Thermostable Alanine Racemase from *Bacillus stearothermophilus*: Molecular Cloning of the Gene, Enzyme Purification, and Characterization

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Received June 3, 1985; Revised Manuscript Received October 28, 1985

ABSTRACT: The alanine racemase (EC 5.1.1.1) gene of a thermophilic bacterium, *Bacillus stearothermophilus*, was cloned and expressed in *Escherichia coli* C600 with vector plasmid pICR301, which was constructed from pBR322 and the L-alanine dehydrogenase gene derived from *B. stearothermophilus*. A coupled assay method with L-alanine dehydrogenase and tetrazolium salts was used to detect visually the alanine racemase activity in the clones. Alanine racemase overproduced in a clone carrying the plasmid pICR4, 12 kilobases of DNA, was purified from cell extracts about 340-fold to homogeneity by five steps including heat treatment. The overproduced enzyme was confirmed to originate from *B. stearothermophilus* by an immunochemical cross-reaction with the enzyme of *B. stearothermophilus*. The purified enzyme has a molecular weight of about 78 000 and consists of two identical subunits of M_r of 39 000. At the optimum temperature (50 °C), the enzyme has a specific activity of 1800 units/mg (V_{max}, D- to L-alanine). Resolution and reconsititution experiments together with the absorption spectrum of the enzyme clearly indicate that alanine racemase of *B. stearothermophilus* is a pyridoxal 5′-phosphate enzyme.

Alanine racemases (EC 5.1.1.1) catalyze the interconversion of L- and D-alanine, providing the latter enantiomer for the construction of the peptidoglycan layer of bacterial cell walls. Since the first demonstration of alanine racemase in *Streptococcus faecalis* (Wood & Gunsalus, 1951), the enzyme has been ubiquitously found in bacterial species. However, few enzymes have been investigated in detail [for reviews, see Adams, (1972, 1976)]. The requirement of cofactor, in particular, has remained obscure; the homogeneous enzyme purified from *Pseudomonas putida* requires pyridoxal 5′-phosphate as the sole cofactor (Adams et al., 1974), whereas those from *Bacillus subtilis* (Diven et al., 1964), *Pseudomonas* sp. 3550 (Free et al., 1967), *Staphylococcus aureus* (Rose & Strominger, 1966), and *Escherichia coli* (Lambert & Neuhaus, 1972) are unclear in cofactor requirement.

Wasserman et al. (1983) have recently found that there are at least two genes, *dadB* and *dal*, encoding alanine racemases in *Salmonella typhimurium*. The enzyme encoded by the *dadB* gene that is essential for utilization of L-alanine as a source of carbon, energy, and nitrogen through the D-alanine dehydrogenase reaction has been purified from *E. coli* cells containing the plasmid pSW12, which carries the cloned *dadB* gene (Wasserman et al., 1984). The physical and kinetic characterization of the racemase as well as its primary structure predicted by DNA sequencing was also reported (Wasserman et al., 1984). The *Salmonella alr* gene encoded alanine racemase (Esaki & Walsh, 1986) probably functions biosynthetically in the bacterial cell wall assembly.

Because of the pivotal role of alanine racemase in cell wall biosynthesis and its unique distribution to prokaryotes, the enzyme has been recognized as a target for antibacterial drugs. In fact, some halogenated derivatives of D-alanine and phosphoalanine-containing dipeptides were found to act as antibacterials 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). Detailed studies of the reaction mechanism of alanine racemase and the chemistry and geometry of its active site are needed to develop racemase-directed antibacterials with more selective toxicity.

Gram-negative and Gram-positive bacteria are often differentially susceptible to antibiotics. Thus, the phosphonate analogue of alanine (1-aminoethyl)phosphonic acid (Ala-P), which is the active component derived by intracellular peptidase cleavage of the antibacterial alaphosphin (L-Ala-L-Ala-P), inactivates alanine racemase from Gram-positive bacteria, *Sh. aureus* and *Sr. faecalis*, in a time-dependent fashion but is a reversible inhibitor of racemases from Gram-negative bacteria, *E. coli* and *Sl. typhimurium* (Atherton et al., 1979). The molecular basis of the mode of irreversible inactivation on racemases from Gram-positive organisms has now been investigated with the pure enzyme from *Sr. faecalis*, and the inactivation was found to be derived from slow dissociation of Ala-P tightly, but noncovalently, bound to the enzyme (Badet & Walsh, 1985). However, the *Sr. faecalis* enzyme had to be purified 20 000-fold to homogeneity, so little protein was available for detailed mechanistic work.

In this paper, we report cloning and expression of the alanine racemase gene from a thermophilic Gram-positive bacterium, *Bacillus stearothermophilus*, in *E. coli*, rapid and simple purification of the thermostable enzyme, and its enzymatic characterization. The availability of pure alanine racemase in quantity has motivated the detailed studies on the mechanism of irreversible inhibition of the Gram-positive bacterium derived racemase by Ala-P as described in the following paper (Badet et al., 1986).

