Catabolic Alanine Racemase from *Salmonella typhimurium*: DNA Sequence, Enzyme Purification, and Characterization†

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ABSTRACT: The alanine racemase encoded by the *Salmonella typhimurium dadB* gene was purified to 90% homogeneity from an overproducing strain. At 37 °C the enzyme has a specific activity of 1400 units/mg (V_{max}, L- to D-alanine). Active enzyme molecules are monomers of M_r 39 000 with one molecule of pyridoxal 5′-phosphate bound per subunit. The K_m's for L- and D-alanine are 8.2 and 2.1 mM, respectively. Measurement of turnover numbers yielded the expected K_{eq} value of 1.0. Determination of 22 of the 25 N-terminal amino acid residues of the purified polypeptide allowed localization of cloned DNA encoding the structural gene. Sequencing of subcloned DNA revealed that the *dadB* gene encodes a polypeptide of 356 amino acids whose calculated molecular weight (apoenzyme) was 39 044.

Alanine racemases (EC 5.1.1.1) are enzymes unique to prokaryotic organisms that interconvert L- and D-alanine (Wood & Gunsalus, 1951). As the sole-identified biosynthetic sources of D-alanine for bacterial cell wall assembly, they have been seen as particularly suitable targets for antibiotic research (Park, 1958). Recently developed drugs, halogenated derivatives of D-alanine and phosphoalanine-containing dipeptides, appear to act predominately, if not solely, by blocking the racemization of L- to D-alanine (Kollonitsch et al., 1973; Manning et al., 1974; Allen et al., 1978; Atherton et al., 1979). Further design and development of racemase-directed antibacterials and, in particular, of agents with more selective toxicity would be greatly facilitated by a detailed description of the chemistry and geometry of the active site of an alanine racemase.

Although several alanine racemases have been detected, few have been investigated in detail [for reviews see Adams (1972, 1976)]. The enzyme from *Pseudomonas putida* is unstable when pure (Adams et al., 1974), that from *Pseudomonas* sp. 3550 is poorly characterized (Free et al., 1967), and that from *P. striata* is uniquely nonspecific for alanine (Soda & Osumi, 1969). Furthermore, the cofactor requirement of the enzyme purified from *Bacillus subtilis* is unclear (Diven et al., 1964; Babu, 1974; Yonaha et al., 1975). Of the purified alanine racemases, only that from *B. subtilis* has an experimentally defined role in alanine metabolism (Berberich et al., 1968).

We have recently described an alanine racemase in *Salmonella typhimurium* that is essential only for L-alanine catabolism, providing substrate for a D-specific alanine dehydrogenase (encoded by the *dadA* gene) (Wasserman et al., 1983). This enzyme, the major source of intracellular alanine racemase activity, is probably a secondary source of D-alanine for cell wall biosynthesis. However, because alanine racemases can detoxify many agents directed against D-alanine metabolism (Wang & Walsh, 1978), the action of this enzyme is relevant to antibiotic research. We now describe the purification of the *S. typhimurium* enzyme encoded by the *dadB* gene, made possible by overproduction from a cloned gene. We also report the physical and kinetic characterization of the racemase as well as its primary structure as predicted by DNA sequencing.

Materials and Methods

Measurement of Enzymatic Activity. All assays were performed at 37 °C. A unit of enzyme was that amount which catalyzed the formation of 1 μmol of product/min. Activities were measured by the continuous, coupled, spectrophotometric assays of Wang & Walsh (1978), except that CHES[1] buffer (100 mM, pH 9.2) was substituted for potassium phosphate. The coupling enzyme D-amino acid oxidase (DAAO) was purified from frozen hog kidneys by the method of Jenkins and co-workers (Jenkins et al., 1979). To follow the course of racemase purification, activity was measured solely in the L to D direction: product D-alanine was converted by D-amino acid oxidase to pyruvate, which was in turn reduced by lactate dehydrogenase and NADH; the loss of absorbance at 340 nm was followed.

For kinetic studies of purified enzyme, activity was also measured in the D- to L-alanine direction: product L-alanine was converted to pyruvate with L-alanine dehydrogenase and NAD; the increase in absorbance at 340 nm was monitored. Racemase activity toward amino acids other than alanine was assayed with a Clark O_2 electrode calibrated by DAAO oxidation of D-alanine. The reaction mixture contained 0.5 unit of DAAO and 20 units of catalase in 1 mL of CHES buffer (100 mM, pH 9.0).

