

Multiple Hydrogen Kinetic Isotope Effects for Enzymes Catalyzing Exchange with Solvent: Application to Alanine Racemase

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ABSTRACT: Alanine racemase catalyzes the pyridoxal phosphate-dependent interconversion of the D- and L-isomers of alanine. Previous studies have shown that the enzyme employs a two-base mechanism in which Lys39 and Tyr265 are the acid/base catalysts. It is thus possible that stereoisomerization of the external aldimine intermediates occurs through a concerted double proton transfer without the existence of a distinct carbanionic intermediate. This possibility was tested by the application of multiple kinetic isotope effect (KIE) methodology to alanine racemase. The mutual dependence of primary substrate and solvent deuterium KIEs has been measured using equilibrium perturbation-type experiments. The conceptually straightforward measurement of the substrate KIE in H₂O is complemented with a less intuitive protium washout perturbation-type measurement in D₂O. The primary substrate KIE in the D → L direction at 25 °C is reduced from 1.297 in H₂O to 1.176 in D₂O, while in the L → D direction it is reduced from 1.877 in H₂O to 1.824 in D₂O. Similar reductions are also observed at 65 °C, the temperature to which the *Bacillus stearothermophilus* enzyme is adapted. These data strongly support a stepwise racemization of stereoisomeric aldimine intermediates in which a substrate-based carbanion is an obligatory intermediate. The ionizations observed in $k_{\text{cat}}/K_{\text{M}}$ pH profiles have been definitively assigned based on the ΔH_{ion} values of the observed $\text{p}K_{\text{a}}$'s with alanine and on the pH dependence of $k_{\text{cat}}/K_{\text{M}}$ for the alternative substrate serine. The acidic $\text{p}K_{\text{a}}$ in the bell-shaped curve is due to the phenolic hydroxyl of Tyr265, which must be unprotonated for reaction with either isomer of alanine. The basic $\text{p}K_{\text{a}}$ is due to the substrate amino group, which must be protonated to react with Tyr265-unprotonated enzyme. A detailed reaction mechanism incorporating these results is proposed.

Bacterial cell walls are rigid structures consisting of polysaccharides cross-linked with peptides (1). They provide protection from osmotic lysis, and thus their biosynthetic pathways are attractive targets for antibiotic development. The peptide component of peptidoglycan contains D-amino acids such as D-alanine and D-glutamate, which are thought to confer resistance to protease digestion (2). D-Alanine is essential in that it plays an important role both in synthesis of the primary pentapeptide and in interstrand cross-linking (1, 2).

There are three potential enzymatic antibacterial targets for blocking the incorporation of D-alanine into peptidoglycans: alanine racemase, D-Ala-D-Ala ligase, and ATP-dependent D-Ala-D-Ala adding enzyme (1, 2). Alanine racemase is a pyridoxal phosphate (PLP)¹-dependent enzyme that catalyzes the first step in the D-alanine branch of peptidoglycan biosynthesis, the reversible isomerization of L- to D-alanine (3). Inhibitors of alanine racemase (4–8) have been pursued as potential antibacterial agents, although apparently no designed inhibitors have yet found clinical success. On the other hand, cycloserine, a natural product, is a good inhibitor of alanine racemase and other PLP-dependent en-

zymes (9–13), and has found wide clinical use in some applications (14–18).

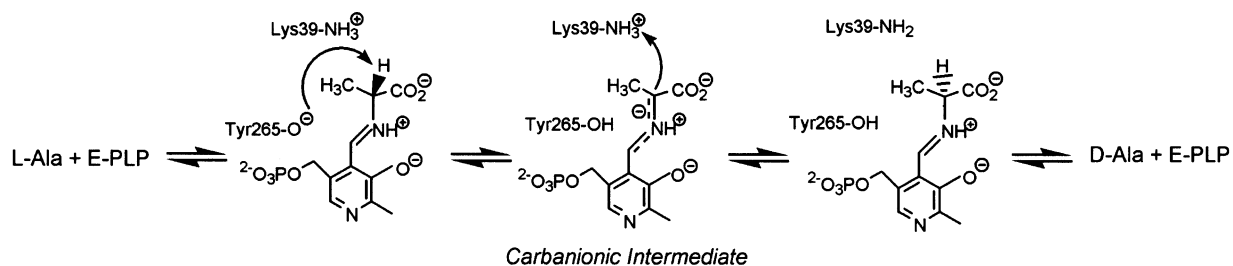
As with all PLP-dependent enzymes, a covalent PLP-amino acid aldimine intermediate is formed in the alanine racemase mechanism, increasing the acidity of the C α proton. Previous work has established that racemization in the *Bacillus stearothermophilus* enzyme takes place via a two-base mechanism, in which Tyr265 and Lys39 act as general acid/base catalysts (19–21). Tyr265 abstracts the C α proton from the aldimine formed from L-alanine (Scheme 1). Reprotonation from the opposite face, with a solvent derived proton from Lys39, yields the antipodal aldimine. In the D → L direction, Lys39 abstracts the substrate proton, while Tyr265 provides the solvent derived proton.

Racemization may take place through a distinct, delocalized PLP-amino acid carbanionic intermediate. Alternatively, the racemization may take place in a single, concerted double proton transfer, avoiding formation of a carbanionic intermediate and the development of negative charge at C α . Several observations are consistent with a concerted reaction mechanism. Many PLP-containing enzymes enhance the electron-sink capabilities of PLP by juxtaposing a highly conserved acidic residue adjacent to the pyridine nitrogen, presumably maintaining it protonated. Uniquely in alanine racemase, an arginine side chain (Arg219) hydrogen bonds to the pyridine nitrogen (22). This interaction effectively

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¹ Abbreviations: PLP, pyridoxal 5'-phosphate; KIE, kinetic isotope effect; CD, circular dichroism; SIE, solvent isotope effect.

Scheme 1: Outline of the Alanine Racemase Mechanism Including a Carbanionic Intermediate.



keeps the pyridine nitrogen from being formally protonated since the pK_a difference between these groups is ~ 8 units. The lack of pyridine nitrogen protonation is expected to destabilize the carbanionic intermediate. Further support for the concerted mechanism is provided by the inability to detect spectroscopically the formation of a carbanionic quinonoid intermediate in the wild-type enzyme (19).

