Hatanaka, M., Yoshimura, N., Murakami, T., Kannagi, R., & Murachi, T. (1984) *Biochemistry 23*, 3272–3276.
Hatanaka, M., Yumoto, N., Sakihama, T., & Murachi, T. (1985) *Biochem. Int. 10*, 187–193.
Hawkes, R., Niday, E., & Gordon, J. (1982) *Anal. Biochem. 119*, 142–147.
Imajoh, S., Kawasaki, H., & Suzuki, K. (1986) *J. Biochem. (Tokyo) 99*, 1281–1284.
Inomata, M., Hayashi, M., Nakamura, M., Imahori, K., & Kawashima, S. (1985) *J. Biochem. (Tokyo) 98*, 407–416.
Ishiura, S., Sano, M., Kamakura, K., & Sugita, H. (1985) *FEBS Lett. 189*, 119–123.
Kido, H., Fukusen, N., & Katunuma, N. (1985) *Arch. Biochem. Biophys. 239*, 436–443.
Kitahara, A., Sasaki, T., Kikuchi, T., Yumoto, N., Yoshimura, N., Hatanaka, M., & Murachi, T. (1984) *J. Biochem. (Tokyo) 95*, 1759–1766.
Klempner, M. S., Mikkelsen, R. B., Corfman. D. H., & Andrē-Schwartz, J. (1980) *J. Cell Biol. 86*, 21–28.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Mellgren, R. L., Repetti, A., Muck, T. C., & Easly, J. (1982) *J. Biol. Chem. 257*, 7203–7209.
Melloni, E., Pontremoli, S., Michetti, M., Sacco, O., Sparatore, B., Salamino, F., & Horecker, B. L. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6435–6439.
Murachi, T. (1983a) *Trends Biochem. Sci. (Pers. Ed.) 8*, 167–169.
Murachi, T. (1983b) in *Calcium and Cell Function* (Cheung, W. Y., Ed.) Vol. 4, pp 377–410, Academic, New York.
Murachi, T., Hatanaka, M., Yasumoto, Y., Nakayama, N., & Tanaka, K. (1981a) *Biochem. Int. 2*, 651–656.
Murachi, T., Tanaka, K., Hatanaka, M., & Murakami, T. (1981b) *Adv. Enzyme Regul. 19*, 407–424.
Ohno, S., Emori, Y., Imajoh, S., Kawasaki, H., Kisaragi, M., & Suzuki, K. (1984) *Nature (London) 312*, 566–570.
Pontremoli, S., & Melloni, E. (1986) *Annu. Rev. Biochem. 55*, 455–481.
Pontremoli, S., Sparatore, B., Salamino, F., Michetti, M., Sacco, O., & Melloni, E. (1985) *Biochem. Int. 11*, 35–44.
Ross, E., & Schatz, G. (1973) *Anal. Biochem. 54*, 304–306.
Sakihama, T., Kakidani, H., Zenita, K., Yumoto, N., Kikuchi, T., Sasaki, T., Kannagi, R., Nakanishi, S., Ohmori, M., Takio, K., Titani, K., & Murachi, T. (1985) *Proc. Natl. Acad. Sci. U.S.A. 85*, 6075–6079.
Suzuki, K. (1987) *Trends Biochem. Sci. (Pers. Ed.) 12*, 103–105.
Tanaka, K., Nakamura, T., & Ichihara, A. (1986) *J. Biol. Chem. 261*, 2610–2615.
Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.
Walloe, H. P. (1966) *Scand. J. Clin. Lab. Invest. 18*, 353–356.
Yoshimura, N., Kikuchi, T., Sasaki, T., Kitahara, A., Hatanaka, M., & Murachi, T. (1983) *J. Biol. Chem. 258*, 8883–8889.
Yoshimura, N., Hatanaka, M., Kitahara, A., Kawaguchi, N., & Murachi, T. (1984) *J. Biol. Chem. 259*, 9847–9852.
Yumoto, N., Kikuchi, T., Sasaki, T., & Murachi, T. (1984) *J. Biochem. (Tokyo) 96*, 1531–1537.

Racemization of Alanine by the Alanine Racemases from *Salmonella typhimurium* and *Bacillus stearothermophilus*: Energetic Reaction Profiles†

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Received September 22, 1987; Revised Manuscript Received December 7, 1987

ABSTRACT: Alanine racemases are bacterial pyridoxal 5′-phosphate (PLP) dependent enzymes providing D-alanine as an essential building block for biosynthesis of the peptidoglycan layer of the cell wall. Two isozymic alanine racemases, encoded by the *dadB* gene and the *alr* gene, from the Gram-negative mesophilic *Salmonella typhimurium* and one from the Gram-positive thermophilic *Bacillus stearothermophilus* have been examined for the racemization mechanism. Substrate deuterium isotope effects and solvent deuterium isotope effects have been measured in both L → D and D → L directions for all three enzymes to assess the degree to which abstraction of the α-proton or protonation of substrate PLP carbanion is limiting in catalysis. Additionally, experiments measuring internal return of α-^{3}H from substrate to product and solvent exchange/substrate conversion experiments in ^3H_2O have been used with each enzyme to examine the partitioning of substrate PLP carbanion intermediates and to obtain the relative heights of kinetically significant energy barriers in alanine racemase catalysis.

The alanine racemases are a group of pyridoxal 5′-phosphate (PLP)[1] containing bacterial enzymes that catalyze the racemization of D- and L-alanine. These enzymes are essential in bacteria, as D-alanine is an important component in the biosynthesis of the cell wall and can be obtained only by isomerization of L-alanine. Work in these laboratories has centered on the racemases from the Gram-negative bacteria *Salmonella typhimurium* (Badet et al., 1984; Esaki & Walsh, 1986) and *Pseudomonas striata* (Roise et al., 1984) and the Gram-positive thermophile *Bacillus stearothermophilus* (Badet et

† This work was supported in part by a grant from the National Science Foundation (PCM 8308969). W.S.F. was supported by a National Institutes of Health postdoctoral fellowship (GM 11471-02).
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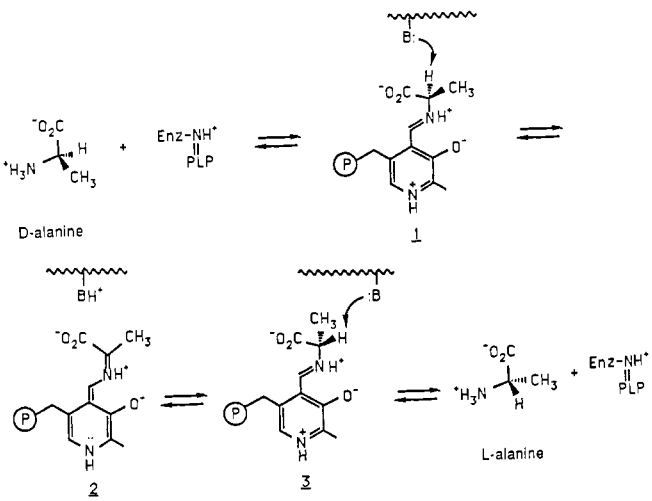
[1] Abbreviations: PLP, pyridoxal 5′-phosphate; DAAO, D-amino acid oxidase; LDH, lactate dehydrogenase; CHES, 2-(*N*-cyclohexylamino)-ethanesulfonic acid; LADH, L-alanine dehydrogenase; CD, circular dichroism; NAD, nicotinamide adenine dinucleotide; NADH, reduced NAD.

0006-2960/88/0427-3267$01.50/0

Scheme I



al., 1986). Cloning and sequencing of the racemase genes from *S. typhimurium* and *B. stearothermophilus* reveal that they possess an identical decapeptide at the active site. Wasserman et al. (1983) have demonstrated that two alanine racemase genes are present in *S. typhimurium* and have ascribed putative anabolic (*alr* racemase) and catabolic (*dadB* racemase) functions for each enzyme. Both gene products show considerable similarity (ca. 40%; Galakatos & Walsh, 1987); however, differences in their steady-state parameters and inactivation kinetics are substantial. Although a great deal of work has been done elucidating the mechanism of action of a number of racemase inhibitors (Badet et al., 1984, 1986; Roise et al., 1984), analysis of the detailed enzyme mechanism of alanine racemase with its natural substrates has remained substantially incomplete.

