Inactivation of the *dadB Salmonella typhimurium* Alanine Racemase by D and L Isomers of β-Substituted Alanines: Kinetics, Stoichiometry, Active Site Peptide Sequencing, and Reaction Mechanism†

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ABSTRACT: The pyridoxal phosphate dependent *Salmonella typhimurium dadB* alanine racemase was inactivated with D- and L-β-fluoroalanine, D- and L-β-chloroalanine, and *O*-acetyl-D-serine. Enzyme inactivation with each isomer of β-chloro[^{14}C]alanine followed by $NaBH_4$ reduction and trypsin digestion afforded a single radiolabeled peptide. In the same manner, NaB^3H_4-reduced native enzyme gave a single labeled peptide after trypsin digestion. Purification and sequencing of these three radioactive peptides revealed them to be a common, unique hexadecapeptide which contained labeled lysine at position 6 in each case. Enzyme which had been inactivated, but not reductively stabilized with $NaBH_4$, released a labile pyridoxal phosphate–inactivator adduct on denaturation. The structure of this adduct suggests that the enzyme was inactivated by trapping the coenzyme in a ternary adduct with inactivator and the active site lysine. Under denaturing conditions, facile α,β-elimination occurred, releasing the aldol adduct of pyruvate and pyridoxal phosphate. Reduction of the ternary enzyme adduct blocked this elimination pathway. The overall mechanism of racemase inactivation is discussed in light of these results.

The alanine racemase encoded by the *dadB* gene in *Salmonella typhimurium* has been overproduced and purified and the primary sequence determined from sequencing of the cloned DNA, as described in the preceding paper (Wasserman et al., 1984). That work has been undertaken in part to allow structural investigations on the mechanism of alanine racemase inactivation by such antibiotics as D-chloroalanine (Manning et al., 1974), D-fluoroalanine (Kollonitsch & Barash, 1976), and *O*-acetyl-D-serine (Wang & Walsh, 1978). We have previously shown that these molecules are mechanism-based inactivators for the partially purified *Escherichia coli* B alanine racemase (Wang & Walsh, 1978, 1981), an enzyme available in limited quantity with no sequence information available. Amino acid racemases process both the D and L isomers of substrates and are inactivated by both D and L isomers of mechanism-based inactivators (Scheme I).

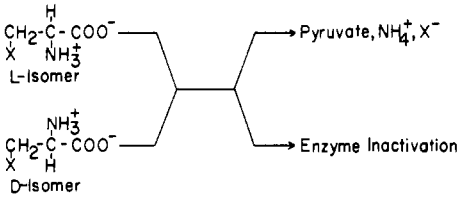
In this paper, and in the following one on the broad spectrum amino acid racemase from *Pseudomonas striata* (Roise et al., 1984), we detail studies on the kinetics and mechanism of racemase inactivation and the isolation and sequence of tryptic peptides labeled with radioactive forms of both isomers of mechanism-based inactivators. The isolation of radioactive tryptic peptides requires borohydride reduction *after* enzyme inactivation. In the absence of such subsequent reduction, the inactive enzyme releases a form of the pyridoxal coenzyme covalently modified by the three-carbon inactivator.

Experimental Procedures

Materials

L- and D-alanine, D-serine, *Bacillus subtilis* L-alanine dehydrogenase (LADH)[1] (EC 1.4.1.1) (27 units/mg in 50% glycerol), CHES, HEPES, and pyridoxal 5′-phosphate were purchased from Sigma. L-Serine was from Cal-Biochem. D- and L-β-chloroalanines were from Vega-Fox Biochemicals. D-

Scheme I



and L-β-fluoroalanines (Kollonitsch & Barash, 1976) were the gift of Dr. J. Kollonitsch of Merck Sharp & Dohme. L-[U-^{14}C]Serine (176 mCi/mmol) and NaB^3H_4 (350 mCi/mmol) were from New England Nuclear. D-[3-^{14}C]Serine (56 mCi/mmol) was from ICN. Hog muscle lactate dehydrogenase (LDH) (EC 1.1.1.27) (550 units/mg, glycerol solution) was purchased from Boehringer-Mannheim. Hog kidneys were from Pel-Freeze Biologicals. TPCK-treated trypsin was from Worthington Biochemicals. Acetonitrile and water used for HPLC were from Omnisolv. Sequencing reagents and solvents were from Beckman. All other chemicals were of analytical reagent grade or of the highest quality commercially available.

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] This compound was synthesized on a 30-mmol scale as described by Schnackerz et al. (1979). After neutralization to pH 7 with

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[1] Abbreviations: LADH, L-alanine dehydrogenase; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-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; Tris, tris(hydroxymethyl)aminomethane; KP_i, potassium phosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; PTH, phenylthiohydantoin; BSA, bovine serum albumin.

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

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$HClO_4$, the resulting concentrated supernatant solution was brought to pH 2 with concentrated HCl. The compound precipitated as a red-orange powder (7.6 g, 75% yield). The compound was pure by TLC and NMR (Likos et al., 1982).

β-Chloro[^{14}C]alanines. β-Chloro[^{14}C]alanines were synthesized according to the Fischer procedure (Fischer, 1907). A total of 105 mg (1.0 mmol) of unlabeled D- or L-serine was added to 0.25 mCi of D- or L-[^{14}C]serine. The resulting solution was lyophilized and dried over P_2O_5. The dried powder was resuspended in 10 mL of dry methanol and saturated with dry HCl. The solvent was removed under vacuum and the residue crystallized in methanol/ether (152 mg, 98% yield). The procedure could be repeated if the esterification was not complete (checked by TLC, silica, 1-propanol/water, 7:3).