Multiple kinetic isotope effects (KIEs) have been widely employed to distinguish between concerted and stepwise mechanisms (23–25). Two independent KIEs must be observed prior to the application of this methodology, and the mutual dependence of these KIEs provides the diagnostic. In the case of alanine racemase, primary deuterium substrate KIEs and D_2O solvent KIEs were previously measured and found to be significantly greater than unity, yet not large (~ 1.5) (19). Thus, the multiple KIE diagnosis of mechanism is applicable to alanine racemase if hydrogen KIEs of high enough precision can be measured.

The equilibrium perturbation method, introduced by Cleland and co-workers for reversible enzyme catalyzed reactions (26), allows the measurement of KIEs with high precision (e.g., $\leq 1\%$ error), and thus is attractive for KIE measurements on alanine racemase. Several excellent reviews of the technique are available (27, 28). The technique has been used to determine primary KIEs with errors as low as 0.38% (29). The equilibrium perturbation technique is particularly amenable to freely reversible systems, especially those with low ΔH_0 values (27). Having an equilibrium constant of unity, and a ΔH_0 value of zero, alanine racemase is particularly well suited to the equilibrium perturbation method. To obtain multiple hydrogen KIEs for alanine racemase, the current study employs “competitive hydron washout” experiments (29, 30), which are a type of equilibrium perturbation experiment, in both H_2O and D_2O .

The data support a stepwise mechanism for alanine racemase at both 25 and 65 °C (the optimal growth temperature of *B. stearothermophilus*). Thus, the carbanionic intermediate is obligatory, and Arg219 may serve to destabilize it to avoid side reactions such as transamination (31). On the basis of the conclusions from the multiple KIE experiments and several k_{cat}/K_M pH profiles, a detailed reaction mechanism for alanine racemase is proposed that includes enzyme and substrate protonation states.

EXPERIMENTAL SECTION

Materials. Deuterated D-alanine was purchased from CDN Isotopes (Quebec, Canada), catalog number D-1274, 98 atom %. Deuterated L-alanine was purchased from Isotec, Inc. (USA), minimum 98 atom %. Protiated D- and L-alanine were purchased from Aldrich Chemical Company (99% pure),

respectively. Deuterium oxide (99.9 atom %) was purchased from Cambridge Isotope Laboratories Inc. The alanine racemase used in this study was prepared as described previously (19). The buffers used were from Fisher.

Amino Acid pK_a Values. The pK_a values for alanine and serine at an ionic strength of 0.125 (equal to that in the pH profile determinations) were determined. Solutions of 10 mL of 10 mM amino acid in 125 mM KCl were titrated with 1 M KOH. The pK_a values were obtained by nonlinear regression fitting to the Henderson-Hasselbach equation. The pH meter was calibrated between pH 4 and 10.

Instrument. A Jasco 720 circular dichroism spectrometer was used for all studies. The wavelengths used in the various studies were chosen such that CD is linear with amino acid concentration, which was found to correspond to a “high tension voltage” (HTV) value below 400 V. Data acquisition was carried out using the Jasco program J-700 under the Windows Hardware Manager, version 1.10.00.

k_{cat}/K_M pL Profiles. Buffers that are optically clear at 210 nm were required. Therefore, depending on the pL, the buffers were either 50 mM potassium borate, 100 mM potassium chloride (between pL 8.0 and 10.9) or 50 mM potassium phosphate, and 100 mM potassium chloride (pL less than 8.0, and greater than 10.9). The pH of the reaction mixtures was measured before and after data collection; differences were generally less than 0.02 units, and not greater than 0.05. The pH value taken after the run was used in the profiles. The $D \rightarrow L$ direction was followed for alanine. As outlined below, this gives k_{cat}/K_M pL profiles that are equivalent to those for the $L \rightarrow D$ direction since the equilibrium constant for the overall reaction is one. The Michaelis–Menten equation reduces to a simple first-order kinetics equation when the concentration of substrate is much less than the value of K_M . This is shown in eq 1. Knowing

$$v_i = \frac{k_{cat}}{K_M} [E]_i [S] = k'_1 [S] \quad (1)$$

the enzyme concentration, this allows the determination of k_{cat}/K_M from progress curves that take the form of single exponentials. The case of a reversible reaction is more complicated since the back reaction must be considered in the approach to equilibrium. The rate equation for a reversible single substrate, single product enzyme catalyzed reaction is given in eq 2 (32). When the substrate and product

$$v_{net} = \frac{V_f \frac{[S]}{K_S} - V_r \frac{[P]}{K_P}}{1 + \frac{[S]}{K_S} + \frac{[P]}{K_P}} \quad (2)$$

concentrations are kept much lower than the values of K_S and K_P , respectively, then eq 2 reduces to eq 3.

$$v_{\text{net}} = \frac{V_f}{K_S}[S] - \frac{V_r}{K_P}[P] \quad (3)$$

This has the same form as a differential rate equation for a reversible first-order mechanism, given the units of V/K are s^{-1} . This can then be integrated to give eq 4 using standard techniques (33). Here, x is the extent of reaction

$$\frac{x_e}{x_e - x} = e^{-\left(\frac{V_f}{K_S} + \frac{V_r}{K_P}\right)t} \quad (4)$$

and x_e is the extent of reaction at equilibrium. This shows that the observed rate constant is equal to the sum of k_{cat}/K_M for the forward and reverse directions multiplied by the enzyme concentration.

Stereoisomerization has an equilibrium constant of unity that is independent of pH. Thus, the values of k_{cat}/K_M in both directions are equal, and this equality is pH independent, i.e., the pH dependence of k_{cat}/K_M is identical for both directions. What one observes here is the pH dependence of either of these, with the observed values of the rate constants being twice that of a single direction.

The observed values of k_{cat}/K_M (i.e., sum of the forward and reverse values) were determined from first-order fits to progress curves for racemization of 0.15 mM D-alanine. This is at all pH values at least 10-fold less than the value of either K_M . The k_{cat}/K_M vs pH data were fitted to eq 5, which describes a bell-shaped curve that approaches zero at the pH extremes. For the 25 °C data, pD was calculated by adding

$$\log(k_{\text{cat}}/K_M) = \log\left[\frac{(k_{\text{cat}}/K_M)_{\text{lim}}}{1 + 10^{(pK_a^1 - pL)} + 10^{(pL - pK_a^2)}}\right] \quad (5)$$

0.40 to the pH meter reading (34). For the 65 °C data, the observed pH meter reading at 65 °C was directly plotted for both H₂O and D₂O solutions, since no accepted H₂O/D₂O difference was found for the pH electrode at the higher temperature. The values of pH used for 65 °C were those measured at 25 °C, given the negligible heats of ionization of the buffers used (35, 36). The observed values of apparent pH measured at 65 °C using a meter calibrated at 25 °C agreed to within 0.1 pH units or, more generally, less with those measured at 25 °C. The enzyme concentrations were 9.2 and 4.6 nM for 25 and 65 °C, respectively. It was necessary to have 15 μM PLP in the 65 °C reaction mixture, or the enzyme is fully inactivated in approximately 10 min. The D → L reaction was also followed at 65 °C.

