was treated at −78 °C with *n*-butyllithium (1.2 equiv). After being warmed up to 0 °C, the resulting orange anion was quenched with 0.2 mL of tritiated water (5 Ci/mL). The organic phase was washed with 50% saturated ammonium chloride solution, and the washings were back-extracted with methylene chloride (20 mL). The solvent was removed, and the oily residue was refluxed with concentrated HCl (10 mL, 36 h). The reaction mixture was washed with benzene (4 times 10 mL) and the solvent removed. The residue was dissolved in ethanol (5 mL) and precipitated by propylene oxide. The compound was recrystallized from ethanol–water to give 0.38 g (60%) of [1-^{3}H]-DL-Ala-P, the specific radioactivity of which was 34.4 Ci/mol.

Assays. Radioactive assays for racemase activity in bacteria were performed on toluenized cells. A 1-mL aliquot of growing cells (50–200 Klett units) was washed and resuspended in 0.1 mL of 50 mM KPO_4, pH 7.5. The cells were then vortexed 0.5 min with 2% toluene and left on ice for 20 min. A 10-μL sample of permeabilized cells was incubated with 5 mM [^{14}C]-L-alanine (0.24 Ci/mol) in 100 mM CHES, pH 9, at 37 °C for 10 min (incubation volume 50 μL) and the reaction stopped by boiling. D-AAO (1 unit) was then added and the solution incubated 30 min at 37 °C; the reaction was stopped by 10 μL of acetic acid and the mixture run through 1 mL of AG 50W-X4, H^+ resin.

Racemase spectrophotometric assay using either D-amino acid oxidase (D-AAO) coupled to lactate dehydrogenase (LDH) or L-alanine dehydrogenase (L-ADH) in 100 mM CHES, pH 9, has been previously described (Badet et al., 1984): in the L to D direction we used 1.7 units of D-AAO, 50 μg of LDH, 0.16 mM NADH, and 4 ng of racemase with 50 mM L-alanine. In the reverse direction, we used 0.3 unit of L-ADH, 10 mM NAD, 20 mM D-alanine, and 8 ng of racemase; 10^{-5} M PLP was systematically included in the assays. A unit of activity is defined as 1 μmol of substrate epimerized per minute.

Ammonia, ethylamine, or acetaldehyde production from Ala-P was assayed with respectively glutamate dehydrogenase, monoamine oxidase, or alcohol dehydrogenase (Bergmeyer, 1974). Phosphate from Ala-P incubation was assayed according to the method of Penney (1975).

Inactivation Assays with Ala-P. Inactivation experiments were run in 0.1 mL of 100 mM HEPES, pH 7.4, at 37 °C with 1 racemase activity unit. The reaction was started by addition of Ala-P; at several time points, 10 μL of the incubation mixture was diluted 10-fold in the same buffer kept at 0 °C. In protection experiments, D-alanine was added just before Ala-P.

In radioactive experiments, 0.42 mg (10 nmol) of enzyme was treated with 1 mM of either [U-^{14}C]-L-Ala-P (6.25 Ci/mol) or [α-^{3}H]-DL-Ala-P (34.4 Ci/mol) at 37 °C in 0.44 mL of 20 mM bis-Tris-propane, pH 7.5, containing 15% glycerol. The UV–visible spectrum was monitored over the incubation period. After 30 min, enzyme was assayed for activity and loaded at 4 °C on a Sephadex G-25 column (20 × 1 cm), which was eluted at 6 mL/h. The specific radioactivity of the protein, clearly separated from the free Ala-P, was determined.

To check α-tritium exchange, 42 μg (1 nmol) of racemase was incubated in 0.1 mL of 20 mM bis-Tris-propane, pH 7.2, 10% glycerol, and 1 mM [α-^{3}H]-DL-Ala-P (7.94×10^6 dpm) for 30 min at 25 °C. The remaining activity after this time

was 18%. The reaction was treated with 6 μL of 1 M NaOH and run through a 1-mL AG 1-X8, $HCOO^-$ column that was washed with 5 mL of water; the radioactivity eluting as tritiated water (3200 dpm) was in the range of the no enzyme control (4700 dpm).

Selection of a S. faecalis O-Carbamyl-D-serine-Resistant Mutant. The general protocol of Reitz et al. (1967) was used; first mutants were selected on dextrose broth plates containing 20 mM drug. They were further selected in liquid medium (dextrose broth) at different drug concentrations; mutants were checked for racemase activity with the radioactive assay. Cells growing at 40 mM *O*-carbamyl-D-serine were selected and repurified on plates without drug: no decrease of racemase activity was observed after this operation. The mutant exhibited a high level of resistance to the drug (>100 mM).

S. faecalis Alanine Racemase Purification. S. faecalis NCIB 6459 (wild type and *O*-carbamyl-D-serine-resistant mutant) was grown in 25-L batches to late exponential phase in dextrose broth for 4 h at 37 °C from a 2% overnight culture inoculum. The cells were concentrated with a Pellicon membrane (Millipore), collected by centrifugation, washed twice with 50 mM $NaPO_4$, pH 7, containing EDTA (0.5 mM), β-mercaptoethanol (1.4 mM), and PLP (0.010 mM) (washing buffer), and frozen. From 125 L we obtained 320 g of cells (wet wt.) from wild strain and 220 g from the *O*-carbamyl-serine-resistant mutant.

All the following steps were conducted at 4 °C except where noted. All buffers contained 0.5 mM EDTA and 1.4 mM β-mercaptoethanol. During the purification, racemase activity was followed by the L to D spectrophotometric assay.

Cells (220 g) were thawed overnight and disrupted by grinding with 550 g of alumina in a mortar. To the white paste, 400 mL of washing buffer was added and the mixture incubated with 6 mg of DNase I for 15 min. The alumina was spun down (5000*g*, 10 min) and washed once with 600 mL of washing buffer, and the combined supernatants were centrifuged at 100000*g* for 30 min. The supernatant (900 mL) was brought to 45% saturation by addition of solid ammonium sulfate; the pellet was discarded, and the supernatant was brought to 75% saturation.

(*A*) *Hydrophobic Chromatography.* The pellet was redissolved in 40% saturated ammonium sulfate in 50 mM $NaPO_4$, pH 7 (starting buffer), and loaded on a 250-mL phenyl-Sepharose column at a flow rate of 60 mL/h. The column was washed with 300 mL of starting buffer and eluted with a 1-L linear gradient of 40–0% ammonium sulfate in 50 mM $NaPO_4$, pH 7; 10-mL fractions were collected. At the end of the gradient, the column was washed with 50 mM $NaPO_4$, pH 7. Active fractions centered at 10% ammonium sulfate were pooled, concentrated 5-fold by ultrafiltration (Amicon apparatus, PM-10 membrane), and then diluted 5-fold with 20 mM bis-Tris-propane, pH 9.