The dried serine ester (152 mg, 0.98 mmol) from the last step was dissolved in 7.5 mL of acetyl chloride; 300 mg (1.44 mmol) of phosphorus pentachloride was slowly added at 0 °C. The solid came slowly into solution, and a white precipitate appeared after a few minutes. After stirring for 1 h at room temperature, the white precipitate was filtered, washed with acetyl chloride (10 mL) and ether (20 mL), and recrystallized in methanol/ether (120 mg, 70% yield).

The β-chloroalanine methyl ester (120 mg, 0.69 mmol) was heated in 1.6 mL of 20% HCl at 85 °C for 1.5 h. After dilution with water, the solvent was removed under vacuum and the residue dried over P_2O_5. Recrystallization in methanol/ether gave 107 mg (0.67 mmol, 97 yield) of pure compound (TLC, silica, 1-propanol/water, 7:3). The compound was neutralized to pH 6 and stored frozen in aqueous solution at −20 °C.

Enzymes. The amino acid racemase (EC 5.1.1.1) was purified from *Salmonella typhimurium* DB9071/pSW12 as described in the preceding paper (Wasserman et al., 1984). 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). A total of 175 mg of pure enzyme was obtained from 3.1 kg of tissue. The specific activity was 13 units/mg under the racemase assay conditions.

Assays. Enzymatic activity of the racemase was assayed spectrophotometrically with either L-alanine or D-alanine as substrate. A unit of activity is defined as 1 μmol of substrate epimerized per min. Production of D-alanine from L-alanine was followed in a standard assay mixture containing 100 mM CHES (pH 9.0), 0.05 mg of LDH, 0.15 mg of DAAO, 0.16 mM NADH, 30 mM L-alanine, and 0.12 μg of racemase in 1 mL at 37 °C. Pyruvate produced by DAAO from D-alanine was reduced by LDH with NADH. Loss of absorbance at 340 nm was measured vs. time.

Production of L-alanine from D-alanine was followed by the formation of NADH during L-alanine dehydrogenase mediated oxidation of L-alanine to pyruvate and ammonia. The assay contained 100 mM CHES (pH 9.0), 0.02 mg of LADH, 10 mM NAD, 30 mM D-alanine, and 0.12 μg of racemase in 1 mL at 37 °C. NADH production was followed at 340 nm. In each case, the reaction was initiated by addition either of racemase or of substrate.

Inactivation assays were performed at pH 7.2 since some of the inactivators were much less stable at higher pH. The assay contained 100 mM HEPES (pH 7.2), 0.05 or 0.10 mg of LDH, 0.16 mM NADH, 0.36–0.6 μg of racemase, and various concentrations of the inactivator in a 1-mL cuvette at 37 °C. The production of pyruvate during processing of the inactivator by the enzyme was measured as NADH consumption by LDH.

Chiral Purity. Serine and alanine derivatives were assayed for chiral purity by using a coupled assay system containing D-amino acid oxidase and lactate dehydrogenase with NADH for the presence of D-isomer contamination or L-alanine dehydrogenase with NAD for L-isomer contamination. Change in absorbance at 340 nm due to the NADH consumption in the former case, or NADH production in the latter, quantified the contaminant.

Chiral purity of the β-chloro[^{14}C]alanines was determined by assay based on the much higher rate of turnover of the D isomer with racemase. For L-β-chloro[^{14}C]alanine, inactivator (0.75 μmol) was incubated with racemase (15 μg, 0.38 nmol) for 5 min in 1 mL of standard incubation buffer (see assays). Comparison of the inactivation rate for this solution with those standard mixtures containing known amounts of D isomer gave the percent of contamination with good precision. The purity of the D ^{14}C-labeled isomer was determined by a 10-min incubation of the inactivator (0.05 umole) with racemase (0.38 nmol) under the same conditions. The resulting solution was acidified with acetic acid (0.05 mL) and run through a 0.5-mL Dowex H^+ column. The radioactivity eluting in the water wash (3 mL) and in the ammonium wash (1 M NH_4HCO_2, 3 mL) was determined and compared to a control incubation without the racemase to give the optical purity.

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

Active Site Labeling of the Racemase. The radioactive labeling experiments were done at 26 °C for the L isomer. A 1.42-mg sample of racemase was diluted to 1.9 mL with 100 mM HEPES (pH 7.2) in a 3-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 0.1 mL of 0.5 M L-β-chloro[^{14}C]alanine (0.231 mCi/mmol) was added and the spectrum between 360 and 500 nm taken at several time points. The extent of the reaction was followed by loss of absorbance at 415 nm and activity assays of 0.01-mL aliquots. After 90 min, $NaBH_4$ (approximately 4 mg) was added in small portions with cooling. After 2 h at room temperature, the sample was applied to a Sephadex G-25 column (20 × 1 cm) and eluted with 100 mM NH_4HCO_3 at 10 mL/h; 0.6-mL fractions were collected. The protein-containing peak eluted before, and was cleanly separated from, the peak containing small molecules. The specific radioactivity of this protein was determined, and it was frozen and lyophilized.

