Kinetic and Stereochemical Comparison of Wild-type and Active-Site K145Q Mutant Enzyme of Bacterial L-Amino Acid Transaminase*

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D-Amino acid transaminase (EC 2.6.1.21), from Bacillus sp. YM-1, a thermostable enzyme with pyridoxal 5'-phosphate as coenzyme and a target for the design of novel antimicrobial agents, catalyzes the reversible transfer of an amino group between D-alanine and α-ketoglutarate to form pyruvate and D-glutamate, respectively. To explore the catalytic role of Lys-145, which binds the coenzyme, a site-specific mutant enzyme, K145Q (in which Lys-145 had been mutated to glutamine) constructed earlier (Futaki, S., Ueno, H., Martinez del Pozo, A., Pospischil, M. A., Manning, J. M., Ringe, D., Stoddard, B., Tanizawa, K., Yoshimura, T., and Soda, K. (1990) J. Biol. Chem. 265, 22306–22312) was compared to the wild-type enzyme for its kinetic parameters. Initial velocity studies and partial reaction isotope exchange experiments showed that the low activity of the mutant enzyme (about 1.5% of the wild-type enzyme with saturating substrates) is an intrinsic property, confirming that contaminating enzymes do not account for the low activity of the K145Q mutant enzyme. The rates of the forward reaction for both wild-type and mutant enzymes were about 30–40 times higher than the rates of the reverse reaction. KM values for the four substrates were about 10 to 100 times higher for the mutant compared to the wild-type enzyme. Whereas D-alanine is preferred over L-alanine by the wild-type enzyme (105 higher kcat/KM for D-over L-alanine), the K145Q enzyme does not efficiently discriminate between L- and D-alanine. Both wild-type and mutant enzymes also catalyze the slow racemization of L- and D-alanine. Proton NMR studies showed that wild-type enzyme catalyzed a time-dependent exchange of the Ca proton of D-alanine with solvent D2O and a slow exchange of the α proton of L-alanine; the latter slow exchange rate is the same for the Ca proton of both L- and D-alanine with the K145Q mutant enzyme. Thus, in addition to binding pyridoxal 5'-phosphate, the active-site Lys-145 of D-amino acid transaminase is involved in several other important functions, i.e., it optimizes catalytic efficiency and it maintains stereochemical fidelity. The steady-state kinetic results on the K145Q mutant enzyme together with the findings on the relative racemization rates and the NMR protein exchange data suggest that an alternate base catalyzes abstraction of the α proton of substrate in this mutant D-amino acid transaminase.

D-Amino acid transaminase from bacteria is a target enzyme for the development of novel antimicrobial agents because it catalyzes the synthesis of D-glutamate and D-alanine, important constituents in the bacterial cell wall (1). Studies on its mechanism of action are important in order to accomplish that goal. Like L-amino acid transaminases (2), it employs pyridoxal 5'-phosphate (PLP) as coenzyme (3, 4). Reactions catalyzed by L-amino acid-specific enzymes proceed via a Ping-Pong kinetic mechanism and consist of two half-reactions, each of which is comprised of three major steps. A similar mechanism is proposed for D-amino acid transaminase in Scheme I, which also outlines some mechanistic questions posed in this study. In the first step, referred to as transaldimination, the coenzyme in an internal aldime structure with the ε-NH2 group of Lys-145 in the wild-type enzyme, forms a Schiff’s base with an amino acid substrate to form an external aldime with the concomitant release of the ε-amino group of Lys-145. The second step, which is the critical 1,3-prototropic shift involving abstraction of a proton from the α carbon of the amino acid and donation of a proton to the aldehydic carbon of the coenzyme, may proceed via a quinonoid intermediate (5) to yield a ketimine product. The final step for the first half-reaction is the hydrolysis of the ketimine and release of the keto acid. The second half-reaction is the reversal of these steps but with a different α-keto acid and the formation of its corresponding amino acid. In L-aspartate transaminase Lys-258 has the dual function of binding the coenzyme and abstracting the α proton of substrate (2). Whether it performs other functions in this enzyme is not known.