Experimental Procedures

Strains and Media. B. stearothermophilus IFO 12550 and *E. coli* C600 $r_K^-m_K^-$*thi thr leu* were used as a donor strain of the gene and the host strain for plasmid constructions, re-

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0006-2960/86/0425-3268$01.50/0

spectively. Transformed *E. coli* cells were grown in L broth (1% polypeptone, 0.5% yeast extract, 0.5% NaCl, and 0.1% glucose, pH 7.2) supplemented with 2% agar and appropriate antibiotics. Antibiotic concentrations used for the selection of transformants were 25 μg/mL of ampicillin and 15 μg/mL of tetracycline. *B. stearothermophilus* was grown at 55 °C in a medium (pH 7.2) containing 1.5% polypeptone, 0.1% glycerol, 0.01% yeast extract, 0.01% meat extract, 0.5% NaCl, 0.1% KH_2PO_4, 0.2% K_2HPO_4, and 0.01% $MgSO_4 \cdot 7H_2O$ with shaking.

DNA Preparation. *B. stearothermophilus* grown in 500 mL was harvested in the late log phase, and the chromosomal DNA was isolated as described by Saito and Miura (1963). Plasmid DNA from *E. coli* was prepared by modification of the method of Oka (1978). After removal of RNA by gel filtration on a Bio-Gel A-5m (Bio-Rad Laboratories) column (1.0 × 40 cm), covalently closed circular plasmids were purified by banding in a cesium chloride–ethidium bromide equilibrium gradient. Ethidium bromide was removed by extraction with 1-butanol, followed by dialysis against 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer (pH 7.5) containing 0.2 mM ethylenediaminetetraacetic acid (EDTA). Minipreparations of recombinant plasmids were obtained by the alkaline-sodium dodecyl sulfate (SDS) lysis procedure (Kado & Liu, 1981).

Digestion of DNA with Restriction Endonucleases. Restriction enzymes (*Sal*I and *Hin*dIII) were obtained from Takara Shuzo Co. (Kyoto, Japan). Digestion was performed under the conditions receommended by the supplier and analyzed on 0.7% agarose gels in a Tris–borate–EDTA buffer system (Meyers et al., 1976) containing 0.5 μg of ethidium bromide/mL. For digestion of plasmid DNA, enzymes were used at 2 units/μg of DNA, and incubations were carried out at 37 °C for 16 h.

Ligation and Transformation. T4 DNA ligase was purchased from Takara Shuzo Co. (Kyoto, Japan). Ligations were carried out for 16 h at 13 °C in 66 mM Tris-HCl buffer (pH 7.6) containing 6.6 mM $MgCl_2$, 10 mM dithiothreitol, and 66 μM ATP. Transformation of *E. coli* was performed as described by Lederberg and Cohen (1974).

Construction of Vector Plasmid. Total genomic DNA (75 μg) from *B. stearothermophilus* was partially digested with *Sal*I (50 units), and the resultant fragments were ligated into the *Sal*I site of pBR322 (12 μg) by T4 DNA ligase (9 units). The ligated mixture was used directly for transformation (Lederberg & Cohen, 1974). Transformants of *E. coli* C600 that contain these hybrid plasmids are resistant to ampicillin but sensitive to tetracycline. To detect the L-alanine dehydrogenase producing colonies among these transformants, the replica printing method developed by Raetz (1975) was modified as follows: colonies of transformant grown on one of duplicate L broth plates were transferred onto a Toyo No. 5C filter paper disc (diameter 8 cm). Colonies grown on the other plate were stored at 4 °C until use. After treatment with lysozyme and EDTA as indicated (Raetz, 1975), filter paper discs in Petri dishes were rapidly frozen in liquid N_2 followed by thawing in a water bath. This freezing and thawing was repeated twice. The filter paper dried in a water bath at 70 °C for 20 min was then transferred to another Petri dish containing 1.5 mL of a reaction mixture for L-alanine dehydrogenase assay. The mixture contained 50 mM glycine–KOH buffer (pH 10.5), 50 mM L-alanine, 0.625 mM NAD, 0.064 mM phenazine methosulfate, and 0.24 mM nitro blue tetrazolium. The colonies producing L-alanine dehydrogenase appeared as blue spots on the replica disc. Plasmid DNA was prepared from the L-alanine dehydrogenase producing cells, which werre grown from the corresponding colonly on the stored plate. Since the obtained plasmid pICR3 was rather large (11.0 kb) for use as a cloning vector, the 3.2-kb *Hin*dIII fragment was deleted by digestion and religation of pICR3 (see Figure 1). Transformed *E. coli* containing the plasmid pICR301 thus obtained produced 2.37 units of L-alanine dehydrogenase/mg of soluble protein. The plasmid pICR301 (7.8 kb) was used as a vector for cloning of the alanine racemase gene.