Radioactive labeling with the D isomer was performed in essentially the same way. A 1.42-mg sample of racemase was inactivated with 0.025 mmol of D-β-chloro[^{14}C]alanine (0.247 mCi/mmol) at 15 °C. More than 95% inactivation had occurred after 2 min.

NaB^3H_4 Reduction of the Racemase. A total of 1.0 mg of racemase was diluted to 1.0 mL with 10 mM KP_i (pH 7) and a UV–visible spectrum taken. Approximately 0.1 mg of 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 at 0 °C, 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. Typically, the lyophilized, salt-free protein was dissolved in 6 M guanidine hydrochloride, 0.35 M Tris (pH 8.1), and 3 mM EDTA to a 1 mg/mL concentration. A 40-fold molar excess of DTT over the expected

number protein thiols was added, and the sample was sealed under a stream of argon. After incubation for 1 h at 37 °C, a 3-fold molar excess of iodoacetamide over DTT was added, keeping the sample under inert atmosphere. The incubation was continued in the dark for an additional hour at 37 °C. The reaction was quenched with a drop of β-mercaptoethanol. Guanidine was removed by extensive dialysis against 100 mM NH_4HCO_3.

Trypsin Digestions. S-Carboxamidomethylated racemase was suspended in 100 mM NH_4HCO_3 to approximately 1 mg/mL concentration. TPCK-treated trypsin was added to yield a final ratio of 2:100 (w/w) trypsin to racemase, and the solution was incubated at 37 °C. The extent of reaction could be followed easily as the initially cloudy solution became clear. After 4 h the reaction was quenched by adding acetic acid to 10% (v/v) final concentration.

HPLC Peptide Purification. The radioactively labeled peptides were purified by reverse-phase HPLC using a modification of the procedure of Mahoney & Hermodson (1980). The separations were performed on a Waters Associates HPLC consisting of two Model M-45 pumps, either a Model 660 solvent programmer or an automated gradient controller, a Model U5K injector, and a Model 441 absorbance detector fitted with a 214-nm filter. A Waters Associates μ-Bondapak 10-μm analytical alkylphenyl column protected by a short C_{18} guard column was used. Separation was achieved at 1.0 mL/min with a 60-min linear gradient running from 10 to 50% acetonitrile in water; 0.1% trifluoroacetic acid and 0.06% (v/v) trifluoroacetic acid were added to the water and acetonitrile, respectively. Peptides were detected by their absorbance at 214 nm. Fractions were collected at 1-min intervals, and aliquots were counted in Liquiscint aqueous counting scintillant by using a Beckman LS-100 liquid scintillation counter. Elution of peptides containing pyridoxyl adducts was followed by using a second detector equipped with a 313-nm filter. Fractions containing radioactivity were checked for purity by rechromatography of an aliquot on a 60-min linear gradient at 1.0 mL/min from 10 to 50% 2-propanol in water. Again, the water contained 0.1% trifluoroacetic acid, and the 2-propanol contained 0.06%.

Peptide Sequencing. The amino acid sequences of the pure peptides were determined on a Beckman 890C sequenator in the laboratory of Prof. Robert Sauer, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA. A double-cleavage, 0.1 M Quadrol program was used (Brauer et al., 1975), and the sequenator cup was treated with 6 mg of polybrene (Tarr et al., 1978) prior to loading the peptide: 3 nmol of a peptide could be routinely sequenced.

2-Anilinothiazolinone amino acids were converted to their phenylthiohydantoin (PTH) derivatives with 1 M HCl at 80 °C under a nitrogen atmosphere (Ilse & Edman, 1963). The PTH derivatives were extracted with ethyl acetate. PTH-norleucine was added to each cycle before conversion as an internal standard.

The PTH-amino acids were identified on a Hewlett-Packard 5830A gas chromatograph with a 10% SP-400 column and also on a Waters Associates HPLC equipped with a Waters RCM-C_{18} column. The gradient program used has been described previously (Sauer et al., 1981). PTH-arginine and PTH-histidine were not extracted under the conditions used.

Identification of Compound Released after Inactivation without Reduction. (1) Thin-Layer Chromatography. A total of 2.0 mg of *S. typhimurium* alanine racemase was inactivated with D-β-chloro[^{14}C]alanine at room temperature for 15 min under the conditions previously described. After gel filtration, the protein (compound **1**) was pooled and lyophilized. The partially denatured protein was resuspended in water (3 mL) and boiled for 2 min. The supernatant solution (11 800 cpm) was separated from precipitated protein (4100 cpm) and lyophilized. The residue was dissolved in methanol/1 M HCl (5:1) and analyzed on TLC (silica gel; ethanol/water, 7:3, and 1-propanol/water/NH_4OH, 7:3:0.4; 700 cpm/spot). Autoradiography and treatment with Gibb's reagent (Waldi, 1965) showed one major spot (ethanol/water, R_f 0.59, and 1-propanol/water/NH_4OH, R_f 0.26) which ran with synthetic compound **2**.

The remaining solution was subjected to alkaline phosphatase treatment for 24 h at 25 °C at pH 9 and the crude mixture analyzed by TLC (silica gel; 1-propanol/water/NH_4OH, 7:3:0.4). Gibb's reagent and autoradiography again showed a single spot (R_f 0.37) which ran with phosphatase-treated compound **2**.