Elucidation of the role(s) of the coenzyme-linked Lys-145 of D-amino acid transaminase has been a focus of our studies on this enzyme. Hence, we have constructed the active-site K145Q mutant enzyme by site-specific mutagenesis (6). With...
this attenuated mutant enzyme, it was possible to study some intermediates in the reaction pathway in a conventional spectrophotometer, e.g., the formation of the external Schiff base with D-alanine, which occurred over the period of several minutes and its subsequent transformation to the ketimine, which took place over a period of hours. These results suggested that some amino acid side chain other than Lys-145, which was absent in the mutant enzyme, was responsible for these slow transformations. In the present study, we compare the kinetic details of the reaction pathway of the wild-type and mutant enzymes in an effort to understand the basis for these observations.

The possibility that the low activity of a mutant enzyme is due to contamination by the wild-type enzyme was rigorously excluded in order to be certain of the conclusions reached through studies on this attenuated enzyme. Another way to distinguish between the intrinsic catalytic activities of the wild-type and mutant enzymes is the use of steady-state kinetic methods. Thus, we have conducted a detailed comparative investigation of the kinetic mechanism of both wild-type and mutant enzymes. We employ initial velocity studies to determine the kinetic parameters for the substrates in the forward and reverse reactions, and partial reaction isotope exchange techniques on D-alanine/pyruvate and D-glutamate/α-ketoglutarate substrates and products, to determine the binding constants for substrates. We have also explored the possible presence of dead-end complexes in the kinetic mechanism of D-amino acid transaminase. Based on these results, a minimal kinetic mechanism for this enzyme has been constructed, and minimal values have been assigned to the individual rate constants.

The ability of a transaminase to catalyze an exchange of the Ca proton of the amino acid substrate has been shown in L-alanine transaminase (7, 8). Those results were confirmed and extended by Oshima and Tamiya (9) and by Cooper (10). Julin et al. (11) reported a 96% exchange of the Ca proton of L-aspartate with water catalyzed by Lys-258 of L-aspartate transaminase. A similar exchange might be expected for D-amino acid transaminase. As shown in Scheme I, all the steps along the reaction pathway are freely reversible. Thus, the catalytic base that abstracts the proton from D-alanine could exchange it with solvent and reprotocate the quinonoid to reform alanine. If carried out in D2O, the resulting product would be [2-D]alanine. The two substrate-product pairs, D-alanine/α-ketoglutarate and pyruvate/D-glutamate, were freshly prepared before each experiment.

Reduced Stereoselectivity in an Attenuated Transaminase
Blue Sepharose affinity chromatography resin, rabbit muscle L-lactic dehydrogenase Type II (EC 1.1.1.27), D-alanine, pyruvate, D-glutamate, α-ketoglutarate, and the other biochemical reagents were products of Sigma. Marfey's reagent was purchased from Pierce Chemical Co. Centricon 10 microconcentration units were from Amicon.

Initial Velocity Studies—Previous studies on the mutant enzyme indicated low amounts of activity that could be detected by the standard spectroscopic assay (6). Assays were performed on a Varian Cary 2200 spectrophotometer or on a Zeiss PMQII instrument both with cuvettes thermostatted at 25 °C. For the assay, a 1-mM reaction mixture contained 5 mM α-ketoglutarate, 200 mM D-alanine, 100 mM NADH and 0.4 mg/ml dehydrogenase in 50 mM bis-Tris, pH 7.2, at 25 °C. The reaction was initiated by addition of enzyme. One unit of enzyme activity is defined as the amount of enzyme necessary to convert 1 μmol of NADH to NAD per min at 25 °C, which corresponds to the reduction of an equivalent amount of pyruvate produced from D-alanine, as described previously (25).

