Inactivation of the *Pseudomonas striata* Broad Specificity Amino Acid Racemase by D and L Isomers of β-Substituted Alanines: Kinetics, Stoichiometry, Active Site Peptide, and Mechanistic Studies†

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ABSTRACT: Mechanism-based inactivators were used to probe the active site of the broad specificity amino acid racemase from *Pseudomonas striata*. Kinetic parameters for the inactivation of the racemase with both stereoisomers of β-fluoroalanine, β-chloroalanine, and *O*-acetylserine were determined. By use of ^{14}C-labeled *O*-acetylserines, the stoichiometry of inactivator binding was found to be one inactivator bound per enzyme subunit. The PLP-dependent enzyme contains one coenzyme per subunit, and after NaB^3H_4 reduction of the PLP-imine bond, followed by trypsin digestion of the protein, the amino acid sequence of the PLP-binding peptide was determined. Trypsin digestion of the enzyme labeled with either L or D isomer of *O*-acetylserine and sequencing of the labeled peptide revealed that the inactivators bind to the same lysine residue which binds PLP in native enzyme. The characterization of a PLP adduct released from inactivated enzyme under some conditions is also described. Implications of the formation of this compound with respect to the overall reaction mechanism of inactivation are discussed.

In a review on amino acid racemases, Adams (1976) noted that at that time very few amino acid racemases had been purified to homogeneity despite their potential as therapeutic targets. Among them were three racemases from pseudomonads: an arginine racemase from *Pseudomonas graveolens* (Yorifugi et al., 1971), an alanine racemase from *P. putida*, an enzyme that was very unstable when pure (Rosso et al., 1969), and a broad specificity amino acid racemase from *P. striata* (Soda & Osumi, 1969). Although the physiological role of this *P. striata* amino acid racemase has not yet been determined, it was obtained in crystalline form and in quantity but has been little studied since. The *P. striata* racemase will process alanine at high rates, and we have now investigated whether it would have similar susceptibility to β-substituted D- and L-alanines as suicide substrates in studies parallel to that reported in the preceding paper on the cloned *Salmonella typhimurium* alanine racemase (Badet et al., 1984a). We report here of *P. striata* racemase inactivation and isolation and characterization of the covalently modified active site peptide.

Experimental Procedures

Materials

Materials used were from the same sources as described in the preceding paper (Badet et al., 1984a). PIC-A solution for paired ion chromatography was purchased from Waters Associates. Serines and alanines were assayed for chiral purity by using a coupled assay system containing D-amino acid oxidase and lactate dehydrogenase with NADH[1] for the presence of D isomer contamination or L-alanine dehydrogenase with NAD for L isomers. Change in absorbance at 340 nm due to NADH consumption in the former case, or NADH production in the latter, indicated the presence of the contaminating stereoisomer.

Methods

N^{ϵ}-(Phosphopyridoxyl)-L-lysine. N^{ϵ}-(Phosphopyridoxyl)-L-lysine was synthesized by the method of Forrey et al. (1971).

4-[2-Methyl-3-hydroxy-5-[(phosphooxy)methyl]-4-pyridinyl]-2-oxo-3-butenoic Acid (Compound **2**).[2] 4-[2-Methyl-3-hydroxy-5-[(phosphooxy)methyl]-4-pyridinyl]-2-oxo-3-butenoic acid was synthesized as described by Schnackerz et al. (1979).

O-Acetyl[^{14}C]serines. The *O*-acetyl[^{14}C]serines were synthesized by a modification of the method of Sakami & Toennies (1942). A total of 0.050 mCi of L- or D-[^{14}C]serine was dried in vacuo over $CaSO_4$ and NaOH and suspended in 0.5 mL of acetic acid. A total of 10.5 mg of unlabeled serine and 0.013 mL of 70% $HClO_4$ were added with stirring, and after the solution had cleared, 0.070 mL of acetic anhyride was added. The solution was stirred at room temperature for 4 h, at which point TLC of the reaction mixture showed complete formation of product. (E. Merck, silica gel 60 F-254, 0.25 mm; 1-propanol/water, 70:30; serine R_f 0.28, *O*-acetylserine R_f 0.38); 60-MHz NMR confirmed the structure of the product. The reaction mixture was dried, and the compound was stored frozen in aqueous solution. Specific radioactivities were 0.593 and 0.600 mCi/mmol for the D and L isomer, respectively.

Enzymes. The amino acid racemase (EC 5.1.1.10) was purified from *Pseudomonas striata* AKU 0813 as previously described (Soda & Osumi, 1969). The enzyme was stored at −70 °C.

D-Amino acid oxidase (DAAO) (EC 1.4.3.3) from hog kidney was purified by the method of Jenkins et al. (1979)

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[1] Abbreviations: LADH, L-alanine dehydrogenase; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; PLP, pyridoxal 5′-phosphate; LDH, lactate dehydrogenase; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; DAAO, D-amino acid oxidase; KP_i, potassium phosphate; SDS, sodium dodecyl sulfate; PTH, phenylthiohydantoin; BSA, bovine serum albumin; TCA, trichloroacetic acid.

[2] Nomenclature of Likos et al. (1982) and Ueno et al. (1982).

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as noted in the preceding paper (Badet et al., 1984a).

Assays. Enzymatic activity of the racemase with D- and L-alanine was measured by using the system described in the previous paper for the *Salmonella* alanine racemase (Badet et al., 1984a). Inactivation assays were performed at pH 6.8, since some of the inactivators were much less stable at higher pH. The assays contained 100 mM KP_i (pH 6,8), 0.05 or 0.10 mg of LDH, 0.16 mM NADH, 0.3–0.6 μg of racemase and various concentrations of inactivators in 1.0 mL at 37 °C. The production of pyruvate during processing of the inactivator by the racemase was measured as NADH consumption by LDH.

