Biosynthetic Alanine Racemase of *Salmonella typhimurium*: Purification and Characterization of the Enzyme Encoded by the *alr* Gene†

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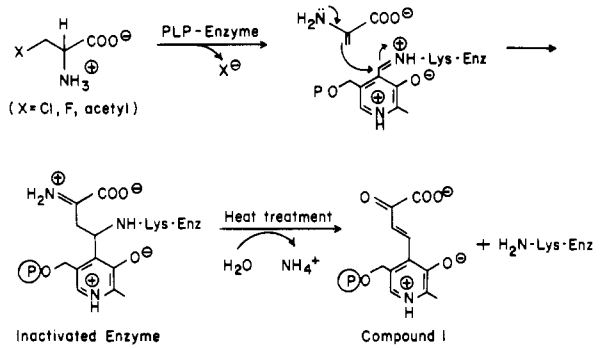
ABSTRACT: An alanine racemase, encoded by the *alr* (*dal*) gene and believed to be the biosynthetic source of D-alanine for cell wall formation, was purified to homogeneity from an overproducing strain of *Salmonella typhimurium* (*dadB*), and the enzymological properties of this enzyme were compared with those of the *dadB* alanine racemase that functions in the catabolism of L-alanine [Wasserman, S. A., Daub, E., Grisafi, P., Botstein, D., & Walsh, C. T. (1984) *Biochemistry 23*, 5182]. The *alr*-encoded enzyme has a monomeric structure with a molecular weight of about 40 000. One mole of pyridoxal 5′-phosphate is bound per mole of enzyme, which is essential for catalytic activity of the enzyme. After the internal Schiff base with pyridoxal 5′-phosphate was reduced with NaB^3H_4, followed by carboxamidomethylation and tryptic digestion of the enzyme, the amino acid sequence of the pyridoxal 5′-phosphate binding peptide was determined. The sequence of 10 amino acid residues around the lysine residue, to which pyridoxal 5′-phosphate is bound, was identical with that of the *dadB* racemase. No homology was found in the amino-terminal amino acid sequence between the two enzymes. The enzyme was inactivated with D- and L-β-fluoroalanine, D- and L-β-chloroalanine, and D-*O*-acetylserine in a mechanism-based fashion with a common partition ratio of about 150. The enzyme was labeled with an equimolar amount of [^{14}C]-D-β-chloroalanine. The inactivator–pyridoxal 5′-phosphate adduct was isolated and shown to be the same structure formed in the *dadB* racemase inactivation [Roise, D., Soda, K., Yagi, T., & Walsh, C. (1984) *Biochemistry 23*, 5195].

D-Alanine is an essential component of the peptidoglycan layer of bacterial cell walls and is thought to be synthesized by an alanine racemase: an enzyme unique to bacteria. In order to design rational antibacterial drugs that specifically target alanine racemases, we have studied the enzymological aspects of alanine racemases (Wang & Walsh, 1978, 1981; Wasserman et al., 1983, 1984; Roise et al., 1984; Badet et al., 1984; Badet & Walsh, 1985) and the synthesis of D-alanine in bacteria. Wasserman et al. (1983) have isolated two different genes specifying alanine racemase activity in *Salmonella typhimurium*. One of these (*dadB*) has been shown to code for an alanine racemase that, apparently, functions primarily in the catabolism of L-alanine (through D-alanine), although it can provide D-alanine for cell wall biosynthesis, as evidenced in *alr* mutants (E. Daub et al., unpublished results). This *dadB* racemase has been purified from *S. typhimurium*, and its enzymological properties and the mechanism of inactivation by various β-substituted alanines have been studied (Wasserman et al., 1984; Badet et al., 1984). Recently, the other gene, designated *alr*, has been isolated and shown to specify the activity of a new alanine racemase that presumably acts in the biosynthesis of D-alanine (E. Daub et al., unpublished results). We had initially named this locus *dal* (Wasserman et al., 1983) before the gene had been fully characterized, but in conformity with the *Escherichia coli* linkage map, we now propose that this gene be renamed *alr* (Wijsman, 1972; Bachmann, 1983).

We here describe the purification of this biosynthetically important *alr*-encoded alanine racemase from a *dadB*⁻ strain of *S. typhimurium* and its enzymological properties. We report also the kinetics and mechanism of inactivation by β-substituted alanines as well as the amino acid sequence at the amino terminus and at the active site to compare with those of the *dadB* racemase. In an accompanying paper, we report the *alr* gene sequence and the predicted polypeptide sequence (Galakatos et al., 1986) with confirmation of that predicted sequence provided by the peptide sequence and amino acid composition data presented here.

Scheme I



Experimental Procedures

Materials. L- and D-alanine, L-*O*-acetylserine, L-alanine dehydrogenase (EC 1.4.1.1) from *Bacillus subtilis* (30 units/mg), D-amino acid oxidase (EC 1.4.3.3) from pig kidney (15 units/mg), pyridoxal 5′-phosphate (PLP), ampicillin (sodium salt), and kanamycin sulfate were purchased from Sigma Chemical Co. Pig muscle lactate dehydrogenase (EC 1.1.1.27, 550 units/mg) was from Boehringer-Mannheim Biochemicals. D- and L-β-chloroalanine were from Vega-Fox Biochemicals. D- and L-β-fluoroalanine were a kind gift from Dr. J. Kollonitsch of Merck Sharp & Dohme (Kollonitsch &

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Barash, 1976). [^{14}C]-D-β-Chloroalanine (0.25 mCi/mmol), 4-[2-methyl-3-hydroxy-5-(phosphooxymethyl)-4-pyridinyl]-2-oxo-3-butenoic acid (Scheme I, compound **1**), and D-*O*-acetylserine were provided by Dr. B. Badet of this laboratory (Badet et al., 1984). NaB^3H_4 (350 mCi/mmol) and NaB^3H_4 (1 mCi/g) were from New England Nuclear. TPCK-treated trypsin was from Worthington Biochemicals. The *dadB* alanine racemase was purified to homogeneity as described previously (Wasserman et al., 1984).