Purification of Alanine Racemase. Alanine racemase was purified by a five-step procedure modified from that of Wang & Walsh (1978). All steps were performed at 4 °C except where noted. Buffers contained 0.5 mM EDTA and 10^{-5} M pyridoxal phosphate (PLP); columns were packed and run by gravity feed. During purification peak column fractions were identified by assay of racemase activity or by monitoring the ratio of absorbance at 280 and 420 nm.

Cell Growth. Bacteriophage P22 was used to transduce plasmid pSW12 into DB9071, a prototrophic strain of *S. typhimurium*, by methods previously described (Wasserman et al., 1983). Strain DB9071/pSW12 was grown to saturation in M9 minimal medium (Miller, 1972) with 1% casamino acids (Difco) as carbon source and 25 μg/mL ampicillin. Cells were collected by centrifugation and chilled to 4 °C. A total of 30–50 g of cell paste was used in a single purification.

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[1] Abbreviations: CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; kb, kilobase.

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Cell Lysis. Cells were washed in 50 mM sodium phosphate buffer (pH 8.0) containing 1.4 mM β-mercaptoethanol (βME). The washed cells were centrifuged and resuspended in 5 volumes of the same buffer and sonicated. Following disruption, the extract was clarified by centrifugation.

Protamine Sulfate Precipitation. One-fifth volume of 2% protamine sulfate (pH 7) was slowly added to the clarified supernatant. The suspension was stirred for 15 min and centrifuged at 25000*g* for 20 min, and the pellet was discarded.

Hydrophobic Affinity Chromatography. The resulting supernatant was diluted with one-fourth volume of buffer (20 mM sodium phosphate, pH 8.0, 1.4 mM βME, 4 M NaCl) and loaded at 60 mL/h on a 250 mL phenyl-Sepharose (LKB) column (4.4 × 16 cm) preequilibrated with the same buffer containing only 0.8 M NaCl. The column was washed with 1 L of the preequilibration buffer, and the enzyme activity was then eluted with a convex gradient consisting of 500 mL of the initial (preequilibration) buffer and 1 L of the final buffer (20 mM sodium phosphate, pH 8.0, 1.4 mM βME, 30% glycerol). The gradient was run at 80 mL/h, and 15-mL fractions were collected. Peak fractions were pooled and concentrated 5-fold by ultrafiltration (Amicon apparatus, PM10 membrane). The concentrate was then diluted with 3 volumes of ethylenediamine buffer (20 mM, pH 7.0) containing 10% glycerol.

Ion-Exchange Chromatography. The diluent was loaded at a flow rate of 10 mL/h onto an 80-mL DEAE-Sephacel (Pharmacia) column (2.5 × 16 cm) preequilibrated with ethylenediamine buffer. The column was washed with 3 bed volumes of the same buffer containing 50 mM NaCl, and the enzyme was eluted with a 400-mL (total volume) linear gradient of 50–225 mM NaCl (25 mL/h flow rate). Fractions (2.5 mL) of the highest specific activity were pooled and concentrated by ultrafiltration.

Gel Filtration. The concentrate (1–2 mL) from the DEAE-Sephacel column was loaded onto a 180-mL Ultrogel AcA54 (LKB) column (1.6 × 90 cm) preequilibrated with gel filtration buffer (50 mM sodium phosphate, pH 8.0, 10% glycerol). Protein was eluted at a flow rate of 6 mL/h. Fractions (2.0 mL) containing purified enzyme were pooled and stored at −70 °C.

Polyacrylamide Gel Electrophoresis. Analytical sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out as described by Laemmli (1970) and modified by Ames (1974). Scanning with laser densitometry of gel proteins visualized with Coomassie brilliant blue indicated that the enzyme was approximately 90% pure. Carbonic anhydrase (M_r 31 000), creatine kinase (M_r 42 000), ovalbumin (M_r 43 000), catalase (M_r 57 500), and bovine serum albumin (M_r 68 000) were used as standard calibration proteins. Plots of molecular weight vs. R_f in 10 and 12% gels indicated that the *dadB* subunit molecular weight is between 38 500 and 40 000.