SDS Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis was performed by using the discontinuous buffer system of Laemmli (1970). Separating gels contained 10% polyacrylamide and stacking gels 4.5% polyacrylamide. Gels were stained by the method of Vesterberg (1971) and destained with 20% ethanol/10% acetic acid in water.

Amino Acid Analysis. All amino acid analyses were performed on a Durrum D500 amino acid analyzer in the laboratory of Prof. Lisa Steiner, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA. For the complete amino acid composition, duplicate samples were hydrolyzed in 6 M HCl in evaculated tubes for 24, 48 and 72 h. Values for serine and threonine were estimated by extrapolation to zero time. The value for tryptophan was calculated by difference from the protein absorbance at 280 nm, assuming that only tyrosine and tryptophan contribute significantly to the absorbance at this wavelength. ϵ_{280} values of 5700 cm^{-1} M^{-1} for tryptophan and 1300 cm^{-1} M^{-1} for tyrosine were used. (Cantor & Schimmel, 1980). No adjustment was made for cysteine lost during hydrolysis.

Protein Determination. Protein concentrations were estimated either from absorbance at 280 nm or by the method of Lowry et al. (1951) using BSA as the standard. The values were adjusted as described under Results.

PLP Determination. PLP concentrations were estimated by using the fluorometric technique of Adams (1979). The concentration of the standard solution was measured spectrophotometrically by using ϵ_{388} = 4900 cm^{-1} M^{-1} for PLP at pH 7.0 (Peterson & Sober, 1954). The racemase solution was dialyzed against 10 mM KP_i (pH 7.0) at 4 °C prior to the determination to remove excess PLP. The PLP standards, as well as the protein samples, were treated with 10% TCA prior to addition of the other reagents.

Active Site Labeling of the Racemase. The radioactive labeling experiments were done at 25 °C. A 0.8-mg sample of racemase was diluted to 0.72 mL with 100 mM KP_i (pH 6.8) in a 1-mL cuvette. A UV–visible spectrum of the solution was taken to determine the A_{280}/A_{415} ratio and an aliquot removed for activity assays. A total of 0.28 mL of 55 mM *O*-acetyl-L-[^{14}C]serine (0.600 mCi/mmol) was added, and at several time points the spectrum between 370 and 500 nm was taken. The extent of reaction was followed by loss of the absorbance at 415 nm. After 15 min there was no further change in 415-nm absorbance, and the sample was applied to a Sephadex G-50 column (18 × 0.9 cm) to separate the protein from small molecules. The column was eluted with 100 mM NH_4HCO_2 at 10 mL/h. Fractions of 1.0 mL were collected. The protein peak was well separated from the major radioactive peak. Aliquots from the protein peak were removed for Lowry assay and scintillation counting. Unlabeled $NaBH_4$ (approximately 1 mg) was added at this point to stabilize the adduct and after 8 h at 4 °C. The solutions were dialyzed extensively against 100 mM NH_4HCO_2 to remove salts. This protein solution was then frozen and lyophilized. Radioactive labeling of the enzyme with the D isomer was performed in essentially the same way. One milligram of racemase was labeled with 0.0168 mmol of *O*-acetyl-D-[^{14}C]serine (0.593 mCi/mmol).

NaB^3H_4 Reduction of the Racemase. A 0.65-mg sample of racemase was diluted to 1.0 mL with 10 mM KP_i (pH 7.0) and a UV–visible spectrum taken. Enzyme having an A_{280}/A_{415} ratio less than 5.7 was dialyzed against 10 mM KP_i (pH 7.0) at 4 °C to remove excess PLP. Approximately 0.1 mg of dry NaB^3H_4 (350 mCi/mmol) was added to the enzyme solution, and the enzyme's characteristic yellow color was bleached instantly. After 2 h on ice the solution was exhaustively dialyzed against 50 mM NH_4HCO_3. Specific radioactivity of the protein was calculated, and the sample was frozen and lyophilized.

Reductive Alkylations, Trypsin Digestions, HPLC Peptide Purification, and Sequencing. These procedures were accomplished essentially as described for the *S. typhimurium dadB* alanine racemase (Badet et al., 1984a).

Release of Unstable Enzyme Adduct. A 1.5-mg sample of protein was inactivated with 0.032 mmol of *O*-acetyl-D-[^{14}C]serine (0.593 mCi/mmol) in 1.1 mL of 100 mM KP_i, pH 6.8 at 22 °C. After 30 min less than 4% of the original activity remained, and the solution was desalted on a Sephadex G-25 column (23 × 1 cm) equilibrated with 100 mM $NH_4CH_3CO_2$. The specific radioactivity of the eluted protein indicated 0.90 inactivator molecule bound per subunit. A portion of the protein-containing solution was dialyzed extensively against 10 mM imidazole, pH 7.0. This solution was placed in boiling water for 2 min and centrifuged to remove precipitated protein. A UV–visible spectrum of the supernatant solution was taken, and a portion was analyzed for radioactivity.

HPLC Separation of the Released Compound. Several HPLC conditions were used to identify the released compound. All separations were done isocratically on a Waters Associates instrument equipped with an Alltech 5-μm analytical C_{18} column. Detection was at 254 nm, and the flow rate was 1.0 mL/min throughout. Four different solvent conditions were used: (1) 0.1% PIC-A, 19% methanol in water; (2) 0.1% PIC-A, 100 mM $NH_4CH_3CO_2$, pH 4.7, 15% methanol in water; (3) 0.1% PIC-A, 100 mM $NH_4CH_3CO_2$, pH 7.0, 15% methanol in water; (4) 0.06% trifluoroacetic acid in water. In each case, standard compound **2** was well resolved from other PLP derivatives.