(2) High-Pressure Liquid Chromatography. A total of 1.3 mg of *S. typhimurium* alanine racemase was inactivated with *O*-acetyl-D-[^{14}C]serine (0.59 mCi/mmol; Roise et al., 1984) under the inactivation conditions previously described. Only 0.5% of the initial activity remained after 30 min. Gel filtration using 10 mM imidazole, pH 7.0, separated protein from small molecules. The protein solution was boiled for 2 min and a UV–visible spectrum of the supernatant solution taken. HPLC of the resulting solution was performed isocratically on an Alltech 5-μm analytic C_{18} column with 0.1% trifluoroacetic acid in water. The boiled enzyme solution contained material which comigrated with authentic compound **2**.

Spectrophotometric Determinations. All UV–visible spectra were recorded either on a Perkin-Elmer Model 554 or on a Perkin-Elmer lambda 3 instrument.

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.

Table I: Kinetic Constants of *Salmonella typhimurium* Alanine Racemase Inactivation by β-Substituted Alanines[a]

inactivator	k_{inact} (min^{-1})	K_m (mM)	partition ratio
β-fluoro-L-alanine	3.49	1.45	770
β-fluoro-D-alanine	3.7	0.072	790
β-chloro-L-alanine	0.49	14	730
β-chloro-D-alanine	1.84	0.077	790
O-acetyl-D-serine	2.55	3.54	760

[a] Inactivations were performed at pH 7.2 as described under Methods. Pyruvate production was measured as NADH consumption by LDH. At each inactivator concentration a replot of pyruvate production activity vs. time on a semilog scale gave the individual k_{inact} values. Intercepts from the double-reciprocal plots were calculated by using k^4 weighting (Wilkinson, 1961). Partition ratios were directly measured from the amount of pyruvate produced.

Results

Kinetics of Inactivation by D *and* L *Isomers of Suicide Substrates.* On the basis of our previous work with the partially purified *E. coli* B alanine racemase (Wang & Walsh, 1978), we anticipated that both D and L isomers of some β-substituted alanines with small β-substituents might function as mechanism-based inactivators for the cloned *dadB* gene product alanine racemase of *S. typhimurium*. Indeed both D- and L-β-chloroalanine and D- and L-β-fluoroalanine inactivate with the kinetics shown in Table I. The two kinetic constants K_I and k_{inact}(max) are compiled in the same table. In competition with the killing reaction, both enantiomeric pairs are processed to pyruvate, and the partition ratios

Table II: Synthesis of β-Chloro[^{14}C]alanines

	β-chloro[^{14}C]alanine				
starting material	optical purity (%)	overall yield (%)	radiochem purity (%)	optical purity (%)	sp radioact. (mCi/mmol)
L-serine	95.8	66.5	95	99.5	0.231
D-serine	93	68	93	93	0.247

(turnovers/inactivation event) are also tabulated. A pattern similar to that seen for the *E. coli* B racemase emerges. While the partition ratios are essentially constant (730–790/1) with this pure *Salmonella* racemase, the K_I values for the D isomers are 20–200-fold lower than the K_I for corresponding L isomers. In particular, in the L series as the substituent is changed from the very small fluoro to the chloro group, the K_I goes from 1.4 to 14 mM. For *O*-acetyl-L-serine there is no inactivation (or elimination to pyruvate) as steric bulk of the acetyl group apparently interferes. For comparison the *O*-acetyl-D-serine has a K_I of 3.5 mM (Table I). In all five entries of the table the common partition ratio argues strongly that the killing species is symmetric at C-2 and is uninfluenced by the initial leaving group ability at C-3. We have suggested (Wang & Walsh, 1978, 1981; Walsh, 1982) that the eneamino–PLP species arising on net α-H,β-X elimination is such a species (see below for example).

Stoichiometry of Racemase Inactivation by D- *and* L-β-*Chloro*[^{14}C]*alanines.* Because the cloned *dadB* gene has been overexpressed in a pBR322 vector (Wasserman et al., 1984), we have been able to obtain pure *S. typhimurium* racemase in sufficient quantity to determine labeling stoichiometry from both the D and L isomers of inactivator (as prelude for sequence work). Although the *O*-acetylserines are synthetically the most convenient enantiomeric pair [see Roise et al. (1984) for the *P. striata* racemase), the L isomer is not a suicide substrate. The enantiomeric fluoroalanines would be ideal but are obtainable only with great synthetic difficulty on radioscale synthesis. Thus, we prepared the D- and L-β-chloro[^{14}C]alanines from the corresponding ^{14}C-labeled serines.

The fact that this *S. typhimurium* racemase shows a 200-fold lower K_I for D- vs. L-β-chloroalanine made chiral purity evaluation important. In particular we wanted L-β-chloro-[^{14}C]alanine of high optical purity. Table II summarizes the preparations. Both the D and L synthesis involved crystallization of β-chloroalanine methyl esters, and this may account for enantiomeric enrichment in the L-β-chloroalanine to 199/200 parts L isomer. While in principle this residual 1 part in 200 of D-β-chloroalanine could be problematic, the inactivation conditions were adjusted to obviate such a problem. For example, we knew the partition ratio (turnovers/inactivation) was about 760/1 for both isomers. By use of only an 800-fold molar excess of L-β-chloro[^{14}C]alanine to enzyme, even if the resultant 4 equiv of D isomer (1 part in 200) were processed with complete kinetic preference, they would still result in at most 4/760 of the possible inactivation events. Thus, only 0.5% of the racemase inactivations could be due to the presence of D-isomer contamination; 99.5% of the racemase molecules would be labeled by the L isomer. Figure 1 shows the change in the UV–visible spectrum of the enzyme vs. time during inactivation with the L isomer. The kinetics were linear during the first 15 min of inactivation. During this time the enzyme had lost 80% of its original activity. The deviation from linearity after this time was not further investigated.