The values of k_{cat}/K_M for the pH profile for serine at 25 °C were also obtained from exponential progress curves. The reaction of L → D serine was followed. The initial concentration of L-serine was 0.15 mM, which is much lower than the published value of 110 mM at pH 9 (37). The progress curves fit well to a first-order rate equation in all cases.

Equilibrium Perturbation-Type Washout Experiments. The wavelength of the CD instrument was set at 215 nm for these experiments. Mixtures containing 10 mM each of D- and L-alanine, in a buffer composed of 50 mM potassium borate and 100 mM potassium chloride, were made and were checked to ensure that the optical activity was zero, as

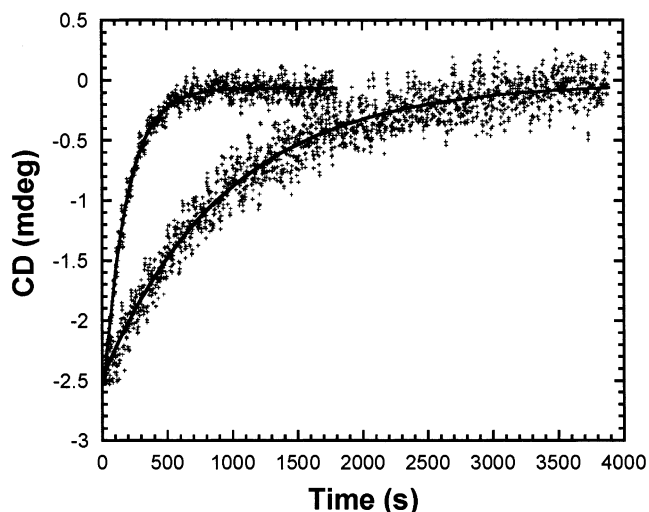


FIGURE 1: Progress curves for racemization of 0.15 mM D-Ala at 25 °C, monitored by CD spectroscopy, at pH 8.0 (upper trace) and 6.6 (lower trace). The data were fitted to a single-exponential equation to give the lines shown. The observed rate constant is equal to twice the value of k_{cat}/K_M for either direction.

expected for a racemic mixture. The enzyme was added to begin the reaction.

For 25 °C data sets, the enzyme concentration was 54 nM. The H₂O perturbations were performed at pH 8.48, while the D₂O perturbations were performed at pD 8.90. On the basis of the pK_a values observed in the pH profiles, this gives enzyme in equivalent protonation states, which is necessary to ensure identical commitment factors. For the 65 °C data set, the enzyme concentration was 22 nM and the reaction mixture contained 15 μM PLP. The H₂O perturbations were performed at a pH of 8.0, while the D₂O perturbations were performed at a pH meter reading of 8.4, which gives enzyme in equivalent protonation states.

The kinetic isotope effects were calculated according to Bahnsen and Anderson (29). These authors give a full treatment of equilibrium perturbation-type washout kinetics and provide the equations necessary for extracting correct KIEs from experiments in which a fractionating isotope is irreversibly washed out into solvent during the course of reaction.

RESULTS

Amino Acid pK_a Values. The pK_a values for the α -amino groups of alanine and serine were measured under the present experimental conditions so that a direct comparison to the observed pK_a values from pH profiles could be made. The measured values are alanine, 9.94 ± 0.02 ; serine, 9.26 ± 0.01 .

Progress Curve Determination of k_{cat}/K_M . Progress curves for alanine racemization are shown in Figure 1. The good fit to a single-exponential equation is evident. Equation 4 predicts that the observed rate constant is equal to twice that of k_{cat}/K_M for either direction. This was checked for pH 8.9 data. The observed exponential rate constant divided by enzyme concentration is equal to 2.4 ± 0.3 times the value of k_{cat}/K_M for the L → D direction obtained in initial rate experiments.

pL Profiles. Figure 2A shows the pH and pD vs $\log(k_{\text{cat}}/K_M)$ profiles for D-alanine racemization, at 25 °C. Both D₂O and H₂O data sets exhibit a bell shaped curve, which is

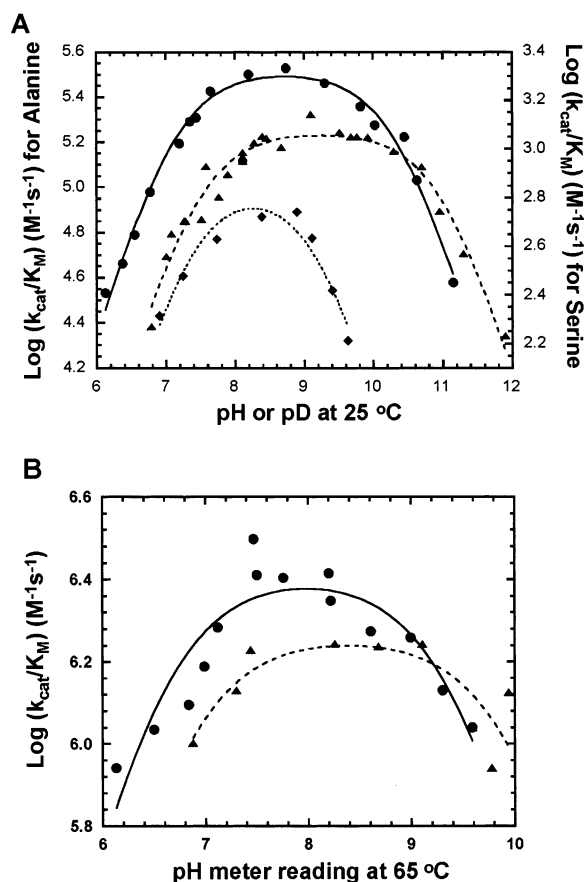


FIGURE 2: $\text{Log}(k_{\text{cat}}/K_M)$ for racemization of D- to L-alanine as a function of pL (pH or pD) of solution. (A) 25 °C. Circles, H₂O; triangles, D₂O; diamonds, serine in H₂O. (B) 65 °C. Circles, H₂O; triangles, D₂O. The pK_a values obtained from the fits are given in Table 1.