Enzyme Assays. (*1*) *Spectrophotometric Method.* L-Alanine produced from the D-alanine was determined in a reaction mixture (1 mL) containing 100 mM Tricine (*N*-[tris(hydroxymethyl)methyl]glycine)/NaOH (pH 8.5), 0.15 unit of L-alanine dehydrogenase, 50 mM D-alanine, 10 mM NAD, and enzyme (0.005–0.013 unit) at 30 °C. Absorbance at 340 nm was followed.

The formation of D-alanine from L-alanine was followed in a reaction mixture (1 mL) containing 100 mM Tricine/NaOH (pH 8.5), 50 mM L-alanine, 0.12 mM NADH, 110 units of lactate dehydrogenase, 1 unit of D-amino acid oxidase, and enzyme (0.006–0.012 unit) at 30 °C. The decrease in absorbance at 340 nm was monitored. A unit of alanine racemase was defined as the amount of enzyme that catalyzes the formation of 1 μmol of L- (or D-) alanine/min.

(*2*) *Radioactive Assay.* Alanine racemase activity with toluene-permeabilized cells was determined by the same manner as described previously (Wasserman et al., 1983).

(*3*) *Determination of Enzymatic Activity with Other Amino Acids as Substrates.* The enzymatic activity on other L-amino acids was determined with a reaction mixture (1.0 mL) containing 40 mM substrate except for tyrosine (1.4 mM), 100 mM Tricine/NaOH (pH 8.0), 1 unit of D-amino acid oxidase, 2×10^{-6} M FAD, and enzyme (0.02 unit). The reaction was carried out at 30 °C for 30 min. Concentrated HCl (0.1 mL) was added to stop the reaction. After centrifugation, 0.5 mL of the supernatant solution was taken out, and 0.25 mL of 2.5 mM 2,4-dinitrophenylhydrazine in 1.2 N HCl was added to it. After 30 min at 23 °C, 1.75 mL of 2 N NaOH was added to the solution, and the visible spectrum was measured.

Reactivity on L-serine was examined with D-serine dehydratase (EC 4.2.1.14) of *Escherichia coli* (Robinson & Labow, 1971). The reaction mixture contained 0.1 M Tricine/NaOH (pH 8.5), 50 mM L-serine, 0.176 mg of NADH, 110 units of lactate dehydrogenase, 10 μg of D-serine dehydratase, and racemase (0.003–0.012 unit) in a final volume of 1 mL.

The enzymatic incorporation of ^3H into various substrates was measured with a reaction mixture (0.1 mL) containing 75 mM Tricine/NaOH (pH 8.5), 30 mM substrate, 50 mCi of ^3H_2O, and enzyme (0.01–0.05 unit). The enzyme was omitted in a blank. After 60 min at 30 °C, the reaction was stopped by addition of 0.1 mL of 2 N HCl. The reaction mixture was then applied to a Dowex 50 H^+ column (0.5 × 1.5 cm), followed by washing with 20 mL of water. The ^3H-labeled amino acids were eluted with 2 mL of 1 N NH_4OH, and the eluate was evaporated to dryness with a Speed-Vac concentrator (Savant). The residue was then dissolved in 1.0 mL of water and counted with Liquiscint (National Diagnostics).

(*4*) *Inactivation Assay.* The inactivation of the enzyme by β-substituted alanines was followed by the measurement of the rate of β-elimination (i.e., pyruvate production) in a 1-mL reaction mixture containing 100 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] (pH 7.2), 110 units of lactate dehydrogenase, 0.12 mM NADH, and enzyme (20–60 μg). Nonenzymatic β-elimination of β-substituted alanines was slow (at least less than 0.1 nmol/min) under the conditions used.

Strain Construction. The plasmid pSW39, a pBR322 derivative, codes for the *alr* gene, under the control of the leftward promotor of λ, and selectable ampicillin resistance. The plasmid pcI857 codes for kanamycin resistance and *c*I857, the temperature-sensitive λ repressor. These plasmids were constructed and analyzed as described elsewhere (Galakatos et al., 1986). The strain DB7818/pSW39/pcI857 was constructed by P22 generalized transduction as described elsewhere (Wasserman et al., 1983).

Purification of alr Alanine Racemase. The strain DB7818/pSW39/pcI857 (*S. typhimurium*) was grown in a fermentor with 25 L of LB broth (Davis et al., 1980) containing 50 μg/mL ampicillin and 20 μg/mL kanamycin sulfate at 30 °C for 4 h. The temperature was then shifted to 42 °C, and the cultivation was continued for another 1.5 h. The cells were harvested with a Millipore membrane filter and washed with 50 mM potassium phosphate buffer (pH 7.2). The cell yield was about 70 g/25 L.

All the purification procedures were carried out at 4 °C unless otherwise stated. Potassium phosphate buffer (20 mM, pH 7.2) containing 2×10^{-5} M PLP, 0.01% 2-mercaptoethanol, and 0.5 mM EDTA (ethylenediaminetetraacetic acid) was used as the standard buffer.