Spectrophotometric Determinations. All UV–visible spectra were run on a Perkin-Elmer Model 554 spectrophotometer. Kinetic experiments were performed either on the Model 554 or on a Perkin-Elmer lambda 3 instrument. Fluorescence was measured on a Perkin-Elmer LS-3 fluorescence spectrometer.

Kinetic Calculations. All K_m, V_{max}, K_I, and k_{inact} values were calculated by using v^4 (or k_{inact}^4) weighting (Wilkinson, 1961). Double-reciprocal plots were drawn, but the intercepts were calculated by using the v^4 weighting method.

Results

Amino Acid Composition and Protein Concentration Analysis. In studies to determine the stoichiometry of suicide substrate covalent binding to the *P. striata* amino acid racemase described below, it was necessary to obtain an accurate relationship of 280-nm absorbance and Lowry assays (Lowry et al., 1951) to protein content. The pure racemase (120 units/mg) was analyzed for amino acid composition as reported in Table I. For each determination 0.0194 mg of protein (by Lowry assay) was hydrolyzed. The amino acid analyses were used for an independent measure of protein.

Table I: Amino Acid Composition of *P. striata* Amino Acid Racemase

amino acid	residues (mean ± SD)[a]
Asp	36.6 ± 0.4
Thr	24.5 ± 1.3
Ser	21.5 ± 0.8
Glu	39.4 ± 0.6
Pro	11.1 ± 0.3
Gly	35.0 ± 0.6
Ala	43.7 ± 0.4
Val	37.5 ± 0.8
Met	9.4 ± 1.0
Ile	14.1 ± 0.3
Leu	35.2 ± 0.3
Tyr	6.8 ± 0.2
Phe	11.5 ± 0.5
His	11.1 ± 0.1
Lys	17.7 ± 1.3
Arg	23.6 ± 1.1
Trp[b]	8.1
total[c]	387

[a] Based on M_r 42 000. [b] Estimated from 280-nm absorbance as described in text. [c] Total number of residues.

These values were used to calculate the actual amount of protein loaded. The average of six amino acid analyses was 0.0114 mg per experiment so all Lowry protein values were multiplied by 11.4/19.4 = 0.59 to obtain corrected protein concentrations. Similar calculations for 280-nm absorbance gave 0.77 mg/mL per absorbance unit.

Subunit Molecular Weight. Subunit molecular weight was estimated by electrophoresis in 10% polyacrylamide gels in the presence of SDS. The single band observed migrated at a rate corresponding to M_r 42 000. Native gel electrophoreses (7.5% gels) and gel filtration experiments suggest that the racemase can aggregate in a concentration-dependent manner though this has not been pursued in detail. Soda & Osumi (1969) reported a native molecular weight by gel filtration of 110 000.

Stoichiometry of PLP Binding. The *P. striata* racemase was purified in buffers containing PLP, so excess PLP was dialyzed away from purified enzyme prior to analysis of coenzyme binding stoichiometry. The A_{280}/A_{415} ratio of various preparations of enzyme ranged from 4.7 to 5.4 before dialysis. Dialysis against 10 mM KP_i (pH 7.0, 4 °C) increased the ratio to between 5.5 and 5.8 with full retention of activity. By use of samples having a corrected protein concentration of 0.52 mg/mL and A_{280}/A_{415} = 5.6, analysis for PLP by the method of Adams (1979) gave 0.0145 mM coenzyme. The enzyme subunit concentration for this solution was 0.0123 mM, giving a value of 1.17 PLP/subunit. The ϵ_{415} for bound PLP is 9000 M^{-1} cm^{-1}.

Kinetic Parameters. The *P. striata* amino acid racemase shows a broad pH optimum centered around pH 9.0 (data not shown). At pH 9.0 the racemase has a V_{max} of 124 units/mg and a K_m of 7.0 mM for L-alanine, while it has a V_{max} of 249 units/mg and a K_m of 7.4 mM for D-alanine. Kinetic parameters could not be determined accurately far away from the pH optimum due to inefficiency of the D-amino acid oxidase or L-alanine dehydrogenase coupling enzymes. However, at the pH where suicide substrate inactivations were performed (pH 6.8), the racemase retained 50% of its pH 9.0 V_{max} with L-alanine and approximately the same K_m value. The inactivations could not be done at pH 9.0 due to instability of some of the inactivators.

Kinetics of Inactivation with β-Substituted Amino Acids. Incubation of *P. striata* racemase with β-substituted amino acids known to possess antibacterial activity (e.g., β-fluoroalanine, β-chloroalanine, *O*-acetylserine) resulted in mechanism-based inactivation. We have noted this phenomenon previously for a partially purified *Escherichia coli* B racemase (Wang & Walsh, 1978) and in the preceding paper for the cloned *dadB* racemase of *Salmonella* (Badet et al., 1984a). In competition with racemase inactivation each of the above six suicide substrates are catalytically processed to pyruvate, as measured by coupled LDH assay. First-order loss of pyruvate production activity was observed at different concentrations of each isomer of β-fluoroalanine, β-chloroalanine, and *O*-acetylserine. In Figure 1 are replots of inactivation data for all six suicide substrates, allowing calculation of K_I values and k_{inact} values (at infinite [inactivator]). These are collected in Table II along with the number of inactivator molecules processed per enzyme inactivation. This ratio of k_{cat}/k_{inact} is a partition ratio for inactivation efficiency, the frequency that initiation of a catalytic cycle ends in loss of activity for the enzyme molecule. The data of Table II show that the fluoroalanine and chloroalanine isomers display essentially identical partition ratios, 830–890 turnovers per inactivation. The variation with *O*-acetylserine isomers is larger, 720–980, but the ratios are still within experimental error of the haloalanine values. This constant ratio, independent of α-carbon config-