The racemization of alanine by alanine racemase (Scheme I) is envisioned to proceed by an initial transaldimination, followed by α-hydrogen abstraction to give the resonance-stabilized carbanion **2**. Reprotonation on the opposite face yields the antipodal aldimine **3** with subsequent release of the isomerized alanine moiety. Uncertainties, however, lie in the detailed mechanistic features. For example, (i) does catalysis proceed via a one-base (flexible) or two-base (oppositely disposed on the two faces of the PLP α-carbanion) mechanism and (ii) what step(s) is (are) rate limiting in the overall reactive process?

As discussed in detail by Adams (1976), and more recently by Soda et al. (1986), the mechanism and stereochemistry of the racemase reaction catalyzed by a number of different racemases are not well understood. Only the broad specificity amino acid racemase from *P. striata* is known to proceed via a one-base mechanism (Shen et al., 1983), as evidenced by internal return of substrate deuterium. α-Amino-ϵ-caprolactam racemase, another PLP-containing enzyme, is also believed to proceed by a one-base mechanism (Ahmed et al., 1986) based on internal return of α-H. However, Adams (1976) has proposed a two-base mechanism for the alanine racemase from *Pseudomonas putida* and *Bacillus subtilis* based on extensive asymmetry observed in tritium-exchange experiments and Michaelis–Menten parameters. Such asymmetry is also observed with the alanine racemases from *S. typhimurium* (Badet et al., 1984; Esaki & Walsh, 1986) and *Escherichia coli* (Wang & Walsh, 1978, 1981) in mechanism-based inhibitor studies with β-substituted D- vs L-alanines. In contrast, Whitman et al. (1985) have demonstrated a considerable amount of symmetry present in the racemization of the α-hydroxy acid mandelate by mandelate racemase (Kenyon & Hegeman, 1979); this enzyme shows little difference in steady-state parameters, isotope effect data, and inhibition rate constants in either direction. These authors used these and other data to conclude that the enzyme proceeds via a one-base mechanism with remarkable symmetry, considering the inherent asymmetry of an enzyme active site.

Proline racemase (Cardinale & Abeles, 1968; Fisher et al., 1986a,b) and diaminopimelic acid (DAP) epimerase (Wiseman & Nichols, 1984), both of which are known not to require cofactors, are distinctive in that they operate via a two-base mechanism. Proton abstraction from one face (by an active site cysteine) is concomitant with proton donation on the other face (by another active site cysteine), followed by product release. A two-base mechanism, however, has not been demonstrated for any PLP-containing amino acid racemase.

Herein we present the data necessary to construct a partial reaction profile for the racemization of alanine by two isozymic alanine racemases from the Gram-negative mesophile *S. typhimurium* (*dadB* and *alr*) and one from the Gram-positve thermophile *B. stearothermophilus*. Substrate and solvent isotope effect experiments and tritium exchange experiments act as probes toward investigating various features of enzyme-catalyzed racemization. This information should provide a greater understanding of the reaction mechanism of these important enzymes and more thoroughly examine the phenomenon of symmetry and asymmetry in enzymic catalysis.

Experimental Procedures

Materials. L-Alanine, D-alanine, L-alanine dehydrogenase (0.9 mg/mL; 20 units/mg) from *B. subtilis*, aminoacylase from porcine kidney, catalase (23 mg/mL; 3000 units/mg) from bovine liver, NAD, 3-acetyl-NAD, NADH, α-ketoglutarate, and CHES were purchased from Sigma Chemical Co. Lactate dehydrogenase (10 mg/mL; 550 units/mg) from porcine muscle, D-amino acid oxidase (5 mg/mL; 15 units/mg) from porcine kidney, porcine heart glutamate–pyruvate transaminase (10 mg/mL; 80 units/mg), and glutamate dehydrogenase (10 mg/mL; 120 units/mg) from bovine liver were from Boehringer-Mannheim Biochemicals. ^3H_2O (5 mCi/mL) was purchased from Amersham Biochemicals. Deuteriated water (^2H_2O) and acetic acid (CH_3COO^2H) were purchased from Aldrich Chemical Co.

D-[^2H]Alanine and L-[^2H]Alanine. These were synthesized by the procedure of Greenstein and Winitz (1961) using deuteriated acetic acid as solvent. This gave enantiomerically pure D-[^{2}H]alanine and L-[^{2}H]alanine containing 96% deuterium at the α-hydrogen as judged by ^{1}H NMR.

D-[^3H]Alanine and L-[^3H]Alanine. To 5.0 mL of a 2 mCi/mL ^3H_2O solution containing 0.1 M ammonium bicarbonate was added 0.55 g of L-alanine, at which time racemase was added and the reaction allowed to proceed until all L-alanine had exchanged with solvent. The ^3H_2O was removed by bulb-to-bulb distillation, and the activity of DL-[^{3}H]alanine was found to be 16 μCi/mmol. A 200-mg (2.25-mmol) aliquot was placed in 20 mL of H_2O containing 0.72 g of Na_2CO_3 (6.8 mmol), 1.2 equiv of acetic anhydride (freshly distilled) was added, and the solution was stirred at 25 °C for 5 h. The solution was then acidified (pH 2) and evaporated under vacuum to dryness. Enzymic resolution of *N*-acetyl-DL-[^{3}H]alanine by hog kidney acylase (Greenstein & Winitz, 1961) gave enantiomerically pure L-[^{3}H]alanine but only 96% enantiomerically pure D-[^{3}H]alanine. The specific activities of both L- and D-[^{3}H]alanine were found to be 14 μCi/mmol.

The *B. stearothermophilus* alanine racemase was prepared according to the method of Neidhart et al. (1987). The *S.*

typhimurium dadB alanine racemase was prepared according to the method of Wasserman et al. (1984), while the *S. typhimurium alr* alanine racemase was prepared according to the method of Galakatos et al. (1986). All three enzymes were purified to >90% homogeneity as determined by polyacrylamide gel electrophoresis, and the concentration of each enzyme was ascertained by the A_{280}/A_{420} ratio as described previously (Wasserman et al., 1984; Esaki & Walsh, 1986; Neidhart et al., 1987).

Analytical Methods. All kinetic experiments were performed at 37 °C in 0.1 M CHES buffer (pH 9.1), unless otherwise stated. Absorption spectra and steady-state reaction rates were measured with a Perkin-Elmer 554 spectrophotometer, while circular dichroism (CD) readings were recorded on a Jasco J500C spectropolarimeter. Radioactivity was measured by dissolving aliquots (≤1.1 mL) into 5 mL of scintillation solution and placing them in a Beckman LS1800 scintillation counter.

Steady-State Kinetics. Michaelis–Menten parameters were obtained by the method of Wilkinson (1961) from initial velocity measurements of the racemization of L-alanine → D-alanine and D-alanine → L-alanine irreversibly under steady-state conditions. To obtain Michaelis–Menten parameters in the D → L direction, the assay mixture consisted of 10 mM NAD and 20 μL of LADH in a total of 1.0 mL of buffer. D-Alanine concentration was between 0.4 and 30 mM, and upon addition of racemase, the increase in absorption at 340 nm corresponding to production of NADH was monitored, where the change in extinction coefficient at this wavelength was taken to be 6220 M^{-1} cm^{-1}. Enzyme concentrations were as follows: *B. stearothermophilus* racemase, 0.1 nM; *dadB* racemase, 0.2 nM; *alr* racemase 13.5 mM. To obtain Michaelis–Menten parameters in the L → D direction, the assay mixture consisted of 0.2 mM NADH, 20 μL of DAAO, and 5 μL of LDH in a total of 1.0 mL of buffer. L-Alanine concentration was between 1.0 and 30 mM, and the reaction was followed by monitoring the decrease in absorption at 340 nm, corresponding to the consumption of NADH occurring in the reduction of pyruvate to lactate. Enzyme concentrations of 0.019, 0.023, and 7.45 nM for *B. stearothermophilus*, *dadB*, and *alr* racemases, respectively, were used.

Measurement of Substrate Deuterium Isotope Effect. To ascertain the contribution of α-proton abstraction to the overall racemization rates, the effect of α-deuteriated D- and L-alanine on the steady-state rate parameters was investigated. In the D → L direction, the reaction conditions are identical with that discussed above except (i) D-[^{2}H]alanine was the substrate and (ii) enzyme concentrations were 0.09, 1.1, and 25 nM for *B. stearothermophilus*, *dadB*, and *alr*, respectively. In the L → D direction, the reaction conditions were identical with that discussed above except L-[^{2}H]alanine was the substrate.