(*B*) *Ion Exchange Chromatography.* The solution was loaded onto an 80-mL DEAE-Sephacel column at a flow rate of 10 mL/h. The column was washed with the same buffer containing 100 mM NaCl and eluted with a 500-mL linear gradient of 100–500 mM NaCl at 25 mL/h; activity eluted at about 350 mM NaCl.

(*C*) *Gel Filtration.* The pooled fractions were concentrated to 5 mL and loaded on a 135-mL AcA 44 (LKB) column that was eluted at 5 mL/h.

(*D*) *FPLC.* The resulting active fractions were then purified by FPLC (Pharmacia) on a strong anion exchange column (Mono Q) at room temperature under two different conditions, 20 mM bis-Tris-propane, pH 7.2, and 30 mM piperazine, pH 9.8, with a 0.1–0.6 M linear NaCl gradient.

Protein Determination. Protein concentrations were estimated from absorbance at 280 nm or by the method of Lowry (1951) or Bradford (1976) with either bovine serum albumine or carbonic anhydrase as the standard. The values were adjusted as described under Results.

Amino Acid Analysis. Amino acid analysis was performed according to the conditions previously described (Roise et al., 1984).

SDS Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis was performed as described by Laemmli (1970).

Autoradiography. Autoradiography of SDS gels was obtained after treatment of the gel with the Enlightening enhancer (New England Nuclear) solution and exposure to a Kodak XAR-5 film at −70 °C for 3–7 days.

HPLC. HPLC separation was performed isocratically on a strong anion exchange column (Partisil SAX, Whatman) and with 250 mM ammonium formate, pH 3.5, as solvent; detection was at 313 nm, and the flow rate was 1 mL/min.

RESULTS

Purification of Alanine Racemase from Streptococcus faecalis. The *S. faecalis* alanine racemase was purified following a procedure similar to that recently established in this laboratory for *Salmonella typhimurium dad B* alanine racemase (Wasserman et al., 1984). Among the several disruption techniques used, alumina grinding gave by far the best results. The enzyme activity was not affected by ammonium sulfate treatment in contrast to that reported for *S. typhimurium* enzyme (Wasserman et al., 1984), which allowed us to make a 45–75% ammonium sulfate cut that was loaded at 40% saturation for phenyl-Sepharose chromatography. Protamine sulfate precipitation was avoided due to partial precipitation of the enzyme.

Ion exchange chromatography was found to be much more efficient at high pH (pH 9), giving typically a 12-fold purification. The purification obtained from gel filtration varied from 4- to 12-fold according to the starting strain (wild type or mutant). At this stage though, the enzyme was only 2–4% pure. The use of fast protein chromatography (FPLC) increased tremendously the efficiency of the purification, giving a 25–40-fold increase of the specific activity.

Starting with 200 g of cells from wild-type *S. faecalis*, we obtained 0.115 mg of 75% pure enzyme, the specific activity of which was 2390 units/mg at 50 mM L-alanine; this represents a 25 000–30 000-fold purification on the basis of the specific activity of the protamine sulfate recovery (this step was avoided in the next preparation). No increase in racemase activity could be obtained by growth of the cells on minimal medium with D- or L-alanine as the sole carbon source. Resistance to *O*-carbamyl-D-serine, reported to inhibit only the alanine racemase in streptococci (Reitz et al., 1967), allowed us to isolate a mutant resistant to high levels of antibiotic (>100 mM). This mutant was stable after several cycles of growth in medium without drug, as checked by radioactive racemase assay, giving a 6-fold increase in the amount of racemase activity for a given cell weight. Purification of the alanine racemase from this mutant, summarized in Table I, gave 1.5 mg of more than 90% pure enzyme, the specific activity of which was 2920 units/mg assayed at 50 mM L-alanine (there is partial inhibition of the enzyme activity at this concentration). As the starting specific activity could not be reproducibly measured in the crude extract (as in the wild-type purification), yield and purification were based on activity from the ammonium sulfate precipitation step. The

Table I: *S. faecalis* Alanine Racemase Purification from the *O*-Carbamyl-D-serine-Resistant Mutant[a]

step	vol (mL)	tot protein (mg)	tot units	sp act. (units/mg)	purification (*x*-fold)
ammonium sulfate, 45–75%	580	9350	6900	0.74	
phenyl-Sepharose	160	3224	6350	2	2.7
DEAE-Sephacel	97	237	7300	24.6	33
gel filtration	20	78	6250	80	110
FPLC	2	1.5	4400	2920	4000

[a] Cells (220 g) were disrupted by alumina grinding as described under Methods. Enzyme activity was followed in the L to D direction at 50 mM L-alanine. Protein concentrations were estimated by the method of Bradford; this value was corrected from amino acid composition for the pure protein.

protein concentrations were determined by the Bradford method due to the high interference of the buffer (bis-Tris-propane) with the Lowry reagent; for the most pure enzyme, protein concentration was corrected as described below.

Amino Acid Composition and Protein Concentration. In studies to determine the stoichiometry of Ala-P binding to the *S. faecalis* alanine racemase described below, it was necessary to obtain an accurate relationship of 280-nm absorbance, Lowry, and Bradford assays to protein content. The pure racemase (2920 units/mg) was analyzed for amino acid composition (data not shown). For each determination, 19.48 μg of protein (by Bradford assay) was hydrolyzed. The average of six amino acids analyses was 13.81 μg per experiment so all Bradford protein values were multiplied by 13.81/19.48 = 0.71 to obtain the corrected protein concentration. Similar calculations gave a correction factor of 0.61 for Lowry protein determination and a value of 0.55 mg/mL per absorbance unit.

Characterization of Enzyme. The subunit molecular weight was estimated by electrophoresis in 10% polyacrylamide in the presence of SDS. The main band migrated at a position corresponding to M_r 42000. The M_r obtained from gel filtration experiments in two different conditions (phosphate buffer, pH 8, or bis-Tris-propane, pH 7.2) was 67000. The enzyme exhibited the typical UV–visible spectrum of pure alanine racemases (Badet et al., 1984; Roise et al., 1984; Wasserman et al., 1984) with a A_{280}/A_{420} ratio between 6.7 and 7.7.

Kinetic Parameters. The pure *S. faecalis* alanine racemase shows a broad pH optimum centered around pH 9 (data not shown). At pH 9, the racemase has a V_{max} of 3570 units/mg and a K_m of 7.8 ± 0.5 mM for L-alanine while it has a V_{max} of 1210 units/mg and a K_m of 2.2 ± 0.2 mM for D-alanine. These numbers are similar to the data reported for *S. typhimurium dad B* racemase (Wasserman et al., 1984) with, however, an 2.5-fold increase in the V_{max}. The specificity of the enzyme, estimated by tritium incorporation from ^3H_2O into the α-position, was found, as in the case of *S. typhimurium* enzyme (Wasserman et al., 1984), to be very narrow: among the 20 amino acids tested, only glycine and α-aminobutyric acid are taken as substrates with low efficiency (respectively 8 and 4.8% of the V_{max} of alanine in this assay).