Initial Velocity Studies—Previous studies on the mutant D-amino acid transaminase indicated low amounts of activity that could be detected by the standard spectroscopic assay (6). Furthermore, with D-amino acid substrates alone there were complete spectral transformations of the enzyme, i.e., from E-PLP to E-PMP, consistent with the intrinsic activity of the mutant enzyme. In the present work we have developed a sensitive assay that employs radiolabeled substrates to provide a convenient method for the determination of kinetic parameters and to permit a study of the reverse reaction catalyzed by D-amino acid transaminase. Since D-alanine and pyruvate were the substrate-product pairs, their concentrations were varied between 0.25 and 4 times the Km values, while the fixed concentration of the other substrate
Reduced Stereoselectivity in an Attenuated Transaminase

\[
\text{CH}_3 - \text{COO}^- \quad \text{BASE II} \quad \text{HB}
\]

\[
\text{CH}_3 - \text{C} = \text{C} - \text{NH}_2 \quad \text{I1}
\]

\[
\text{H} - \text{C} - \text{C} = \text{O}' \quad \text{q}
\]

\[
\text{H} - \text{N} : \text{H} : \text{B}
\]

\[
\text{C} - \text{H}
\]

\[
\text{Lys-14s}
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\[
\text{I}
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Internal Aldimine

\[
\text{H}-\text{N}
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\text{ti}
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External Aldimine

\[
\text{SCHEME I. Mechanism of transamination and racemization in D-amino acid transaminase.}
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\[
\text{E-PLP.Glu} \quad k_6 \quad \text{E-PLP} \quad k_1 \quad \text{E-PLP.Ala} \quad k_7
\]

\[
k_5 \quad k_{-5} \quad k_{-1}
\]

\[
k_4 \quad k_3 \quad k_2
\]

\[
\text{E-PMP.Kg} \quad k_8
\]

\[
\text{E-PLP.Kg} \quad k_9
\]

\[
\text{E-PMP} \quad k_{-3} \quad \text{E-PMP.Pyr}
\]

\[
\text{E-PMP} \quad k_{-4} \quad \text{E-PMP}
\]

SCHEME II. Minimal kinetic mechanism for wild-type and mutant D-amino acid transaminase.

was maintained at 5 times its \( K_M \) value. To determine the kinetic parameters for the forward reaction, a 100-\( \mu \)l reaction mixture contained D-alanine, \([\alpha-5-^{14}\text{C}]\)ketoglutarate (200,000 cpm) in 50 mM bis-Tris-HCl, pH 7.2 at 25 °C. To investigate possible dead-end complexes in the kinetic mechanism of D-amino acid transaminase, one substrate was maintained below its \( K_I \) value, and the second was varied between 0.5 and 10 times its \( K_I \) value. For the \( E\text{-PMP. Ala} \) dead-end complex, a 100-\( \mu \)l reaction mixture contained 0.01-0.70 M D-alanine, \([\alpha-5-^{14}\text{C}]\)ketoglutarate (200,000 cpm), and \( \alpha\)-ketoglutarate (1 \( \mu \)M for the wild-type, 100 \( \mu \)M for the K145Q mutant enzyme). For the \( E\text{-PMP. Glu} \) experiments, a 100-\( \mu \)l reaction mixture contained 0.5-7 mM D-glutamate, \([1-^{14}\text{C}]\)pyruvate (200,000 cpm), and pyruvate (1 \( \mu \)M with the wild-type enzyme and 50 mM with the K145Q enzyme). For the \( E\text{-PLP. Fyr} \) dead-end complex experiments, a 100-\( \mu \)l reaction mixture contained 5-50 mM pyruvate, \([1-^{14}\text{C}]\)pyruvate (200,000 cpm), and D-glutamate (100 \( \mu \)M with the wild-type enzyme). The spectroscopic assay method was employed for the determination of the \( K_I \) value for the \( E\text{-PLP. Kg} \) complex in wild-type D-amino acid transaminase. A 1 ml reaction mixture contained 0.5-15 mM Kg, 5-100 mM alanine, 100 \( \mu \)M NADH, and 0.4 mg/ml lactate dehydrogenase in 50 mM bis-Tris-HCl, pH 7.2, at 25 °C.