Enzyme and Protein Assays. Enzyme assays were performed at 50 °C. A unit of enzyme is that amount that catalyzes the formation of 1 μmol of product/min at pH 9.0. The specific activity is expressed as units per milligram of protein. Activity was measured in the D- to L-alanine direction by monitoring the production of NADH at 340 nm on a Union Giken SM401 recording spectrophotometer as the L-alanine was converted to pyruvate and ammonia by L-alanine dehydrogenase. A saturating amount of L-alanine dehydrogenase (ca. 50 units) was added to the assay mixture during the course of racemase purification, but the addition was not necessary when the racemase activity in transformants which carried a plasmid encoding the L-alanine dehydrogenase gene, was assayed. A standard assay contained 100 μmol of D-alanine, 100 μmol of glycine–KOH buffer, 2.5 μmol of NAD, 50 units of L-alanine dehydrogenase, and alanine racemase at pH 9.0 in a 1-mL volume. L-Alanine dehydrogenase obtained from *B. stearothermophilus* (about 20% pure) was provided by Y. Sakamoto in this laboratory. Racemase activity was assayed also by measuring the change in optical rotation at 365 nm with a Perkin-Elmer 241 polarimeter. A photocell with a 10-cm light path contained 100 μmol of an amino acid, 250 μmol of potassium phosphate buffer (pH 8.0), and alanine racemase in 1 mL. The values of molar rotation $(\Phi)_{365}$ are 1.08°, 7.44°, −0.80°, and −1.78° for L-alanine, L-lysine, L-serine, and L-methionine, respectively. Protein was estimated by the biuret method (Gonall et al., 1949), with bovine serum alubumin as a standard. For most column fractions, the protein elution patterns were determined by absorption at 280 nm.

Enzyme Purification. All operations were carried out at 0–5 °C. Potassium phosphate buffer (pH 7.2) containing 10 μM pyridoxal 5′-phosphate and 0.01% 2-mercaptoethanol was used as the buffer throughout the purification except where noted.

(*Step 1*) *Preparation of Crude Extract.* Cells of *E. coli* C600 carrying pICR4 harvested by centrifugation were washed twice with 0.85% NaCl. The washed cells (20 g wet weight) were suspended in 100 mL of 10 mM buffer and disrupted by sonication at 0 °C for 30 min with a 19-KHz Kaijo Denki oscillator (Tokyo, Japan). The intact cells and debris were removed by centrifugation.

(*Step 2*) *Heat Treatment.* The supernatant solution was kept at 70 °C for 1 h and was centrifuged after cooling in ice.

(*Step 3*) *Ammonium Sulfate Precipitation.* The supernatant solution (80 mL) was brought to 60% saturation with solid ammonium sulfate. After being allowed to stand for 1 h the precipitate was collected by centrifugation and dissolved in 20 mL of 10 mM buffer followed by dialysis against 2 L of the same buffer.

(*Step 4*) *DEAE-Toyopearl Column Chromatography.* The dialyzed solution was applied to a DEAE-Toyopearl 650M (Toyo Soda Co., Tokyo, Japan) column (1.5 × 10 cm) equilibrated with 10 mM buffer. After the column was washed with 100 mL of the buffer containing 20 mM KCl, the enzyme

was eluted at a flow rate of 150 mL/h with the buffer containing 50 mM KCl. The active fractions were combined and concentrated with an Amicon 202 ultrafiltration unit.

(Step 5) Sephadex G-150 Column Chromatography. The enzyme solution was applied to a column (2 × 100 cm) of Sephadex G-150 (Pharmacia, Sweden) equilibrated with 10 mM buffer and eluted at a flow rate of 10 mL/h. The active fractions were pooled concentrated by ultrafiltration.

Ultracentrifugal Analysis. The purity of the purified enzyme and its sedimentation coefficient were determined with a Spinco Model E ultracentrifuge equipped with a phase plate as a schlieren diaphragm. The molecular weight of the enzyme was determined by the ultracentrifugal sedimentation equilibrium method according to the procedure of Van Holde and Baldwin (1958). The experiments were carried out in a Spinco Model E ultracentrifuge equipped with Rayleigh interference optics. Multicell operations were employed in order to perform the experiment on four samples of different initial concentrations ranging from 0.9 to 3.8 mg/mL with the use of An-G rotor and double cells of different side-wedge angles. The rotor was centrifuged at 8225 rpm for 20 h at 20 °C. Interference patterns were photographed at intervals of 30 min to compare and make sure that the equilibrium was established. The relation between the concentration of the enzyme and the fringe shift was determined with the synthetic boundary cell.

Spectrophotometry. Absorption spectra were taken with a Union Giken SM401 recording spectrophotometer or a Shimazu UV3000 recording spectrophotometer. Circular dichroism measurement was done in a JASCO J-20 automatic recording spectropolarimeter.

Amino Acid Analysis. Amino acid analysis was performed according to the method of Spackman et al. (1958) with a Hitachi 835 high-performance amino acid analyzer. The enzyme was hydrolyzed in 6 N HCl at 110 °C under reduced pressure for 24, 48, and 72 h. The hydrolysates were evaporated to dryness and subjected to amino acid analysis in duplicate. Half-cystine was determined as cysteic acid after performic acid oxidation and hydrolysis. Tryptophan and tyrosine were determined spectrophotometrically by the method of Edelhoch (1967).