Measurement of Solvent Deuterium Isotope Effect. To determine whether protonation of intermediate **2** in Scheme I is in the rate-determining step, steady-state experiments were performed in D_2O. The conditions were identical with that stated above except the buffer system was 0.1 M CHES in D_2O at pD 9.1 [pD readings were obtained by adding 0.40 to pH meter readings (Glasoe & Long 1960)] and substrate was D-[^{1}H]alanine (D → L direction) or L-[^{1}H]alanine (L → D direction). Enzyme concentrations were 0.1, 0.45, and 11.5 nM for *B. stearothermophilus*, *dadB*, and *alr*, respectively.

Equilibrium Perturbation Experiment. The method employed was identical with that described in Whitman et al. (1985) and Cleland (1977). A total of 100 μL of a 20 mM L-[^{1}H]alanine solution and 100 μL of a 20 mM D-[^{2}H]alanine solution was placed in a 0.1-cm path length CD cell (250-μL volume), and the contents were mixed thoroughly. The solution showed a null at 220 nm in the spectropolarimeter indicative of the presence of an equilibrium mixture. This wavelength (220 nm) was used as it provided optimal resolution between D- and L-alanine at the concentrations used in the experiment. Racemase (final concentrations were 10, 25, and 61 nM for *B. stearothermophilus*, *dadB*, and *alr*, respectively) was added to the mixture, and the CD was monitored at 220 nm. To measure the perturbation in the opposite direction, 100 μL of a 20 mM L-[^{2}H]alanine solution and 100 μL of a 20 mM D-[^{1}H]alanine solution were placed in the CD cell. The contents were mixed thoroughly and racemase was added as described above. Control reactions were done in both directions in which racemase was added to solutions of L-[^{1}H]alanine and D-[^{1}H]alanine in buffer; no perturbation was observed.

Internal Return Experiments. In order to determine the extent of exchange between the substrate-derived proton and solvent upon racemization, experiments that monitor the degree of internal return from substrate to product were conducted. D-[^{3}H]Alanine, containing 96% D α-[^{3}H] and 4% L α-[^{3}H] as described above, was dissolved in 1.0 mL of buffer in both H_2O and D_2O at concentrations of 7–10 mM. The amounts of D-alanine and L-alanine were accurately measured by using DAAO/LDH and LADH assay systems, respectively. To the D-[^{3}H]alanine solution was added 4.0 mg of NAD, 20 μL of LADH, and 10 μL of LDH, and the mixture was placed in a 37 °C water bath for 2 h. Assay for lactate according to the method of Everse (1975) revealed that >95% of the L-[^{3}H]alanine present had been converted to L-[^{3}H]lactate. At this time, 10 μL of racemase (final concentrations of 0.5, 1.6, and 60 nM for the *B. stearothermophilus*, *dadB*, and *alr* enzymes, respective) was added and the mixture incubated at 10 °C for 14 h. The mixture was placed in boiling water for 5 min (to inactivate the racemase) and allowed to cool to room temperature, at which time D-alanine and lactate were assayed quantitatively. Aliquots containing 20 μL of DAAO and 2 μL of catalase were added and the mixture was incubated at 37 °C for an additional 6 h. This led to the complete destruction of all the remaining D-alanine; purification of the remaining lactate from the radioactivity in the solvent was achieved by anion-exchange column chromatography. The mixture was thus added to a Bio-rad AG1-X8 anion-exchange column (1.0 × 2.2 cm; acetate form) and washed with water until the radioactivity fell to background level. Lactate was eluted from the column by addition of 1.0 M NaCl. Fractions (1.0 mL) were collected, assayed for lactate, and counted by liquid scintillation. In order to demonstrate that L-[^{3}H]alanine stoichiometrically gives L-[^{3}H]lactate under the experimental conditions, the following control was performed. To 1.0 mL of buffer solution in D_2O containing 5 mM L-[^{3}H]alanine was added 20 μL of LADH, 10 μL of LDH, and 4 mg of NAD, and the reaction was allowed to proceed until all L-alanine was consumed. Purification by anion-exchange chromatography as described above led to isolation of lactate that contained exactly the same amount of radioactivity (~16 μCi/mmol) as the starting L-[^{3}H]alanine.

Discrimination Experiments. D-Alanine → L-Alanine. To 1.0 mL of HTO (specific activity ca. 1 mCi/mL), containing buffer at pH 9.1, were added 20 μL of LADH, 10 μL of LDH, 4 mg of NAD, and 10 μL of D-[^{1}H]alanine (final concentrations of 3.4, 5, and 10 mM). This was incubated at 37 °C for 15 min, at which time racemase was added. After roughly 2 mM substrate had been consumed, the reaction was

Table I: Steady-State Parameters for Racemization of Alanine Catalyzed by Alanine Racemase

enzyme	D → L direction		L → D direction	
	K_m (mM)	k_{cat} (min^{-1})	K_m (mM)	k_{cat} (min^{-1})
B. stearothermophilus	2.7 (±0.2)	7.0 (±0.2) × 10^4	4.4 (±0.2)	1.1 (±0.1) × 10^5
dadB	2.2 (±0.2)	1.6 (±0.1) × 10^4	11.0 (±0.3)	7.3 (±0.2) × 10^4
alr	0.5 (±0.05)	2.6 (±0.2) × 10^2	1.7 (±0.1)	9.7 (±0.3) × 10^2

quenched by submersion in boiling water for 5 min. Upon cooling, 20 μL of DAAO and 2 μL of catalase were added, and the reaction was incubated at 37 °C until all D-alanine had been consumed. A 10-μL aliquot was assayed for radioactivity in order to obtain a precise number for the specific activity of the HTO. Purification and assay of lactate were identical with that described in the internal return experimental procedure above.

Exchange vs Conversion Experiments. L-Alanine → D-Alanine. To 1.0 mL of HTO (specific activity ca. 1 mCi/mL), containing buffer at pH 9.1, were added 20 μL of DAAO, 5 μL of LDH, 3 mg of NADH, and 25 μL of L-[^{1}H]alanine (final concentration of 5 mM). This was incubated at 37 °C for 15 min, at which time racemase was added. In order to ensure that virtually no D-alanine accumulated, the amount of racemase added was chosen so as to give a net flux of roughly 12 μM min^{-1} in the L → D direction, which was well below the coupling enzyme rate. Conversion was measured by monitoring the decrease in absorbance at 340 nm, corresponding to NADH consumption. At the appropriate time, the reaction was quenched by placing the mixture in a boiling water bath for 5 min. When the reaction had cooled, 10 μL of DAAO was added to ensure conversion of any D-alanine that may have accumulated. A 10-μL aliquot was then assayed for radioactivity in order to obtain a precise number for the specific activity of the T_2O. Purification of the L-alanine was achieved by adding the mixture to a Bio-Rad AG50-X4 cation-exchange column (1.0 × 1.5 cm; H^+ form), which was washed with water until the radioactivity had reached background level. L-Alanine was eluted from the column by addition of 1.0 M NaCl, and 1.0-mL fractions were collected, assayed for L-alanine, and counted by liquid scintillation.

D-Alanine → L-Alanine. To 1.0 mL of HTO (specific activity ca. 1.25 mCi/mL), containing buffer at pH 9.1, were added 25 μL of glutamate–pyruvate transaminase, 5 μL of LDH, 4 mg of NADH, and 3.5 mg of α-ketoglutarate. This was incubated at 37 °C, at which time racemase was added. As described above, one must avoid L-alanine accumulation; thus, the amount of racemase added was chosen so as to give a net flux of 10 μM min^{-1} in the D-alanine → L-alanine direction, far below the coupling enzyme rate. Conversion was monitored by following the decrease at 340 or 366 nm ($\Delta\epsilon$ = 3300 M^{-1} cm^{-1}), and at the appropriate interval, the reaction was quenched by submersion into boiling water for 5 min. Upon cooling, 10 μL of the transaminase solution was added in order that any residual L-alanine present would be consumed. A 10-μL aliquot was then assayed for radioactivity in order to obtain a precise value for the specific activity of the T_2O. Purification of D-alanine was identical with that described for L-alanine above; however, one must also assay for L-glutamate formed, as any glutamate present will have the specific activity of the solvent. This was done by monitoring the increase at 340 nm upon dilution into an assay mixture containing 10 mM NAD and 20 μL of glutamate dehydrogenase solution. The majority of the L-glutamate eluted after the D-alanine from the Bio-Rad AG50-X4 column.

Determination of Oversaturation. A 200-μL of aliquot of an L-[^{1}H]alanine solution (final concentrations of 20, 50, 100 and 200 mM) containing buffer was placed in a 1.5-mL Eppendorf centrifuge tube. To this was added a small aliquot of racemase (final concentrations of 0.06–0.3 μM for the *B. stearothermophilus* and 0.05–0.2 μM for the *dadB* enzyme), and the mixture was immediately added to the CD cell incubated at 25 °C. The ellipticity at 220 nm was observed as a function of time as the racemization of L-alanine to D-alanine ensued.