For the labeling experiments with the D isomer, chiral purity was less critical. With our D-β-chloro[^{14}C]alanine preparation, 7% of the flux could possibly come from the L isomer. However, we know it is the D isomer with a 200-fold lower K_I that is strongly kinetically favored as an inactivator. These worries about chiral purity are especially important for subsequent protein sequencing experiments. One wants to be sure that a residue labeled on reaction with L-β-chloro[^{14}C]alanine actually arose from reaction with the L isomer and not a D-isomeric contaminant.

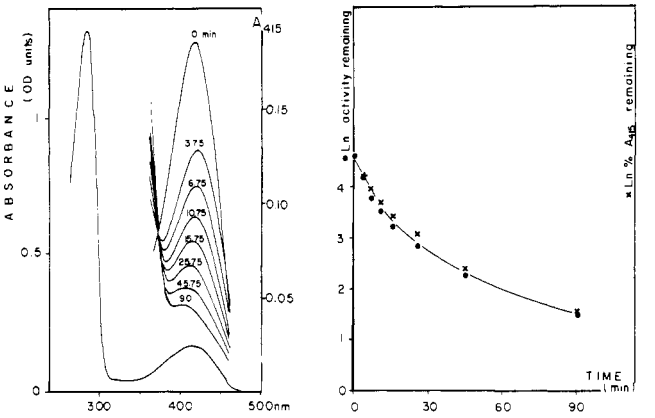


FIGURE 1: Inactivation of *Salmonella typhimurium* alanine racemase with L-β-chloro[^{14}C]alanine. The experiment was performed at 26 °C as described under Active Site Labeling of the Racemase. (Left panel) The UV–visible spectrum between 360 and 500 nm at several time points during the inactivation. (Right panel) Replots of 415 nm absorbance and activity remaining vs. time on the semilog scale.

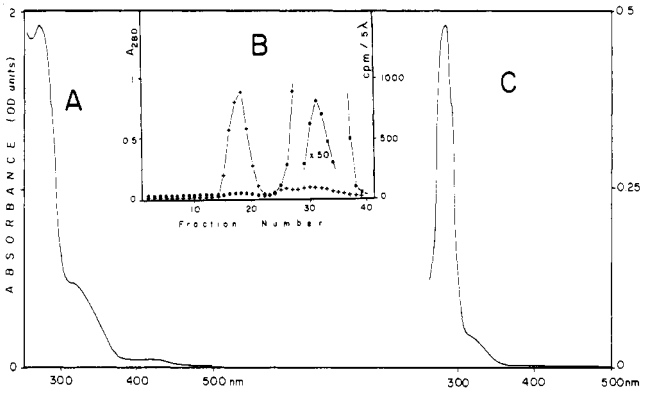


FIGURE 2: (A) UV–visible spectrum of inactivated enzyme (see Figure 1) after $NaBH_4$ reduction. (B) Elution profile of L-β-chloro[^{14}C]-alanine inactivation mixture on Sephadex G-25 (20 × 1 cm). (C) UV–visible spectrum of pooled protein from Sephadex G-25 separation.

After inactivation, enzyme inactivated from either labeled chloroalanine isomer was passed through a Sephadex G-25 gel filtration column, the radioactivity determined, and the protein concentration calculated. Figure 2 shows the column profile and UV–visible spectra of the protein before and after removal of small molecules. As measures of protein concentration the 280-nm absorbance readings were 2-fold high (e.g., assuming BSA as standard) and the Lowry readings 1.5-fold high. The values were corrected by knowledge of the amino acid composition from the encoding DNA sequence (Wasserman et al., 1984). Both D-β-chloro[^{14}C]alanine and L-β-chloro[^{14}C]alanine incorporate between 0.88 and 1.14 labels per mol of M_r 39 000 racemase monomer, indicating that a single inactivator is bound per subunit. Thus, the inactivation is specific.

NaB^3H_4 Reduction of Native Enzyme. Given the racemase primary sequence predicted from the encoding DNA (Wasserman et al., 1984), we wanted to locate the active site lysine in the sequence. We used the standard technique of reduction of PLP–lysine imine in native enzyme by NaB^3H_4 (Tanase et al., 1979) followed by tryptic digestion and HPLC isolation

Table III: Amino Acid Sequence of Labeled Tryptic Peptides from NaB^3H_4 Reduction of Native Enzyme or from Inactivation by L- or D-β-Chloro[^{14}C]alanine

from DNA sequence	-^{30}Val-Trp-Ser-Val-Val-^{35}Lys- Ala-Asn-Ala-Tyr-^{40}Gly-His-Gly-Ile-Glu-^{45}Arg-
NaB^3H_4 reduced[a]	H_2N-^{30}Val-Trp-Ser-Val-Val-^{35}Xxx[b]-Ala-Asn-Ala-Tyr-^{40}Gly-Xxx-Gly-Ile-Glu-
L-β-chloro[^{14}C]alanine inactivated	H_2N-^{30}Val-Trp-Ser-Val-Val-^{35}Xxx[b]-Ala-Asn-Ala-Tyr-^{40}Gly-Xxx-Gly-Ile-Glu-
D-β-chloro[^{14}C]alanine inactivated	H_2N-^{30}Val-Trp-Ser-Val-Val-^{35}Xxx[b]-Ala-Asn-Ala-Tyr-

[a] In all the sequencing runs, the PNP–lysine adduct (residue 6) was not directly identified. Hydrolysis of NaB^3H_4-reduced enzyme (HCl, 110 °C, in vacuo) gave a single radioactive compound which comigrated on TLC with the unidentified hydrolysis product of authentic PNP–lysine (data not shown). [b] Residue containing all the radioactivity.

of the [^{3}H]PNP-containing peptide. The UV–visible spectrum of the reduced racemase is identical with the spectrum of purified, inactivated protein shown in Figure 2C.