Partial Reaction Isotope Exchange—For the D-alanine/pyruvate exchange experiments, substrate concentrations were varied between 0.25 and 4 times \( K_I \) values (see legend to Table I). The reactions (150 \( \mu \)l containing D-alanine, pyruvate, \([1-^{14}\text{C}]\)pyruvate (200,000 cpm) in 50 mM bis-Tris-HCl, pH 7.2, at 25 °C were initiated by the addition of mutant or wild-type enzymes (0.2-10 \( \mu \)g) and terminated at the
VALUES FOR AMINO ACID SUBSTRATES FOR TRANSAMINASES ARE NOT UNUSUAL, POSSIBLE SLOW OFF-RATE FOR ONE OF THE PRODUCTS OF THE REVERSE REACTION (D-ALANINE OR a-KETOGLUTARATE). THE AND THE SAME ENZYME FROM PIG HEART HAS A $K_M$ OF 28 mM FOR L-ALANINE WITH L-ALANINE TRANSAMINASE FROM RAT LIVER (356 ± 14 UNITS/ mg). THIS RESULT WAS RATHER UNUSUAL AND SUGGESTED A DECREASED ABILITY OF L-ALANINE TO ACT AS SUBSTRATE IS NOT DUE TO INFERIOR BINDING COMPARED TO D-ALANINE BUT RATHER THAT ITS PROTON IS APPARENTLY NOT CLOSE ENOUGH TO LYS-145. THE $K_M$ VALUES FOR THE KETO ACIDS, a-KETOGLUTARATE AND D-Glutamate Were 525 AND 8, SIGNIFYING ABOUT A 10-FOLD INCREASE COMPARED TO THE WILD-TYPE ENZYME (TABLE I). THE $K_M$ VALUES FOR THE KETO ACIDS, a-KETOGLUTARATE AND PYRUVATE, WERE 65 AND 8.4 mM, ABOUT A 100-FOLD INCREASE OVER THE WILD-TYPE ENZYME. THE 1.5% ACTIVITY OF THE K145Q MUTANT ENZYME COMPARED WITH THE WILD-TYPE ENZYME IS HIGHER THAN THE VALUE REPORTED PREVIOUSLY BECAUSE THE CURRENT STUDIES WERE PERFORMED AT SATURATING CONCENTRATIONS OF SUBSTRATES.

WE DETERMINED THAT ALTHOUGH WILD-TYPE D-AMINO ACID TRANSAMINASE EMPLOYS D-ALANINE AS THE PREFERRED SUBSTRATE ($K_M$ = 48 mM, $k_{cat}/K_M$ = 2.7 x 10^4 M^{-1} s^{-1}$), IT ALSO ACCEPTS L-ALANINE AS SUBSTRATE, ALTHOUGH IT IS A POOR ONE ($K_M$ = 35 mM, $k_{cat}/K_M$ = 1.18 x 10^3 M^{-1} s^{-1}$). THE $K_M$ VALUES OF EACH ENANTIOMER INDICATE THAT THE ENZYME-SUBSTRATE INTERACTIONS FOR BINDING ARE THE SAME. THE SIGNIFICANT DIFFERENCE IS IN $k_{cat}$. THIS, THE DECREASED ABILITY OF L-ALANINE TO ACT AS SUBSTRATE IS NOT DUE TO INFERIOR BINDING COMPARED TO D-ALANINE, BUT RATHER THAT ITS PROTON IS APPARENTLY NOT CLOSE ENOUGH TO LYS-145. THE K145Q MUTANT ENZYME ACCEPTS L-ALANINE ($K_M$ = 570 mM, $k_{cat}/K_M$ = 1.16 x 10^3 M^{-1} s^{-1}$) AS WELL AS D-ALANINE AS SUBSTRATES WITH NEARLY EQUAL EFFICIENCY, I.E., SIMILAR $k_{cat}/K_M$ VALUES. THE $K_M$ VALUE IS THE SAME FOR L-ALANINE AND D-ALANINE.