Native Molecular Weight Determination. The native molecular weight of the enzyme was determined by gel filtration chromatography using the column and conditions described above. The void volume was determined with blue dextran 2000 (Pharmacia). Standard proteins were cytochrome *c* (M_r 12 300), myoglobin (M_r 17 000), carbonic anhydrase (M_r 31 000), ovalbumin (M_r 43 000), and bovine serum albumin (M_r 68 000). From plots of molecular weight vs. K_{av}, the *dadB* alanine racemase was seen to elute with an apparent molecular weight of 50 000.

Pyridoxal 5′-Phosphate Determination. Pyridoxal 5′-phosphate was determined by the fluorometric method of Adams (1979) by using a Perkin-Elmer LS-3 fluorescence spectrophotometer. The enzyme solution used contained 200 μg/mL (corrected concentration; see below) alanine racemase. The PLP content was found to be 4.5 nmol/mL. Using a molecular weight of 39 000 and taking into account that the racemase was 90% pure, the PLP content was calculated to be 0.98 molecules/monomer.

Subcloning of dadB Alanine Racemase Gene. To facilitate DNA sequencing of the *dadB* gene in plasmid pSW12 (Wasserman et al., 1983), this plasmid was further subcloned by using DNA manipulations as described by Davis et al. (1980) and by Maniatis et al. (1982), as illustrated in Figure 2 (top). The first step was to delete the 2.7-kb *Nde*I–*Nde*I fragment covering the junction between pBR322 DNA and the DNA 5′ to the *dadB* gene. DNA from pSW12 was digested with *Nde*I, phenol extracted, precipitated with ethanol, and then ligated at low DNA concentration (3 μg/mL). It was then recut with *Pvu*II to destroy the integrity of any plasmids retaining the 2.7-kb fragment. The digestion mixture was used to transform *Escherichia coli* strain HB101 (Shortle et al., 1980) to ampicillin resistance. Plasmid pSW30, which lacks the 2.7-kb fragment, was identified by restriction analysis of one such transformant.

Subsequently, the 0.4-kb *Sal*I–*Hin*dIII fragment of *S. typhimurium* DNA that had been 3′ to the *dadB* insert was removed. DNA from pSW30 was restricted with *Hin*dIII and *Sal*I, phenol extracted, and ethanol precipitated. The ends were filled in with the large (Klenow) fragment of DNA polymerase and deoxynucleotide triphosphates. The polymerase was heat inactivated and the DNA ligated at 2 μg/mL DNA concentration. The ligation mixture was cut with *Sal*I to linearize any plasmids in which the *Sal*I site had not been destroyed, and this DNA was used to transform *E. coli* strain BNN45 (Davis et al., 1980) to ampicillin resistance. Restriction analysis of transformant DNA allowed identification of strains bearing plasmids missing the 0.4-kb *Sal*I–*Hin*dIII fragment. One milligram of one such plasmid, pSW31, was prepared and used for DNA sequencing.

DNA Sequence Analysis of the dadB Region. Restriction fragments of plasmid pSW12 or pSW31 were 3′ end labeled, usually by using deoxynucleotide [α-^{32}P]triphosphates and the large fragment of DNA polymerase I (Maniatis et al., 1982). On some occasions 3′ end labeling utilized cordycepin 5′-[α-^{32}P]triphosphate and terminal transferase as described by Tu & Cohen (1980). Labeled DNA was eluted from polyacrylamide gels by grinding in buffer (Maxam & Gilbert, 1980) or by electroelution. Sequences of labeled fragments were determined by the chemical cleavage method of Maxam & Gilbert (1980) utilizing their G, T+C, and C reactions and an A+G reaction using formic acid (Maniatis et al., 1982).

Results

Enzyme Purification. The *S. typhimurium dadB* alanine racemase was purified from cells containing the plasmid pSW12, which carries the cloned *dadB* gene, as well as the ampicillin resistance gene and origin of replication from pBR322. Exponential phase cells bearing pSW12 produce 20–30-fold higher levels of the *dadB* gene product than parental cells (Wasserman et al., 1983).