Table 1: Kinetic Parameters Obtained from k_{cat}/K_M pH Profiles^a

pH profile	pK_{a1}	pK_{a2}	limiting value of k_{cat}/K_M
alanine in H ₂ O (25 °C)	7.14 (0.04)	10.31 (0.05)	$3.24 (0.16) \times 10^5$
alanine in D ₂ O (25 °C)	7.48 (0.05)	10.99 (0.06)	$1.75 (0.07) \times 10^5$
alanine in H ₂ O (65 °C)	6.56 (0.11)	9.41 (0.13)	$2.56 (0.21) \times 10^6$
serine in H ₂ O (25 °C)	7.35 (0.13)	9.19 (0.13)	700 (100)

^a Reaction conditions: 50 mM buffer, 100 mM KCl, temperatures as indicated. Standard errors from nonlinear regression are given in parentheses.

well described by eq 5. The pK_a and limiting k_{cat}/K_M values obtained from the nonlinear regression fits to eq 5 are given in Table 1. Due to an equilibrium isotope effect on proton dissociation, the exchange of H₂O for D₂O produces an upward shift in the observed pK_a values. The observed ΔpK_a values at 25 °C are 0.34 and 0.68 for the acidic and basic ionizations, respectively. These values are in the characteristic range for weak organic acids, which have ΔpK_a values of $0.5 + 0.2$ (38).

The k_{cat}/K_M pH profile for serine is also shown in Figure 2A. Serine is 460-fold worse as a substrate than is alanine based on the limiting values of k_{cat}/K_M obtained from the pH profiles (Table 1). The acidic pK_a value for the serine profile is, within error, very close to that for the alanine profile, while the basic pK_a value is reduced by ~ 1 unit. This suggests that the basic ionization is due to the substrate α -amino group, since serine has a 0.7 unit lower pK_a value.

The pL profiles for alanine at 65 °C are shown in Figure 2B. Similar bell-shaped curves are observed and the pK_a values for H₂O are reported in Table 1. The observed pK_a values for the D₂O profile at 65 °C are 6.8 ± 0.2 and 10.0 ± 0.2 . These values are not comparable to the other pK_a values since no pH meter correction was available for 65 °C and uncorrected meter readings were used. These will remain uninterpreted due to this uncertainty. They are given here for completeness. Nonetheless, the profile fully serves the purpose of determining the apparent pH value required to obtain enzyme in equivalent protonation states in H₂O and D₂O.

On the basis of the H₂O data presented in Figure 2A and B, the ΔH values for the two ionizations can be calculated. These are 6.5 ± 0.4 and 10.5 ± 0.7 kcal/mol for the acidic and basic ionizations, respectively. The former compares favorably to a ΔH_{ion} of 6 kcal/mol for tyrosine (36) and 5.6 kcal/mol for phenol (39) suggesting that the first ionization might be ascribed to Tyr265. The latter compares favorably to the ΔH_{ion} of 10.9 kcal/mol for alanine (36) and 10–13 kcal/mol for amino groups in general (39).

Multiple KIE Determinations. The excellent fits of bell shaped curves (eq 5) to the k_{cat}/K_M pL profiles indicate that two ionizable groups control the rate of the enzymatic reaction. The deuterated and protiated forms of the enzyme/substrate pair must be in the same ionic state to obtain accurate isotope effects, due to the influence of enzyme and substrate ionizations on commitment factors (34). The pK_a values in Table 1 allow one to calculate the pL values at which the enzyme is in equivalent protonation states in H₂O and D₂O. These were calculated to be 8.48 and 8.90 for H₂O and D₂O, respectively, at 25 °C.

Precise kinetic isotope effects in reversible enzyme catalyzed reactions can be measured using the equilibrium perturbation technique (26). In this method, the reactant and product concentrations are set at their equilibrium values, with one of the species containing a heavy atom substitution. The system is thus at “chemical” but not “isotopic” equilibrium. The difference in reaction rate between the light and heavy isotopes produces a transient perturbation away from the equilibrium concentrations, from which the KIE on k_{cat}/K_M can be calculated (26).

Alanine racemase presents a more complicated kinetic system. Here, one KIE is engendered by solvent deuteration and the other by alanine deuteration at C α . The latter site on both isomers is eventually fully equilibrated with the large pool of solvent isotope since: (i) the antipodal aldimine is formed through donation of a solvent derived hydron from one acid/base catalyst in a two-base mechanism (19), and (ii) washout of the original hydron from the second acid/base catalyst into solvent is relatively rapid (40). This is illustrated in the upper panel of Figure 3 for the H₂O case.

This incorporation of solvent derived isotope into the reactant and product pools creates multiple kinetic manifolds (Figure 3). A perturbation in this case can be obtained from rate differences between the two kinetic manifolds, which is the origin of the racemization “overshoot” phenomenon originally observed with proline racemase (41). The system eventually relaxes to a single manifold of solvent hydron composition. Thus, the reactant with isotopic composition initially different than that of solvent is the fractionating species. This would be the deuterated D isomer in H₂O as