Susceptibility of S. faecalis Enzyme to Racemase Inhibitors. (A) β-Substituted Alanines. Incubation of the enzyme with β-substituted amino acids known to possess antibacterial activity, i.e., β-fluoroalanines (Kollonitsch & Barash, 1976), β-chloroalanines (Manning et al., 1974), and *O*-acetyl-D-serine (Wang & Walsh, 1978), resulted in mechanism-based inactivation. The suicide substrates are processed, in kinetic competition with enzymic killing, to pyruvate, which was measured by the coupled LDH assay:

Table II: Kinetic Constants of *S. faecalis* Alanine Racemase Inactivation by β-Substituted Alanines[a]

inactivator	K_I (mM)	k_{inact} (min^{-1})	partition ratio
β-fluoro-D-alanine	0.08 ± 0.008	2.9 ± 0.1	880
β-fluoro-L-alanine	0.72 ± 0.15	3.4 ± 0.3	860
β-chloro-D-alanine	0.67 ± 0.24	5 ± 1	880
β-chloro-L-alanine	16 ± 6	1.2 ± 0.3	920
O-acetyl-D-serine	9.9 ± 2.4	5.6 ± 0.9	890
O-carbamyl-D-serine	11.6 ± 3.8	3.9 ± 0.8	800

[a] Inactivations were performed at pH 7.4 in 100 mM HEPES with 1.5 μg of racemase. Pyruvate production was measured as NADH consumption by LDH. At each inactivator concentration, a replot of pyruvate production activity vs. time on semilog scale gave the individual k_{inact} values. Intercepts from the double-reciprocal plots were calculated with v^4 weighting (Wilkinson, 1961). Partition ratios were directly measured from the amount of pyruvate produced.

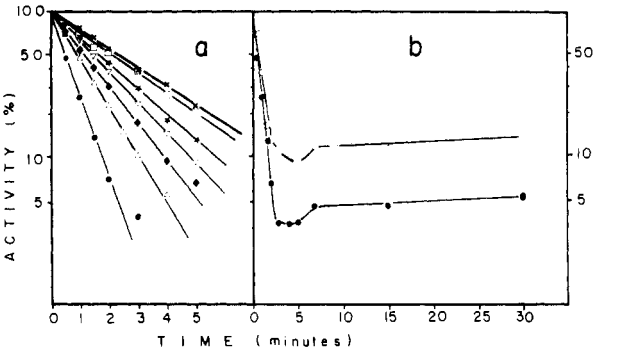
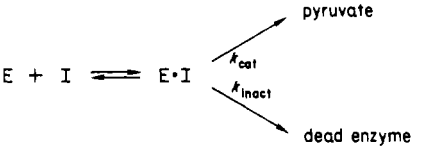


FIGURE 1: Time-dependent inactivation of *S. faecalis* alanine racemase (a) with L-Ala-P at (●) 1, (△) 0.55, (♦) 0.4, (▽) 0.3, (solid star) 0.2, (□) 0.12, and (×) 0.1 mM in 100 mM HEPES, pH 7.4, and (b) with 1 mM L-Ala-P (●) or D-Ala-P (○) in 100 mM HEPES, pH 7.4, as described under Methods.



In each case a first-order loss in pyruvate production activity was observed. Table II gives the K_I and the $k_{inact,max}$ values deduced from replots of inactivation data. The observed partition ratio that represents the ratio k_{cat}/k_{inact} (Walsh, 1980) is constant within experimental error, 870 turnovers per inactivation. This value is remarkably similar to that found for the *S. typhimurium dad B* encoded racemase (730–790) (Wasserman et al., 1984) and for the *Pseudomonas striata* (720–980) enzyme (Roise et al., 1984). It is worth noting that *O*-carbamyl-D-serine is an irreversible inactivator, as observed for *Escherichia coli* alanine racemase (Wang & Walsh, 1978). The K_I values obtained here are in the range of those for the *dad B* enzyme except for β-chloro-D-alanine, which exhibits a 10-fold increase. The L isomer of *O*-acetylserine is rejected from catalysis, again a situation similar to the *S. typhimurium* case where bulky β-substituents on L isomers are not tolerated for processing, catalytic or killing, by the *dad B* alanine racemase. In these attributes, the Gram-positive and Gram-negative racemases are highly analogous.

(B) (1-Aminoethyl)phosphonic Acids. As shown in Figure 1a, *S. faecalis* alanine racemase is inactivated in a time-dependent manner by L-Ala-P and D-Ala-P (data not shown for D isomer). Substrate protection (10 mM D-alanine) considerably slowed down the inactivation rates (2.5-fold at 0.5 mM

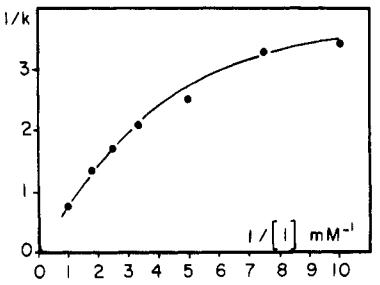
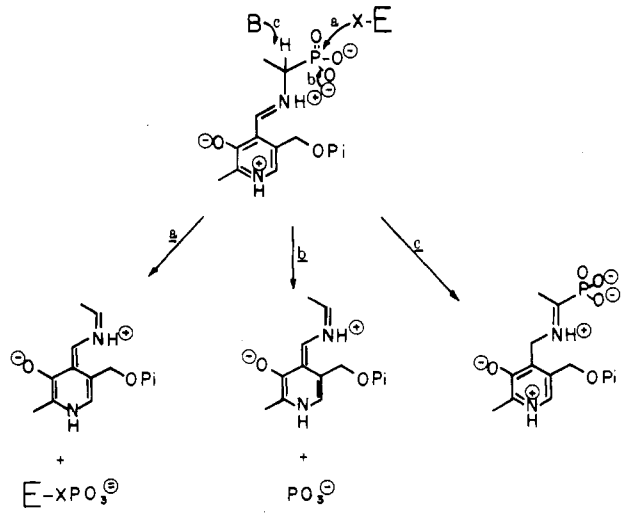


FIGURE 2: Double-reciprocal plot of k_{inact} vs. L-Ala-P concentration; extrapolated inactivation rate constant was calculated by the v^4 weighting method of Wilkinson (1961) by using the first four points.

Scheme I



L-Ala-P, 4-fold at 1 mM L-Ala-P). This confirms time-dependent loss of racemase activity in crude extracts of this bacterial strain observed by Atherton et al. (1979).

However, the inactivation does not go to completion as shown in Figure 1b. We observed during the late time points a regain of activity during the enzymatic assay itself; the same observation was made even after dialysis of the inactivated enzyme. Remaining activity, checked with 50 mM L-alanine, was 10% for L-Ala-P-inactivated enzyme and 30% for D-Ala-P-inactivated enzyme. Replot of $1/k$ $1/[I]$ at each inhibitor concentration (Figure 1a) gave a parabolic curve (Figure 2) whereas a replot of k vs. [I] was linear (data not shown).