Table I traces the course of a typical enzyme preparation in which the *S. typhimurium* alanine racemase was purified 300-fold from crude extract with a 36% final recovery. During purification protein was determined with the folin phenol reagent (Lowry et al., 1951). However, quantitative amino acid analysis of the final preparation indicated that, for the purified alanine racemase, the folin phenol method may ov-

Table I: Summary of *dadB* Alanine Racemase Purification

procedure/fraction	volume (mL)	activity (units)	protein (mg)[a]	sp act. (units/mg)	purification (x-fold)	yield (%)
100000*g* supernatant	150	11000	3750	3.0		
protamine supernatant	216	12000	3020	4.0	1.3	100
phenyl-Sepharose pool	130	9100	78	120	40	76
DEAE, Amicon pool	1.85	4600	12	390	130	38
Ultrogel AcA54 pool	11.7	4300	4.7	910	300	36

[a] Protein was determined with the folin phenol reagent (Lowry et al., 1951). For the purified racemase the protein concentration determined by the folin phenol method was 1.5-fold too high (see text). Therefore, the correct final specific activity was 1400 units/mg (50 mM L-alanine substrate), and the final yield of protein was 3.1 mg.

(MET)-THR-(ARG)-PRO-ILE-GLN-ALA-SER-LEU-ASP-LEU-GLN-VAL

1 2 3 4 5 6 7 8 9 10 11 12 13

MET-LYS-GLN-ASN-LEU-ALA-ILE-VAL-(ARG,ARG)-ALA-ALA

14 15 16 17 18 19 20 21 22 23 24 25

FIGURE 1: Amino-terminal sequence of the purified *dadB* racemase. Residues not in parentheses were identified in each of two sequence determinations by automatic Edman degradation of 0.7 nmol of enzyme (Putney et al., 1981). The sequence thus determined (designated residues 2–25) contained three undetermined residues. The fractional (0.05–0.15) amount of methionine present at the amino terminus was revealed by dansylation. Assignment of the three undetermined residues as arginines was made on the basis of the DNA sequence.

erestimate protein concentration by as much as 1.5-fold. When this corrected concentration was used, the racemase specific activity was calculated to be 1400 units/mg at pH 9.2 with 50 mM L-alanine as substrate.

Physical and Kinetic Characterization. Comparison of the denatured (39 000) and native (50 000) molecular weights clearly indicates that the active enzyme, as isolated, is monomeric. By use of a molecular weight of 39 000, one PLP molecule was bound per enzyme monomer, as determined by the fluorometric method of Adams (1979). Although pyridoxal 5′-phosphate (PLP) was included in the purification procedure, its presence had no effect on the A_{280}/A_{420} ratio for the purified enzyme. The extinction coefficient at 420 nm was calculated to be 10 000 M^{-1} cm^{-1}, within the range expected for the Schiff's base formed between PLP and an active site lysine (Snell & DiMari, 1972). The amino-terminal sequence of the purified *dadB* alanine racemase is shown in Figure 1. Greater than 90% of the amino-terminal methionine was apparently removed in vivo. The amino-terminal protein sequence was used in conjunction with DNA sequence data to precisely locate the *dadB* gene in a plasmid subclone (see below).

The Haldane relationship (Briggs & Haldane, 1925) predicts that

$$K_{eq} = \frac{K_m(\text{D-Ala})V_{max}(\text{L-Ala})}{K_m(\text{L-Ala})V_{max}(\text{D-Ala})} = 1$$

for the chemically symmetric reaction L-alanine ↔ D-alanine. The following k_m and V_{max} values were calculated from double-reciprocal plots by using data obtained at pH 9.2 in 100 mM CHES buffer: for L-alanine, k_m = 8.2 mM and V_{max} = 1500s^{-1}; for D-alanine, k_m = 2.1 mM and V_{max} = 380 s^{-1}. When these values were used, the calculated K_{eq} for alanine racemization was 1.01, in close agreement with the theoretical value. Relative activities toward alternative substrates (20 mM) measured at pH 9.0, relative to L-alanine (1.0), were as follows: L-norvaline, 0.06; L-serine, 0.02; L-lysine, <0.01; L-α-aminobutyrate, <0.01.