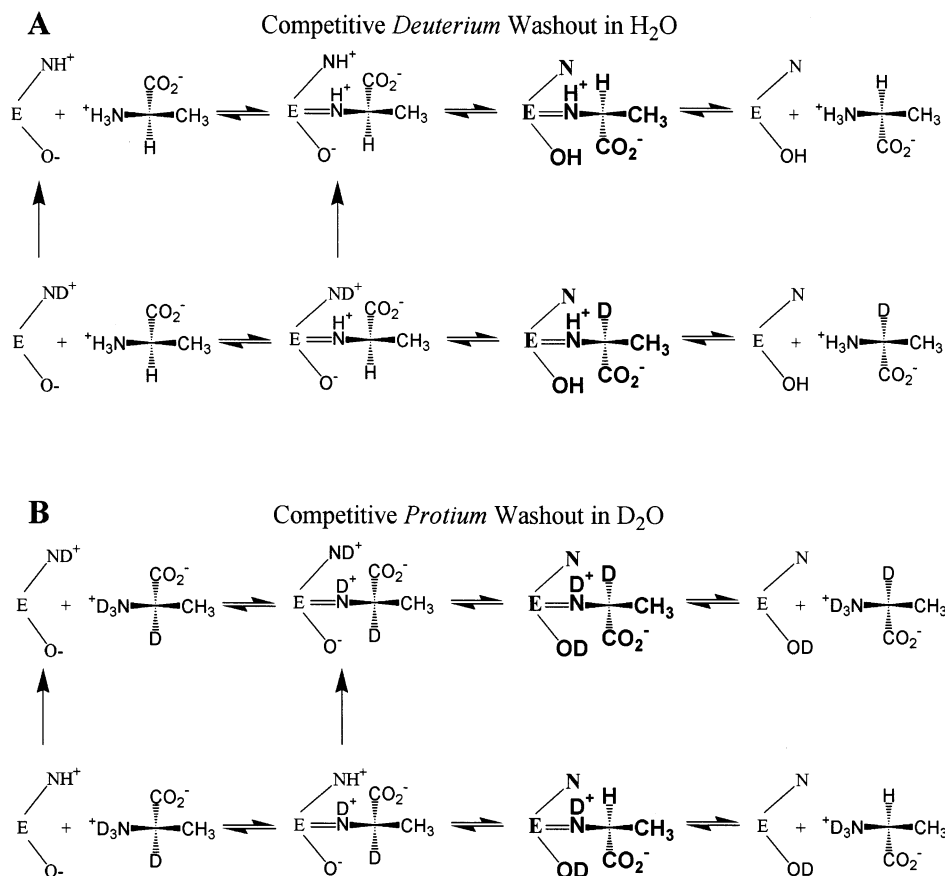


FIGURE 3: Schematic representations of D-alanine equilibrium perturbation-type washout experiments. (A) Washout of deuterated D-alanine in H₂O from equal starting concentrations of [²H]-D-alanine and [¹H]-L-alanine. At initiation of the perturbation, only the [²H]-D-alanine (lower manifold) and [¹H]-L-alanine enzyme complexes (upper manifold) are present. The D isomer is transiently accumulated, due to slower racemization caused by the heavier isotope. The deuterium in [²H]-D-alanine is washed out into the solvent pool during the course of racemization. At equilibrium, only the upper manifold exists, in which forward and reverse racemization rates are equal. The primary substrate KIE is due to the difference in rates of racemization of the [²H]- and [¹H]-D-alanine enzyme complexes, shown in bold. (B) Washout of [¹H]-D-alanine in D₂O from equal starting concentrations of [¹H]-D-alanine and [²H]-L-alanine. The multiple kinetic isotope effect is obtained by comparing the magnitudes of the KIEs from the H₂O and D₂O perturbations.

drawn in the upper panel of Figure 3. This requires that the D isomer in D₂O (lower panel of Figure 3) be *protiated* for it to fractionate.

In the H₂O case, the initially deuterated (fractionating) isomer reacts slower than the protiated isomer, giving a transient accumulation of the fractionating isomer. In the D₂O case, the initially protiated (fractionating) isomer reacts faster than the deuterated isomer, giving a transient accumulation of nonfractionating isomer. *The perturbations for a given fractionating isomer in H₂O vs D₂O will thus be of opposite directions.* This prediction is borne out by the experimental data presented in Figure 4.

Table 2 presents multiple KIEs obtained for both D- and L-isomers. In all cases, the primary substrate KIE is decreased by solvent deuteration. This is diagnostic for a stepwise mechanism (25). Table 3 compares the observed SIEs, obtained under the same conditions as the perturbations, to the SIEs calculated from the reduction in the substrate derived KIEs using eq 6 (25). Here, ${}^{\text{H}_2\text{O}}(V/K)_D$ is the substrate

$$\text{SIE} = \frac{[{}^{\text{H}_2\text{O}}(V/K)_D - 1]}{[{}^{\text{D}_2\text{O}}(V/K)_D - 1]} \quad (6)$$

derived KIE in H₂O, while ${}^{\text{D}_2\text{O}}(V/K)_D$ is the substrate derived

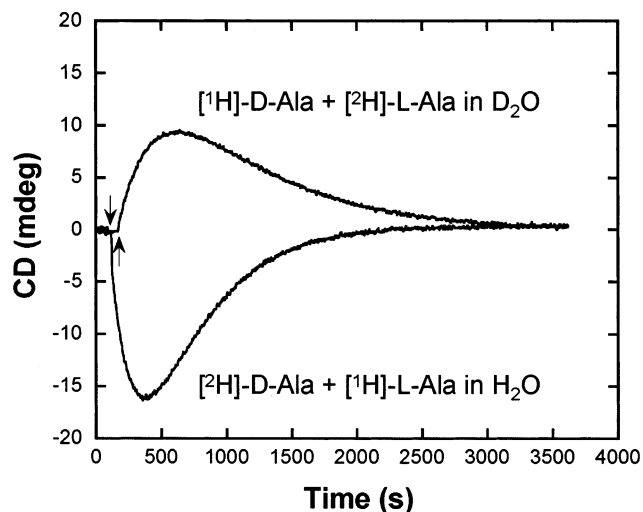


FIGURE 4: Equilibrium perturbation-type washout experiments. The isotopic compositions of the reactants are indicated. At 25 °C, H₂O solutions were pH 8.48 and D₂O solutions were pD 8.90, which gives enzyme in the same ionic state.

KIE in D₂O. The observed and calculated SIEs are in excellent agreement. The larger SIE in the D → L direction elicits a larger reduction in the substrate derived KIE than that observed in the L → D direction.

Table 2: Multiple Kinetic Isotope Effects from Equilibrium Perturbation-Type Experiments^a

fractionating isomer	25 °C ^b		65 °C ^c	
	H ₂ O	D ₂ O	H ₂ O	D ₂ O
D-alanine	1.297 (0.012)	1.176 (0.004)	1.355 (0.005)	1.265 (0.002)
L-alanine	1.877 (0.007)	1.824 (0.006)	1.491 (0.006)	1.486 (0.009)

^a Perturbations were treated as in Bahnson and Anderson (29) in which hydron washout is considered irreversible. The KIEs represent averages of triplicate determinations. Standard deviations for replicate measurements are given in parentheses. ^b H₂O solutions were at pH 8.48 and D₂O solutions were at pD 8.90, which correspond to the same ionic state of the enzyme, indicated by the data in Figure 2A. ^c At 65 °C, H₂O solutions were at pH 8.0 and D₂O solutions had a pH meter reading of 8.4, which corresponds to the same ionic state of the enzyme, indicated by the data in Figure 2B.