The cells (190 g) were resuspended in 130 mL of the standard buffer and disrupted by French Press under 12000 psi. The cell debris was removed by ultracentrifugation at 27000*g* for 20 min. Eighty milliliters of 2% protamine sulfate (pH 7.4) was added dropwise to the supernatant (300 mL) over 15 min. After centrifugation at 12000*g* for 20 min, the supernatant solution (370 mL) was mixed with 74 mL of the standard buffer supplemented with 4 M NaCl and applied to a phenyl-Sepharose column (4.4 × 20 cm). The column was washed successively with 1 L each of the standard buffer containing 0.8 M NaCl, the standard buffer, and the standard buffer containing 30% (v/v) glycerol. The enzyme was then eluted with the standard buffer supplemented with 40% (v/v) glycerol. The active fractions were combined and dialyzed against the standard buffer and concentrated with an Amicon PM-10 membrane.

The enzyme was applied to a DEAE-Sephacel column (2.5 × 13 cm) equilibrated with the standard buffer. After the column was washed successively with the standard buffer (480 mL) and with 0.04 M NaCl/standard buffer (90 mL), the enzyme was eluted with 0.09 M NaCl/standard buffer (220 mL). The enzyme solution was concentrated with an Amicon PM-10 membrane and applied to an Ultrogel AcA54 column (1.6 × 90 cm). The main fractions were combined and concentrated in the same manner. The side fractions were combined and rechromatographed with the same Ultrogel column. In some preparations, the enzyme was contaminated by α,ϵ-diaminopimelate epimerase (EC 5.1.1.7), which is easily removed by a Reactive Blue 2–agarose column (Wiseman & Nichols, 1984). The enzyme was stored frozen in the standard buffer supplemented with 15% glycerol at −70 °C until use.

Molecular Weight Determination. The molecular weight was determined in the presence of sodium dodecyl sulfate (SDS) by disc gel electrophoresis according to the method of Laemmli (1970). Bovine serum albumin (M_r 68000), bovine liver catalase (M_r 58000), bovine liver glutamate dehydrogenase (M_r 53000), egg albumin (M_r 43000), rabbit muscle aldolase (M_r 40000), pig muscle lactate dehydrogenase (M_r 36000), bovine erythrocyte carbonic anhydrase (M_r

29 000), bovine pancreatic trypsinogen (M_r 24 000), and bovine milk β-lactoglobulin (M_r 18 400) were used as standard proteins. The native molecular weight was also determined by gel filtration chromatography with an Ultrogel AcA54 column (1.6 × 90 cm). *Pseudomonas fluorescens* β-galactose dehydrogenase (M_r 150 000), bovine serum albumin (M_r 67 000), egg albumin (M_r 45 000), pig kidney D-amino acid oxidase (M_r 37 000), bovine erythrocyte carbonic anhydrase (M_r 29 000), and horse heart cytochrome *c* (M_r 12 400) were used as standard proteins.

Protein Determination. Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard protein. For most column fractions, the protein elution patterns were estimated by their 280-nm absorbance. Concentration of the purified enzyme was determined from the absorbance at 280 nm. The absorption coefficient ($A^{1\%}_{1cm,280nm}$ = 14.9) was estimated from the molecular weight and the amino acid composition of the enzyme as described below.

PLP Determination. PLP was determined by the fluorometric method of Adams (1979). The enzyme was dialyzed against 20 mM potassium phosphate buffer (pH 7.2) at 4 °C prior to determination of the PLP content.

Determination of Amino Acid Composition. Amino acid analysis was performed with a Durrum D500 amino acid analyzer. Duplicate samples of the enzyme were hydrolyzed in 6 N HCl in evacuated tubes at 120 °C for 24, 48, and 72 h. Serine and threonine were estimated by extrapolation to zero time of hydrolysis. Half-cystine was determined as *S*-(carboxymethyl)cysteine with the carboxamidomethylated enzyme prepared as follows. The enzyme (0.35 mg) was dissolved in 0.4 M Tris-HCl (pH 8.2) containing 6 M guanidine hydrochloride and 3 mM EDTA (final volume 1.0 mL). After the enzyme solution was flushed with Ar, 16 μmol of dithiothreitol was added, followed by incubation at 23 °C for 30 min. Monoiodoacetamide (9.3 mg, Eastman) was then added to the solution, which was subsequently incubated at 23 °C for 90 min. The alkylation was quenched by addition of one drop of 2-mercaptoethanol. The enzyme thus treated was dialyzed against several changes of 100 mM NH_4HCO_3 (1 L each) at 4 °C and subjected to amino acid analysis after hydrolysis in 6 N HCl for 24 h. Tryptophan was determined spectrophotometrically at 280 and 288 nm in the presence of 6 M guanidine hydrochloride by the method of Edelhoch (1967). Tyrosine was also determined spectrophotometrically by measurement of the absorbance at 295 and 300 nm at pH 6.5 and 12.5 according to the method of Bencze and Schmidt (1957).

Analysis of Amino Acid Sequence. The amino acid sequence analysis was carried out by automated Edman degradation with a Beckman 890C sequencer. A single-cleavage 0.1 M Quadrol program was used for the amino-terminal sequence analysis. The purified peptides were analyzed by a double-cleavage program. Polybrene was added to the sequenator cup to minimize extractive losses of the sample. 2-Anilinothiazolinone amino acids were converted to their phenylthiohydantoin (PTH) derivatives with 1 M HCl at 80 °C under N_2. The PTH amino acids were identified with a Hewlett-Packard 5830A gas chromatograph (with a 10% SP-400 column) and also with a Waters Associates HPLC equipped with a Waters RCM C-18 column.