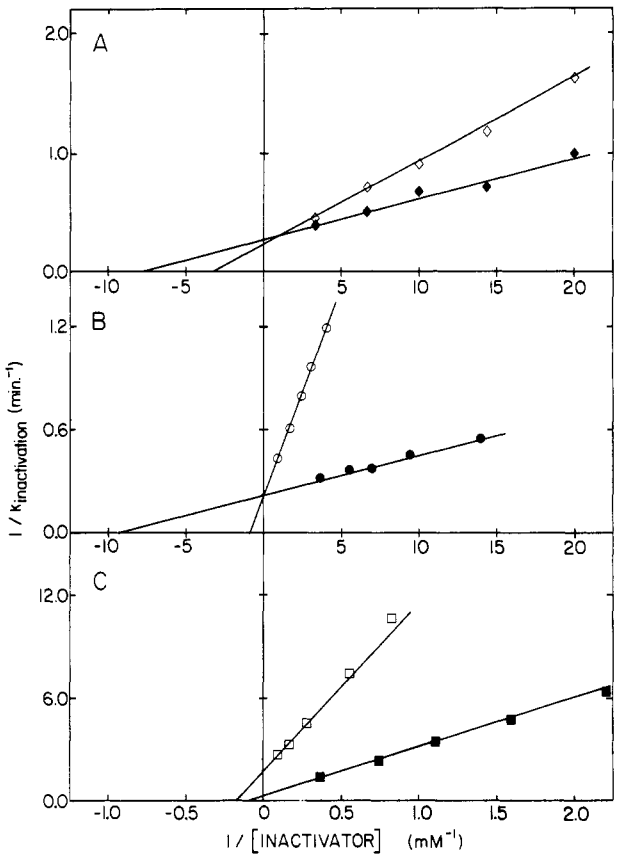


FIGURE 1: Double-reciprocal plots of k_{inact} vs. inactivator concentration. (A) (◇) L-β-Fluoroalanine and (◆) D-β-fluoroalanine. (B) (○) L-β-Chloroalanine and (●) D-β-chloroalanine. (C) (□) *O*-Acetyl-L-serine and (■) *O*-acetyl-D-serine. Intercepts were calculated by the v^4 weighting method of Wilkinson (1961).

Table II: Inactivation Parameters for *P. striata* Racemase

	k_{inact} (min^{-1})	K_I (mM)	partition ratio
L-fluoroalanine	3.7	0.23	870
D-fluoroalanine	3.8	0.14	830
L-chloroalanine	4.9	1.2	890
D-chloroalanine	4.5	0.10	830
O-acetyl-L-serine	0.65	7.1	720
O-acetyl-D-serine	2.9	7.9	980

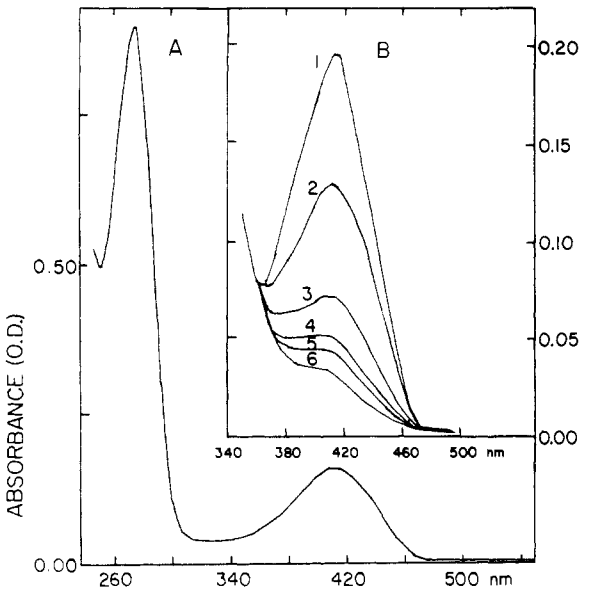


FIGURE 2: UV-visible spectra of *P. striata* racemase. (A) Native enzyme. (B) PLP-imine absorbance during inactivation with 16.7 mM *O*-acetyl-L-[^{14}C]serine. The time at each 415nm peak was (1) 0, (2) 0.88, (3) 1.88, (4) 2.88, (5) 3.88, and (6) 10.38 min after addition of the inactivator.

uration and β-carbon leaving group, has suggested to us previously with *E. coli* B racemase (Wang & Walsh, 1978, 1981) and in the preceding paper with *Salmonella* racemase (Badet et al., 1984a) that a common killing species, symmetric at the α- and β-carbon, is responsible. The partition ratio for the *Salmonella dadB*-encoded racemase is 730–790/1, remarkably similar to that found here despite the fact that the *dadB* racemase has narrow specificity for alanine racemization while the *P. striata* racemase will process a dozen amino acids. One might have thought that this broad specificity indicated more steric tolerance in the active site (and a higher partition ratio).

A major distinction between the *Salmonella* racemase and *P. striata* racemase susceptibility to mechanism-based inactivators does show up in the latter's equal susceptibility to both D and L isomers of *O*-acetylserine. In both *Salmonella* and *E. coli* B alanine racemases, L isomers with large β-substituents are rejected for catalysis and so *O*-acetyl-L-serine does not inactivate. The broad specificity *P. striata* racemase can uniquely accommodate *O*-acetyl-L-serine, allowing the use of radioactive D and L isomers of *O*-acetyl[^{14}C]serine in probe inactivation stoichiometry and active site labeling. It could also mean that *O*-acetyl-L-serine would be a good reagent for killing this pseudomonal enzyme in vivo.