Table 3. Observed and Calculated k_{cat}/K_M Solvent Isotope Effects

	25 °C ^b		65 °C ^c	
	D → L	L → D	D → L	L → D
SIE calculated for stepwise mechanism ^a	1.69	1.07	1.35	1.01
SIE from progress curves	1.63 (0.09)	1.06 (0.08)	1.30 (0.09)	1.02 (0.05)

^a Calculated from the two primary effects in Table 1 assuming a stepwise mechanism, as described by O'Leary (25). ^b H₂O data was obtained at pH 8.48, while D₂O runs were at a pD of 8.90, which corresponds to the same ionic state of the enzyme. Standard deviations for replicate measurements are given in parentheses. ^c At 65 °C, H₂O data was obtained at pH 8.0, while D₂O runs had a pH meter reading of 8.4, which corresponds to the same ionic state of the enzyme. Standard deviations for replicate measurements are given in parentheses.

Table 2 also presents multiple KIEs obtained for both D and L isomers at 65 °C. The changes in the KIEs are smaller than for 25 °C, and the D → L direction gives the only statistically significant change; a reduction of the substrate KIE in D₂O is observed. The multiple KIE diagnosis for 65 °C is thus the same as for 25 °C, a stepwise mechanism. Table 3 shows that the experimental SIE for the D → L direction at 65 °C agrees well with that calculated from the reduction in the substrate KIE.

DISCUSSION

Stepwise Double Proton-Transfer Catalyzed by Two Acid/Base Catalysts. The dependence of a KIE on the isotopic composition of a second site has been applied as a diagnostic for stepwise vs. concerted bond making/breaking steps in a number of enzyme catalyzed reactions (23, 24, 42, 43). This multiple KIE type of experiment requires the prior observation of two independent KIEs. In the case of alanine racemase, two independent hydrogen KIEs were previously measured using initial rate techniques (19). These were obtained in separate experiments using alanine deuterated at C α , and D₂O. The observation of two independent KIEs opens the possibility that both isotopes are being transferred in the same transition state, i.e., in a concerted double proton transfer.

The X-ray structure of alanine racemase shows that two active site residues are well positioned to act as acid/base catalysts. These are Tyr265 and Lys39, which lie on opposite faces of and close to C α in the external aldimine formed

from alanine phosphonate (44) and in the two isomeric phosphopyridoxylalanine structures (45). It has been demonstrated by mutagenesis that these are the two acid/base catalysts in a two-base racemization mechanism. Watanabe et al. (46) showed that the Y265A mutant is 1000-fold less active than the wild-type enzyme, while the K39A mutant was shown to be completely inactive but is rescued by the addition of methylamine (21). Mutants at Arg219, which hydrogen bonds to the pyridine nitrogen of PLP, have also been characterized (19). This latter work showed that the hydrogen bond to the pyridine nitrogen is worth only 40-fold to the catalytic mechanism based on limiting k_{cat}/K_M values from pH profiles. The lowering of the Tyr265 phenolic hydroxyl pK_a from ~10 to ~7 via interaction through His166 is the major role of this residue. Thus, a two-base mechanism for alanine racemization, employing Lys39 and Tyr265, is well established, which is a requirement for a concerted double proton transfer.

Evidence disfavoring a stepwise mechanism has also been obtained. The quinonoid resonance form ($\lambda_{\text{max}} \sim 500 \text{ nm}$, $\epsilon \sim 40\,000 \text{ M}^{-1} \text{ cm}^{-1}$) of a carbanionic intermediate, which is central to a stepwise mechanism, is not observed with the wild-type enzyme either in stopped-flow experiments (19), or in static experiments in which 50 mM (saturating) of each D- and L-alanine were added to 50 μM wild type subunits (data not shown). These data allow one to calculate that the quinonoid carbanionic intermediate is $\geq 4.5 \text{ kcal/mol}$ higher in energy than the aldimine intermediates. Yet, with the R219E mutant quinonoid absorbance is readily observed (19), as it is with the R219A mutant containing *N*-methylPLP (Toney, unpublished results).

The magnitude of the primary substrate and solvent KIEs measured previously with alanine racemase (~ 1.5) are both small (19). This requires the use of a high precision technique for measuring the multiple KIEs, since the expected decrease in the first KIE by substitution at the second site would be smaller than could be accurately determined by routine steady-state techniques. Published methods for high precision steady-state kinetic studies were cost prohibitive for the present study due to coupling enzyme costs (47).

Given the reversibility of the alanine racemase catalyzed reaction, the equilibrium perturbation technique (26) appeared suited to the purpose. Bahnson and Anderson (29) provide a straightforward theoretical treatment of equilibrium perturbation-type experiments where a substrate derived hydron is irreversibly lost to solvent during the course of the reaction, as is the case with alanine racemase. Fisher et al. (30) also treated such a system, but, to the present authors, their analysis is much less accessible than that of Bahnson and Anderson.

The primary substrate KIE for a single reaction direction (e.g., D → L) can be obtained from equilibrium perturbation-type washout experiments (29). Thus, one can perform multiple KIE analyses by measuring the effect of substitution of D₂O for H₂O on the primary substrate KIE. At the risk of being redundant of the explanation given in Results, the following attempts to provide a qualitative understanding of the equilibrium perturbation-type experiments required to obtain the multiple KIEs.

The primary substrate KIE measurement in H₂O is conceptually straightforward. If, as is shown in Figure 3, the D isomer is initially deuterated and the L isomer is

protiated, the L isomer reacts faster to D than does the D isomer to L if a primary substrate KIE is expressed on the $D \rightarrow L$ reaction. If no deuterium were present in the D isomer initially, then no perturbation from equilibrium (i.e., deviation from zero optical rotation in these experiments) would be observed when enzyme is added.

The primary substrate KIE measurement in D_2O is not as conceptually straightforward. Simply changing the solvent from H_2O to D_2O in the above experiment in which the D isomer is initially deuterated *does not* yield the desired KIE. In this case, the D isomer remains deuterated throughout the course of the reaction since deuterium is provided by solvent for incorporation into D isomer that is produced from L. On the other hand, the L isomer will not remain protiated during the course of the reaction, but will eventually become fully deuterated. As L is converted to D, the substrate derived proton is lost to solvent by exchange and the nascent D isomer acquires a deuteron from solvent. Eventually, an all deuterated equilibrium is reached. In this case, *isotopic discrimination is determined by the nonsolvent isotope that is present initially but lost to solvent at equilibrium, i.e., the protium on the L isomer.*

The primary substrate KIE for the D isomer fractioning in D_2O , required for the multiple KIE comparison to deuterated D isomer in H_2O , is thus obtained by employing initially *protiated* D isomer in D_2O . This reacts faster than the deuterated L isomer in the reverse reaction *due to the primary KIE in the $D \rightarrow L$ direction*. The signs of the perturbations will be opposite since in H_2O the D isomer accumulates transiently while in D_2O the L isomer accumulates transiently, even though the same primary KIE is being measured. This remarkable prediction is observed experimentally as shown by the data in Figure 4.