NaB^3H_4 Reduction, Carboxamidomethylation, and Tryptic Digestion of Enzyme and HPLC Separation of the Radioactive Peptides. About 0.1 mg of solid NaB^3H_4 (350 mCi/mmol) was added to 3.4 mL of the enzyme solution (1.0 mg/mL), followed by incubation at 23 °C for 30 min. The yellow color from the PLP cofactor disappeared soon after the addition of NaB^3H_4. The enzyme solution was dialyzed against seven changes of 1 L of 100 mM NH_4HCO_3 (pH 8.2) at 4 °C and evaporated to dryness with a Speed-Vac concentrator. The residue was dissolved in 1.0 mL of 1.2 M Tris-HCl (pH 8.1) containing 6 M guanidine hydrochloride. About 3 mg of dithiothreitol was added to the solution under Ar, and the enzyme was alkylated with 8 mg of monoiodoacetamide as described above. TPCK-trypsin (40 μL of a 0.5 mg/mL solution) was added to the enzyme suspension (3.2 mL). The solution became almost clear after 6 h at 37 °C, and glacial acetic acid was added (10% v/v) to the solution to stop the reaction, followed by evaporation with a Speed-Vac concentrator. Radioactive peptides were isolated and purified by HPLC with a reverse-phase column (Alltech C-18, 10 μm) on a Du Pont HPLC system. The column was programmed with a 60-min linear gradient from 10 to 50% acetonitrile in water at a flow rate of 1.5 mL/min. Trifluoroacetic acid was added at concentrations of 0.1 and 0.06% (v/v) to water and acetonitrile, respectively. Peptides and N^{ϵ}-pyridoxyllysine were detected at 217 and 313 nm, respectively. Fractions were collected every minute, and radioactivity was counted in Liquiscint with a Beckman LS 1800 liquid scintillation counter.

Enzyme Inactivation by [^{14}C]-D-β-Chloroalanine and Analysis of the Enzyme Adduct. The enzyme (1.5 mg) was incubated with 20 μmol of [^{14}C]-D-β-chloroalanine (0.25 mCi/mmol) in 0.58 mL of 0.1 M HEPES (pH 7.2) at 30 °C. After 30 min, less than 2% of the original enzyme activity remained. The reaction mixture was applied to a Bio-Gel P6DG column (1 × 12 cm) equilibrated with 10 mM imidazole buffer (pH 7.1). The ^{14}C-labeled protein was collected and boiled for 2 min. After centrifugation, the supernatant solution was passed through a Centricon-30 microconcentrator (Amicon). The filtrate was subjected to HPLC analysis with a Videk 10-μm C-18 column and 0.06% trifluoroacetic acid at a flow rate of 1.0 mL/min.

RESULTS

Purification of the alr Alanine Racemase. The *S. typhimurium* strain DB7818 has a Tn*10Δ16Δ17* transposon (Foster et al., 1981) in the *dadB* alanine racemase gene so none of this racemase will be produced (Wasserman et al., 1983), thus making this strain a suitable strain from which to purify the *alr*-encoded alanine racemase. The *alr* gene was present on the multicopy plasmid pSW39 (Galakatos et al., 1986). The plasmids were introduced into DB7818 by P22 generalized transduction as described elsewhere (Wasserman et al., 1983). When DB7818/pSW39/pcI857 was grown at 30 °C in LB medium (with 50 μg/mL ampicillin and 20 μg/mL kanamycin sulfate), alanine racemase activity of 0.01–0.03 unit/mg was detected (by the radioactive assay with the toluene-permeabilized cells). This was about 100-fold higher than the racemase activity of strain DB7818 lacking all of the above plasmids. When the temperature was shifted to 42 °C in the early exponential phase of growth as described under Experimental Procedures, the enzyme activity was further increased to 0.04–0.07 unit/mg. The temperature-sensitive λ repressor *c*I857 is inactivated at 42 °C, and thus, the *alr* racemase was effectively overproduced, as the gene is under the control of p*L* promoter of λ. The increase in activity seen in DB7818/pSW39/pcI857 over wild-type activity at 30 °C was probably a result of the natural *alr* promoter, which is still on the plasmid pSW39.

The enzyme was purified by a simple method with good yield (85%) as summarized in Table I. The chromatographic procedures used are essentially the same as described previ-

Table I: Summary of Purification of *alr* Alanine Racemase

step	vol (mL)	total protein (mg)[a]	total act. (units)[b]	sp act. (units/mg)
crude extract	300	22000	930	0.042
protamine sulfate	370	12000	870	0.073
phenyl-Sepharose	330	440	850	1.9
DEAE-Sephacel	220	150	960	4.4
Ultrogel AcA54	10	36[c]	790	22

[a] Protein was determined by the method of Lowry. [b] Activity was measured by the direction from D- to L-alanine. [c] A corrected value by multiplication of 0.8 (see text).

Table II: Amino Acid Composition of *alr* Amino Acid Racemase

amino acids	residues (mean ± SD)	amino acids	residues (mean ± SD)
Asp	29.5 ± 0.07	Ile	15.9 ± 0.6
Thr[a]	17.1	Leu	28.3 ± 1.1
Ser[a]	18.7	Tyr	7.3 ± 0.5
Glu	41.2 ± 1.0	Phe	10.7 ± 0.2
Pro	24.3 ± 0.9	Trp[b]	7.2
Gly	34.6 ± 0.3	His	10.0 ± 0.3
Ala	46.0 ± 1.2	Lys	13.0 ± 1.3
Val	30.9 ± 1.2	Arg	24.6 ± 0.8
Met	8.6 ± 1.0	Cys[c]	3.7

[a] Determined by extrapolating to zero time of hydrolysis. [b] Determined spectrophotometrically. [c] Determined as *S*-(carboxymethyl)cysteine.

ously for the *dadB* alanine racemase purification (Wasserman et al., 1984). Ammonium sulfate (25% saturation) can be substituted for NaCl (0.8 M) in the phenyl-Sepharose chromatography without any decrease in recovery of total activity. The enzyme is more stable than the *dadB* racemase in the presence of ammonium sulfate (Wasserman et al., 1984). Although the *alr* racemase can be bound to the phenyl-Sepharose column in the absence of NaCl or ammonium sulfate, recovery of the activity decreased significantly (less than 40%). At present, the reason for this is not known.