PARTIAL REACTION ISOPOREXCHANGES—THE ABILITY OF AN ENZYME TO CATALYZE THE EXCHANGE OF RADIOLABEL BETWEEN EACH SUBSTRATE-PRODUCT PAIR IN THE ABSENCE OF THE OTHER SUBSTRATE-PRODUCT PAIR IS AN IMPORTANT CRITERION TO ESTABLISH A PING-PONG KINETIC MECHANISM (32, 33). PARTIAL REACTION ISOPOREXCHANGE EXPERIMENTS WERE PERFORMED WITH BOTH SUBSTRATE-PRODUCT PAIRS D-ALANINE/PYRUVATE AND D-GLUTAMATE/DR-KETOGLUTARATE.
The partial reaction isotope exchange experiments allow for the establishment of Ping-Pong type kinetics for the enzyme and the determination of the dissociation constants for the enzyme-substrate complexes in wild-type and K145Q D-amino acid transaminase. The $K_i$ values obtained by this method represent true dissociation constants (32, 34). These exchanges, which were readily detected, confirm a Ping-Pong type kinetic mechanism for wild-type and mutant D-amino acid transaminase. Neither exchange occurred in the absence of the enzyme nor when the enzyme was denatured by boiling in 1% SDS. The rates for the exchanges are summarized in Table I. For the wild-type and mutant enzymes, the rate of the D-alanine/pyruvate exchange was similar to the forward reaction rate ($V_{\text{FOR}}$), whereas the D-glutamate/a-ketoglutarate exchange rate was 30- to 50-fold slower. This result is consistent with Equation 2 (35) in which the sum of the reciprocal values of the forward ($V_{\text{FOR}}$) and reverse ($V_{\text{REV}}$) reaction is the slow, rate-determining step. The dissociation constants for substrates with wild-type D-amino acid transaminase end mutant are summarized in Table I. In the K145Q mutant enzyme the dissociation constants for the amino acids D-alanine and D-glutamate are about 11-fold higher than for the wild-type enzyme. The $K_i$ value for a-ketoglutarate is 153-fold higher and that for pyruvate 95-fold higher than the wild-type enzyme. These results are indicative of a difference in the active-site geometry of wild-type and K145Q mutant D-amino acid transaminase. Although the attenuated K145Q mutant enzyme is significantly less efficient than the wild-type enzyme, it remains a competent enzyme that proceeds via Ping-Pong kinetic mechanism in the same manner as its wild-type counterpart.

**Dead-end Complexes**—The formation of dead-end complexes between the enzyme and one substrate is characteristic of enzymes that proceed via Ping-Pong kinetics. For example, in L-aspartate transaminase, the E-PLP-alpha-ketoglutarate and E-PMP-aspartate dead-end complexes are formed (36). Initial velocity experiments have been employed to investigate the possible formation of dead-end complexes in the kinetic mechanism for wild-type and mutant D-amino acid transaminases. In these experiments one substrate is maintained at a low concentration ($< K_i$), while the concentration of the second is varied from 0.5 to 10 times $K_i$. A decrease in the maximal overall reaction rate at high concentration of the varied substrate would suggest the formation of an enzyme-substrate dead-end complex (32). For wild-type enzyme at low concentrations of D-alanine (in relation to its $K_i$) and high concentration of Kg (relation to its $K_i$), there was inhibition in the maximal reaction rate ($V_{\text{mac}}$), indicating the formation of a possible E-PLP-Kg dead-end complex. The $K_i$ value for that complex was determined by the method of Segel (32) to be 3.4 mM. Under our experimental conditions, the formation of E-PLP-Pyr ($K_i > 50$ mM), E-PMP-Ala ($K_i > 0.7$ m), or E-PMP-Glu ($K_i > 7$ m) dead-end complexes was not detected since there was no inhibition of the maximal reaction rate by high concentrations (relative to $K_i$) of substrate forming the complex. In the case of the mutant enzyme no dead-end complexes were detected, although limits of the binding constants of such complexes can be assigned, i.e. 20 mM, 700, 660, and 7 mM for the E-PLP-Kg, E-PMP-Ala, E-PLP-Pyr, and E-PMP-Glu complexes, respectively (see Materials and Methods for details).