With an understanding of the required experiments in hand, the collection of the data was straightforward and high precision was obtained from triplicate measurements. The multiple KIE data presented in Table 2 provide strong evidence that alanine racemase employs a stepwise not a concerted mechanism for the two proton transfers required to effect racemization of the external aldimine intermediate. The data for the $D \rightarrow L$ direction at 25 °C show a reduction of ~10% in the primary KIE, well outside the error of the determinations. The KIE reductions for the $L \rightarrow D$ direction are smaller but nonetheless statistically significant.

The enzyme employed in this study is from *B. stearothermophilus*, a thermophilic bacterium whose optimal growth temperature is ~65 °C. Thus, the multiple KIEs were measured at this higher temperature also to probe the mechanism at the temperature to which the enzyme has been adapted, as a comparison to the “cold” regime of 25 °C. Again, a reduction of the primary substrate KIE of ~10% is observed for the $D \rightarrow L$ direction, supporting a stepwise mechanism at this higher temperature. The KIE change in the $L \rightarrow D$ direction is not statistically significant at 65 °C. Apparently, alanine racemase preserves its basic chemical mechanism, as well as its free energy profile as gleaned from the similar KIEs, over this 40 °C temperature range.

The formation of the external aldimine from the internal aldimine plus substrate requires at least two proton-transfer steps (see below). Thus, it is possible that the SIEs observed with alanine racemase are due to one of these proton transfers that are not involved in the stereoisomerization step. If this

were the case, then the observed reduction in primary substrate KIEs would not be indicative of a stepwise interconversion of stereoisomeric aldimines, rather it would simply be due to an increase in the appropriate commitment factor due to solvent substitution in transamination.

The following reasoning suggests that internal/external aldimine interchange is not significantly rate limiting under the present conditions, and that the interpretation of the multiple KIEs as supporting a stepwise mechanism is correct. Linear proton inventories on k_{cat} (KIEs ~ 1.5) were observed *in both directions* for the wild-type enzyme, indicating a single solvent derived proton in flight in the solvent sensitive step that occurs after enzyme–substrate complexation in either reaction direction (19). This proton was assumed to be the one being donated to $C\alpha$ in stereoisomerization in the above discussion. This assumption is supported by the fact that, *in both directions*, primary substrate KIEs on k_{cat} and k_{cat}/K_M are significant and similar (~1.5) (19). The solvent derived proton being transferred to $C\alpha$ in one direction (e.g., $D \rightarrow L$) is mechanistically equivalent to the substrate derived proton in the other direction (e.g., $L \rightarrow D$), and this solvent derived proton must be that observed in the proton inventories given the substrate KIEs. Additional evidence that weighs against this possibility is the observation that the wild-type enzyme shows no dependence on solvent viscosity at pH 8.9 (Toney et al., unpublished results).

The high specificity of alanine racemase for racemization vs transamination (31) would be cleanly explained by a concerted double proton transfer were it enforced. In the context of a stepwise mechanism, the relative destabilization of the carbanionic quinonoid intermediate by employing Arg219 to interact with the PLP pyridine nitrogen instead of an aspartate or glutamate at this position would also tend to disfavor transamination or other side reactions. Transamination, for example, requires a 1,3-prototropic shift from $C\alpha$ to $C4'$. This requires a relatively long quinonoid lifetime to be efficient, such that the acid/base catalyst can move to deliver the proton to the second site. The shorter lifetime of the carbanionic intermediate in alanine racemase, coupled with the prepositioning of the proton donor on the opposite face of $C\alpha$, would tend to favor 1,1-proton transfer as occurs in racemization.

Protonation States of Catalytically Productive Enzyme and Substrate. The mechanisms of PLP-dependent enzymes require the formation of a covalent substrate-coenzyme Schiff base (external aldimine intermediate). This is formed via a transamination reaction in which, ultimately, a protonated Lys-PLP Schiff base (internal aldimine) is attacked by a deprotonated substrate amino group. Interestingly, it was shown that with aspartate aminotransferase this can occur by either of two routes (48). The unprotonated internal aldimine enzyme can bind the protonated amino acid substrate, with subsequent proton transfer between these to get the reactive form. Alternatively, the protonated enzyme can bind the unprotonated substrate, with direct reaction between these to form the external aldimine.

Alanine racemase exists only in the form in which the Lys39-PLP Schiff base is protonated from pH 6–10.5, since no spectral changes in the coenzyme spectra are observed over this pH range (data not shown). Thus, either the unprotonated substrate must be captured by the enzyme for