Amino Acid Composition and Protein Concentration Analysis. The relationship between 280-nm absorbance and protein concentration measured by the Lowry method was determined as follows. An enzyme sample that had an absorbance of 0.39 at 280 nm gave a value of 0.327 mg/mL by the Lowry method. Two 50-μL samples were hydrolyzed separately in 6 N HCl for 24, 48, and 72 h and analyzed with an amino acid analyzer (Table II). The actual amount of protein hydrolyzed was calculated from the molecular weights (subtracting 18 for H_2O) and the amounts of each amino acid found. The average of six amino acid analyses gave an estimated weight of 13.06 μg for the protein hydrolyzed. Thus, $A^{1\%}_{1cm,280nm}$ of the enzyme was calculated to be 14.9 (i.e., A_{280} multiplied by 0.67 gives an actual protein concentration in terms of mg/mL). When the Lowry method is used to determine protein concentration, the Lowry value should be multiplied by 0.80 to obtain the corrected value for the protein concentration.

Molecular Weight. SDS–polyacrylamide gel electrophoresis of the purified enzyme gave a single band, which had an estimated M_r of 42000. This value is anomalously high as noted from the *alr* gene sequence (Galakatos et al., 1986). When a mixture of *dadB* and *alr* racemases were electrophoresed in the presence of SDS, *dadB* migrated faster than *alr*. A M_r of 39000 was obtained for the *dadB* enzyme, which is consistent with the value determined from the DNA sequencing of the *dadB* gene (Wasserman et al., 1984). The

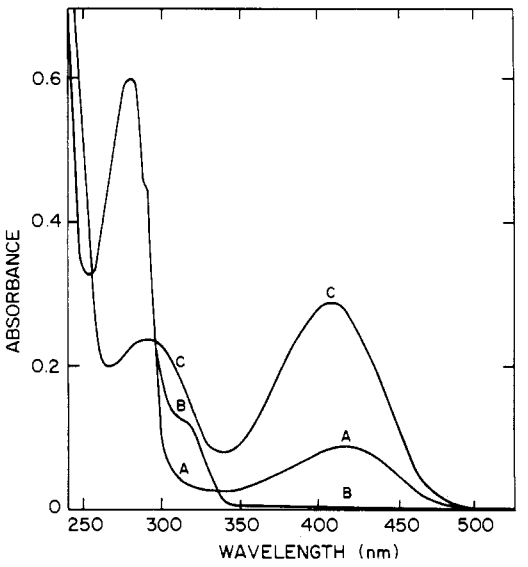

FIGURE 1: UV–visible spectra of the native *alr* racemase (A), enzyme after inactivation by [^{14}C]-D-β-chloroalanine and chromatography by Bio-Gel P6DG (B), and enzyme adduct isolated by boiling of the chloroalanine-inactivated enzyme in 10 mM imidazole buffer (pH 7.1) (C).

native molecular weight of the *alr* enzyme was estimated to be about 43000 by Ultrogel AcA54 chromatography. These results indicate that the enzyme is composed of a single polypeptide chain. Wasserman et al. (1984) also reported a monomer structure for the *dadB* racemase; very few other PLP enzymes are monomeric.

Cofactor Requirements. Figure 1A shows the absorption spectrum of the enzyme, which is characteristic of a PLP enzyme. The A_{280}/A_{420} ratio was about 6.7, and the extinction coefficients at 280 and 420 nm were calculated to be 62000 and 9300 M^{-1} cm^{-1}, respectively, from the $A^{1}_{1cm,280nm}$ (see above). The enzyme solution, which had A_{280} of 0.197, was found to contain 3.3 μM PLP when determined fluorometrically. Using the extinction coefficient at 280 nm, we calculated the PLP content to be 1.0 mol/mol of enzyme.

The enzyme incubated with 10 mM NH_2OH (pH 7.2) at 30 °C for 20 min showed almost no activity (remaining activity was about 1.8%) in the absence of added PLP and no longer exhibited the absorption maximum at 420 nm. Activity was restored to 68% of the original activity by the addition of 5 × 10^{-6} M PLP. The K_m value for PLP was estimated to be 3.3 × 10^{-8} M. The reconstituted enzyme showed a A_{280}/A_{420} ratio of about 7.1, which is slightly higher than that of the original holoenzyme.

Amino-Terminal Amino Acid Sequence. Nine residues were determined from the amino-terminal end by Edman degradation (Table III). Only one major PTH-amino acid was detected in each cycle up to cycle 9. The calculated amount of the amino-terminal PTH-methionine is probably higher than the actual amount due to experimental error. But the results strongly suggest that the enzyme is composed of a single polypeptide chain. The enzyme has no similarity to the *dadB* racemase in terms of amino-terminal amino acid sequence (Wasserman et al., 1984). The *alr* N-terminal nonapeptide sequence is matched exactly by that predicted from the gene sequence (Galakatos et al., 1986), as is the active site peptide sequence reported below.