Analysis for Products from Ala-P. Given time-dependent loss of *S. faecalis* racemase activity on exposure to either enantiomer of Ala-P, we began to test for catalytic processing by the enzyme of Ala-P to account for inactivation. In order to test the possible mechanisms shown in Scheme I, we assayed for either inorganic phosphate, ethylamine, or acetaldehyde production as described under Methods; none of these compounds were detected as enzymic products from Ala-P.

Studies of Inactivation Mechanisms. (A) α-Deuterio-L-Ala-P Inactivation Kinetics. Inactivation kinetic experiments were performed with α-protio- and α-deuterio-L-Ala-P. At either 0.11 or 1.1 mM inactivator concentration, the kinetic isotope effect observed in 100 mM HEPES, pH 7.4, was 1.2 ± 0.2; no kinetic isotope effect was detectable running the same reaction in 100 mM CHES, pH 9. If the C_α–H or C_α–^{2}H linkage is being cleaved during inactivation, that does not occur in a kinetically significant transition state.

(B) α-Tritio-DL-Ala-P Inactivation. Initial attempts to analyze enzyme-mediated reversible C_α–H cleavage during inactivation focused on exchange of ^{3}H from ^3H_2O into L-Ala-P. However, α-hydrogen exchange could not be clearly detected by inactivation in tritiated water due to the high radioactive background and the low partition ratio documented above. It became clear that the reciprocal experiment, [α-^{3}H]Ala-P incubation in H_2O, would be more determinative.

α-Tritio-DL-Ala-P was synthesized through the imine adduct with benzaldehyde followed by α-proton abstraction and quenching in ^3H_2O; this route allowed us to obtain the desired compound with a high specific activity (34.4 Ci/mol). Incubation of 1 nmol of enzyme for 30 min at 25 °C with 1 mM labeled Ala-P resulted in 82% racemase inactivation. No counts above the background (~4000 dpm) were detected by eluting ^3H_2O from [^{3}H]Ala-P adsorbed to an anion exchange column. If one C_α–^{3}H bond in Ala-P had been cleaved and the $^3H^+$ released to solvent for each enzyme molecule inactivated, then 75000 dpm tritium should have been released from Ala-P. Less than 0.04 equiv was detected. Since there is no k_H/k_D on [α-^{2}H]Ala-P inactivation as noted above, a significant k_H/k_T is unlikely. This experiment pointed very strongly against any mechanism involving α-proton abstraction during Ala-P-mediated *S. faecalis* alanine racemase inactivation, even though C_α–H cleavage is an early obligate step in racemase catalysis with alanine as substrate.

Inactivations were then performed on a scale large enough to determine stoichiometric labeling of the enzyme during and after inactivation; 10 nmol of racemase was incubated at 37 °C with 1 mM of either [U-^{14}C]-L-Ala-P or [α-^{3}H]-DL-Ala-P. In both cases, no change in the 420-nm absorbance was observed during the 30-min incubation period, arguing against transaminative inactivation (even though that would have required C_α–H cleavage). The remaining activity, checked with 50 mM L-alanine, was respectively 10 and 20%, which is in agreement with the 10 and 30% remaining activity noticed above for respectively L- and D-Ala-P inactivation in kinetic experiments with 100-fold less concentrated enzyme. The protein samples were separated from small molecules by gel filtration, and the specific radioactivity of labeled enzyme was determined. On the basis of the specific radioactivity of the inactivator used (6.25 Ci/mol for [U-^{14}C]-L-Ala-P and 34.4 Ci/mol for [α-^{3}H]-DL-Ala-P), a stoichiometry of 0.99 inactivator per enzyme for [U-^{14}C]-L-Ala-P and 1.06 inactivator per enzyme for [α-^{3}H]-DL-Ala-P was found. This indicates that a single inactivator molecule is bound per enzyme molecule and per PLP coenzyme. The ^{14}C or ^{3}H radioactivity associated with the protein was stable for days at 4 °C but was quantitatively released by enzyme denaturation (see below).

Gel Autoradiography of [U-^{14}C]-L-Ala-P-Inactivated Enzyme. Racemase inactivation on a 5-μg (0.12-nmol) scale with [U-^{14}C]-L-Ala-P (126 nmol) was performed in the same conditions as described above; as a positive control, the same amount of enzyme was incubated with 150 nmol of *O*-acetyl[^{14}C]-D-serine (as a DL mixture). After 30 min, the samples were extensively dialyzed against 50 mM NH_4HCO_3, and half of each sample was treated with sodium borohydride. The four samples were then dried down and analyzed by SDS–PAGE. Figure 3a shows the coomassie blue staining and Figure 3b the autoradiography of the gel. The protein was radioactive only after *O*-acetylserine but not after Ala-P inactivation (lanes 1 and 2 vs. 3 and 4). Moreover, borohydride reduction affixed definitively the label to the enzyme in the first case, as expected from our previous work with *P. striata* and *S. typhimurium* (Badet et al., 1984; Roise et al., 1984) but did not produce the bound radioactivity in the latter. The experiment with [^{14}C]-L-Ala-P was repeated on a 1-nmol scale

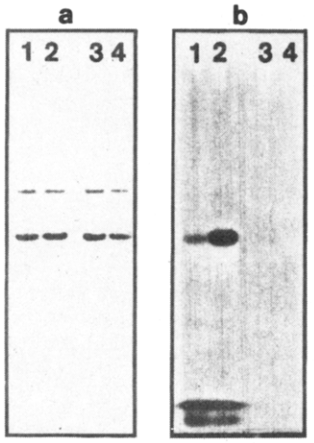


FIGURE 3: (a) SDS gel electrophoresis of wild-strain racemase (2.5 μg) inactivated with [U-^{14}C]-*O*-acetyl-D-serine (lanes 1 and 2) and [U-^{14}C]-L-Ala-P (lanes 3 and 4) as described in the text (10% remaining activity after 30 min in each case). (Lanes 2 and 4) Samples reduced with $NaBH_4$ after inactivation. (b) Autoradiography (72 h, −70 °C) of the gel shown in (a).

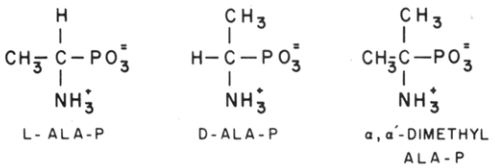
to take into account the lower specific activity of the phosphono amino acid (6.25 Ci/mol) compared to *O*-acetylserine (50 Ci/mol). It gave the same result: no protein-associated radioactivity on the gel from [^{14}C]-L-Ala-P.