Table II: Deuterium Isotope Effect Results (on V/K) for Racemization of Alanine by Alanine Racemase

enzyme	L → D direction		D → L direction	
	$(V/K)_H/(V/K)_D$	$(V/K)_{H_2O}/(V/K)_{D_2O}$	$(V/K)_H/(V/K)_D$	$(V/K)_{H_2O}/(V/K)_{D_2O}$
B. stearothermophilus	1.4 ± 0.05	1.0 ± 0.05	1.2 ± 0.05	1.0 ± 0.05
dadB	1.3 ± 0.05	2.4 ± 0.10	2.9 ± 0.20	1.0 ± 0.05
alr	1.4 ± 0.05	1.65 ± 0.10	2.0 ± 0.20	1.1 ± 0.05

RESULTS

Comparison of the Steady-State Rate Values. The Michaelis–Menten constants for each alanine racemase in both directions are listed in Table I. The *B. stearothermophilus* racemase values were very similar to the values reported earlier (Inagaki et al., 1986), as were those for the *dadB* racemase (Wasserman et al., 1984). The values for the *alr* racemase from *S. typhimurium* differed slightly from those obtained by Esaki and Walsh (1986), and this is ascribed to a different enzyme preparation and different conditions under which the steady-state values were obtained. With these values, the K_{eq} was found to be between 1.04 and 1.09, in accordance with what is predicted for racemases by the Haldane equation (Briggs & Haldane, 1925).

Isotope Effect Studies. The values for $(V/K)_{^1H}/(V/K)_{^2H}$ and $(V/K)_{H_2O}/(V/K)_{D_2O}$ obtained from both equilibrium perturbation experiments and steady-state experiments are shown in Table II. It is apparent that abstraction of the α-proton (for both D- and L-alanine) is not rate limiting for the *B. stearothermophilus* enzyme. The presence of a very small isotope effect in both directions indicates that, after PLP aldimine formation (Scheme I), the α-proton is sufficiently acidic such that abstraction (as measured by substrate isotope effects) or protonation (solvent isotope effects) has little or no bearing on the overall reaction rate. Comparison of the steady-state constants indicates that both K_m and k_{cat} remain the same regardless of the isotope (α-[^{1}H] or α-[^{2}H]) in substrate or in solvent. Thus, it may be assumed that some step not involving proton transfer to and from the substrate α-carbon is the rate-determining step in catalysis for the *B. stearothermophilus* alanine racemase.

The *dadB* enzyme provides a striking contrast to the *B. stearothermophilus* racemase (Table II). In the D → L direction, one observes a strong isotope effect accompanying abstraction of the α-deuteron (V/K = 2.9); however, no isotope effect is observed in the protonation (D_2O) step. Abstraction of the α-deuteron in this direction appears to be partially rate determining, while protonation (with deuterium) of the carbanion intermediate **2** [Scheme I, shown as the quinone-like

Table III: Deuterium Isotope Effect Results (on V) for Racemization of Alanine by Alanine Racemase

enzyme	L → D direction		D → L direction	
	V_H/V_D	V_{H_2O}/V_{D_2O}	V_H/V_D	V_{H_2O}/V_{D_2O}
B. stearothermophilus	1.5 ± 0.05	1.2 ± 0.05	1.1 ± 0.05	1.2 ± 0.05
dadB	1.3 ± 0.05	2.45 ± 0.10	3.0 ± 0.10	1.0 ± 0.05
alr	1.1 ± 0.10	2.45 ± 0.10	2.6 ± 0.10	1.4 ± 0.05

structure (Kallen et al., 1984)] has little effect on the reaction rate. In the opposite direction (L → D), abstraction of the α-deuteron leads to virtually no isotope effect (V/K = 1.3), while protonation occurs with a larger isotope effect [V/K (H_2O/D_2O) = 2.4]. Thus, it appears as if abstraction of the α-deuteron of the D-alanyl-PLP aldimine **1** (Scheme I) *and* protonation of the carbanion intermediate **2** to give the D-alanyl-PLP aldimine are more difficult (hence, detectably rate limiting) than those of the L isomer. The isotope effect values for the *dadB* enzyme are also due entirely to changes in V_{max}, as K_m has remained virtually the same (Table III).

Analysis of the isotope effects observed with the *alr* racemase (Table II) shows similarity to the *dadB* enzyme, but a few subtle differences are present. Abstraction of the α-deuteron in D-alanine appears to be partially rate limiting, as an isotope effect is seen; however, it is substantially lower than the *dadB* enzyme: 2.0 vs 2.9. The presence of a small solvent isotope effect in the L → D direction [V/K (H_2O/D_2O) = 1.65] suggests partial rate-limiting protonation of the D α-deuteron; however, this value is much smaller than the corresponding *dadB* racemase value of 2.4. These values become more illuminating when one compares $V_{^1H}/V_{^2H}$ and $V_{(H_2O}/V_{D_2O}$ for both the *dadB* and *alr* enzymes (Table III). The isotope effects on k_{cat} are very similar (columns 2 and 3 of Table III), suggesting a similar catalytic mechanism; however, binding (K_m) has been affected, which leads to the poor correlation between V/K parameters.

Internal Return. Experiments to detect internal transfer of substrate α-tritium to product were utilized to monitor the degree of exchange between the enzyme base and solvent protons. Detection of internal transfer (detection limit in this study was reliably 3–5%) would be strong evidence that a one-base mechanism is operative. The lack of any internal transfer can be interpreted as resulting from either (i) a two-base mechanism or (ii) a one-base mechanism in which rapid exchange of enzyme base with solvent occurs. Studying the reaction in the D → L direction with D-α-[^{3}H]alanine as described under Experimental Procedures, we were unable to detect any internal transfer.

Discrimination. By following the racemization of unlabeled D-alanine in T_2O under irreversible conditions, provided by coupling enzymes, and analyzing L-alanine radioactivity, one may obtain a measure of the extent of discrimination against solvent tritium during protonation of the carbanion intermediate **2** (Scheme I) to yield L-alanine. As visualized in Scheme II, if equilibration between enzyme base and solvent is rapid, then discrimination experiments can provide information as to the relative rates of k_2^H and k_2^T. As demonstrated below, exchange vs conversion experiments can provide data on the relative rates of k_2^H and k_1^T as well as the rates of k_1^H and k_2^T. These experiments allow a detailed insight into the partioning of intermediates and the mechanism of racemization.

Discrimination experiments performed with all three racemases in the D → L direction yielded virtually identical results. As shown in Table IV, the carbanion intermediate shows very little discrimination (~1.4-fold) between ^{3}H and ^{1}H (k_2^T and

Scheme II

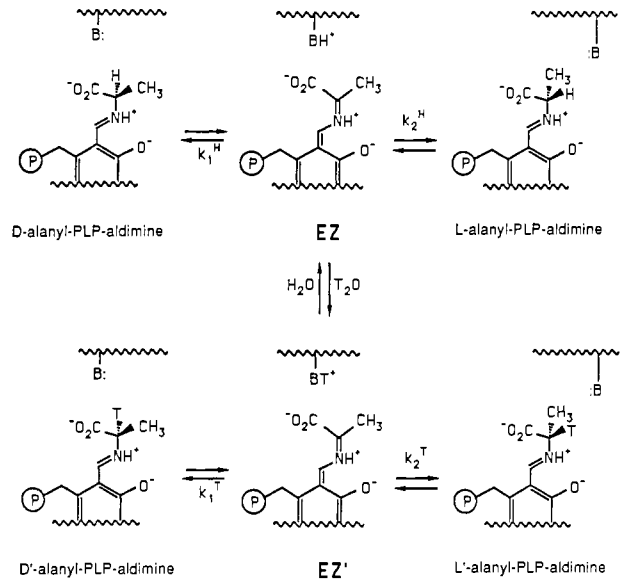


Table IV: Incorporation of Tritium into Product L-Lactate during Racemase-Catalyzed Reaction of D-Alanine to L-Alanine under Irreversible Conditions

enzyme	fractional extent of reaction	specific radioactivity (μCi/mmol)		isotope content of product
		solvent	lactate	
B. stearothermophilus	0.21	8.0	5.4	0.68
	0.30	8.0	5.5	0.69
	0.38	8.2	5.9	0.70
	0.50	8.2	5.4	0.66
dadB	0.21	9.4	5.1	0.55
	0.40	9.2	6.5	0.70
	0.52	9.2	6.3	0.67
	0.60	8.2	5.5	0.67
alr	0.18	8.2	4.4	0.55
	0.40	8.6	5.6	0.65
	0.50	8.2	6.0	0.73
	0.70	8.1	5.4	0.67

k_2^H in Scheme II) when it accepts a proton at the α-carbon to give the PLP aldimine of L-alanine. Since a primary tritium isotope effect would lead to a rate decrease of roughly 6–20-fold (Knowles & Albery, 1977) and the results show that $k_2^H \approx k_2^T$ (1.4 ≈ 1), one may conclude that proton transfer to the α-carbanion is quite rapid with respect to release of L-alanine (i.e., in Scheme I, **2** → **3** is more rapid than **3** → L-alanine + enzyme). Thus, both abstraction of the L-alanyl-PLP α-H and protonation back to the L-alanyl α-locus are quite rapid, in agreement with the conclusions derived from deuterium isotope effect experiments (discrimination experiments were not performed in the L → D direction).