**Analysis of Kinetic Mechanism**—Based on the steady state kinetic parameters, minimal values for the rates of individual steps of the D-amino acid transaminase-catalyzed reaction were calculated (Table I). From $V_{\text{FOR}}$, a $k_{\text{cat}}$ of 129 s$^{-1}$ (based on a molecular weight of 32,000 per subunit) was calculated for the wild-type enzyme. The corresponding $k_{\text{cat}}$ for the K145Q enzyme was 1.8 s$^{-1}$, about 70-fold lower than that for the wild-type enzyme. For the wild-type enzyme, taking $k_{\text{cat}}/K_d$ of $2.7 \times 10^3$ M$^{-1}$ s$^{-1}$ as the minimal on-rate for D-alanine ($k_1$, see Scheme II) and the $K_i$ value ($k_{\text{cat}}/K_m$) obtained from the D-alanine/pyruvate isotope exchange (Table I), the off-rate ($k_{-1}$) for D-alanine from E-PLP was calculated to be 199 s$^{-1}$ (Table II). The $k_{\text{cat}}/K_d$ for D-alanine for the K145Q mutant enzyme was calculated to be 3.4 M$^{-1}$ s$^{-1}$, which is about 800-fold lower than that for the wild-type. This rather large effect on $k_{\text{cat}}/K_d$ may reflect the inability of the mutant enzyme to stabilize the transition state. From the on-rate of D-alanine ($k_1$, see Table II) and $K_i$ of 780 mM, an off-rate of 2.7 s$^{-1}$ was calculated. Based on a molecular weight of 32,000, D-alanine/pyruvate isotope exchange rates of 356 (wild-type) and 3.8 (K145Q) units/mg yield rate constants of 178 and 1.9 s$^{-1}$. Thus, these rate constants can be assigned to the central complex interconversion ($k_2$ and $k_{-2}$), and to the off-rate of pyruvate ($k_5$) from E-PMP (Scheme II). Since in both wild-type and K145Q mutant enzyme the $V_{\text{mac}}$/pyruvate exchange rate is faster than the $k_{\text{cat}}$, neither central complex interconversion ($k_2$ and $k_{-2}$) nor product off-rates ($k_{-3}$ and $k_3$) are rate-limiting in either enzyme. As described earlier, the one for pyruvate ($k_3$) can be calculated from $k_{\text{cat}}/K_m$ to be $2.4 \times 10^4$ and 3.0 M$^{-1}$ s$^{-1}$ for the wild-type and mutant enzymes, respectively (Table II). From the $k_{\text{cat}}/K_d$ values of $1.6 \times 10^5$ M$^{-1}$ s$^{-1}$ (wild-type) and 28 M$^{-1}$ s$^{-1}$ and 3.4 values of 15 M$^{-1}$ s$^{-1}$ and 2.5 M$^{-1}$ s$^{-1}$ for wild-type and mutant enzyme, off-rates ($k_{-4}$) of 2.4 and 0.006 s$^{-1}$ were calculated. For both enzymes, this off-rate is rather slow compared to $k_{\text{cat}}$. However, it reflects the slow off-reverse reaction rates and slow a-ketoglutarate/D-glutamate isotope exchange rate in both enzymes. Thus, the reverse reaction in both enzymes is limited by a slow off-rate of Kg (Scheme II). The forward reaction may at least be partially limited by an off-rate for D-glutamate. Since net catalysis proceeds with $k_{\text{mac}}$ values of 129 s$^{-1}$ (wild-type) and 18 s$^{-1}$ (K145Q), these values represent minimal rate constants.
for $k_b$, $k_{o-b}$, and $k_e$ (Glu off-rate). The on-rate for D-glutamate ($k_{a}$) was calculated from $K_{a}^{D}$ determined from the D-glutamate/o-κetoglutarate partial reaction isotope exchange experiments. Thus, while the reaction rates are slower and substrate binding constants are higher in the K145Q mutant enzyme, the kinetic mechanism is similar to the wild-type enzyme, i.e. Ping-Pong type with a slow reverse reaction rate resulting from a slow Kg off-rate.