Analysis and Identification of Tritiated Species from S. faecalis Alanine Racemase Inactivated with [α-^{3}*H*]-DL-*Ala-P.* For the tritiated enzyme isolated by gel filtration from [α-^{3}H]-Ala-P incubations, the radioactivity was quantitatively released by heating the enzyme at 100 °C for 3 min in 50 mM ammonium bicarbonate; the released radioactive material migrated on TLC (cellulose; *n*-BuOH–AcOH–H_2O, 6:2:2) as a single radioactive spot, which comigrated with authentic Ala-P visualized with ninhydrin.

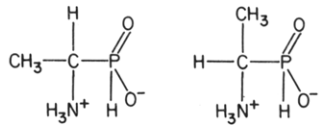
An aliquot (167 000 dpm) of [^{3}H]Ala-P-inactivated enzyme after gel filtration was boiled, and 100 mg of cold DL-Ala-P was added to the supernatant. The solution was then percolated through a 3-mL Dowex H^+ (Ala-P did not bind in these conditions) column; after evaporation, the residue was heated with 2 mL of 12 N HCl and purified as described in the synthesis of [α-^{3}H]-DL-Ala-P under Methods. The specific radioactivity of the DL-Ala-P after each crystallization step was monitored: crystallization to constant specific radioactivity occurred after two of the five steps. Further TLC analysis confirmed the tritiated material was pure Ala-P.

Attempt at Borohydride Reduction of Ala-P-Treated Racemase. Native *S. faecalis* alanine racemase is readily reduced by BH_4^- as monitored by loss of the characteristic PLP-aldimine chromophore at 420 nm. However, when sodium borohydride was added to the gel-filtered [^{3}H]Ala-P-inactivated enzyme, no disappearance of the 420-nm peak occurred, even in the presence of large excess of reducing agent. The radioactivity was released from the BH_4^--exposed protein as above by brief boiling, and HPLC injection of the supernatant confirmed that no (*N*-phosphopyridoxyl)-Ala-P was present: all the ^{3}H radioactivity eluted in the void volume as [^{3}H]Ala-P, 2 min before an authentic sample of PNP-Ala-P.

Studies on Other Phosphorus-Containing Analogues of Alanine. As a racemase the *S. faecalis* enzyme can recognize and process both D and L isomers of alanine and, as noted above, is inhibited in a time-dependent manner by D and L isomers of Ala-P. Although the binding determinants for this racemase are not yet well mapped, if the carboxylate and amino groups of the substrate are common determinants, then the methyl group of D- and L-alanine will fill different quadrants of space in the initial binding complex. For the Ala-P cases, this would suggest the α,α′-dimethyl analogue should be able to bind reasonably well (one CH_3 D-like; the other L-like in the E·I complex). If indeed there is no C_α–H cleavage required in enzyme inactivation by D- or L-Ala-P, then α,α′-dimethyl-Ala-P should also be a time-dependent inhibitor. This was confirmed by the inactivation kinetics giving at 0.5 mM concentration $t_{1/2}$ values of 1.15 and 2.03 min for respectively L-Ala-P and the α,α′-dimethyl analogue.



To analyze the effect of oxidation state of the phosphorus atom on irreversible inhibition, we turned to the phosphonous (phosphinic) analogue of Ala-P, the D and L isomer having been previously synthesized by Dingwall and colleagues at Ciba-Geigy (Baylis et al., 1981):



In the phosphinates there is still a C–P bond, but one of the P–O bonds is instead a P–H bond. We might have anticipated the PHO_2^- group to be more like a COO^- group than the phosphonate group PO_3^{2-} in enzymic studies at pH 7.4. In fact, neither D- nor L-phosphino analogues of alanine (at 5 mM) showed any time-dependent inhibition or even any substantial reversible inhibition of the *S. faecalis* alanine racemase. The phosphonate oxidation state in Ala-P, but not the α-H then, is a crucial determinant in irreversible inhibition of this Gram-positive racemase.

Discussion

Alanine racemases from Gram-positive bacteria have not been well characterized even though the first identified alanine racemase (in crude extracts and 30-fold purified) was from *Streptococcus faecalis* (Wood & Gunsalus, 1951). In this organism alanine racemase was later shown to be the primary site of action of the antibiotic *O*-carbamylserine (Lynch & Neuhaus, 1966), and mutants resistant to this drug were found to be overproducers of the enzyme by crude extract assays (Reitz et al., 1967). The enzyme in *Bacillus subtilis* has been purified from two strains (Diven et al., 1964; Yonaha et al., 1975) and the alanine racemases from *Lactobacillus fermenti* (Johnston & Diven, 1969) and *Staphylococcus aureus* (Roze & Strominger, 1966) have also been studied; however, in only two cases has the enzyme been purified to more than 50% homogeneity (Johnston & Diven, 1969; Yonaha et al., 1975).

The alanine racemase we have now isolated from wild-strain *S. faecalis* NCIB 6459, purified 28 000-fold, is about 75% pure with a specific activity of 2390 units/mg at 50 mM L-alanine. The enzyme isolated from the *O*-carbamyl-D-serine-resistant mutant we selected was purified 4000-fold (~6-fold more enzyme in crude extracts) and is more than 90% pure with a specific activity of 2920 units/mg. Both enzymes have the same Michaelis constants and the same behavior in all aspects studied.

Kinetic constants for alanine racemization by this racemase are similar in magnitude to those of the enzymes isolated from *S. aureus* (Roise, 1984), *S. typhimurium* (Wasserman et al., 1984), and *Pseudomonas striata* (Roise et al., 1984) and fit

quite well the Haldane relationship (Briggs & Haldane, 1925), giving $K_{eq} = 0.82$ in agreement, within experimental error, with the predicted value of 1. Given the spectroscopic ratio $A_{280}/A_{420} = 7$ seen with three other pure alanine racemases in this laboratory (Wang & Walsh, 1978; Roise et al., 1984; Wasserman et al., 1984), we assume one PLP molecule bound per enzyme monomer. The presence of added PLP had no effect either on the A_{280}/A_{420} ratio of purified enzyme or on the activity. In contrast to what was previously observed in partial purification (70-fold by harsh methods) of *S. faecalis* (Wood, 1955) and *L. fermenti* (Johnston & Diven, 1969), no resolution of the PLP cofactor occurred during the purification. The use of FPLC allowed a further 40-fold purification unreachable by conventional methods; the specific activity of the so obtained nearly pure enzyme is 2920 units/mg (L to D direction), which is comparable to the values obtained for other narrow specificity, highly purified alanine racemases, for example, *S. typhimurium* of 1400 units/mg (Wasserman et al., 1984), *B. subtilis* of 1500 units/mg (Yonaha et al., 1975), or *L. fermenti* of 1200 units/mg (Johnston & Diven, 1969). At 50 mM L-alanine, the enzyme does not work at its V_{max} because of partial substrate inhibition. From the molecular weights obtained by SDS–PAGE (42 000) and gel filtration (67 000) experiments, the enzyme is believed to be a nonspherical monomer in its native state.