When frozen at −70 °C in buffer containing 10% glycerol, the enzyme retained greater than 90% of its initial activity for

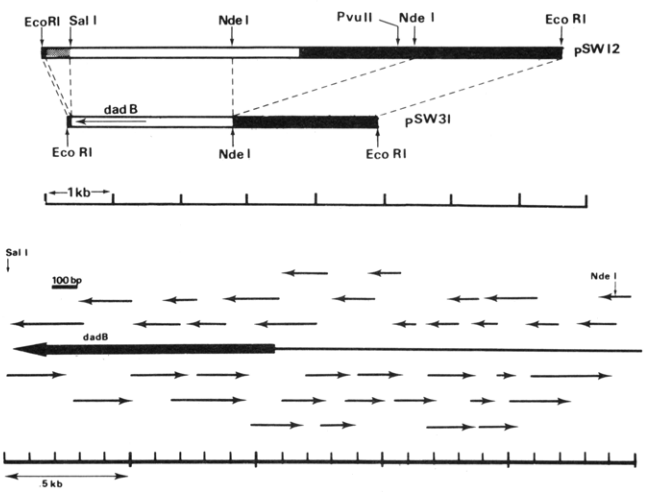

FIGURE 2: (Top) Derivation of *dadB* subclone from pSW12. Diagrammatic representation of the two-step construction of plasmid pSW31 from pSW12 showing location and orientation of *dadB* gene. Blackened regions indicate pBR322-derived DNA. Shaded region represents *S. typhimurium* DNA that, although subcloned with *dadB*, was derived from a nonadjacent piece of genomic DNA. (Bottom) DNA sequencing scheme for *S. typhimurium dadB* region. Vertical arrows indicate the ends of the pSW31 *Sal*I–*Nde*I cloned insert. The position and extent of sequence derived from the reaction of a single-labeled fragment is represented by a small horizontal arrow. Sequence was determined from the 3′ end of each fragment, starting from the point marked by the tip of the arrowhead (scale indicated). The large horizontal arrow illustrates the size and orientation of the *dadB* coding region.

at least 2 years. Enzymatic activity was stable in 30% ammonium sulfate but was irreversibly diminished by exposure to ammonium sulfate concentrations near or above 40%, where precipitation occurred.

Enzyme Inactivation. Both isomers of β-fluoroalanine inactivated the racemase in a time-dependent manner. This enzyme inactivation by suicide substrates is examined in detail in an accompanying paper (Badet et al., 1984).

Subcloning and DNA Sequencing of the dadB Region. Initial Maxam–Gilbert sequencing used fragments of pSW12 generated by digestion with restriction enzymes recognizing six-base sequences. The gene was located and oriented by using the amino-terminal sequence, and the *dadB* region was accordingly subcloned as a 2.4-kb fragment in the pBR322-derived plasmid pSW31 (Figure 2, top) to facilitate fragment isolation. By use of restriction enzymes recognizing six-, five-, or four-base sequences, sequencing of the insert was completed on both strands and across all restriction sites. The sequence scheme is shown in Figure 2 (bottom).

dadB Amino Acid Sequence and Composition. The sequence of the *dadB* gene with translated polypeptide is shown in Figure 3. The gene was found to be 1068 bp in length, encoding a protein of 356 amino acids. The predicted amino-terminal protein sequence exactly matched that obtained from sequencing of the purified gene product (Figure 3). The