Isolation of Radioactive Peptides and Sequencing. To determine the site of enzyme modification on NaB^3H_4 reduction and to see whether or not D- and L-β-chloro[^{14}C]-alanines capture distinct enzyme residues on enzyme inactivation, the radiolabeled enzymes were prepared for tryptic digestion. However, on dialysis of the β-chloro[^{14}C]alanine-treated protein solutions after reductive alkylation of thiols in the presence of guanidine, the radioactivity was lost from the protein. We will return to this problem subsequently. Borohydride reduction of the radiolabeled enzymes after gel filtration and before guanidine denaturation stabilized the radiolabel with the protein such that reduction, alkylation, and exhaustive trypsin digestion was successful. The tryptic peptides of all three radiolabeled proteins were then separated by reverse-phase HPLC as shown in Figure 3. Monitoring the elution at 313 nm located the PNP-containing peptide in each case. A single radiolabeled peptide was produced from all three labeling methods. Equally important, the peptide from either suicide substrate isomer, or from NaB^3H_4 treatment, had the same retention time on HPLC. The radioactive peptide eluted in each case was further purified by isocratic HPLC elution and subjected to N-terminal sequencing. The sequencing data are summarized in Table III.

Analysis of the sequence data for all three peptides of Table III revealed that a common tryptic peptide was being sequenced in each of the three cases. For the NaB^3H_4-reduced and the L-β-chloro[^{14}C]alanine-derived peptide, neither residue 12 nor residue 16 of the hexadecapeptide was identified, but the DNA sequence noted in the preceding paper (Wasserman et al., 1984) predicts histidine at position 12 and arginine at the C-terminus of this hexadecapeptide, and as noted under Methods neither PTH-arginine nor PTH-histidine is extracted under the standard sequenator conditions. All other 14 residues found are as predicted by the DNA sequence for the unique tryptic peptide encompassing valine-30 through arginine-45 in the 356-residue subunit. Thus lysine-35 is the active site lysine. During sequencing of the NaB^3H_4-reduced tryptic peptide, the radioactivity (35 600 cpm) appeared in the HCl layer of cycle 6, as expected for lysine at position 6 in the tryptic petide and position 35 in the enzyme sequence.

For the peptide from L-β-chloro[^{14}C]alanine inactivation, 13 out of 16 residues were positively identified. Only a small amount of radioactivity was detected in the HCl layer of residue 6. This is probably due to instability of the enzyme–PLP–inactivator adduct, since there was no radioactivity at any other residue either. However, as shown by protection of the poential trypsin cleavage site, lysine-35 was modified. For the D-β-chloro[^{14}C]alanine an initial sequencing run gave a poor yield and allowed only identification of the first five residues (Val-Trp-Ser-Val-Val). When the D-β-chloro[^{14}C]-alanine inactivation and peptide isolation was repeated, the trypsin used for digestion was contaminated with chymotrypsin. The single radioactive peptide isolated had a shorter