Optical Spectrum of P. striata Amino Acid Racemase during Inactivation. During inactivation with *O*-acetyl-L-[^{14}C]serine the optical spectrum of the racemase was monitored in the region from 370 to 500 nm at 25 °C (Figure 2). The 415-nm chromophore in the initial holoenzyme, reflecting the PLP–enzyme aldimine complex, disappears in time-dependent fashion that is first order for at least 90% of the reaction. The parallel experiment with *O*-acetyl-D-[^{14}C]serine inactivation also induces loss of the PLP–chromophore concomitant with inactivation but at a faster rate. This is as anticipated from the 4.5-fold higher k_{inact} rate constant of Table II. The rate constants for loss of 415-nm absorbance are not directly comparable with the data in Table II because of different temperature and buffer conditions, but the 415-nm loss tracks with inactivation. On complete loss of the 415-nm peak in such incubations, the enzyme contained less than 4% of its initial racemase activity.

Stoichiometry of Mechanism-Based Inactivation. Racemase samples inactivated with either *O*-acetyl-D-[^{14}C]serine or *O*-acetyl-L-[^{14}C]serine were separated by gel filtration from small molecules, and the specific radioactivity of labeled enzyme was determined. Protein inactivated with the D isomer contained 0.51 mCi/mmol of enzyme subunit. For the L isomer there was 0.50 mCi bound/mmol of subunit. On the basis of the specific radioactivity of the inactivators used (0.60 mCi/mmol for L isomer and 0.59 mCi/mmol for D isomer), a stoichiometry of 0.85 inactivator per enzyme for D isomer and 0.83 inactivator per enzyme for L isomer was found. This indicates that a single inactivator molecule is bound per subunit and per PLP coenzyme from either L or D isomer, the minimal stoichiometry anticipated for specific, mechanism-based inactivation. The radioactivity associated with this inactivated enzyme was stable for days at 4 °C.

As part of the procedure for generation of tryptic peptides, the enzyme was denatured in guanidine, and it was observed that radioactivity was quantitatively lost from the protein [as noted for the *Salmonella* enzyme (Badet et al., 1984a)]. This issue is addressed in a separate section. If, however, inactive, radiolabeled enzyme were subjected to subsequent $NaBH_4$ reduction, as noted under Methods, then the radiolabel was stably affixed to the protein for tryptic digestion, peptide isolation, and sequencing.

Reduction of Active, Native Enzyme with NaB^3H_4. Native enzyme (15 nmol) was dialyzed free of excess PLP, reduced by NaB^3H_4, and extensively dialyzed against 100 mM ammonium acetate to yield an optical spectrum lacking the 415-nm peak and exhibiting a shoulder on the A_{280} peak at 325 nm (data not shown). This indicates reduction of the PLP–lysine aldimine link to the hydrolytically stable PNP–lysyl secondary amine linkage. This enzyme had a specific radioactivity of 43 mCi/mmol, suitable for partial hydrolysis and sequencing.

Isolation of Labeled Peptides. The enzyme samples from NaB^3H_4 reduction and from *O*-acetyl[^{14}C]serine inactivation (and then $NaBH_4$-reductive stabilization) were separately digested with trypsin and the peptides separated on HPLC. Representative chromatograms are shown in Figures 3. The peptide elution patterns are highly reproducible, each demonstrating a single major radioactive peptide, in good analogy to the *Salmonella* enzyme case described in the preceding paper (Badet et al., 1984a). The radioactive peak in each case also showed 325-nm absorbance, reflecting a pyridoxamine-type adduct in the peptides. The same absorbance was also seen in the UV–visible spectra of inactivated or reduced proteins (data not shown). The ^{14}C-containing peptides were homogeneous as judged by rechromatography on a second gradient system. The ^{3}H-containing peptide required rechromatography on the second system before it was pure enough for sequencing. Yields of peptides recovered from each run were quite low, ranging from 50 to 80% (based on radioactivity).

Sequence of Labeled Active Site Peptides. Automated Edman degradation was used to determine the amino acid sequence of the three labeled peptides. Since NaB^3H_4 labels the PLP-binding lysine (Tanase et al., 1979), the reduced, tritiated peptide was used to find the sequence of the region of the active site that binds PLP. The sequence of the two peptides labeled by the inactivators was then determined for comparison. The results from all three sequencing runs were the same. This amino acid sequence is indicated in Table III. From the NaB^3H_4-derived peptide, a 16-residue, N-terminal

Table III: Sequence Homology of the Two Racemase Active Site Peptides

Pseudomonas striata	H_2N-[1]Leu-Thr-Ala-*Val*-Leu-*Lys*[a]-*Ala*-Asp-*Ala*-[10]*Tyr*-*Gly*-XXX-*Gly*-*Ile*-Gly-Leu-
Salmonella typhimurium	H_2N-[1]Val-Trp-Ser-*Val*-Val-*Lys*[a]-*Ala*-Asn-*Ala*-[10]*Tyr*-*Gly*-His[b]-*Gly*-*Ile*-Glu-Arg-COOH

[a] Location of bound radioactivity. [b] Based on DNA sequence. See Wasserman et al. (1984).