Exchange vs Conversion. As elegantly demonstrated with triosephosphate isomerase, Knowles and Albery (1977) showed how exchange (of α-H with solvent protons) vs conversion experiments can be used to provide insight for defining the energetics of the reaction profile. Three types of behavior may be distinguished depending on the reaction profile under proper conditions. The first behavior is described as "equilibration", in which isotope exchange is fast relative to conversion (i.e., fast exchange followed by rate-limiting conversion), and is seen in Figure 1a. The second behavior is "accumulation", in which isotope exchange is slow relative to conversion (i.e., rate-limiting step is also proton transfer step), and is seen in Figure 1b. The third behavior is "intermedate" and is depicted in Figure 1c [for a more detailed discussion, see Albery and Knowles (1976a)].

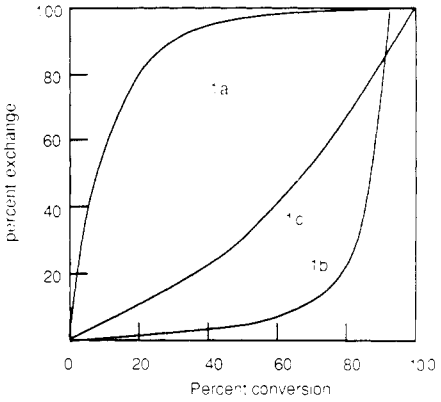


FIGURE 1: Extent of isotopic content of reactant during the course of reaction as a function of the fraction of substrate remaining [taken from Albery and Knowles (1976a)].

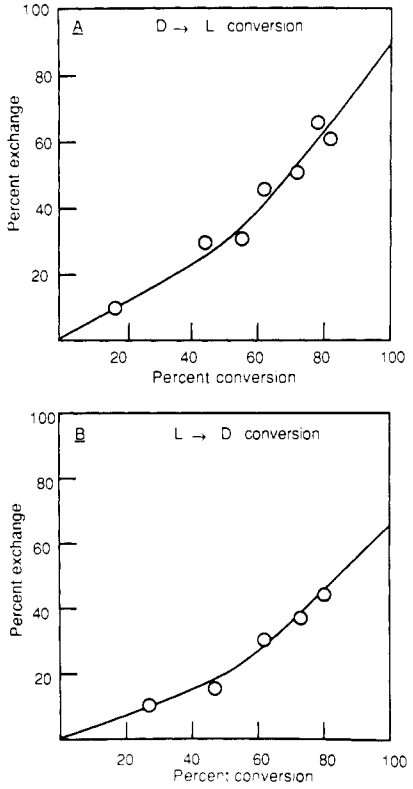


FIGURE 2: Incorporation of ^3H from tritiated water into remaining substrate D-alanine (A) or L-alanine (B) as a function of the extent of the reaction for the *B. stearothermophilus* racemase. The isotopic content of the remaining substrate (ordinate) is plotted as a function of conversion to product (abscissa).

Treatment of the data obtained with unlabeled substrate, starting from D- or from L-alanine, in T_2O with the alanine racemase from *B. stearothermophilus* is seen in Figure 2. Both D- and L-alanine can be interpreted as following an intermediate behavior, consistent with the deuterium and tritium isotope effect data. The initial slope of the line (as determined by a least-squares fit of all points up to ca. 50% conversion) in Figure 2A (in the D → L direction) shows that the carbanion intermediate undergoes conversion to L-alanine 2 times faster than it experiences exchange (to give D-[^{3}H]alanine). Measurement of the initial slope of the line in Figure 2B (in the L → D direction) shows that the PLP–substrate α-carbanion intermediate undergoes conversion to D-alanine 3 times faster than it experiences exchange of solvent tritium (to give back L-[^{3}H]alanine). These data, along with that obtained in the discrimination experiments described above, provide the requisite information to construct a reaction profile as discussed later.

Scheme III: Mechanism of Release of Tritiated (D′L′) and Normal (DL) D- and L-Alanine from the Carbanion Intermediate (EZ and EZ′) in Which the Enzyme Base Contains a Proton (EZ) or Tritium (EZ′) As Depicted in Scheme II

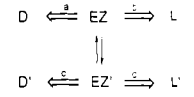


Table V: Relative Rates of Tritium Exchange and Product Conversion for D-Alanine (DD′) and L-Alanine (LL′) According to Scheme III

enzyme	rate *a*	rate *b*	rate *c*	rate *d*
B. stearothermophilus	4 ± 0.3	2 ± 0.2	1 ± 0.1	1.5 ± 0.1
dadB	11 ± 1	3.5 ± 0.2	1 ± 0.1	2.5 ± 0.2
alr	7 ± 0.5	2.5 ± 0.2	1 ± 0.1	2.0 ± 0.2

As shown by Fletcher et al. (1976), the exchange/conversion and discrimination experiments allow evaluation of the relative rates *a*–*d* of Scheme III and are collected in Table V. Exchange vs conversion experiments allow one to obtain the ratio c/b (for D → L conversion) and d/a (for L → D conversion) through measurement of initial slope, while discrimination experiments allow one to obtain the ratio b/d (for D → L conversion) (Knowles & Albery, 1977; Fletcher et al., 1976). As shown in Table V, the relative values of the rates for the *B. stearothermophilus* racemase can be calculated and provide a qualitative picture for the reaction profile. There is only a small tritium isotope effect in either direction, $a/c = 4$ for EZ → D while $b/d = 1.3$ for EZ → L. One detects a 2-fold difference in the rate of release of D-alanine (rate *a*) vs L-alanine (rate *b*) from the carbanion intermediate **2** (Scheme I).

Similar experiments performed with unlabeled D-alanine in T_2O with the *dadB* racemase were suggestive of accumulation (Figure 1b). After nearly 60% completion, little radioactivity was detected in substrate but subsequently rose very quickly (Figure 3A). This shows that as the reaction proceeds, the tritium content in the substrate grows steadily greater as D-[^{1}H]alanine is preferentially consumed until the last few percent are virtually all tritiated. Measurement of the initial slope shows that the carbanion intermediate **2** (Scheme I) is converted to L-alanine 3.5 times faster than it experiences exchange of solvent tritium.

Analysis of the exchange/conversion data obtained with unlabeled L-alanine in T_2O (L → D direction) with the *dadB* enzyme proved intriguing in that intermediate behavior was observed (Figure 3B). Measurement of the initial gradient as described above shows that carbanion intermediate **2** (Scheme I) is converted to D-alanine 4 times faster than it experiences exchange of solvent tritium. The exchange/conversion experiments and the discrimination experiment allow one to calculate the relative racemization rates for the *dadB* enzyme in the same manner described for the *B. stearothermophilus* enzyme. As shown in Table V, a large primary tritium isotope effect of 11/1 is seen in the EZ → D (Scheme III, rate *a*) direction while virtually no isotope effect (1.4/1) is apparent in the EZ → L (Scheme III, rate *b*) direction, which is expected from the deuterium isotope effect studies. It is also observed that release of unlabeled D-alanine from EZ is substantially easier (ca. 3-fold) than release of unlabeled L-alanine from EZ (ratio a/b). The lack of an isotope effect in T_2O in the EZ → L direction (1.4/1 for the ratio b/d) leads one to assume that the rate-determining step of the racemization reaction with unlabeled substrate in H_2O is *release* of

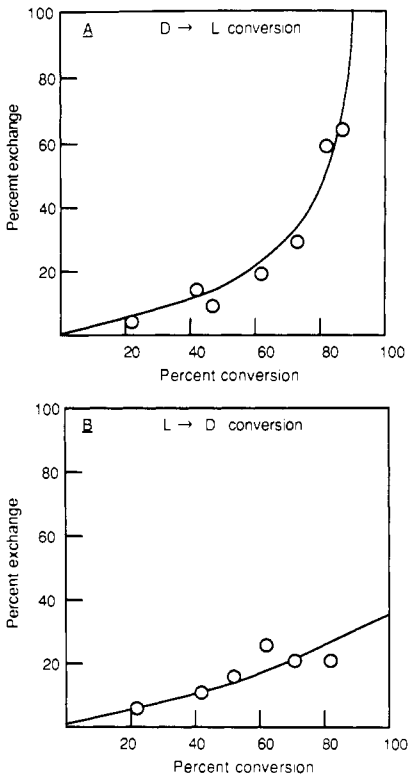


FIGURE 3: Incorporation of ^3H from tritiated water into remaining substrate D-alanine (A) or L-alanine (B) as a function of the extent of reaction for the *dadB* racemase. The isotopic content of the remaining substrate (ordinate) is plotted as a function of conversion to product (abscissa).