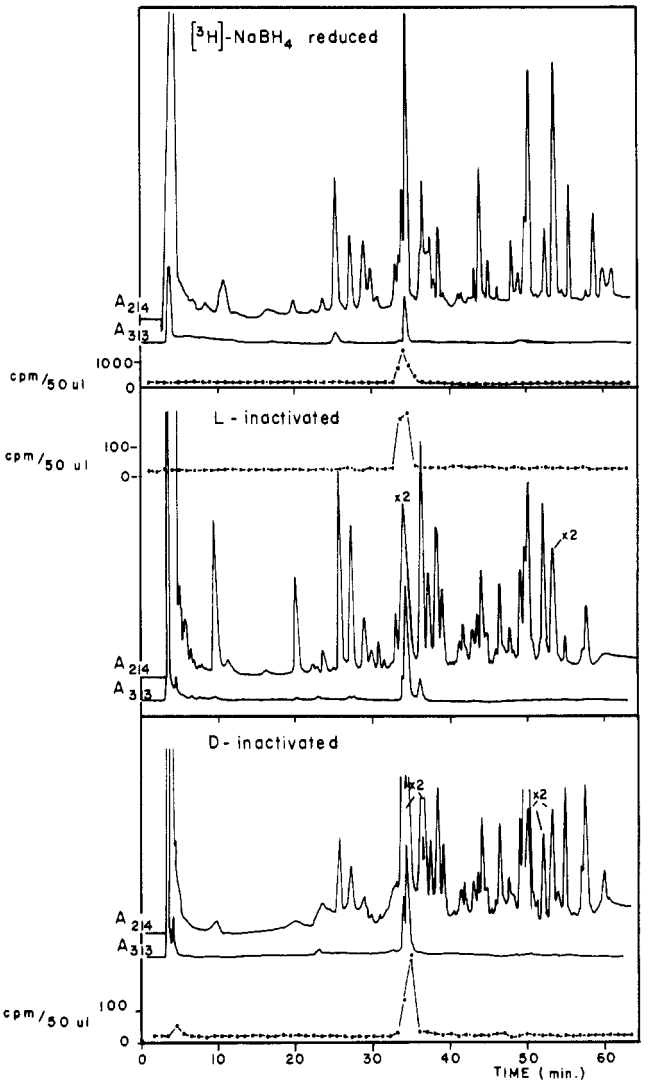


FIGURE 3: HPLC separation of *S. typhimurium* alanine racemase trypsin digests. NaB^3H_4 reduced: Native racemase was reduced by ^{3}H-labeled $NaBH_4$ as described in text. L inactivated: Racemase sample was inactivated with L-β-chloro[^{14}C]alanine (0.23 mCi/mmol) and then $NaBH_4$ reduced prior to trypsin digestion. D inactivated: Racemase was inactivated with D-β-chloro[^{14}C]alanine (0.25 mCi/mmol) and $NaBH_4$ reduced.

retention time on reverse-phase HPLC and had the sequence of the octapeptide, residues 32–39. Again only a small amount of radioactivity appeared in cycle 6. However, there is no doubt that a single active site peptide is labeled from each chloroalanine isomer.

Isolation of Radioactive Adduct from β-Chloro[^{14}C]alanine-Inactivated Enzyme in the Absence of Reductive Stabilization. It was noted above that unless inactivated enzyme was reduced with $NaBH_4$ before denaturation for tryptic hydrolysis, no radioactive peptides were isolable. To analyze the nature of the labile adduct, 2.0 mg of racemase inactivated with D-β-chloro[^{14}C]alanine was isolated by gel filtration and

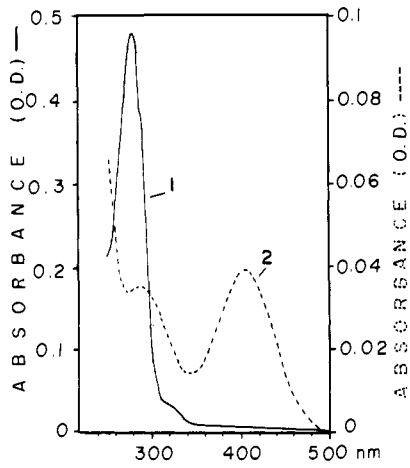


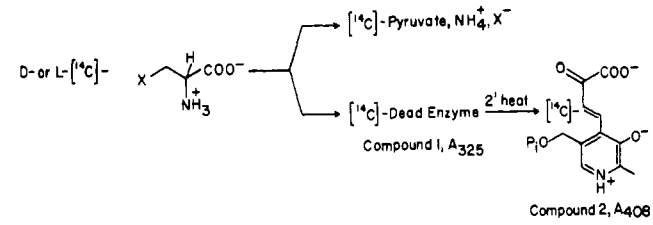
FIGURE 4: UV–visible spectrum of *O*-acetyl-D-serine-inactivated *S. typhimurium* alanine racemase (curve 1; see Methods) after gel filtration with 10 mM imidazole buffer. Note the 325-nm absorbance characteristic of unconjugated pyridoxyl (Scheme II). The solution was boiled for 2 min, the precipitated protein removed by centrifugation, and a UV–visible spectrum of the supernatant solution taken (curve 2). The 408-nm absorbance is typical of compound **2** (Scheme II; A_{max} = 408 nm, ϵ = 8000 cm^{-1} M^{-1}). The concentration of compound **2** in this solution (5 μM, from 408-nm absorbance) is in good agreement with the concentration determined from recovered radioactivity (5.5 μM). The presence of compound **2** was established by HPLC; 36% of the original radioactivity coeluted with authentic compound **2** under conditions described in the text.

lyophilized. The dry sample was resuspended in 3 mL of water and heated for 2 min at 100 °C. The supernatant solution contained 75% of the radioactivity and was lyophilized. TLC of the redissolved residue in two systems (see Methods) gave after autoradiography a single major spot which comigrated with 4-[2-methyl-3-hydroxy-5-[(phosphooxy)methyl]-4-pyridinyl]-2-oxo-3-butenoic acid (compound **2**), an adduct recently prepared by Schnackerz et al. (1979). Treatment of labeled adduct with alkaline phosphatase at pH 9 for 10 h gave again a major radioactive spot migrating with the phosphatase-treated compound **2**, as visualized with Gibb's reagent.

The experiment was repeated for HPLC analysis. Protein eluted from gel filtration was boiled for 2 min to release more than 80% of protein-associated radioactivity. However, only 20% of the radioactivity eluted with standard compound **2** on HPLC. Various conditions were used to release the adduct from the protein, most giving irreproducible results. Enzyme labeled with either β-chloro-D-alanine or *O*-acetyl-D-serine gave the same outcome. After many trials, it was found that dialysis of the gel-filtered protein solution against either water or 10 mM imidazole, pH 7, followed by a 2-min heat treatment released more than 90% of the radioactivity. Of the 60% of counts recovered from HPLC, 60% coeluted with authentic standard, a 32% overall yield.