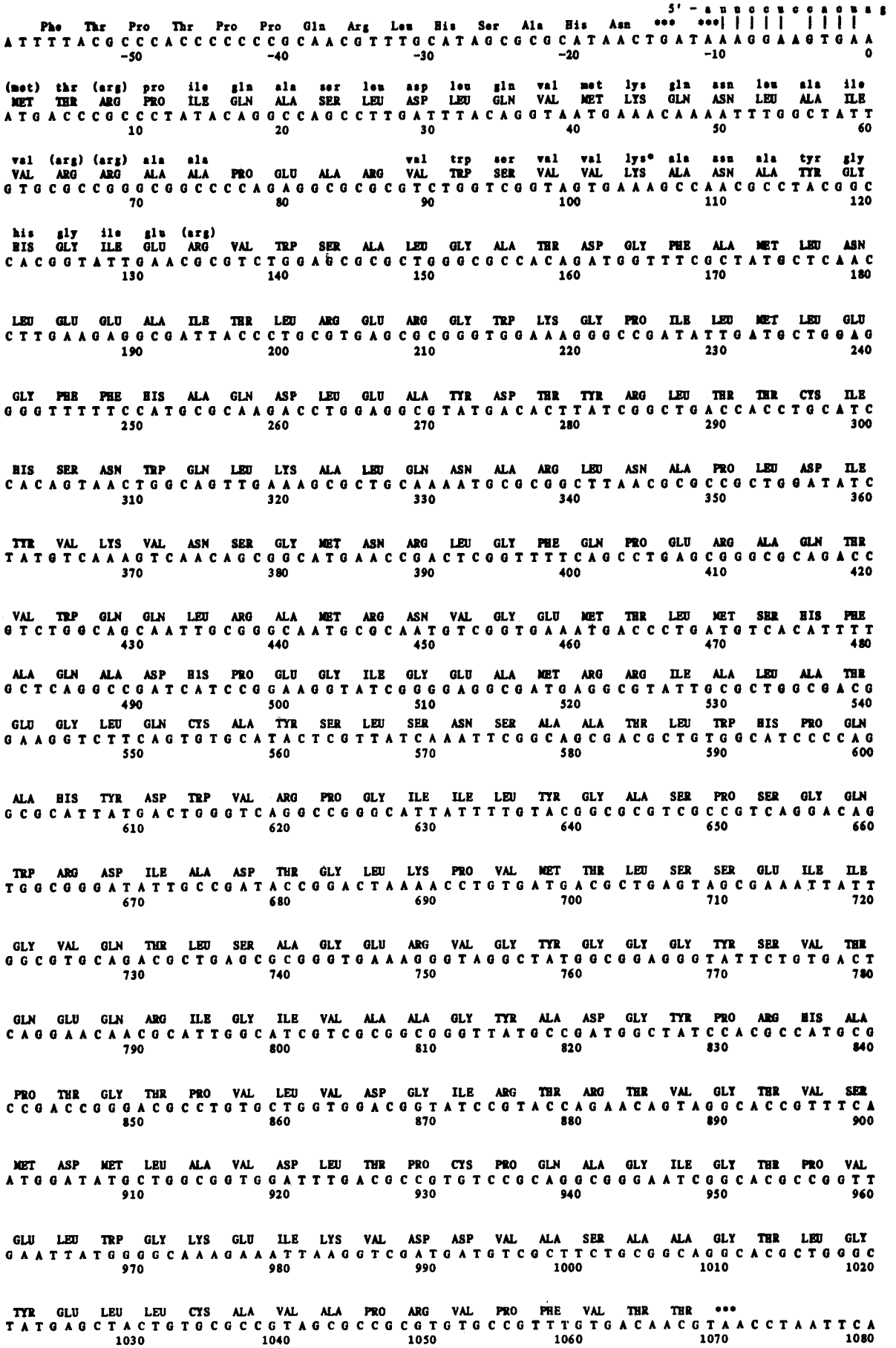

FIGURE 3: *dadB* DNA sequence and translated polypeptide. Position 1 of the DNA sequence corresponds to the first base of the *dadB* coding region. The translated *dadB* polypeptide is indicated in capital letters above the DNA sequence. Two peptide sequences are shown in small letters above DNA bases 1–75 (amino-terminal sequence) and bases 88–135 [active site peptide sequence (Badet et al., 1984)]. The lysyl residue involved in Schiff base formation with PLP is designated with an asterisk. Potential base pairing between the DNA at positions −10 to −1 and the 3′ end of 16S rRNA (small letters) is represented by vertical bars.

Table II: Predicted and Observed *dadB* Amino Acid Composition[a]

amino acid residue	mol/mol of enzyme predicted	observed
Ala	41	38
Arg	23	24
Asn	10	
Asx	25	26
Asp	15	
Cys	4	ND[b]
Gln	18	
Glx	36	35
Glu	18	
Gly	34	32
His	8	7
Ile	19	14
Leu	33	33
Lys	8	9
Met	12	11
Phe	6	8
Pro	18	18
Ser	17	15
Thr	24	21
Trp	9	ND
Tyr	12	11
Val	27	26
total residues	356	

[a] Predicted values were derived from the translated DNA sequence of the *dadB* gene. Observed values were obtained from a single determination on a Durrum amino acid analyzer following hydrolysis of enzyme in 6 N HCl at 110 °C for 24 h. [b] ND = not determined.

calculated molecular weight from the amino acid sequence is 39044.