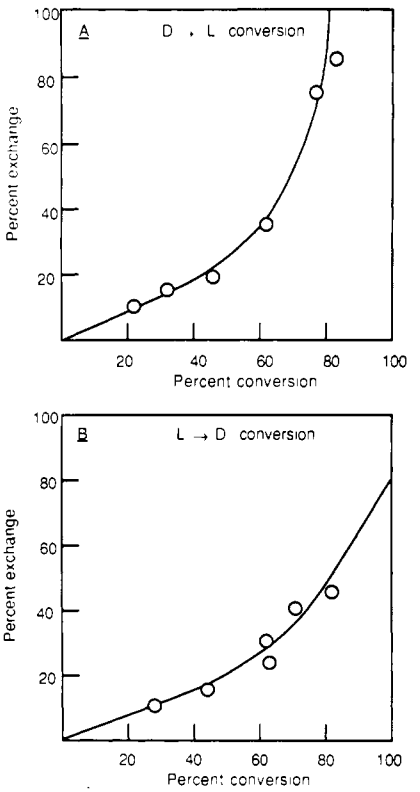


FIGURE 4: Incorporation of ^3H from tritiated water into remaining substrate D-alanine (A) or L-alanine (B) as a function of the extent of reaction for the *alr* racemase. The isotopic content of the remaining substrate (ordinate) is plotted as a function of conversion to product (abscissa).

L-alanine (**3** → L-alanine + enzyme in Scheme I), a conclusion that could not be drawn through isotope rate effect studies.

The results from exchange/conversion experiments performed with the *alr* racemase were very similar to those observed with the *dadB* enzyme. As seen in Figure 4A, monitoring substrate (D-alanine) radioactivity as a function of conversion led to a curve that rises dramatically after roughly 50% completion. This corresponds to accumulation behavior, indicative of an isotope effect. Measurement of the initial slope (as described above) of the line in Figure 4A (D → L direction) shows that carbanion intermediate **2** (Scheme I) is converted to L-alanine 2 times faster than it experiences exchange with solvent tritium to yield D-α-[^{3}H]alanine.

Experiments performed with the other enantiomer, unlabeled L-alanine, in T_2O with the *alr* racemase showed intermediate behavior and are seen in Figure 4B. Measurement of the initial slope of the line shows that the intermediate **2** (Scheme I) is converted to D-alanine 3 times faster than it experiences exchange with solvent tritium. Calculation of the ratios described above allows one to assign relative rates for Scheme III with the *alr* racemase. These values are shown in Table V. A primary tritium isotope effect of 7/1 is seen in protonation of the carbanion intermediate to give the D-alanyl-PLP aldimine (Scheme II); however, it is lower than the 11/1 isotope effect observed for the *dadB* racemase. The rate-limiting step in the racemization of alanine by the *alr* enzyme appears to be *release* of L-alanine (Scheme I, **3** → L-alanine + enzyme) as there is virtually no isotope effect (1.25/1) in the EZ → L direction.

Oversaturation. In the case of proline racemase, analysis of the racemization of L-proline under oversaturating conditions corroborated the results of Abeles (Cardinale & Abeles, 1968; Rudnick & Abeles, 1975), which demonstrate that there are two forms of the free enzyme, one that binds D-proline and one that binds L-proline. Fisher et al. (1986a,b) were able to detect the presence of the two forms very elegantly utilizing a simple experimental procedure and were also able to determine the rate of interconversion and substrate concentration at which oversaturation occurred.

As described under Experimental Procedures, an L-alanine concentration of 20–200 mM (which is above saturation) was used and the ellipticity at 220 nm followed as a function of time. A plot of the results from the *B. stearothermophilus* and *dadB* racemases following the equations of Fisher et al. (1986a) showed no oversaturation; thus, the data do not support a mechanism in which the enzyme has two distinct forms, one that binds D-alanine and the other that binds L-alanine. However, one cannot definitively rule out the two-base mechanism on the basis of these experiments, as fast interconversion of two enzyme forms [as seen by Fisher et al. (1986a,b) in proline racemase in ammonium bicarbonate buffer] may preclude observation of such distinct species.

DISCUSSION

The kinetic constants obtained for all three alanine racemases studied here show that they obey the Haldane equation, i.e., $K_{eq} = 1$, confirming the enzymes are true racemases but revealing little per se about the molecular details of the reaction. Analysis of the individual Michaelis–Menten parameters in both directions shows marked differences in binding of each isomer, with the *S. typhimurium* enzymes exhibiting the greatest difference. This suggests that the alanine racemases from *S. typhimurium* and *B. stearothermophilus* have a considerable amount of asymmetry at the active site, which is evidenced directly in catalysis. In contrast, the alanine racemase from *P. striata* (Roise et al., 1984) and mandelate racemase from *P. putida* (Kenyon & Hegeman, 1979; Sharp et al., 1977; Whitman et al., 1985), both of which are known

to operate via a one-base mechanism, show considerable similarity in the Michaelis–Menten parameters in either direction. In mandelate racemase, Whitman et al. (1985) have demonstrated a high degree of active site symmetry (perhaps surprising due to the inherent chirality of the enzyme) in which the enzyme appears to show no distinction between (*R*)- and (*S*)-mandelate in binding or catalysis.

The asymmetry for substrate enantiomer recognition and turnover is clearly seen upon analysis of the results from the isotope effect experiments with the alanine racemases from *S. typhimurium*. With the *dadB* enzyme, abstraction of the D-alanyl-PLP aldimine α-^{2}H and protonation of the carbanion intermediate **2** (Scheme I) to give the D-alanyl aldimine appear to be partially rate limiting in the racemization reaction. This suggests that the base that abstracts α-H from the D-alanyl-PLP aldimine (and, conversely, donates the proton to the carbanion intermediate **2** to give the D-alanyl-PLP aldimine) behaves differently than the base that abstracts the L-alanyl-PLP aldimine α-proton. Whether this difference is due to a dual-base mechanism in which one base is stronger than the other or a single-base mechanism in which the conformation of the base is distorted such that abstraction of the D α-proton is more difficult is not resolvable at this time. It is known that triosephosphate isomerase, an enzyme that catalyzes the isomerization of dihydroxyacetone phosphate (DHAP) to glyceraldehyde 3-phosphate (GAP), requires more energy to abstract the 1-*pro-R* proton of DHAP than the 2-proton of GAP even though the reaction proceeds via a one-base mechanism (Knowles & Albery, 1977).

This apparent asymmetry of the racemization reaction was also evident with the *alr* enzyme. As with the *dadB* enzyme, the base that abstracts α-H from the D-alanyl-PLP aldimine was much less effective than the base that abstracts α-H from the L-alanyl-PLP aldimine. However, the isotope effect was less pronounced in the *alr* enzyme and is visualized more clearly through isotope effects on V rather than V/K. This indicates subtle differences in the active site environment between the *dadB* and *alr* racemase that have been detected previously in k_{cat} and in partition ratios with β-substituted alanine suicide substrates (Esaki & Walsh, 1986).

The virtual absence of any isotope effect in the racemization of alanine by the Gram-positive *B. stearothermophilus* enzyme distinguishes this racemase from the *S. typhimurium* enzymes. It is unlikely that the rate-limiting step in racemization is simple diffusion (the rate at which substrate and enzyme form the noncovalent Michaelis complex) as values for formation of the initial enzyme–substrate complex tend to vary between 10^6 and 10^8 M^{-1} s^{-1} (Hammes & Schimmel, 1970) and the bimolecular process (k_{cat}/K_m) for the three racemases with alanine is $\sim 10^3$–10^5 M^{-1} s^{-1}. Thus, one is left with transaldimination as the most likely candidate for the kinetically significant transition state and rate-determining step.