Isolation and Sequencing of N^{ϵ}-(Phosphopyridoxyl)lysyl Peptide. PLP enzymes studied so far have unique lysine residues that bind PLP through an aldimine linkage at the active site (Snell & DiMari, 1972). The Schiff base has been

Table III: Sequential Edman Degradation of Native and Peptide Fragments of the *alr* Alanine Racemase

protein or peptide (nmol)	cycle no.[a]									
	1	2	3	4	5	6	7	8	9	
alr	Met-	Gln-	Ala-	Ala-	Ser-	Val-	Val-	Ile-	Asp-	
(5.2)	5.2	5.0	3.4	3.4	ND[b]	2.6	2.4	2.9	4.3	
	1	2	3	4	5	6	7	8	9	10
F1	Leu-	Val-	Ala-	Val-	Val-	Lys[d]-	Ala-	Asn-	Ala-	Tyr-
(2.2)[c]	2.1	1.7	1.7	1.2	1.2	0.49	1.1	0.67	0.96	1.4
	1	2	3	4	5	6	7	8	9	10
F2	Leu-	Val-	Ala-	Val-	Val-	Lys[d]-	Ala-	Asn-	Ala-	Tyr-
(4.2)[c]	3.8	3.6	2.7	3.4	2.4	0.90	2.3	1.7	1.4	1.8
	11	12	13	14	15	16	17	18		
	Gly-	His-	Gly-	Leu-	Leu-	Gln-	Thr-	Ala-		
	0.62	ND[b]	0.46	0.72	0.72	0.51	ND[b]	0.59		

[a] Numbers shown below each residues are amount of PTH-amino acids determined by HPLC. [b] Not determined. [c] Calculated from the radioactivity. [d] Location of bound PLP. Recovery was measured by the radioactivity.

reduced with NaB^3H_4 to analyze the active site sequences (Tanase et al., 1979). We also have labeled the PLP binding lysine of *alr* racemase with NaB^3H_4, and sequenced the N^{ϵ}-(phosphopyridoxyl)lysyl peptide as follows. The reduced enzyme (79 nmol), with a specific radioactivity of 68 μCi/mmol, was digested with trypsin after carboxamidomethylation as described under Experimental Procedures. We found most of the original radioactivity (3.9 μCi; 73%) in the supernatant after centrifugation. When the tryptic digest was chromatographed by HPLC, two radioactive peaks both absorbing at 313 nm were obtained. Radioactivity found in the two fractions was as follows: F1 (the fraction that appeared first), 0.54 μCi; F2 (the second fraction), 1.3 μCi. These fractions were purified further by isocratic conditions with 2-propanol/water and acetonitrile/water. After purification, 0.15 μCi of F1 and 0.29 μCi of F2 were obtained, each of which are shown to be homogeneous peptides by analytical HPLC runs with several solvents systems.

The automated Edman degradation of the peptide F2 was carried out for 18 cycles with a sufficient repetitive yield. No PTH-amino acids were found in reasonable amounts beyond the 18th cycle. The majority of the radioactivity was found in the acidic aqueous phase after the conversion step at the sixth cycle of degradation. In addition, the aqueous phase exhibited an absorption band at 325 nm at pH 7.2, which is characteristic of a pyridoxyl derivative. The identity of the residue as the phenylthiohydantoin derivative of N^{ϵ}-(phosphopyridoxyl)lysine was confirmed by the same manner as described previously (Tanase et al., 1979). The main product at the 12th cycle was found in the aqueous phase and identified as PTH-histidine by HPLC: the retention time was 5.5 min with a 10-μm Videx C-18 column and 10% 1-propanol in 50 mM sodium acetate (pH 7.0) at a flow rate of 1.0 mL/min. Thus, the sequence was determined as shown in Table III.

The sequence of the F1 peptide was also determined by the same manner up to the 10th cycle. The radioactivity was released at the sixth cycle, and the sequence was found to be identical with that of the F2 peptide from the first residue through the tenth. The formation of this small peptide is probably ascribed to chymotrypsin contamination of trypsin. Tanase et al. (1979) also obtained two N^{ϵ}-(phosphopyridoxyl)lysyl peptides from pig heart alanine aminotransferase (EC 2.6.1.2). Their shorter peptide lacks a part from the amino terminal through the histidine residue present in the longer peptide.

Table IV: Inactivation Parameters for *alr* Alanine Racemase

inactivator	k_{cat} (min^{-1})[a]	K_I (mM)[a]	partition ratio
β-fluoro-L-alanine	0.10	0.19	160
β-fluoro-D-alanine	0.13	0.048	160
β-chloro-L-alanine	0.077	2.3	170
β-chloro-D-alanine	0.093	0.13	160
O-acetyl-D-serine	0.094	1.3	140

[a] Determined by double-reciprocal plots of inactivation rate against inactivator concentration.

Substrate Specificity. The enzyme was found to show maximum activity around pH 8.5 in Tricine/NaOH when examined in various kinds of buffers at several pH values between 6.5 and 9.1. The following kinetic parameters were determined at pH 8.5 with Tricine/NaOH: L-alanine, K_m = 2.7 mM, V_{max} = 36 units/mg (1500 min^{-1}); D-alanine, K_m = 2.0 mM, V_{max} = 27 units/mg (1100 min^{-1}). With these values, the K_{eq} from the Haldane relationship (Briggs & Haldane, 1925) was calculated to be 1.0, which is identical with the theoretical value for a racemase reaction. The K_m values are similar to those for *dadB* racemase, but the V_{max} values are much lower (2.5 and 6.5% for L- and D-alanine, respectively) than those for the latter enzyme (Wasserman et al., 1984).

When ^3H incorporation into amino acids was examined in ^3H_2O, only the following amino acids were found to be substrates of the enzyme: L-alanine, 14000 dpm/μmol (100%); D-alanine (93); glycine (160); L-cysteine (6.3); L-homoserine (29); L-serine (66). No significant radioactivity was incorporated into L-asparagine, L-aspartic acid, L-glutamine, L-glutamic acid, L-histidine, L-leucine, L-threonine, D-valine, L-cystine, L-isoleucine, D-*allo*-isoleucine, L-homocysteine, L-homocystine, L-lysine, L-arginine, L-ornithine, α,ϵ-diaminopimelate, α-aminopimelate, β-aminobutyrate, β-alanine, DL-alanyl-DL-alanine, L-*O*-acetylserine, D-β-fluoroalanine, γ-glutamyl-L-alanine, and N^{ϵ}-(DL-2-amino-2-carboxyethyl)-L-lysine (Sigma). Enzymatic determination with D-amino acid oxidase also showed that the following L-amino acids were inert: α-aminobutyrate, leucine, methionine, norvaline, proline, phenylalanine, tyrosine, and tryptophan. L-Serine is the only substrate found that undergoes racemization at a significant rate: relative to L-alanine, about 15%.