Susceptibility of Gram-positive alanine racemases to β-substituted alanines is poorly documented. The *B. subtilis* racemase has been reported (Henderson & Johnston, 1976) to be reversibly inhibited by β-chloroalanines: D-chloroalanine was a competitive inhibitor working at low concentrations (K_i = 5 μM), and L-chloroalanine was noncompetitive and worked only at high concentrations (K_i = 1.7 mM). However, those assays, as performed, would not have detected irreversible inactivation and did not allow for processing of the chloroalanine by the racemase, so interpretation of these results is difficult. The behavior of the 100-fold purified *S. aureus* alanine racemase (Roze & Strominger, 1966), which we estimate to be ~1% pure (D. Roise, unpublished observations), toward D-cycloserine and β-(aminooxy)alanine was studied, and D isomers were found to be competitive inhibitors.

Table II summarizes the inactivation of *S. faecalis* alanine racemase with β-substituted alanines; all inactivations showed first-order kinetics. The enzyme displays a reactivity similar to that from Gram-negative *S. typhimurium* (Badet et al., 1984) and *P. striata* (Roise et al., 1984) and from Gram-positive *S. aureus* (Roise, 1984). As we have seen for the other racemases, D isomers inactivate faster than L isomers, and fluoroalanine inactivation is faster than that of chloroalanine, which is faster than *O*-acetyl- or *O*-carbamylserine; partition ratios are similar to those previously observed with Gram-negative enzymes. We have recently noted that, in the Gram-negative cases, racemase inactivation by this class of antibacterials occurs by capture of the PLP aldimine carbon by nascent aminoacrylate product. The invariant partition ratio also seen here suggests the common killing mechanism.

In 1979, the effectiveness of a new class of antibiotics, the phosphono peptides, was reported by a research group at Roche Products Ltd. in England (Atherton et al., 1979; Allen et al., 1979). They showed that a variety of dipeptides contaning the L-(aminoethyl)phosphonic acid at the C terminus were effective antibacterial agents in vitro; this fact was related to the ability to deliver a high intracellular concentration of free L-Ala-P (Atherton et al., 1980) by intracellular dipeptidase action on the transported dipeptide. Evidence was given that L-Ala-P acts on alanine racemase as the primary intracellular target and that this inhibition, while reversible in Gram-negative cells, is time dependent and irreversible in Gram-positive cells. However, the enzymes used for these studies were only slightly purified (~2-fold), and as such, no molecular details of the reaction were determined. We have purified the *S. faecalis* alanine racemase here to try to elucidate the mechanism of irreversible inhibition by the Ala-P enantiomers.

The *S. faecalis* racemase is covalently labeled with radioactive *O*-acetyl-D-serine after BH_4^- reduction (to stabilize the presumed ternary adduct of inactivator–PLP–enzyme, Figure 3b, lanes 1 and 2) as were the *S. typhimurium* and *P. striata* enzymes (Badet et al., 1984; Roise et al., 1984). An interesting difference between these enzymes is the relative stability of the unreduced adduct in *S. faecalis* racemase. For both the *Salmonella* and the *Pseudomonas* cases, inactivated enzyme nonreduced with $NaBH_4$ quantitatively lost the label during a 2-min boiling in water or 10 mM imidazole, pH 7. For the inactivated *S. faecalis* racemase, some radioactivity remained bound even after a 15-min heating period at 120 °C in 1% SDS.

The D and L isomers of Ala-P cause no inactivation of the *Salmonella* or *Pseudomonas* alanine racemases (data not shown). However, Ala-P causes rapid time-dependent loss of *S. faecalis* racemase activity to a level where approximately 10% of the original activity remains for L isomer and 30% for D isomer (Figure 1b). These residual activities and the discrepancy in their amount are not yet understood. No [^{14}C]-L-Ala-P remains bound to the enzyme (Figure 3b, lanes 3 and 4) after denaturation either with or without borohydride reduction.

There are several possible mechanistic interpretations for irreversible inactivation by this alanine analogue (Scheme I). One intriguing possibility was enzyme phosphorylation by direct nucleophilic attack of an enzyme residue (pathway a), a mechanism related to the breakdown of ciliatine [(2-aminoethyl)phosphonic acid] by phosphonoacetaldehyde hydrolase (La Nauze et al., 1977), or, alternatively, by metaphosphate expulsion (as analogue to decarboxylation), generating a highly reactive electrophilic species (pathway b) able to react with any nucleophile in or near the active site (Satterthwait & Westheimer, 1979; Buchwald & Knowles, 1982).

Mechanism c would leave the racemase in the incompetent oxidation state by invoking transamination of Ala-P to produce pyridoxamine and acetylphosphonate; the later is susceptible to decompose in water with production of orthophosphate ions (Cassaigne et al., 1971).

Nonenzymatic pyridoxal-catalyzed dephosphonylation of 2-amino-3-phosphonopropionic acid is known to occur (Martell & Langohr, 1977) as the imine intermediate is a P–XYZ system (Z as electronegative group) as defined by Clark (Clark & Hutchinson, 1968); in our case, the pyridoxyl ring would be used as a electron sink (pathway a).

When we assayed the incubation reaction for phosphate, ethylamine, or acetaldehyde, none of these compounds were found in conditions where we could have detected a turnover of 20–30 product molecules per enzyme inactivation event. Nonenzymatic transamination (pathway c) has been shown to be about 10-fold slower with Ala-P than with alanine (Cassaigne et al., 1971); there remained the possibility that the Gram-positive racemase still could cleave the C_α–H bond of Ala-P in analogy to normal processing of alanine. However, the lack of release of any ^{3}H on inactivation of enzyme appeared to rule out that possibility as well.

Almost quantitative recovery of intact Ala-P from inactivated racemase as shown by TLC and cocrystallization with

cold material ruled out the hypothetical pathways a and c. This result shows that intact Ala-P is bound tightly but noncovalently to native enzyme and can be released only by denaturation. Of the radioactivity associated with [^{14}C]-L-Ala-P-inactivated enzyme, 7% can be released by subsequent gel filtration; this value increases to 13% when enzyme was preincubated for 30 min at 37 °C with 50 mM L-alanine, suggesting a slow release process.

As no modification occurred in the UV–visible spectrum of the enzyme during inactivation, we can postulate either binding of the inactivator in the active site without interference with the PLP–ϵ-NH_2 lysine aldimine link of resting enzyme or rapid transimination of the bound PLP cofactor by Ala-P to give an Ala-P–PLP aldimine, which would be the real inactivator. In both cases this binding must result in a conformational change in the enzyme, explaining the nonaccessibility of the imine to BH_4^- ions, i.e., no disappearance of the 420-nm peak. The stronger basicity of the amino group of Ala-P or of α,-α'-dimethyl-Ala-P, pK = 10.3 (Wozniak et al., 1972), compared to alanine, pK = 9.7, the low basicity of the amino groups of the phosphinic analogues, pK = 8.3, and the results of compared chemical reactivities of Ala-P vs. alanine with PLP ($k_{Ala\text{-}P} = 9k_{Ala}$) (Cassaigne et al., 1971) are in favor of the second hypothesis.