Previous work by Shen et al. (1983) found that the nonspecific alanine racemase from *P. striata* gave some internal return (indicative of a one-base mechanism) while the specific alanine racemase from *E. coli* did not show any internal return. α-Amino-ϵ caprolactam racemase, another PLP-containing racemase, has recently been shown to exhibit some degree of internal return, consistent with a one-base mechanism (Ahmed et al., 1986). It thus appears to be a recurrent theme that isomerases and racemases that proceed by a carbanion mechanism (abstraction of the proton) operate via a one-base mechanism when the carbanion is stabilized (e.g., resonance-stabilized PLP carbanion) and by a two-base mechanism when no resonance stabilization is present (Rose, 1966; Wiseman & Nichols, 1984). Proline racemase (Cardinale & Abeles, 1968) and DAP epimerase (Wiseman & Nichols, 1984), two enzymes that contain no cofactors and proceed by a two-base mechanism, have no obvious means to stabilize the developing negative charge (on the α-carbon) and would thus create a highly destabilized intermediate. A concerted reaction in which proton abstraction is concomitant with proton donation provides a lower energy alternative for such noncofactor racemases. However, mandelate racemase (Whitman et al., 1985) and the *P. striata* alanine racemase (Shen et al., 1983) can both form resonance-stabilized carbanions upon abstraction of the α-proton, which could presumably accommodate catalysis by a mobile single base. Therefore, it is reasonable that the racemization of alanine by the three enzymes analyzed in this study may proceed via a one-base mechanism in which exchange from the conjugate BH^+ is very rapid.

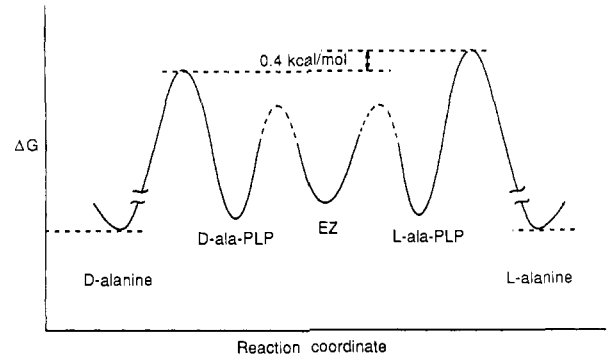


FIGURE 5: Partial reaction profile of alanine racemization by *B. stearothermophilus*. Dotted lines indicate kinetically insignificant steps. The reaction profile shows differences in transition state rather than ground state simply for clarity.

To obtain an overall reaction profile of each enzyme, thereby providing a basis in which one can compare and contrast the various thermophilic and mesophilic racemases, exchange/conversion and discrimination experiments were employed. These experiments had been used to determine the relative barrier heights (Knowles & Albery, 1977; Albery & Knowles, 1976b) with great success with triosephosphate isomerase (Maister et al., 1976; Fletcher et al., 1976) and allow one to obtain information on kinetically insignificant steps. The data presented in Table V allow one to sketch the relative barrier heights pertaining to the mechanism of racemization for each of the three enzymes.

A qualitative reaction profile for racemization of D-alanine to L-alanine with the Gram-positive *B. stearothermophilus* enzyme is seen in Figure 5. One can also construct a reaction profile for the alanine racemases from *S. typhimurium*. For the *dadB* enzyme, the results from Table V lead to the energetic diagram in Figure 6; this profile also models the reaction scheme of the *alr* racemase. The dotted lines in both profiles are estimates since the data obtained could not provide information on the relative heights of the respective barriers.

As discussed by Knowles and Albery (1977), exchange/conversion experiments allow one to analyze the reaction in more detail by introducing an isotope in the middle of the reaction and monitoring its presence in remaining substrate or in product as a function of reaction progress. We have found, as did Knowles and Albery (1977), that these experiments allow insight to steps in a reaction that were previously unobservable. With the *dadB* racemase, abstraction of the deuteron in α-deuterio-D-alanine is partially rate determining, giving an observed isotope effect of 2.9 compared with the calculated effect (from Table V, the ratio a/c) of 5.4 [deuterium isotope effects can be calculated from tritium isotope

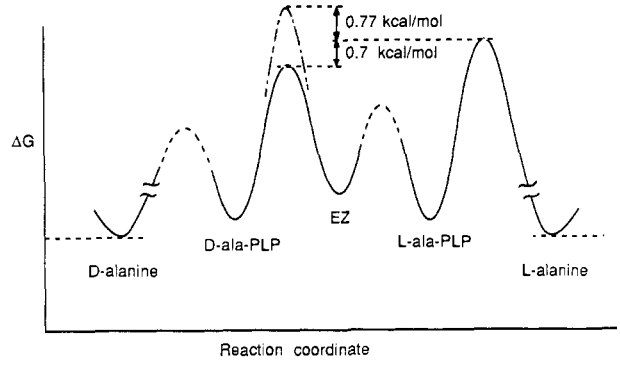


FIGURE 6: Partial reaction profile of alanine racemization by *S. typhimurium* (*dadB* racemase). Dotted lines indicate kinetically insignificant steps, while dot–dash lines indicate kinetic tritium isotope effect. The reaction profile shows differences in transition state rather than ground state simply for clarity.

effects by using the Swain equation (Swain et al., 1958) as described below]. The full extent of the intrinsic isotope effect is presumably masked by the L-alanine transaldimination step, which is rate determining under normal conditions. It is important to realize that because L-alanine and D-alanine are isoenergetic, the roughly 3-fold rate difference (in Scheme III, the ratio a/b) can be applied both going to and coming from the substrate–PLP carbanion intermediate.

It is also possible to compare the solvent deuterium isotope effect observed with steady-state experiments with those obtained through exchange vs conversion experiments. The kinetic deuterium isotope effect for L-alanine to D-alanine in D_2O was found to be 2.4 (Table I), while the tritium isotope effect (from exchange/conversion) for the same reaction (Scheme III, the ratio of b/c) was found to be 3.5. By utilization of the relationship derived by Swain et al. (1958) in which $k_H/k_T = (k_H/k_D)^{1.44}$, it is apparent that the experiments are in agreement.

Upon comparing and contrasting the three alanine racemases used in this study, one finds some similarity in the enzyme mechanism. Transaldimination is a kinetically important step and it appears that transaldimination of L-alanine is the rate-limiting step in catalysis by all three enzymes. The functional asymmetry of the racemase active sites is quite apparent upon analysis of the data and is most pronounced in the Gram-negative *S. typhimurium* racemases. Both *dadB* and *alr* transaldiminate D-alanine very readily, and abstraction of the α-proton is much more difficult, while transaldimination of L-alanine is more difficult than abstraction of the L α-proton. This suggests that binding of D-alanine is energetically different than the binding of L-alanine due to specific interactions between the chiral enzyme and the substrate.

This apparent active site asymmetry is also evident with the β-substituted alanine inhibitors (Wang & Walsh, 1978, 1981). Badet et al. (1984) have found that inactivation of the *dadB* alanine racemase by fluoroalanine, chloroalanine, and *O*-acetyl-D-serine shows some degree of asymmetry in the kinetics of inactivation between the different enantiomers. These authors have concluded that binding of the L isomers is strongly dependent on the β-substituent size while binding of the D isomers is less dependent, suggestive of a "functional asymmetry" at the enzyme active site. It is perhaps the unfavorable interactions of the L-alanine methyl group that lead to poorer binding of L-alanine (i.e., 5-fold higher K_m) and a low rate for transaldimination, as orbital alignment necessary for nucleophilic attack may not be optimal.

The similarity between the *dadB* and *alr* enzymes is not surprising when one considers the extent of identity (43%) between the two enzymes (Galakatos & Walsh, 1987) and the fact that they are produced by the same *S. typhimurium* strain [although both genes map to widely separate loci on the chromosome and appear to be differentially regulated (E. Daub, C. T. Walsh, and D. Botstein, unpublished results)]. The question arises as to why these two enzymes would differ so widely in their Michaelis–Menten parameters and partition ratio upon β-haloalanine inactivation (Badet et al., 1984; Esaki & Walsh, 1986). Steady-state values show that binding (K_m) of alanine by the *alr* racemase is ~5–7-fold stronger than *dadB* while racemization (k_{cat}) is ~50–70-fold less. This shows that subtle differences present at the active site have little bearing on the overall reaction mechanism but do affect the individual rate constants. This difference is also apparent on analysis of the partition ratio between inactivation and turnover of β-haloalanine by both *S. typhimurium* enzymes. Badet et al. (1984) demonstrated that the *dadB* racemase will turn over chloroalanine 800 times (to give pyruvate and ammonium ion) before inactivation while *alr* is found (Esaki & Walsh, 1986) to turn over chloroalanine only 160 times before inactivation.