Kinetics of Inactivation by β-Substituted Alanines. The *alr* alanine racemase was inactivated by β-substituted alanines in the same manner as alanine racemase of *E. coli* (Wang & Walsh, 1978, 1981), the *dadB* alanine racemase (Badet et al., 1984), and the amino acid racemase with broad specificity from *Pseudomonas striata* (Roise et al., 1984). The *alr* racemase, as well as the other racemases, catalyzed β-elimination of β-substituted alanines to produce pyruvate, which was measured by coupling the reaction to lactate dehydrogenase. The enzyme activity was lost with time by pseudo-first-order kinetics during incubation with D and L isomers of β-fluoro- and β-chloroalanines and D-*O*-acetylserine, but L-*O*-acetylserine did not affect the enzyme activity after prolonged incubation. Table IV summarizes kinetic parameters of enzyme inactivation. All the inactivators gave essentially the same partition ratio, which is the average number of the β-elimination events catalyzed per molecule of enzyme before the enzyme is inactivated (i.e., k_{cat}/k_{inact}). This indicates

that a common species caused inactivation that is symmetric at the at the α-carbon and is not influenced by the initial leaving group ability at the β-carbon. Badet et al. (1984) have shown that the *dadB* racemase shows a higher partition ratio (about 800), lower affinity for L isomers of β-fluoroalanine (K_I = 1.45 mM) and β-chloroalanine (14 mM), but much higher (10–30-fold) inactivation rates (k_{cat}) than the *alr* racemase. This is compatible with the relative reactivity between these enzymes on the normal substrates, D- and L-alanine. The selective inactivation by the D isomer of *O*-acetylserine is a common feature for both the enzymes.

Isolation of Enzyme Adduct with [^{14}C]-D-β-Chloroalanine and Its Analysis. The enzyme inactivated with [^{14}C]-D-β-chloroalanine was chromatographed on Bio-Gel P6DG to remove small molecules. The enzyme (1.4 mg, 33.6 nmol) was found to be labeled with 9.8 μCi (39.6 nmol) of [^{14}C]-D-β-chloroalanine: a stoichiometry of 1.2 mol of the inactivator/mol of enzyme. This indicates that essentially a single inactivator molecule is bound to the enzyme. The inactivated enzyme no longer exhibited the absorption maximum at 420 nm, and a new chromophore absorbing around 320 nm appeared (Figure 1B). Similar spectral changes were observed for the *dadB* racemase (Badet et al., 1984) and the amino acid racemase with broad specificity of *P. striata* (Roise et al., 1984).

When the ^{14}C-labeled enzyme was boiled for 2 min in imidazole buffer (pH 7.1), most of the radioactivity (7.7 μCi; 79% recovery) was released from the protein. The absorption spectrum of the supernatant (Figure 1C) was identical with that of authentic 4-[2-methyl-3-hydroxy-5-(phosphooxymethyl)-4-pyridyl]-2-oxo-3-butenoic acid (Scheme I, compound **1**). The amount of compound obtained was calculated to be 34.1 nmol on the basis of the extinction coefficient of the authentic compound at 405 nm (8000 M^{-1} cm^{-1}). The identity of the compound was further confirmed by HPLC in the same manner as reported previously (Badet et al., 1984; Roise et al., 1984). Only one major peak appeared at 6.8 min, which cochromatographed with authentic compound **1**. About 60% of the radioactivity injected was recovered with the peak.

DISCUSSION

Since our demonstration by genetic analysis and gene isolation in 1983 that *S. typhimurium* contains two genes for the interconversion of L-alanine and D-alanine (Wasserman et al., 1983), we have been interested in the molecular and physiological characterization of the two gene products. A similar system of two alanine racemase genes also exists in *E. coli* (Wijsman, 1972; Wild et al., 1985) and may well be general since the L-alanine–D-alanine couple can be viewed as a branch point in bacterial amino acid metabolism, providing D-alanine for the D-alanine branch of the peptidoglycan biosynthetic pathway or providing D-alanine for oxidative deamination and energy metabolism by the membrane-bound D-alanine dehydrogenase (the *dadA* gene product; Wild et al., 1985). We were successful first in isolating the *dadB* gene, which maps at minute 36 on the *S. typhimurium* chromosome next to the *dadA* gene, apparently in a catabolic operon for L-alanine degradation, and we have reported the sequencing of the *dadB* gene, the overproduction of the *dadB* alanine racemase, and its purification to homogeneity (Wasserman et al., 1984). Furthermore, we have uncovered the molecular basis of action of β-substituted alanines as antibacterials on the isolated *dadB* gene product via α,β-HX elimination and attack of nascent aminoacrylate on the PLP-Lys aldimine at the aldehydic carbon to give a ternary adduct where the α-carbon of the inactivator is covalently tethered to the PLP-Lys secondary amine group, now a nonhydrolyzable linkage (Roise et al., 1984).