The predicted and experimentally determined amino acid compositions were also in good agreement, the average error being only 10% (Table II). The largest error was seen for phenylalanine, the least abundant residue determined and therefore one of the most susceptible to detectable contamination in the 90% homogeneous sample used. The amounts of isoleucine, serine, and threonine were below expected values, but since a time course was not followed, no correction for hydrolytic breakdown was made.

Discussion

Using the cloned *dadB* gene, we have purified the *dadB* alanine racemase from *Salmonella typhimurium*. The amount of racemase in these cells is estimated to be 0.1–0.2% of the soluble cellular protein (based on the specific activity in the S100 supernatant as compared to the specific activity of the purified racemase), which is elevated 20–25-fold over the wild-type level. More recently, we have noted that with pSW31, the smaller plasmid used for DNA sequencing, we see another 3–6-fold amplification such that the *dadB* racemase is 0.5–1% of the soluble cellular protein. The N-terminal sequencing results allowed us to set the reading frame in the *dadB* gene and led to the predicted primary sequence reported here.

In our previous work, we have shown that *S. typhimurium* mutants with a transposon insertion in *dadB* can still grow and make a cell wall, so the *dadB* racemase cannot be the only source of D-alanine in the cell (Wasserman et al., 1983). The function of the *dadB* racemase appears to be catabolic, not biosynthetic. Nevertheless, the availability of substantial quantities of purified enzyme makes this racemase a good candidate for detailed mechanistic studies as a model for biosynthetic alanine racemases.

The only other racemases purified to homogeneity from Gram-negative bacteria are a *Pseudomonas putida* alanine racemase (unstable when pure) and a broad specificity amino acid racemase from *Pseudomonas striata* [Soda & Osumi, 1969; see also Roise et al. (1984)]. Alanine racemases from *E. coli* B and *E. coli* W have been partially purified from cells grown on L- or D-alanine; it is likely that these enzymes are functionally equivalent to the *dadB* racemase.

The pure *dadB* alanine racemase has a turnover number of 930–1000 s^{-1}, whereas Wang & Walsh (1978, 1981) had estimated the *E. coli* B enzyme, from inactivation stoichiometry of partially purified enzyme with fluoro[^{14}C]alanine, would have a turnover number of 50 s^{-1} in the L to D direction at 100% purity. The reason for this 20-fold difference in turnover numbers is not known. The *dadB* racemase can be considered to be an efficient catalyst based on its turnover number of 1000 s^{-1}, but it has a k_{cat}/K_m value of 1.1×10^5 M^{-1} s^{-1}, which is considerably below that of the essentially "perfect" enzyme, triosephosphate isomerase, at 3×10^8 M^{-1} s^{-1} (Knowles & Albery, 1977). The isomerase has an equivalent turnover number of 10^3 s^{-1} but has a K_m value in the 10^{-5} range. The K_m value of 8 mM for L-alanine for the *dadB* racemase is consistent with a catabolic function rather than a biosynthetic one; the enzyme is expected to make D-alanine only at high levels of intracellular L-alanine.

The availability of the DNA sequence and quantities of pure *dadB* enzyme facilitated the mechanistic studies on active site structure and sites of inactivation by D and L isomers of β-haloalanines, compounds that show antibacterial activity by blocking D-alanine production. Although the *dadB* racemase is not the only source of D-alanine in *S. typhimurium* (Wasserman et al., 1983), these studies are interesting because it is reasonable that the haloalanines inactivate both catabolic and biosynthetic racemases by similar mechanisms. The accompanying two papers document the kinetic and structural features of mechanism-based inactivation by D- and L-haloalanine and of D- and L-*O*-acetylserine isomers with, first, the *dadB* racemase and, then, the broad specificity *P. striata* racemase (Badet et al., 1984; Roise et al., 1984).

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