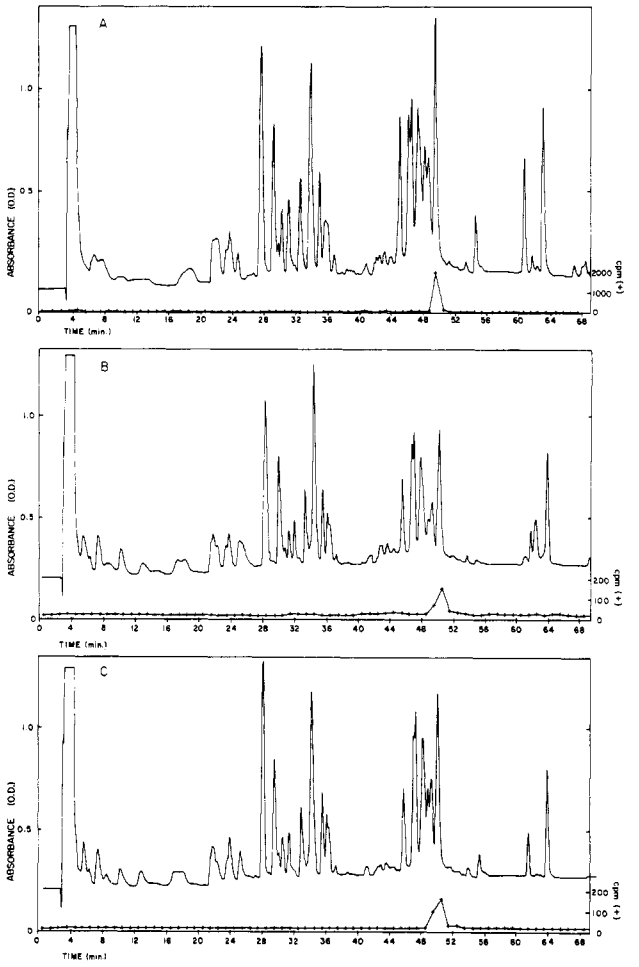


FIGURE 3: HPLC separation of radioactive peptides from trypsin digest of labeled racemase. Absorbance at 254 nm was recorded. (A) Enzyme reduced with NaB^3H_4. (B) Enzyme inactivated with *O*-acetyl-L-[^{14}C]serine and then reduced with unlabeled $NaBH_4$. (C) Enzyme inactivated with *O*-acetyl-D-[^{14}C]serine and then reduced with unlabeled $NaBH_4$.

sequence was determined with a high yield of the first residue leucine (87%) and a very good repetitive yield. We were unable to accurately determine the sequence beyond the 16th position. Residue 6 is the enzyme's active site lysine, derivatized as PNP-ϵ-lysine. The majority of the radioactivity appeared in the HCl layer of cycle 6.

The sequence from the *O*-acetyl-L- and *O*-acetyl-D-[^{14}C]-serine-derived peptides was that of the same peptide, confirming active site covalent modification. However, only minor amounts of radioactivity washed out of the sequencer cup at residue 6, suggesting instability of the inactivator-derived adduct to sequencing conditions. No other significant PTH-amino acid was identified at this locus. The common sequence of these three peptides and the blockage of a potential cleavage site for trypsin at position 6 suggest modification of the lysine by the two inactivators. From the 325-nm absorbance peak we knew those peptides also contained a PNP group so the mechanism-based inactivation had resulted in an adduct between the active site lysine, the coenzyme B_6, and the three-carbon suicide substrate. Insufficient material was available for further characterization of the derivitized lysine at that stage.

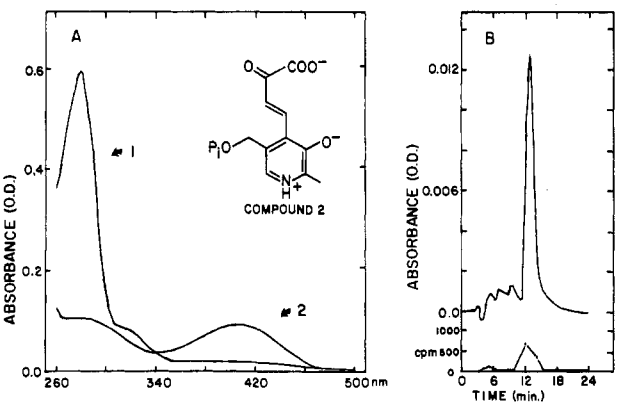


FIGURE 4: (A) Curve 1 shows the UV–visible spectrum of *P. striata* racemase which had been inactivated with *O*-acetyl-D-[^{14}C]serine but not stabilized by reduction with $NaBH_4$. Curve 2 shows the UV–visible spectrum of the supernatant solution after heat denaturation in imidazole and removal of protein. The structure of compound **2** is also shown. (B) HPLC separation of the supernatant solution described in panel A. Absorbance at 254 nm and the position of eluted radioactivity are shown. Trifluoroacetic acid (0.06%) in water was used on an Alltech 10-μm C_{18} column. Authentic compound **2** had an identical retention time.

In none of the three runs was residue 12 identified. However, on the basis of the dramatic sequence homology to the *dadB*-encoded *S. typhimurium* alanine racemase active site peptide (Badet et al., 1984a), it is likely that histidine occurs at this position. PTH-histidine was not extracted under the conditions used.

*Analysis of Radiolabeled Material Released from O-Acetyl-*D*-[^{14}C]serine-Inactivated Racemase.* As noted earlier, the active site peptie labeled by *O*-acetyl-L- or *O*-acetyl-D-[^{14}C]serine could be isolated only if $NaBH_4$ was added to inactive enzyme before denaturation. Otherwise, the radioactivity was labile and was lost as the protein unfolded. Essentially quantitative release of label was obtained with a 2-min heating at 100 °C in water, ammonium acetate or imidazole buffer. Substantial release also occurred after exposure first to pH 12 and then to low pH to precipitate the protein, a modification of the treatment of Likos et al. (1982) and Ueno et al. (1982) of glutamate decarboxylase and aspartate transaminase. The released material was neither starting material nor pyruvate. The optical spectrum of material released into the supernatant solution on heating in imidazole is shown in Figure 4A (curve 2). For comparison the spectrum of the inactive labeled protein before the 2-min boil appears as curve 1, displaying the presence of a 325-nm peak typical of a PNP chromophore, but lack of a 415-nm absorbance. The heat-extracted supernatant solution contains a modified PLP chromophore with absorbance maximum at 405–415 nm, distinct from free PLP at 388 nm. The spectrum of this material is indistinguishable from that of a compound (compound **2**, Figure 4A) prepared by Schnackerz et al. (1979) and recently isolated from aspartate transaminase and glutamate decarboxylase inactivation with equivalent three-carbon suicide substrates (Ueno et al., 1982; Likos et al., 1982). In Figure 4A, the protein concentration from curve 1 computes to 0.013 mM. By use of the known extinction coefficient for compound **2** (ϵ_{405} = 8000 M^{-1} cm^{-1} at pH 7.0), 0.012 mM of this C_3-aldol species was released into supernatant. The radioactivity re-

leased corresponded to 0.011 mM from the known starting specific radioactivity of the *O*-acetyl-D-[^{14}C]serine. This demonstrates quantitative release of inactivator and B_6 coenzyme from the enzyme as a covalent adduct.