Preparation of Antiserum. One milliliter of 0.5 mg of pure enzyme in 10 mM potassium phosphate buffer (pH 7.2) was emulsified with 1 mL of Freund's complete adjuvant (Difco) and injected into a multiple subcutaneous site on the back of a young, male rabbit. After 4 weeks, the animal received a booster injection subcutaneously on the neck with 0.5 mg of the antigen homogenized in an equal volume of the complete adjuvant. On the 7th and 14th day after the booster injection, blood was collected from the ear vein and allowed to clot. The serum was centrifuged at 6000 *g* for 10 min and stored at −20 °C. Ouchterlony plates were made (Ouchterlony, 1953) with 1% agar in 10 mM potassium phosphate buffer (pH 7.2) containing 0.85% NaCl.

Results

Construction of B. stearothermophilus Genomic Library. Total DNA was isolated from *B. stearothermophilus* IFO 12550 and digested with the restriction endonuclease *Hin*dIII to yield a population of fragments of average sizes of about 1–20 kilobases (kb). The fragments were then ligated into the *Hin*dIII site in the L-alanine dehydrogenase encoded vector pICR301 and transformed into *E. coli* C600. A library of some 6000 transformant (Amp^rTc^s) clones was obtained.

Alanine Racemase Screening of Cloned Library. The recombinant *E. coli* library was screened for the expression of *B. stearothermophilus* alanine racemase gene with an activity staining assay. Colonies were replicaplated, lysed in situ, and probed with the L-alanine dehydrogenase assay described under Experimental Procedures except that D-alanine (50 mM) was used as a substrate instead of L-alanine. This screening technique is based on the replica printing method developed by Raetz (1975), and recombinant *E. coli* cells producing alanine racemase are expected to show blue color of the reduced nitro blue tetrazolium as a result of electron transfer from phenazine methosulfate and NADH produced by L-alanine dehydrogenase in the recombinants (Scheme I). In order to avoid nonspecific color development caused by respiratory chain enzymes and alanine racemase from the host *E. coli*, the assay procedure involves heat treatment at 70 °C for 20 min; both L-alanine dehdyrogenase and alanine racemase from *B. stearothermophilus* are heat-stable. Of approximately 6000 Amp^rTc^s recombinants examined, only one colony turned blue on the replica plate. A plasmid band of about 4.2 kb larger than the vector pICR301 was found in this positive clone upon agarose gel electrophoresis of a minipreparation of recombinant plasmid. The plasmid DNA was isolated from the clone and designated pICR4 (12.0 kb, see Figure 1). The cell-free extract of the clone showed a high alanine racemase activity (see below).

Scheme I

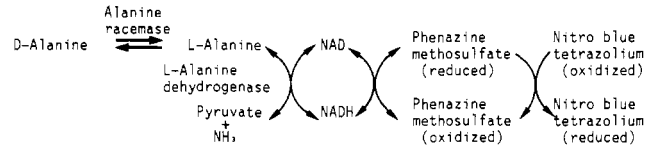

Table I: Alanine Racemase Activity in Crude Extracts of *B. stearothermophilus* and *E. coli* Clones before and after Heat Treatment[a]

strain	sp act. (units/mg) before heat treatment	sp act. (units/mg) after heat treatment
B. stearothermophilus IF012550	0.16	0.20
E. coli C600	0.15	0.0
E. coli C600 pICR4	4.81	33.5
E. coli C600 pICR5	5.21	35.3

[a] Cells (0.5–2 g) were disrupted by sonication as described under Experimental Procedures. The enzyme activity was determined in the D to L direction at 100 mM D-alanine before and after heat treatment at 70 °C for 1 h. The precipitated protein formed by the heat treatment was removed by centrifugation before assaying the enzyme.

Restriction Mapping and Subcloning of pICR4. Digestion of pICR4 with *Hin*dIII restriction endonuclease yielded a 4.2-kb fragment in addition to a 7.8-kb fragment corresponding to the vector plasmid pICR301. Thus, a 4.2-kb fragment derived from *B. stearothermophilus* genomic DNA was inserted into the *Hin*dIII site of pICR301. By evaluating the electrophoretic patterns of pICR4 digested with single- and double-restriction endonucleases, the internal restriction map of the plasmid pICR4 DNA was obtained as shown in Figure 1.

The 4.2-kb *Hin*dIII fragment containing the alanine racemase gene was excised from pICR4 and recloned into the *Hin*dIII site of pBR322 to reduce the size of the plasmid by removing the L-alanine dehydrogenase gene and thereby to facilitate future analysis of the alanine racemase DNA sequence. One of the Amp^rTc^s transformants obtained was found to express a high level of the alanine racemase activity (see Table I) and contain a plasmid, designated pICR5, which was mapped with restriction sites as shown in Figure 1. The transformant carrying pICR5 did not show the L-alanine dehydrogenase activity.