The quantitative recovery of intact [2-^{3}H]-Ala-P by heat denaturation of inactive enzyme along with the lack of any ^{3}H release on inactivation show that Ala-P enantiomers are time-dependent inactivators of the *S. faecalis* Gram-positive alanine racemase due to slow dissociation phenomena of the E·Ala-P enantiomers complexes. This behavior has been termed "slow binding" by Morrison and colleagues (Williams & Morrison, 1979; Morrison, 1982), and one obvious model is isomerization of an initial E·I complex to a new (E·I)* complex where the k_3/k_4 ratio is much larger than unity:

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E{\cdot}I \overset{K_I}{\underset{k_4}{\overset{k_3}{\rightleftharpoons}}} (E{\cdot}I)^* \qquad K_I^* = \frac{k_3}{k_4}$$

While detailed kinetic studies remain to be undertaken, one can make the following observations now. For slow binding inhibitors, plots of initial velocity in incubations containing substrate and inhibitor, initiated by addition of enzyme, yield curved plots of which the data in Figure 4 are typical. The lag period of 2–3 min observed, which is the result of low efficiency in the D-AAO coupling, has not to be taken into account. A double-reciprocal plot of the first-phase velocities yields a K_I value (k_2/k_1) of 0.45 mM for the initial phase of reversible inhibition prior to isomerization. An estimate of k_3 can be made from the extrapolated inactivation rate constants to infinite I concentration as in Figure 2. Because the plot is anomalously nonlinear, one must be cautious, but such extrapolation yields $k_{inact} = 5.3 \pm 0.9$ min^{-1}, which is so slow we assigned it to k_3. To get an estimate of k_4, one can monitor the regain of active enzyme from (E·I)*. When the $t_{1/2}$ is long, this becomes difficult because of problems with concomitant inactivation of E or E·I or (E·I)* by a denaturation process. In this case, we saw a net linear 10% regain of activity over a period 3–80 h after high dilution of (E·I)* into excess S. This would extrapolate to a $t_{1/2}$ of ≥19 days and a net k_{off} from (E·I)* of 2.5×10^{-5} min^{-1}, which is so slow it must be completely dominated by k_4. This would suggest $k_3/k_4 = 2.1 \times 10^5$ and reflects the propensity for accumulation of the isomerized (E·I)* complex. The value of $K_I K_I^* = 2.1 \times 10^{-9}$ M has to be compared to ca. 2×10^{-3} M for alanine K_m values; all the extra binding energy for L-Ala-P comes from the ca. 10^6 value for accumulation of (E·I)* over E·I. This overall

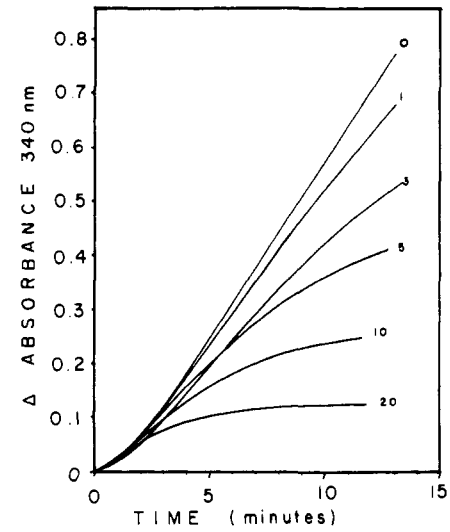
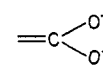


FIGURE 4: Reaction progress curves for L-alanine racemization by *S. faecalis* racemase in the presence of increasing concentrations (1–20 mM) of L-Ala-P in 100 mM CHES, pH 9, at 37 °C; assays were initiated by adding 3.8 ng of racemase to a 1-mL final solution containing 50 mM L-alanine.

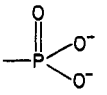
binding constant can be obtained from the plots of steady-state velocities in reactions initiated by addition of the substrate after preincubation of the enzyme with the inhibitor; in our case, however, the sensitivity of the racemase to long incubation periods precluded such an experiment.

This value of about 10^6 is larger than the size of the corresponding rate ratios for isomerization of initial to subsequent tight complex in the inhibition of pyruvate decarboxylase by avidin (Duggleby et al., 1982) and for inhibition of aconitase by the tight binding nitronate analogue (1-hydroxy-2-nitro-1,3-propanedicarboxylic acid) of an isocitrate C_3 carbanion (Schloss et al., 1980). Neither of these cases involves such long life times of the (E·I)* complex as in this alanine racemase. Pierce et al. have noted a $t_{1/2}$ of 16 days for the slow binding inhibition of ribulosebisphosphate carboxylase/oxygenase by 2-carboxy-D-arabinitol 1,5-bisphosphate (Pierce et al., 1980).

Two types of questions come immediately to mind. Why are Ala-P isomers such slow dissociating inhibitors as to be functionally irreversible? And why are only alanine racemases from Gram-positive bacteria [we have seen this behavior with the purified *S. aureus* racemase (Roise, 1984) and the *B. stearothermophilus* enzyme (B. Badet et al., unpublished results)] but not from Gram-negative bacteria sensitive to these phosphono analogues? The latter question will almost surely require at least high-resolution X-ray structures of both types of racemases to be solved. The first question may draw on the tight binding nitronate analogues as mimics specifically of the aci forms of substrate carbanions in the aconitase case noted above, but also for enolase, fumarase, and isocitrate lyase (Anderson et al., 1984; Porter & Bright, 1980; Schloss & Cleland, 1982). The PLP-dependent alanine racemases are supposed to stabilize alanine α-carbanion species as well for stereorandom reprotonation to equilibrate α-carbon configuration. If an aci form were a significant contributor to the Ala–carbanion–PLP structure, the aci form would have a doubly charged



The phosphonate of Ala-P, but not the phosphinate analogue, is doubly charged



under inactivation conditions. However, while the various nitronates analogues are also good planar mimics of the planar aci form of carbanions, the phosphonate group is certainly not planar. How important phosphonate charge and geometry are to the 19 days half-life in slow dissociation of Ala-P from the *S. faecalis* alanine racemase will be an interesting avenue to explore for this class of antibacterial agents.

ACKNOWLEDGMENTS

We thank especially Dr. C. Hassall (Roche Products Ltd., U.K.) for providing us information and material that made this study possible.