Identification of the radiolabeled PLP derivative as compound **2** was established by HPLC. Depending on the conditions used to denature the protein, the stability of the released compound was variable. Under all HPLC conditions used, radioactive material that coeluted with authentic compound **2** was present in the supernatant solution after protein denaturation. Figure 4B shows an HPLC elution profile of the compound released by boiling in 10 mM imidazole; 80% of the released radioactivity eluted in the main peak. The same experiment performed in pure water resulted in 55% yield under this peak. The compound was somewhat labile to the heat step, but no other conditions caused quantitative release of radioactivity in a form suitable for HPLC analysis.

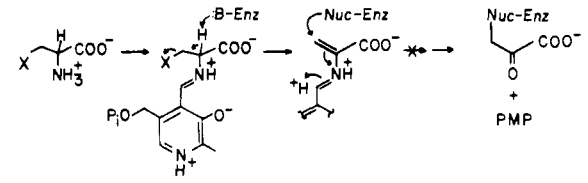
Discussion

The results reported here with the broad specificity amino acid racemase from *Pseudomonas striata* are strikingly similar to those of the companion work on the narrow specificity alanine racemase from *Salmonella* typhimurium in both inactivation efficiency by suicide substrates and that the active site lysine and the B_6 coenzyme are derivatized in each case. It is likely that this will be the general molecular mechanism for inactivation of amino acid racemase involved in cell wall biosynthesis by such antibacterial agents as D-β-fluoroalanine (Kollonitsch & Barash, 1976), D-β-chloroalanine (Manning et al., 1974), and *O*-acetyl-D-serine (Wang & Walsh, 1978).

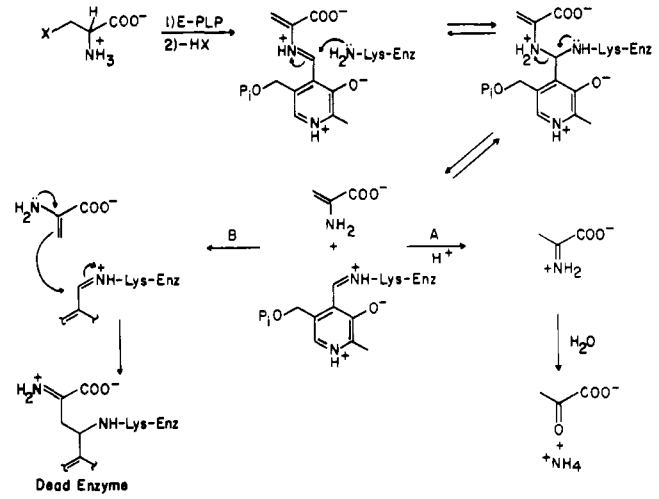
These papers provide the first set of active site sequence data on this therapeutically important class of enzymes, and Table III shows the comparison between *P. striata* and *S. typhimurium* racemase active site peptides. From residue 4 (Val) through residue 14 (Ile), eight residues are identical, and there are two conservative substitutions. Other PLP active site peptide comparisons show no great homology except they resemble the Ser-X-X-Lys placement in the *S. typhimurium* racemase (Tanase et al., 1979). Jansonius and co-workers suggest that the analogous serine in pig heart mitochondrial aspartate aminotransferase helps bind the 5′-phosphate of coenzyme (Ford et al., 1980). In contrast, the *P. striata* racemase has the sequence Thr-X-X-X-Lys, a pattern conserved in the PLP-binding peptides of two other *Pseudomonas* enzymes (Tanase et al., 1979). Given the extensive homology between the peptides from the two racemases, enzymes having markedly different substrate specificities, one wonders if the additional residue between the hydroxy amino acid and active site lysine provides the space to allow the *P. striata* enzyme to racemize longer chain amino acids. We know from the *dadB* gene sequence that the *S. typhimurium* racemase's active site lysine is at residue 35 of 356 amino acids in the M_r 39 000 subunit. The *P. striata* subunit in this work is about M_r 42 000, but no additional sequence data are yet available. It would be interesting to know if the similarity between these two enzymes extends beyond their PLP-binding peptide homology.

The molecular details of racemase inactivation may be relevant for subsequent rational design of antibiotics and so warrant analysis. It is highly likely that the proposal recently suggested by Metzler and colleagues (Likos et al., 1982; Ueno et al., 1982) for aspartate transaminase and glutamate decarboxylase inactivation is operant in these racemases. In their two cases and in these two cases, the label can be quantitatively released and identified as the pyruvate–PLP aldol condensation adduct first synthesized by Schnackerz (Schnackerz et al., 1979).

Scheme I



Scheme II

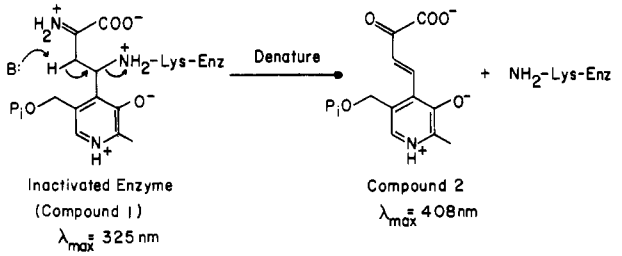


The major consequence of such an inactivation mechanism is that inactivation occurs with *reversal of polarity* from the conventionally assumed mechanism. We, among others, had advanced the hypothesis that an active site *nucleophile* attacks the *electrophilic* β-carbon of the acrylic acid–PLP complex to produce a pyruvylated enzyme nucleophile, e.g., the ε-NH_2 of the active site lysine (Scheme I).