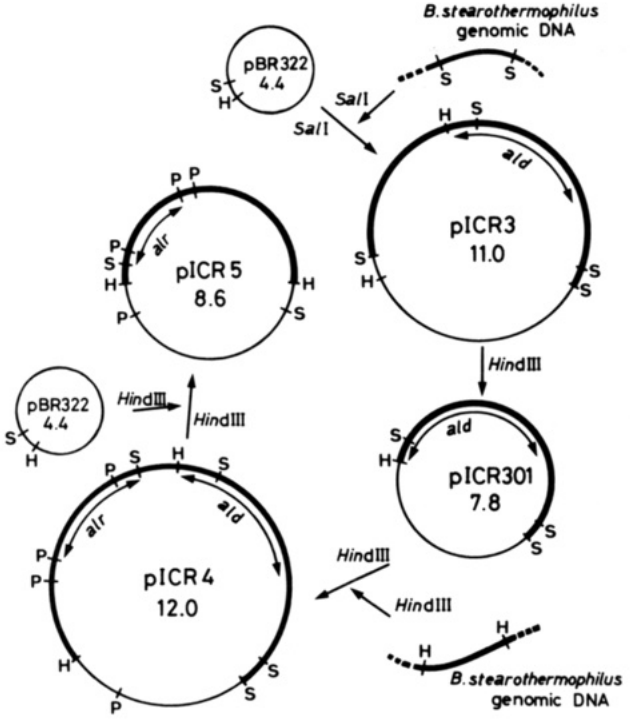

FIGURE 1: Construction scheme for and restriction maps of plasmids pICR4 and pICR5. The thin and heavy lines represent DNA fragments from pBR322 and *B. stearothermophilus* gene, respectively. The restriction maps given here were derived from the known restriction map of pBR322 and from the experiments described in the text. Restriction endonuclease cleavage sites are *Hin*dIII (H), *Sal*I (S), and *Pst*I (P). The DNA regions corresponding to the L-alanine dehydrogenase gene (*ald*) and the alanine racemase gene (*alr*) are represented arbitrarily, and the distances given between the restriction sites are for illustrative purposes only. The numbers below the name of each plasmid are the sizes of the plasmids in kilobases.

Expression of Thermostable Alanine Racemase Gene in E. coli. The crude extract prepared from *E. coli* cells containing the plasmid pICR4, which carries the cloned *B. stearothermophilus* alanine racemase gene, as well as the alanine dehydrogenase gene, the ampicillin resistance gene, and the origin of replication from pBR322, showed about 30-fold higher level of alanine racemase activity than those from *B. stearothermophilus* cells and plasmidless *E. coli* C600 cells (Table I). Although the host *E. coli* C600 cells contain appreciable alanine racemase activity that is indistinguishable from the activity of the *B. stearothermophilus* enzyme by the assay method employed in this study, the 30-fold higher activity in *E. coli* pICR4 suggests the high expression of the *B. stearothermophilus* alanine racemase gene. In addition, a marked increase in specific activity was observed when the cell extract from *E. coli* pICR4 was heated at 70 °C for 1 h. Such heat treatment precipitated most proteins including alanine racemase derived from *E. coli*, the thermostable enzyme activity being left in the supernatant. Thus, the specific activity of the *E. coli* C600 pICR4 extract was 33.5 units/mg of protein after the heat treatment, whereas no detectable activity was found in the heat-treated cell extract of the host *E. coli* C600. This result indicates that the thermostable alanine racemase encoded by a gene from *B. stearothermophilus* is overproduced to about 0.3% of the soluble protein when carried on the plasmid pICR4 in *E. coli*. A high level of the gene expression was also found in *E. coli* C600 harboring the subcloned plasmid pICR5.

Purification of Alanine Racemase from E. coli Carrying pICR4. Starting with 20 g cells of *E. coli* C600-pICR4, the *B. stearothermophilus* alanine racemase was purified by five steps including two chromatographic procedures (Table II). The heat treatment of cell-free extract was found to be very effective for the enzyme purification as described above: 5–7-fold purification was achieved without loss of any enzymatic activity. Ion exchange chromatography with DEAE-Toyopearl was essential in the following procedure, giving typically a 20-fold purification. The enzyme purified 340-fold with a 64% final yield appeared to be homogenous by the criteria of polyacrylamide gel electrophoresis and analytical ultracentrifugation.

Table II: Purification of Alanine Racemase from *E. coli* C600 Carrying pICR4

step	total protein (mg)	total units	sp act. (units/mg)	yield (%)
crude extract	134/	6298	4.7	100
heat treatment	230	6072	26.4	96
ammonium sulfate, 0–60%	120	5664	47.2	90
DEAE-Toyopearl	5.0	5275	1055	84
Sephadex G-150	2.5	4025	1610	64

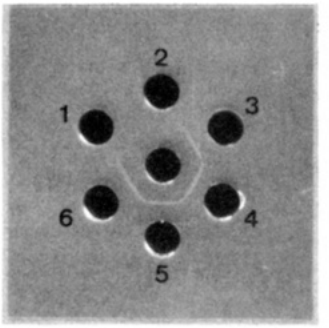

FIGURE 2: Ouchterlony double-diffusion analysis of alanine racemase. The center well contains the antiserum against alanine racemase purified from *E. coli* C600 containing pICR4. Wells 1–4 each contain 10 μL of crude extracts of *E. coli* C600 carrying various plasmids at a protein concentration of ca. 1 mg/mL: (1) no plasmid; (2) pICR301; (3) pICR4; (4) pICR5. Wells 5 and 6 contain respectively homogeneous alanine racemase purified from *E. coli* C600 pICR4 and partially purified alanine racemase of *B. stearothermophilus* IFO 12550 at 4 μg each.