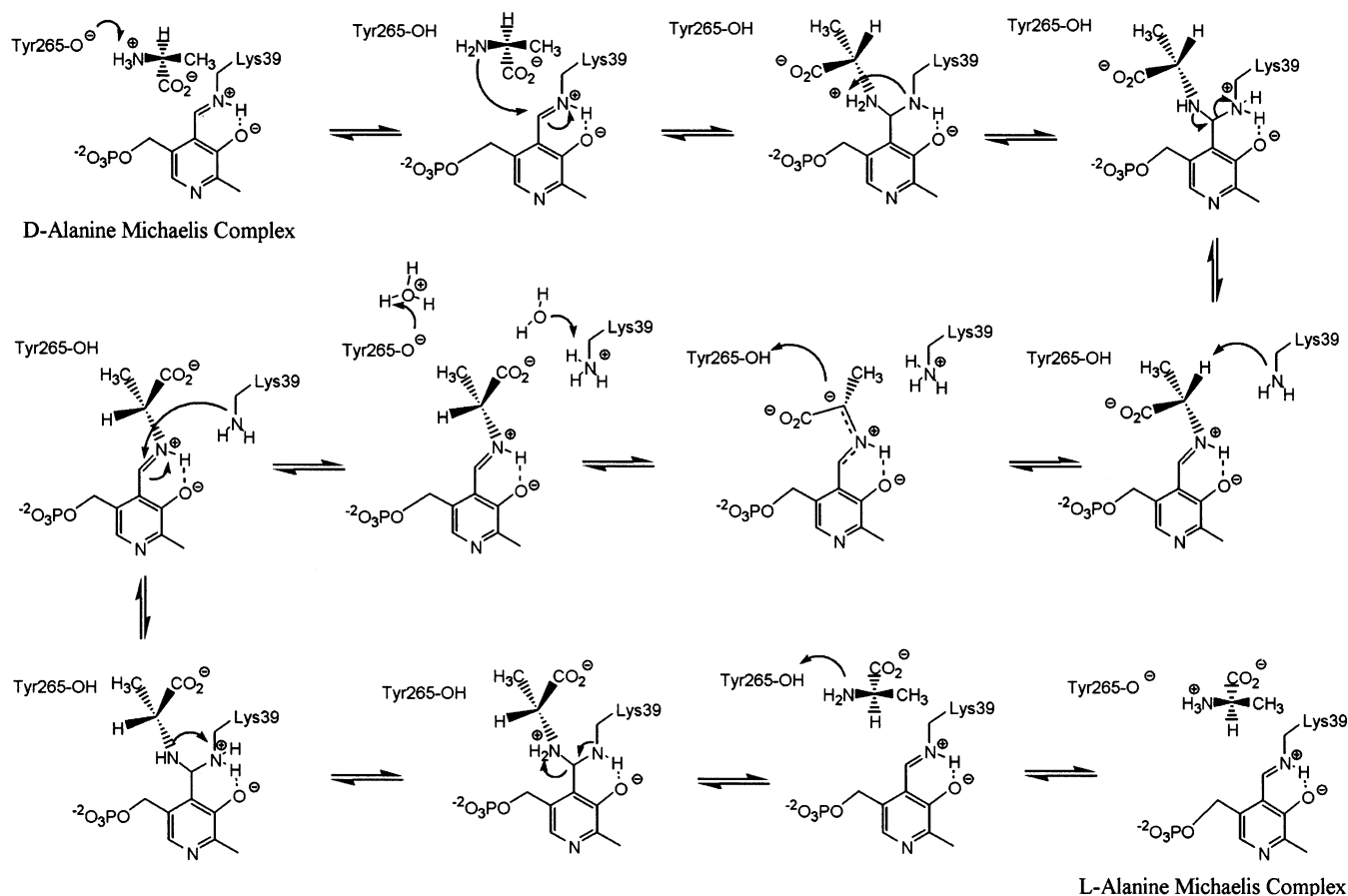


FIGURE 5: Detailed mechanism for the alanine racemase catalyzed reaction. For simplicity, proton exchanges at Lys39 and Tyr265 are shown to occur from the same intermediate. This is not a necessity.

reaction to occur, or an active site residue in its basic form must deprotonate the predominantly protonated amino acid substrate near neutral pH values.

Previous mutagenesis and isotope studies (19) showed that Arg219 lowers the pK_a of an active site residue responsible for the acidic limb of the bell shaped k_{cat}/K_M pH profile of the wild type enzyme. This is expected to be Tyr265 based on its known role as an acid/base catalyst and its electrostatic connection to Arg219 through His166. The ionization controlling the basic limb was more difficult to assign. The coincidence of the higher pK_a value with that of the free substrate was suggestive, yet mutations at Arg219 apparently increased this pK_a weighing against it being the substrate.

The data presented here allow clean assignments of the two ionizations observed in the k_{cat}/K_M pH profile for wild type. The pK_a values obtained at different temperatures allow the calculation of the ΔH_{ion} values, while the use of serine as an alternative substrate allows perturbation of the pK_a value of the substrate α -amino group. The ΔH_{ion} value for the acidic pK_a is very close to that observed for free tyrosine and phenol. This further supports the prior assignment of Tyr265 as the residue responsible for this ionization. The basic pK_a has a ΔH_{ion} that is consistent with it being an amino group, either the substrate or Lys39 in the internal aldimine since PLP aldimine ΔH_{ion} values are also ~ 10 kcal/mol (49). Due to the β -hydroxyl group, serine has a substantially lower pK_a than alanine, and is a reasonably good (460-fold less active) substrate compared to alanine. The serine k_{cat}/K_M pH profile was thus measured and the pK_a values obtained show

an unaltered (within experimental error) acidic pK_a and a substantially lowered basic pK_a value, equal to that of free serine. The combination of data (i.e., perturbed acidic pK_a values with R219 mutants, internal aldimine ionization not observed in spectral titrations of wild type, ΔH_{ion} values, and pK_a values from alanine and serine pH profiles) demonstrate that the acidic pK_a is due to Tyr265 and the basic pK_a is due to the α -amino group of the amino acid substrate.

The initial productive interaction between alanine racemase and either isomer of alanine must then occur between an enzyme with the Lys39-PLP Schiff base protonated and Tyr265 unprotonated, and a substrate whose α -amino group is protonated, which is the predominant form at physiological pH. An active site residue must deprotonate the amino acid before it can attack the protonated Lys39-PLP Schiff base. On the basis of the active site structure of the enzyme, Tyr265 is likely to fulfill this role.

Figure 5 gives a detailed mechanism that incorporates these conclusions. After alanine binds and is deprotonated by Tyr265, it attacks the protonated Lys39-PLP Schiff base to give the *gem*-diamine intermediate which goes on to the external aldimine after a proton transfer. For both isomers, the external aldimine initially formed has Lys39 unprotonated and Tyr265 protonated. This is the protonation state that is catalytically active for $D \rightarrow L$ isomerization. If the substrate is the L isomer, proton transfers at Tyr265 and Lys39 must occur before stereoisomerization of the external aldimine intermediate can occur. Watanabe et al. (45) proposed proton transfer between Tyr265 and Lys39 as an intrinsic component

of a mechanism in which a protonated α -carboxyl is employed. This is an unusual proposition since the substrate carboxylate is stabilized by hydrogen bonds from the side chain guanidino group of Arg136 and from the main chain amide of Met312 (50). Given the large number of examples of enzyme catalyzed reactions in which protons are rapidly exchanged from reaction intermediates, there is no mechanistic imperative to propose such a proton exchange/transfer mechanism. Rather, the mechanism in Figure 5 simply assumes that the dynamic water molecules and hydrogen bonding interactions in the active site allow rapid equilibration of active site protons with the solvent, based on their pK_a values.

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