The *alr* alanine racemase gene has now been mapped at minute 91 on the *S. typhimurium* chromosome (E. Daub et al., unpublished results). This locus has been shown to be identical with the 8.4kb *Hin*dIII fragment of *S. typhimurium* DNA we had independently cloned as a locus conferring a phenotype of growth at restrictive temperature of an *E. coli* mutant that requires D-alanine at 42 °C. Initially, we had named this locus *dal*, but genetic analysis has shown this locus to be analogous to the *alr* locus in *E. coli* (Wijsman, 1972; Bachmann, 1983). In the interest of conformity, we propose that the name of this gene be changed to *alr*. We have now purified this presumed biosynthetic alanine racemase from an overproducing strain and determined its primary structure (by gene sequencing; Galakatos et al., 1986), catalytic properties, and susceptibility to inactivators. The overproduction is about 15-fold to 0.3% of soluble cell protein and has allowed us to isolate 36 mg of pure enzyme from 190 g wet weight of cells. (Given the completion of the gene sequence reported in the preceding paper, we have now constructed a better overproducing plasmid using an *Eco*RI site 8 base pairs upstream of the *alr* gene and have about 6% of the cell protein as *alr* racemase; N. Galakatos, unpublished data.) The purification of the enzyme was performed from strain DB7818, which has a Tn*10*Δ*16*Δ*17* insertion (Foster et al., 1981) in the chromosomal *dadB* gene (Wasserman et al., 1983), so the only alanine racemase produced is clearly encoded by the *alr* gene on either the chromosome or the plasmid.

The molecular weights of the *alr* and *dadB* racemases are very similar; both are PLP-containing monomers of M_r 39000, 359 amino acids (the *alr* racemase; Galakatos et al., 1986), and 356 residues (the *dadB* racemase; Wasserman et al., 1984). The K_{eq} for both enzymes is unity, as it must be for amino acid racemization, and the K_m values for alanine isomers are in the low millimolar range for each alanine racemase. While these values may seem high for a biosynthetic function for the *alr* alanine racemase, the K_m values for other peptidoglycan synthetic enzymes are as follows: D-alanyl-D-alanine ligase (EC 6.3.2.4), K_m = 4.8 mM (Neuhaus, 1962); UDP-*N*-acetylmuramate:L-alanine ligase (EC 6.3.2.8), K_m = 0.15 mM for L-alanine (Mizune & Ito, 1973). Antibodies raised against either racemase do not cross-react, and this may permit separate studies on intracellular localization and compartmentalization. Neither the *dadB* nor the *alr* racemase, when overproduced, is found in the periplasmic space, nor are there detectable signal sequences.

A difference is seen in the catalytic efficiency between the *dadB*- and *alr*-encoded alanine racemases, with the *dadB* enzyme a 20–60-fold faster catalyst depending on which racemization direction is assayed. The molecular basis of this catalytic difference is as yet unobvious, especially given the identical active site decapeptide sequences for the two enzymes. It does appear that even when the *dadB* gene is repressed, enough *alr* alanine racemase molecules are present in the *S. typhimurium* cell to provide all the D-alanine needed for peptidoglycan synthesis (E. Daub et al., unpublished results).

There is also a quantitative difference in susceptibility of the two alanine racemases to antibacterials of D-β-fluoroalanine (Kollonitsch et al., 1975) or the D-*O*-acetylserine type (Wang & Walsh, 1978; Neuhaus & Hammes, 1982), which act as suicide substrates for each racemase by generating nascent aminoacrylate product molecules in the active site as a consequence of the α-H, β-X elimination sequence catalyzed by these PLP-linked enzymes. We have shown here that the *alr*

racemase is inactivated to give the same ternary coenzyme–enzyme inactivator adduct that we had previously deduced for the *dadB* racemase (Roise et al., 1984; Badet et al., 1984). This inactivation arises from kinetic competition between protonation of the aminoacrylate at the anionic α-carbon and rare nucleophilic capture of the aldehydic carbon of the PLP-Lys aldimine at residue 35 (the *dadB* racemase; Wasserman et al., 1984) or 34 (the *alr* racemase; Galakatos et al., 1986) of the racemases. While we have previously isolated a radiolabeled peptide from [^{14}C]chloroalanine-inactivated, BH_4^--reduced *dadB* racemase and shown that it contains ^{14}C at the active site Lys-35 position, we have not repeated this with the *alr* racemase. We know Lys-35 is the active site lysine in the an aldimine linkage to PLP, and the three-carbon inactivator is in turn covalently linked to the PLP from the subsequent identification of the PLP–inactivator adduct. The partition ratio of turnover (protonation of intermediate) to inactivation (PLP aldimine capture) is a measure of how much drug is needed to inactivate a given racemase molecule. On average, 800 molecules of D-β-fluoroalanine or D-*O*-acetylserine are turned over to pyruvate per killing event with the *dadB* racemase but only 160/1 for the *alr* alanine racemase. This 5-fold difference is a sensitive measure of the subtle distinctions in the active site environments of the *dadB* and *alr* racemases (e.g., is the *alr* racemase active site more sequestered from solvent protons?) but also has potential practical implications. It takes 5-fold less drug to kill a *alr* alanine racemase molecule than a *dadB* racemase molecule; the biosynthetic racemase target is intrinsically more susceptible to the antibacterials, and this is in the therapeutically useful direction. However, when the *dadB* gene is derepressed, not only does the number of racemase targets increase, but depending on how much *dadB* alanine racemase is synthesized, the amount of drug needed to cause inactivation (and subsequent inhibition of growth) will increase up to 5-fold. The regulation of expression of the *alr*- and *dadB*-encoded racemase genes will be of fundamental and practical interest and is an ongoing subject of investigation in these laboratories. There appear to be no additional alanine racemase genes in this prototypic Gram-negative organism (Wasserman et al., 1983, 1984; E. Daub et al., unpublished results).

Registry No. PLP, 54-47-7; D-alanine, 338-69-2; L-β-fluoroalanine, 35455-21-1; D-β-fluoroalanine, 35455-20-0; L-β-chloroalanine, 2731-73-9; D-β-chloroalanine, 39217-38-4; *O*-acetyl-D-serine, 44901-25-9; alanine racemase, 9024-06-0; L-alanine, 56-41-7.

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