Registry No. L-Ala-P, 60687-36-7; D-Ala-P, 66068-76-6; [1-^{3}H]-DL-Ala-P, 94843-78-4; α,α'-dimethyl-Ala-P, 5035-79-0; *N*-(phosphopyridoxyl)-L-Ala-P, 94843-79-5; *N*-Cbz-DL-Ala-P, 60668-25-9; *N*-Cbz-DL-Ala-P dimethyl ester, 94843-81-9; Ala-P dimethyl ester, 94843-82-0; Ala-P imine dimethyl ester, 94843-83-1; *O*-carbamyl-D-serine, 3819-76-9; *O*-acetyl-D-serine, 44901-25-9; β-fluoro-D-alanine, 35455-20-0; β-fluoro-L-alanine, 35455-21-1; β-chloro-D-alanine, 39217-38-4; β-chloro-L-alanine, 2731-73-9; 1,1'-carbonyldiimidazole, 530-62-1; *N*-Cbz-D-serine benzyl ester, 53933-06-5; *O*-carbamyl-*N*-Cbz-D-serine benzyl ester, 94843-80-8; L-alanine, 56-41-7; D-alanine, 338-69-2; alanine racemase, 9024-06-0.

REFERENCES

Allen, J. G., Atherton, F. R., Hall, M. J., Hassal, C. H., Holmes, S. W. Lambert, R. W., Nisbet, L. J., & Ringrose, P. S. (1979) *Antimicrob. Agents Chemother. 15*, 684.

Anantharamaiah, G. M., & Sivanandaiah, K. M. (1977) *J. Chem. Soc., Perkin Trans. 1*, 490.

Atherton, F. R., Hall, M. J., Hassal, C. H., Lambert, R. W., Lloyd, W. J., & Ringrose, P. S. (1979) *Antimicrob. Agents Chemother. 15*, 696.

Atherton, F. R., Hall, M. J., Hassal, C. H., Holmes, S. W., Lambert, R. W., Lloyd, W. J., & Ringrose, P. S. (1980) *Antimicrob. Agents Chemother. 18*, 897.

Badet, B., Roise, D., & Walsh, C. (1984) *Biochemistry 23*, 5188.

Baylis, E. K., Campbell, C. D., Dingwall, J. G., & Pickles, W. (1981) *ACS Symp. Ser. No. 171*, 183.

Bergmeyer, H. U. (1974) *Methods of Enzymatic Analysis*, Academic Press, New York.

Buchwald, S. L., & Knowles, J. R. (1982) *J. Am. Chem. Soc. 104*, 1438.

Cassaigne, A., Lacoste, A. M., & Neuzil, E. (1971) *Biochim. Biophys. Acta 252*, 506.

Clark, V. M., & Hutchinson, D. W. (1968) *Prog. Org. Chem. 7*, 75.

Diven, W. F., Scholz, J. J., & Johnston, R. B. (1964) *Biochim. Biophys. Acta 85*, 322.

Forrey, A. W., Olsgard, R. B., Nolan, C., & Fisher, N. E. (1971) *Biochimie 53*, 269.

Henderson, L. L., & Johnston, R. B. (1976) *Biochem. Biophys. Res. Commun. 68*, 793.

Huber, J. W., Gilmore, W. F., & Robertson, L. W. (1975) *J. Med. Chem. 18*, 106.

Johnston, R. B., Scholtz, J. J., Diven, W. F., & Shepard, S. (1968) in *Pyridoxal Catalysis: Enzymes and Model System* (Snell, E. E., Braunstein, A. E., Severin, E. S., & Torschinsky, Y. M., Eds.) p 537, Wiley-Interscience, New York.

Johnston, M. M., & Diven, W. F. (1969) *J. Biol. Chem. 244*, 5414.

Kollonitsch, J., & Barash, L. (1976) *J. Am. Chem. Soc. 98*, 5591.

Kudzin, Z. H., & Stec, W. J. (1978) *Synthesis*, 469.

Lambert, M. P., & Neuhaus, F. C. (1972) *J. Bacteriol. 110*, 978.

La Nauze, J. M., Coggins, J. R., & Dixon, H. B. F. (1977) *Biochem. J. 165*, 409.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.

Lynch, J., & Neuhaus, F. C. (1966) *J. Bacteriol. 91*, 449.

Manning, J. M., Merrifield, N. E., Jones, W. M., & Gotschlich, E. C. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 417.

Martell, A. E., & Langohr, M. F. (1977) *J. Chem. Soc., Chem. Commun.*, 342.

Morrison, J. F. (1982) *Trends Biochem. Sci. (Pers. Ed.)*, 102.

Neuhaus, F. C. (1967) in *Antibiotics: Mechanism of Action* (Gottlieb, D., & Shaw, P. D., Eds.) Vol. 1, p 40, Springer-Verlag, New York.

Nicholson, D. A., Cilley, W. A., & Quimby, O. T. (1970) *J. Org. Chem. 35*, 3149.

Penney, C. L. (1976) *Anal. Biochem. 75*, 201.

Pierce, J., Tolbert, N. E., & Barker, R. (1980) *Biochemistry 19*, 934.

Porter, D. J. T., & Bright, H. J. (1980) *J. Biol. Chem. 255*, 4772.

Reitz, R. H., Slade, H. D., & Neuhaus, F. C. (1967) *Biochemistry 6*, 2561.

Roise, D. (1984) Ph.D. Thesis, MIT.

Roise, D., Soda, K., Yagi, T., & Walsh, C. (1984) *Biochemistry 23*, 5195.

Roze, U., & Strominger, J. L. (1966) *Mol. Pharmacol. 2*, 92.

Satterthwait, A., & Westheimer, F. H. (1980) in *Phosphorus Chemistry Directed Towards Biology* (Stec, W. J., Ed.) p 117, Pergamon Press, Oxford.

Schloss, J. V., & Cleland, W. W. (1982) *Biochemistry 21*, 4420.

Schloss, J. V., Porter, D. J. T., Bright, H. J., & Cleland, W. W. (1980) *Biochemistry 19*, 2358.

Skinner, C. G., McCord, T. J., Ravel, J. M., & Shive, W. (1956) *J. Am. Chem. Soc. 78*, 2412.

Waldi, D. (1965) in *Thin Layer Chromatography* (Stahl, E., Ed.) p 489, Academic Press, New York.

Walsh, C. (1980) *Tetrahedron 38*, 871.

Wang, E. A., & Walsh, C. (1978) *Biochemistry 17*, 1313.

Wasserman, S. A., Daub, E., Grisafi, P., Botstein, D., & Walsh, C. (1984) *Biochemistry 23*, 5182.

Wilkinson, G. N. (1961) *Biochem. J. 80*, 324.

Williams, J. W., & Morrison, J. F. (1979) *Methods Enzymol. 63*, 437.

Wood, W. A. (1955) *Methods Enzymol. 2*, 212.

Wood, W. A., & Gunsalus, I. C. (1951) *J. Biol. Chem. 190*, 403.

Wozniak, M., Nicole, J., & Tridot, G. (1972) *Bull. Soc. Chim. 11*, 4445.

Yonaha, K., Yorifuji, T., Yamamoto, T., & Soda, K. (1975) *J. Ferment. Technol. 53*, 579.