**Exchange of pHJAlanine with Solvent D$_2$O**—Whereas the kinetic experiments described above were conducted under steady state conditions with both substrates present, the Ca proton exchange studies were performed with d-alanine alone employing relatively long incubation times (in hours) and higher enzyme concentrations. The exchange of the Ca proton of L- and D-alanine with solvent D$_2$O catalyzed by wild-type and mutant K145Q enzyme was monitored by NMR by observing the loss of the Ca quadruplet at 3.84 ppm. As shown in Fig. 1, the wild-type enzyme catalyzes a relatively fast exchange of the Ca proton of d-alanine with solvent (k = 0.46 h$^{-1}$). The wild-type enzyme also catalyzes a very slow exchange of the Ca proton of L-alanine (k = 0.034 h$^{-1}$). With a 10-fold increase in the concentration of the mutant enzyme, there was a slow exchange of the Ca proton of L-alanine (k = 0.045 h$^{-1}$) and D-alanine (k = 0.034 h$^{-1}$) (Fig. 2). These constants were calculated taking into account the difference in the concentrations of wild-type and K145Q enzymes. Thus, the Ca proton exchange rates are similar for both L- and D-alanine with the mutant enzyme and close to the value for the exchange rate of the α proton of L-alanine by the wild-type enzyme. This signal, which appears as a doublet at 1.54 ppm, becomes a singlet upon exchange of the Ca proton with D$_2$O. In control experiments, there was no decrease in this signal indicating no exchange of β protons. Furthermore, no exchange of the Ca proton was found in the absence of enzyme.

**Racemization of Alanine**—Donation of a proton to the opposite side of the Ca carbon of the quinonoid complex (Scheme I) would lead to an amino acid of opposite stereochemistry, i.e. racemization. Compared to the Ca proton solvent exchange, racemization catalyzed by wild-type and mutant D-amino acid transaminase was found to be lower than the proton exchange. Thus, the rates of racemization of L- and D-alanine catalyzed by the wild-type enzyme were nearly equivalent and approximately 50-fold slower than the corresponding Ca proton exchange rates, i.e. over a period of 10 h during which 50% of Ca proton of D-alanine had exchanged, only 1–2% of the L-isomer of alanine was found. The K145Q mutant enzyme also catalyzed the racemization of L- and D-alanine at rates 50-times slower than the Ca proton exchange rates. Thus, after a 14-h period when 50% of the Ca protons of D-alanine had been exchanged with solvent, the amount of racemization of L-alanine formed was also about 1%. To eliminate the possibility that the racemization was due to bacterial growth, experiments were conducted under sterile conditions after sterile filtration in a UNIFLO 0.2-μm microfiltration unit in a biological safety cabinet. After completion of the experiment, reaction samples were plated on LB agar media (37). No bacterial growth was observed on the plates when incubated at 37 °C for 2 days.

**FIGURE 2. Exchange of Ca proton of L- and D-alanine with solvent D$_2$O. A 0.5-ml reaction mixture contained 1.2 mg of K145Q mutant enzyme, 100 mM L- or D-alanine in 100 mM potassium phosphate, pH 7.6, in D$_2$O. NMR spectra (details in "Materials and Methods") were taken after 0–16 h. □, L-alanine; ▲, D-alanine.**

**DISCUSSION**

The results in this study demonstrate that although the K145Q mutant D-amino acid transaminase has low catalytic efficiency, i.e. it is an attenuated enzyme whose kinetic profile proceeds via a Ping-Pong mechanism like the wild-type enzyme (Scheme II). The mutation of Lys-145 leads to an enzyme in which there are changes in active-site geometry as indicated by the increases in binding constants and a loss of stereochemical integrity. Unusual for both wild-type and mutant enzymes were the findings that the reverse reaction rate ($V_{REV}$) was 30-fold slower than the forward reaction rate ($V_{FOR}$). However, detailed partial reaction isotope exchange studies revealed a slow α-ketoglutarate/D-glutamate exchange rate and a fast D-alanine/pyruvate exchange rate. The slow reverse reaction rate and Kg/Glu isotope exchange rate were attributed to a slow off-rate for α-ketoglutarate as reflected in its $K_{i}$ (15 μM). Similar studies on the K145Q mutant enzyme reveal that it has a slow Kg off-rate in the reverse reaction.