Immunochemical Analysis. The antiserum against thermostable alanine racemase purified from *E. coli* pICR4 was used to investigate its immunochemical identity with the *B. stearothermophilus* enzyme. The antiserum reacted with the partially purified alanine racemase from *B. stearothermophilus* as well as with the purified enzyme from *E. coli* pICR4, crude extract of *E. coli* pICR4, and crude extract of *E. coli* pICR5, producing a single line of precipitation with complete fusion (Figure 2). No cross-reaction against the antiserum was observed with the cell-free extract of *E. coli* C600 and *E. coli* containing the vector plasmid pICR301. The result indicates that the alanine racemase produced by *E. coli* carrying pICR4 or pICR5 is immunochemically identical with the *B. stearothermophilus* enzyme and therefore confirms that the *B. stearothermophilus* alanine racemase gene is expressed in *E. coli*.

Physical and Kinetic Characterization. The sedimentation coefficient ($S^0_{20,w}$) of the enzyme was calculated to be 5.4 S. With the assumption of a partial specific volume of 0.74, the molecular weight of 78 000 ± 2000 was obtained by the sedimentation equilibrium method. The subunit structure was examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Laemmli, 1970). The sodium dodecyl sulfate treated enzyme was subjected to electrophoresis in the presence of 0.1% sodium dodecyl sulfate and migrated as a single protein band (Figure 3). The molecular weight of the band was

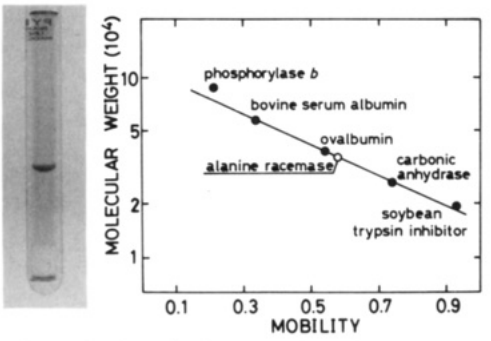

FIGURE 3: Determination of molecular weight of the alanine racemase subunit by sodium dodecyl sulfate gel electrophoresis. After the purified enzyme (25 μg) was treated with 1.0% sodium dodecyl sulfate in 10 mM sodium phosphate buffer (pH 7.2) containing 1.0% 2-mercaptoethanol and 25% glycerol at 37 °C overnight, the treated enzyme preparation was subjected to electrophoresis in the presence of 0.1% sodium dodecyl sulfate with 10% polyacrylamide gels in the Tris–glycine buffer system (Laemmli, 1970) (right). The molecular weight was estimated from a semilogarithmic plot of molecular weight against mobility with phosphorylase *b* (M_r 94 000), bovine serum albumin (M_r 67 000), ovalbumin (M_r 43 000), carbonic anhydrase (M_r 30 000), and soybean trypsin inhibitor (M_r 20 100) as standards (left).

estimated to be about 39 000 on the basis of its mobility relative to those of standard calibration proteins (Figure 3). These results show that the enzyme is composed of two subunits identical in molecular weight.

The enzyme showed maximum reactivity at around 50 °C with a V_{max} value of 1800 units/mg (D- to L-alanine). The temperature dependence of V_{max} was analyzed by Arrhenius plots, and the activation energy E_a was 1.8 kcal/mol with calculated values of ΔH^*, ΔG^*, and ΔS^* = 1.18 kcal/mol, 18.46 kcal/mol, and −55.7 cal/(mol·deg), respectively. At 37 °C in 100 mM 2-(*N*-cyclohexylamino)ethanesulfonic acid buffer (pH 9.0), the K_m for D-alanine is 2.67 ± 0.2 mM and V_{max} for racemization (D to L) is 1400 units/mg. in the L-alanine to D-alanine direction, K_m of 4.25 ± 0.2 mM and V_{max} of 2550 units/mg were obtained by the D-amino acid oxidase coupled assay (Badet & Walsh, 1985). When these values were used, the calculated K_{eq} for alanine racemization was 1.14, in good agreement with the theoretical value (1.0) for the chemically symmetric reaction L-alanine ⇄ D-alanine (Briggs & Haldane, 1925). Alanine is the exclusive substrate for the enzyme; L-serine, L-methionine, L-lysine, L-valine, L-homoserine, and L-α-aminobutyrate (100 mM) were not racemized when examined by the polarimetric assay. Enzymatic activity was found quite stable upon heat treatment at 70 °C for 80 min in 10 mM potassium phosphate buffer (pH 7.2).

Amino Acid Composition. The amino acid composition of the purified enzyme is given in Table III. The predominant residues of the enzyme protein were glutamic acid, aspartic acid, arginine, alanine, and leucine. No other striking feature for a thermostable enzyme was observed except that the enzyme contained only two half-cystine residues per monomeric unit. The total of integral numbers of each amino acid residue gave a calculated molecular weight of about 38 000 for the polypeptide chain. The amino acid composition is quite similar to those of amino acid racemases from Gram-negative bacteria, *Sl. typhimurium* (Badet et al., 1984), and *P. putida* (=*P. striata*) (Roise et al., 1984). Thus, a statistical analysis of the amino acid compositions in a mole percent basis by the method of Harris et al. (1969) yielded low deviation functions ($D = [\sum(X_{1i} - X_{2i})^2]^{1/2}$ between the *B. stearothermophilus* alanine racemase and the *Sl. typhimurium dadB* alanine racemase (D = 0.056) and between the *B. stearothermophilus* alanine racemase and the *P. putida* amino racemase (*D* = 0.065).