On comparing Figures 5 and 6, one can see the difference in the mechanism of racemization between the Gram-positive *B. stearothermophilus* and Gram-negative *S. typhimurium* enzymes. The *B. stearothermophilus* profile shows much greater symmetry, with transaldimination of both D-alanine and L-alanine being rate limiting. Although it appears that transaldimination of D-alanine is slightly easier for both Gram-positive and Gram-negative racemases, the large differences between transaldimination and proton abstraction for both isomers in the *S. typhimurium* racemases are not seen in the *B. stearothermophilus* enzyme. The differences in the active site environment between the *B. stearothermophilus* and *S. typhimurium* enzymes are at present best seen through inhibitor studies. Badet et al. (1986) have shown that (1-aminoethyl)phosphonate (Ala-P) is a tight-binding inhibitor of *B. stearothermophilus* [as well as the alanine racemase from another Gram-positive bacterium, *Streptococcus faecalis* (Badet & Walsh, 1985)] in which the half-life for reactivation is 25 days. However, Ala-P is found to be only a competitive inhibitor of the *S. typhimurium* racemases, with no formation of a long-lived, slowly dissociating enzyme complex.

It is apparent that the three alanine racemases presented in this study show some similarity in their overall reaction profile but differ widely in their kinetic parameters as well as their inactivation mechanism with various inhibitors. This suggests that although a common reaction mechanism may be operant in all three enzymes, relatively minor changes at the active site can lead to drastic differences in kinetic parameters and inactivation mechanism. The asymmetry in the reaction profile is very interesting and suggests that D- and L-alanine are bound differently in the active site. Adams (1976) also noted the unusual asymmetry with the alanine racemase from *P. putida* in both Michaelis–Menten parameters and tritium exchange reactions. Thus, the mechanism of racemization proposed may extend to other PLP-containing racemases as well. Presently, work is in progress to elucidate the crystal structure of the *B. stearothermophilus* racemase (Neidhart et al., 1987) that should lead to a greater structure/function understanding of the mechanism of racemization by this enzyme.

ACKNOWLEDGMENTS

We thank G. Verdine for critical review of the manuscript.

Registry No. ^3H_2O, 14940-65-9; alanine racemase, 9024-06-0; D-alanine, 338-69-2; L-alanine, 56-41-7; DL-[^{3}H]alanine, 31024-95-0;

acetic anhydride, 108-24-7; *N*-acetyl-DL-[^{3}H]alanine, 113474-84-3; acylase, 9012-37-7; L-[^{3}H]alanine, 26434-63-9; D-[^{3}H]alanine, 113532-27-7.

References

Adams, E. (1976) *Adv. Enzymol. Relat. Areas Mol. Biol. 44*, 69–138.

Ahmed, S. A., Esaki, N., Tanaka, H., & Soda, K. (1986) *Biochemistry 25*, 385–388.

Albery, W. J., & Knowles, J. R. (1976a) *Biochemistry 15*, 5588–5600.

Albery, W. J., & Knowles, J. R. (1976b) *Biochemistry 15*, 5627–5631.

Badet, B., & Walsh, C. T. (1985) *Biochemistry 24*, 1333–1341.

Badet, B., Roise, D., & Walsh, C. T. (1984) *Biochemistry 23*, 5188–5194.

Badet, B., Inagaki, K., Soda, D., & Walsh, C. T. (1986) *Biochemistry 25*, 3275–3282.

Briggs, G. E., & Haldane, J. B. S. (1925) *Biochem. J. 19*, 338–339.

Cardinale, G. J., & Abeles, R. H. (1968) *Biochemistry 7*, 3970–3978.

Cleland, W. W. (1977) in *Isotope Effects on Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrup, D. B., Eds.) pp 153–175, 252–279, University Park Press, Baltimore, MD.

Esaki, N., & Walsh, C. T. (1986) *Biochemistry 25*, 3261–3267.

Everse, J. (1975) *Methods Enzymol. 41B*, 41–44.

Fisher, L. M., Albery, W. J., & Knowles, J. R. (1986a) *Biochemistry 25*, 2529–2537.

Fisher, L. M., Belasco, J. G., Bruice, T. W., Albery, W. J., & Knowles, J. R. (1986b) in *Mechanisms of Enzymatic Reactions: Stereochemistry* (Frey, P. A., Ed.) pp 205–216, Elsevier Science, New York.

Fletcher, S. J., Herlihy, J. M., Albery, W. J., & Knowles, J. R. (1976) *Biochemistry 15*, 5612–5617.

Galakatos, N. G., & Walsh, C. T. (1987) *Biochemistry 26*, 8475–8480.

Galakatos, N. G., Daub, E., Botstein, D., & Walsh, C. T. (1986) *Biochemistry 25*, 3255–3260.

Glasoe, P. K., & Long, G. A. (1960) *J. Phys. Chem. 64*, 188–191.

Greenstein, J. P., & Winitz, M. (1961) *Chemistry of the Amino Acids*, Vol. 3, Wiley, New York.

Hammes, G. G., & Schimmel, P. R. (1970) *Enzymes, 3rd Ed. 2*, 67–114.

Inagaki, K., Tanizawa, K., Badet, B., Walsh, C. T., Tanaka, H., & Soda, K. (1986) *Biochemistry 25*, 3268–3274.

Kallen, R. G., Korpela, T., Martell, A. E., Matsushima, Y., Metzler, C. M., Metzler, D. E., Morozov, Y. V., Ralston, I. M., Savin, F. A., Torchinsky, Y. M., & Ueno, H. (1984) in *Transaminases* (Christen, P., & Metzler, D. E., Eds.) pp 37–108, Wiley, New York.

Kenyon, G. L., & Hegeman, G. D. (1979) *Adv. Enzymol. Relat. Areas Mol. Biol. 50*, 325–360.

Knowles, J. R., & Albery, W. J. (1977) *Acc. Chem. Res. 10*, 105–111.

Maister, S. G., Pett, C. P., Albery, W. J., & Knowles, J. R. (1976) *Biochemistry 15*, 5607–5612.

Neidhart, D. J., Distephano, M. D., Tanizawa, K., Soda, K., Walsh, C. T., & Petsko, G. A. (1987) *J. Biol. Chem. 262*, 15323–15326.

Roise, D., Soda, K., Yagi, T., & Walsh, C. T. (1984) *Biochemistry 23*, 5195–5201.

Rose, I. A. (1966) *Annu. Rev. Biochem. 35*, 23–56.

Rudnick, G., & Abeles, R. H. (1975) *Biochemistry 14*, 4515–4522.

Sharp, T. R., Hegeman, G. D., & Kenyon, G. L. (1977) *Biochemistry 16*, 1123–1128.

Shen, S. J., Floss, H. G., Kumagai, H., Yamaka, H., Esaki, N., Soda, K., Wasserman, S. A., & Walsh, C. (1983) *J. Chem. Soc., Chem. Commun.*, 82–83.

Soda, K., Tanaka, H., & Tanizawa, K. (1986) in *Vitamin B_6 Pyridoxal Phosphate* (Dolphin, D., Poulson, R., & Avramovic, O., Eds.) Part B, pp 223–251, Wiley, New York.

Swain, C. G., Stevers, E. C., Reuwer, J. F., & Schaad, L. J. (1958) *J. Am. Chem. Soc. 80*, 5885–5892.

Wang, E., & Walsh, C. T. (1978) *Biochemistry 17*, 1313–1321.

Wang, E., & Walsh, C. T. (1981) *Biochemistry 20*, 7539–7546.

Wasserman, S. A., Walsh, C. T., & Botstein, D. (1983) *J. Bacteriol. 153*, 1439–1450.

Wasserman, S. A., Daub, E., Grasafi, P., Botstein, D., & Walsh, C. T. (1984) *Biochemistry 23*, 5182–5187.

Whitman, C. P., Hegeman, G. D., Cleland, W. W., & Kenyon, G. L. (1985) *Biochemistry 24*, 3936–3942.

Wilkinson, G. N. (1961) *Biochem. J. 80*, 324–332.

Wiseman, J. S., & Nichols, J. S. (1984) *J. Biol. Chem. 259*, 8907–8914.