This mechanism fulfills the restrictions that the ultimate inactivating species not contain the initial β-leaving group or recall intial α-carbon configuration but cannot account for quantitative release of compound **2**. Instead, one must postulate, as did Metzler and co-workers (Likos et al., 1982; Ueno et al.,1982), that inactivation does not occur until the acrylic acid–PLP complex has undergone transaldimination by the active site lysine. This regenerates resting PLP–ε-lysyl enzyme aldimine and nascent aminoacrylate (Scheme II). Aminoacrylate can suffer two fates. It can undergo eneamine–imine tautomerization (route A), which occurs with some stereoselectivity at C_3 protonation in the enzyme active site (Badet et al., 1984b), on the harmless route to pyruvate and ammonia. But once in 850 catalytic turnovers, it can attack and covalently modify the $C_{4'}$ carbon of PLP, resulting in inactivation of the racemase (route B). This reaction requires that the eneamine β-carbon act as a nucleophile in attacking the enzyme. The only electrophile offered by the enzyme's active site is in fact the aldehydic carbon of the PLP cofactor. No amino acid side chain is electrophilic; none is competent to be captured in this chemistry. This mechanism has profound consequences for design of antibacterials targeted as alanine racemase inactivators.

The product of aldol condensation of aminoacrylate on PLP–lysyl enzyme aldimine is a ternary adduct with a nonhydrolyzable secondar amine linkage: a dead enzyme species. We see the 325-nm peak expected for a PNP oxidation state. This adduct is stable to decomposition as long as the inactive enzyme is not denatured extensively. On such denaturation we, as did Metzler and colleagues (Likos et al., 1982; Ueno et al., 1982), anticipate that one of the protons on the carbon

Scheme III



which was intially at the β-position of inactivator is now acidic enough to be removed by a general base from solvent. This abstraction is followed by elimination of the ϵ-N of the enzyme's active site lysine, creating the highly conjugated PLP derivative (compound **2**) (Scheme III). Release of this 412-nm chromophore into solution is highly diagnostic of mechanism.

Our peptide sequence data from both racemases, from both L and D suicide substrate isomers, document that the inactivator has trapped active site lysine and a PNP oxidation state in stoichiometric amounts. That both L isomer and D isomer suicide substrates give the identical adduct agrees with the idea that a symmetric species at the α- and β-carbons, i.e., free aminoacrylate, is the killing species. We had been puzzled by the constant partition ratio for killing efficiency in *Salmonella* and *Pseudomonas* racemases given the great difference in substrate specificity. But this inactivation mechanism suggests the efficiency of killing may be very insensitive to active site structure and in particular to the local concentration of nucleophilic amino acid side chains. They cannot intercept amino acrylate. The probability for capture of the enzyme's ϵ-PLP–lysine may be controlled solely by rotational isomerization and diffusion rates of the nascent aminoacrylate within racemase active sites.

In summary this set of three papers (Wasserman et al., 1984; Badet et al., 1984a) provides the first structural information on pyridoxal phosphate linked amino acid racemases, with one complete primary sequence and two active site peptide sequences. Structural studies on the active site lysine derivatized and on the mechanism of inactivation suggest a reversal in polarity from conventional mechanistic expectation. These results should condition design strategies of antibacterial agents for this therapeutically important class of target enzymes.

References

Adams, E. (1976) *Adv. Enzymol. Relat. Areas Mol. Biol. 44*, 69.

Adams, E. (1979) *Methods Enzymol. 62*, 407.

Badet, B., Roise, D., & Walsh, C. T. (1984a) *Biochemistry* (second paper of three in this issue).

Badet, B., Lee, K., Floss, H. G., & Walsh, C. T. (1984b) *J. Chem. Soc., Chem. Commun.*, 838.

Cantor, C. R., & Schimmel, P. R. (1980) *Biophysical Chemistry*, p 380, W. H. Freeman, San Francisco.

Forrey, A. W. Olsgaard, R. B., Nolan, C., & Fischer, E. H. (1971) *Biochimie 53*, 269.

Jenkins, W. T., Behlen, A., & Rogers, P. J. (1979) *Anal. Biochem. 94*, 105.

Laemmli, U. K. (1970) *Nature (London) 227*, 680.

Likos, J. J., Ueno, H., Feldhaus, R. W., & Metzler, D. E. (1982) *Biochemistry 21*, 4377.

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

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

Peterson, E. A., & Soder, H. A. (1954) *J. Am. Chem. Soc. 76*, 169.

Rosso, G., Takashima, K., & Adams, E. (1969) *Biochem. Biophys. Res. Commun. 34*, 134.

Sakami, W., & Toennies, G. (1942) *J. Biol. Chem. 144*, 203.

Schnackerz, K. D., Ehrlich, J. H., Giesemann, W., & Reed, T. A. (1979) *Biochemistry 18*, 3557.

Soda, K., & Osumi, T. (1969) *Biochem. Biophys. Res. Commun. 35*, 363.

Tanase, S., Kojima, H., & Morino, Y. (1979) *Biochemistry 18*, 3002.

Ueno, H., Likos, J. J., & Metzler, D. E. (1982) *Biochemistry 21*, 4387.

Vesterberg, O. (1971) *Biochim. Biophys. Acta 243*, 345.

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

Wang, E., & Walsh, C. T. (1981) *Biochemistry 20*, 7539.

Wasserman, S. A., Daub, E., Grisafi, P., Botstein, D., & Walsh, C. T. (1984) *Biochemistry* (first paper of three in this issue).

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

Yorifuji, T., Ogata, K., & Soda, K. (1971) *J. Biol. Chem. 246*, 5085.