Table III: Amino Acid Composition of the Cloned *B. stearothermophilus* Alanine Racemase[a]

amino acid	no. of residues (mol/mol of subunit)	amino acid	no. of residues (mol/mol of subunit)
Asp	28	Leu	30
Thr	19	Tyr	10
Ser	16	Phe	13
Glu	29	Lys	11
Pro	19	His	12
Gly	22	Arg	24
Ala	33	Trp	14
Val	22	Cys	2
Met	7		
Ile	17	total no. of residues	328

[a] The protein was hydrolyzed at 110 °C for 24, 48, and 72 h. The results were averaged, and the integral number of the amino acid is presenated. Detials for the experiment are given in the text.

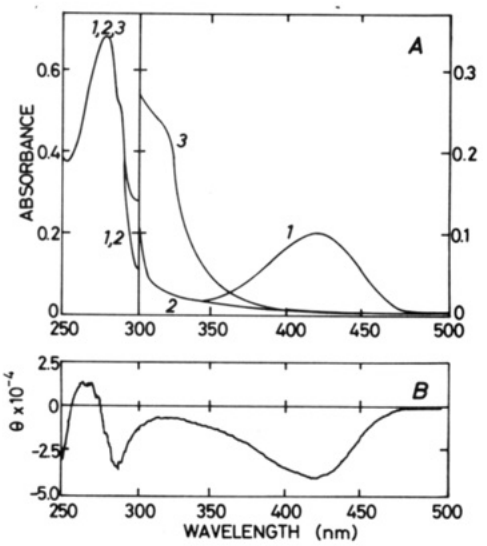

FIGURE 4: Absorption (A) and circular dichroic (B) spectra of alanine racemase. (A) Absorption spectra were taken in 10 mM potassium phosphate buffer (pH 8.0) at the enzyme concentration of 0.6 mg/ml: (1) holoenzyme; (2) apoenzyme; (3) $NaBH_4$-reduced enzyme. (B) Circular dichroic spectrum was taken in 10 mM potassium phosphate buffer (pH 8.0) at the enzyme concentration of 2.5 mg/mL.

Cofactor Analysis and Spectrometric Properties. Although some earlier studies showed that the alanine racemases from *Sh. aureus* (Rose & Strominger, 1966), *Pseudomonas* sp. (Free et al., 1967), and *E. coli* (Lambert & Neuhaus, 1972) did not required pyridoxal 5′-phosphate as a cofactor, recent reports have clearly indicated its presence in homogenous alanine racemase preparations (Badet et al., 1984; Roise et al., 1984; Wasserman et al., 1984; Badet & Walsh, 1985). We also determined the pyridoxal 5′-phosphate content in the purified *B. stearothermophilus* alanine racemase by the phenylhydrazine method of Wada and Snell (1961), after the enzyme solution (0.34 mg/mL) was thoroughly dialyzed against 10 mM potassium phosphate buffer (pH 7.4). An average pyridoxal 5′-phosphate content of 2.3 mol/78 000 g of protein was obtained, indicating that 2 mol of pyridoxal 5′-phosphate is bound per mole of enzyme molecule (dimer).

The purified enzyme inhibits absorption maximum at 420 nm in the visible region (Figure 4A), showing a typical Schiff base formed between the enzyme protein and pyridoxal 5′-phosphate. The extinction coefficient at 420 nm was calculated

to be 8450/[M(monomer)·cm] with the A_{280}/A_{420} ratio of about 5.6. No appreciable spectral shifts occurred by varying the pH (6.0–9.0). Reduction of the enzyme with sodium borohydride by the dialysis method of Matsuo and Greenberg (1959) caused a disappearance of the 420-nm peak and an increase in absorbance at about 330 nm (curve C) in Figure 4A) with a concomitant loss of the enzyme activity. The holoenzyme was converted to the inactive apoenzyme (curve B in Figure 4A) by treatment with 10 mM NH_2OH (pH 8.0) and then dialysis against 10 mM potassium phosphate buffer (pH 8.0) containing 0.1% 2-mercaptoethanol. Incubation of the apoenzyme with various concentrations of pyridoxal 5′-phosphate at 30 °C for 2 h followed by measurement of the enzyme activity gave an apparent Michaelis constant for pyridoxal 5′-phosphate in the range of 1.5–2.0 μM. Circular dichroic spectrum of the enzyme showed a negative peak at 420 nm (Figure 4B), corresponding to the absorption peak. A negative circular dichroic peak caused by bound cofactor has also been observed for the broad specificity amino acid racemase of *P. putida* (K. Tanizawa and K. Soda, unpublished results) and suggests that optical properties around the active site of these racemases are similar with each other. This similarity is reconciled with a dramatic sequence homology in the active site peptide of amino acid racemases (Roise et al., 1984; Badet & Walsh, 1985) including the *B. stearothermophilus* alanine racemase as reported in the following paper (Badet et al., 1986).