The UV–visible spectrum of inactivated *S. typhimurium* alanine racemase is shown in Figure 2C. Inactivation involves loss of initial 420-nm absorbance in kinetics consonant with inactivation rate. The 325-nm peak is stable to gel filtration, but on treatment for 2 min at 100 °C followed by centrifugation to remove protein, the supernatant has the 410-nm chromophore characteristic of compound **2** [Figure 4; see also Roise et al. (1984) regarding the *Pseudomonas striata* amino acid racemase). It is clear that the *S. typhimurium* alanine racemase is covalently modified to yield a species that on brief heating releases the label as a modified PLP, a result recently seen also by Metzler and colleagues for glutamate decarboxylase and L-aspartate transaminase inactivation by β-substituted alanines (Likos et al., 1982; Ueno et al., 1982).

Scheme II



Discussion

The availability of the cloned *dadB* gene of *Salmonella typhimurium* (Wasserman et al., 1983) has permitted DNA sequencing, racemase overproduction, and enzyme purification to homogeneity as described in the preceding paper (Wasserman et al., 1984). This in turn has allowed the structural work on mechanism-based inactivation of alanine racemase described here.

The first notable finding is the constant partition ratio (catalytic turnover vs. enzyme inactivation) for three enantiomeric pairs of β-substituted alanines. The ratios are constant despite up to 200-fold differences in K_I (K_m) values for D and L isomers of β-chloroalanine. As with our earlier work on the partially purified *E. coli* B alanine racemase (Wang & Walsh, 1978, 1981), we interpret this constant ratio to reflect a common inactivating species from all the β-substituted amino acid species. The aminoacrylyl-PLP produced on α,β-HX elimination is the first such common species but the free eneamino acid, aminoacrylate, is a second one. As noted below and more extensively in the following paper on the *P. striata* broad specificity amino acid racemase (Roise et al., 1984), aminoacrylate appears now to be the ultimate inactivator.

Since no lag time was observed during the inactivation reaction, it seems likely that the aminoacrylate is not released into solution but rather reacts immediately with the Schiff base at the enzyme's active site. Aminoacrylate is a highly reactive compound and adds to a number of chemical groups faster than it is hydrolyzed to ammonia and pyruvic acid (Cavallini et al., 1973; Sid Masri & Friedman, 1982). When the inactivation was performed in the presence of β-mercaptoethanol (10 mM) or pyridoxal phosphate (0.10 mM), both known to react with aminoacrylate, there was no change in the kinetics of inactivation. This suggests that inactivation occurs by nascent aminoacrylate directly in the active site.

The unitary stoichiometry of inactivation by both D-β-chloro[^{14}C]alanine and L-β-chloro[^{14}C]alanine was consistent with specific mechanism-based loss of activity. The inactive enzyme contained radioactivity in a stable manner for days at 4 °C, provided extensive protein unfolding had not occurred. Upon denaturation by guanidine, by brief heating or by exposure to high pH, the radioactivity was largely released as a single compound, an aldol adduct of pyruvate and pyridoxal phosphate previously prepared by Schnackerz et al. (1979). This covalently modified coenzyme (compound **2**) is similarly released by base treatment of L-aspartate transaminase or glutamate decarboxylase which had been inactivated by β-substituted amino acids (Likos et al., 1982; Ueno et al., 1982). Its detection is highly diagnostic of mechanism, as elaborated in the following paper on *P. striata* racemase (Roise et al., 1984), and has strong implications for future work on design of antibacterial agents targeted against alanine racemases (Scheme II).

The radiolabeled inactive racemase can be stabilized (both in *Salmonella* and *Pseudomonas striata* cases) by BH_4^- treatment after inactivation but before denaturation. This presumably reduces a carbonyl or imino group on the en-

zyme-PLP-inactivator adduct and suppresses the elimination reaction leading to compound **2** by decreasing the acidity of the adjacent protons. The BH_4^--stabilized radioactive enzyme from both D-chloroalanine and L-chloroalanine yielded a single radioactive peptide which on sequencing proved unambiguously to be the tryptic fragment encompassing residues 30–45 in the racemase sequence (Table III). This hexadecapeptide was independently isolated by $B^3H_4^-$ reduction of active, native enzyme. The lysine at position 6 of that peptide, and position 35 in the overall sequence (Wasserman et al., 1984), was shown to be the active site lysine in imine linkage with the B_6 coenzyme. This peptide releases radioactivity at cycle 6 of the sequenator run. Finally, the radiolabeled peptide from each inactivation case contained PNP as judged by absorbance on HPLC at 313 nm. These data, taken together with the quantitative release of compound **2** under denaturing conditions without BH_4^- reduction, indicate that the inactive enzyme with a 325-nm chromophore is a ternary adduct with C_3 of the inactivator linked to the active site lysine via the $C_{4'}$ position of enzyme-bound pyridoxamine phosphate. This issue is examined more fully in the following *P. striata* racemase paper (Roise et al., 1984).

Thus, this work has shown that both D and L isomers of β-chloroalanine capture a single active site residue, and it is the ubiquitous active site lysine. However, the capture of that lysine ϵ-amino group is only indirect, by a tethering through the B_6 coenzyme. The inactivator is covalently linked to the pyridoxal phosphate, not to any enzymic residue, with C_3 of the inactivator attached to the aldehydic carbon of the coenzyme in an aldol type adduct. That aldol adduct in its formation traps the PLP-lysyl aldimine of active enzyme as a nonhydrolyzable secondary amine group in a pyruvyl-PNP–lysine–enzyme ternary adduct. Likos et al. (1982) and Ueno et al. (1982) had recently suggested this inactivation mechanism may be more common than had been previously recognized.

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