The possibility that the observed activity in the mutant enzyme was due to contamination by wild-type or endogenous enzymes has received consideration from several perspectives, and the results are completely consistent with the initial observation (6). Since the binding constants obtained for the K145Q mutant D-amino acid transaminase in this communi-
cation are strikingly different from those of the wild-type enzyme, the activity observed in the kinetic experiments with the K145Q mutant enzyme must be due to its inherent activity. Furthermore, the possibility that any putative contaminating enzymes would catalyze the partial isotope exchanges, which fit Equation 2, is remote.

The attenuated enzymatic activity of the K145Q enzyme and its ability to catalyze the exchange of the CO proton of D- and L-alanine with solvent indicate that another amino acid side chain but not Lys-145, which is absent in the mutant enzyme, catalyzes the proton abstraction (1.3 prototropic shift) from the amino acid substrate in the mutant enzyme. The low enzymatic activity of the mutant enzyme could arise from an improper anchoring of the coenzyme due to the absence of the internal aldime with Lys-145, similar to the Y70F mutant of Escherichia coli L-aspartate aminotransferase where a conservative mutation of Tyr-70, which interacts with the coenzyme, to phenylalanine leads to an 85% loss in km (38-40). The mutation of Lys-145 to glutamine in about a 10-fold increase in the KM of the amino acids and a 100-fold increase in the KM for the κ2-keto acids. Similar increases in the KI values were also observed. These observations suggest that Lys-145 may also be important in ground state substrate binding. More significantly, the km/kI ratio, which reflects transition-state binding, decreases 104- and 105-fold for the amino acids and keto acids, respectively, in the K145Q mutant enzyme. Thus, the low activity in the K145Q mutant enzyme may reflect its inability to stabilize effectively a transition state in the K145Q mutant enzyme reaction mechanism.

Our results indicate that it is likely that Lys-145 is the basic residue that abstracts the CO proton (1.3 prototropic shift) in wild-type enzyme with optimum efficiency. However, the activity of the K145Q mutant enzyme must be due to another residue, which has been designated BASE II in Scheme I. This view is supported by several results presented in this work, including the rates of proton exchange with solvent for both D- and L-alanine. Thus, L- or D-alanine may bind to the enzyme in a similar way as indicated by similar KM values and the 1.3 prototropic shift with L-alanine as substrate could be carried out by BASE II at a slow rate (Scheme I). In the absence of Lys-145 in the K145Q mutant enzyme, the substrate (or the coenzyme-substrate complex) has greater flexibility in movement and a second base (BASE II) may catalyze the 1.3 prototropic shift from either L- or D-alanine and could abstract the CO proton from either L- or D-alanine. A single base mechanism involving a quinonoid complex that changes its orientation has been proposed for alanine racemase from Bacillus subtilis and is referred to as the swinging door mechanism (12).

The proton exchange results are also consistent with the second base responsible for abstraction of the α proton of L-alanine for the wild-type enzyme and the α proton of D- or L-alanine for the mutant enzyme (Scheme I). With the wild-type enzyme, the external aldime between coenzyme and D-alanine permits the most efficient proton abstraction by Lys-145, thus the relatively high exchange rate. Binding of L-alanine allows for an abstraction of the proton by BASE II and thus the low exchange rate. In the mutant K145Q enzyme, the external aldime between coenzyme and alanine cannot interact with the absent Lys-145. Hence, BASE II abstracts the proton from L- as well as D-alanine at the same slow exchange rate for both isomers. Therefore, Lys-145 must play an important role in conferring stereochemical integrity to D-amino acid transaminase. However, it is not known at present if BASE II functions in the wild-type enzyme. Although our studies strongly suggest that a second catalytic base is present in the active-site of D-amino acid transaminase, its nature remains to be confirmed. It could conceivably be a water molecule. We have recently shown on the basis of chemical modification studies that Lys-267 of D-amino acid transaminase is labeled by β-mercaptosuccinic acid (18). We are currently investigating whether “BASE II” is this side chain. Having reliable kinetic constants for this enzyme, we can now employ more sophisticated kinetic methods (36) to further investigate the chemistry of D-amino acid transaminase.

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