Discussion

A *B. stearothermophilus* gene coding for alanine racemase has been cloned and expressed in *E. coli* C600 with the vector plasmid pICR301 carrying the thermostable L-alanine dehydrogenase gene. Clones were selected for the ability to reduce nitro blue tetrazolium with NADH, which was generated inside *E. coli* cells by the action of L-alanine dehydrogenase on NAD and L-alanine produced from D-alanine by alanine racemase. Although the respiratory chain enzymes and alanine racemase contained in the host *E. coli* cells caused undesirable color development of the reduced nitro blue tetrazolium in the replica printing assay, the heat treatment of the printed colonies was quite effective to minimize the background color, destroying most of thermolabile enzyme activities in *E. coli*. The amount of racemase in the isolated recombinant cells is estimated to be about 0.3% of the soluble cellular protein (based on the specific activity in the *E. coli* pICR4 supernatant as compared to the specific activity of the purified racemase), which is elevated 30-fold over the wild-type level. The alanine racemase purified to homogeneity from *E. coli* pICR4 was confirmed to be immunochemically identical with the enzyme of *B. stearothermophilus* (see Figure 2).

Since alanine racemases generally function constitutively in the cell wall biosynthesis and their intracellular content is very low, attempts to purify the enzymes to a homogeneous state from wild bacterial strains have been limited or unsuccessful (Diven et al., 1964; Rose & Strominger, 1966; Yonaha et al., 1975). Badet and Walsh (1985) had to purify the *Sr. faecalis* enzyme 25 000–30 000-fold to homogeneity, little protein being available for extensive work. In this respect, the cloning of a thermostable enzyme gene in *E. coli* is also of great advantage for purification of the expressed gene product. As demonstrated in this paper, the heat treatment of cell-free extracts raised the specific activity of alanine racemase 5–7-fold without loss of total activity. Thus, subsequent purification procedures were greatly facilitated. Gene cloning of such thermostable enzymes and rapid purification by the heat treatment of *E. coli* cell extract have been successfully employed for 3-isopropylmalate dehydrogenase from an extreme thermophile, *Thermus thermophilus* (Nagahari et al., 1980; Tanaka et al., 1981), and L-leucine dehydrogenase from a moderate thermophile, *B. stearothermophilus* (S. Nagata and K. Soda, unpublished results). These results would provide a good system for purification of various thermostable enzymes of thermophilic bacteria.

The homogeneous alanine racemase of *B. stearothermophilus* isolated from the overproducing *E. coli* recombinant cells is now available in quantity and allows us to compare its enzymological properties with those of other well-characterized amino acid racemases. The dimeric structure with a molecular weight of about 78 000 and some other physicochemical properties (e.g. amino acid composition, absorption and circular dichroism spectra) are very similar to those of the broad-specificity amino acid racemase from *P. putida* (Soda & Osumi, 1969). In contrast, the alanine racemase encoded by the *Sl. typhimurium dadB* gene (Wasserman et al., 1984) and the enzyme of *Sr. faecalis* (Badet & Walsh, 1985) are believed to be a nonspherical monomer with a molecular weight of around 40 000 in their native state. Kinetic parameters for alanine racemization by the *B. stearothermophilus* enzyme are similar in magnitude to those of the enzymes from *Sl. typhimurium* (Wasserman et al., 1984), *Sr. faecalis* (Badet & Walsh, 1985), and *P. putida* (Roise et al., 1984) and fit well to the Haldane relationship (Briggs & Haldane, 1925), giving $K_{eq} = 1.14$ in agreement with the predicted value of 1.0. The extensive homology of the active site phosphopyridoxyl peptide among the *B. stearothermophilus* enzyme reported in the following paper (Badet et al., 1986), the *Sl. typhimurium* enzyme (Badet et al., 1984), and the *P. putida* enzyme (Roise et al., 1984) strongly suggests that these racemases have been evolved from a common progenitor.

Recent studies with a newly developed alanine racemase inhibitor, (1-aminoethyl)phosphonic acid (Ala-P), have revealed that the inhibition by this compound is time-dependent and irreversible for the enzymes of Gram-positive bacteria, while reversible for the enzymes of Gram-negative bacteria (Atherton et al., 1980; Badet & Walsh, 1985). Althought the mode of irreversible inactivation of alanine racemases from one Gram-positive bacterium has now been characterized in detail (Badet & Walsh, 1985; Badet et al., 1986), the molecular basis of the distinct action of Ala-P on enzymes from two types of bacteria has been unknown and will require three-dimensional structures of these racemases to be clarified. The thermal stability of the *B. stearothermophilus* racemase would thus make it a good candidate for high-resolution X-ray analysis, and initial crystals have been obtained (G. Petsko, D. Niedhart, and C. T. Walsh, unpublished results). DNA sequence to provide the encoded primary structure of the *B. stearothermophilus* racemase is under way.

Acknowledgments

We thank Dr. Nobuyoshi Esaki for helpful discussions and comments.

Registry No. Alanine racemase, 9024-06-0; L-alanine dehydrogenase, 9029-06-5; pyridoxal 5′-phosphate, 54-47-7.

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