Partial Reactions of Bacterial D-Amino Acid Transaminase with Asparagine Substituted for the Lysine That Binds Coenzyme Pyridoxal 5′-Phosphate†

Tohru Yoshimura, Mohit B. Bhatia, and James M. Manning*

The Rockefeller University, New York, New York

Dagmar Ringe

Brandeis University, Waltham, Massachusetts

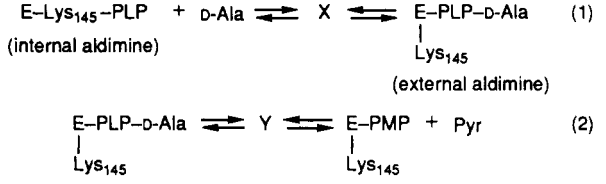
Kenji Soda

Kyoto University, Uji, Kyoto, Japan

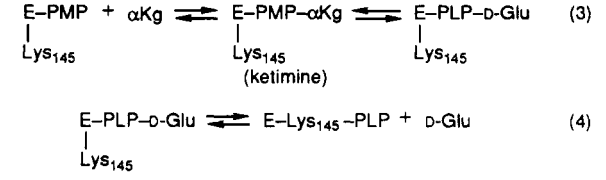
Received April 21, 1992; Revised Manuscript Received September 8, 1992

ABSTRACT: In bacterial D-amino acid transaminase (EC 2.6.1.21) replacement of Lys-145, which is covalently linked to the coenzyme pyridoxal 5′-phosphate in the wild-type enzyme, by an Asn residue gave a mutant enzyme (K145N) that slowly performed each half-reaction, as determined by spectral measurements. With the wild-type enzyme, the kinetics of these events were so rapid that pre-steady-state conditions were needed for their determination. The *internal* aldimine between coenzyme and Lys-145 was rapidly reduced with $NaCNBH_3$ in the wild-type enzyme, whereas in the mutant enzyme the coenzyme, which is not covalently linked to the protein, was more resistant to reduction; the reduced forms of both wild-type and mutant enzymes were inactive. With large amounts of the K145N mutant enzyme and either amino acid or keto acid substrate alone, the formation of some reaction intermediates, i.e., the external aldimine with D-alanine and the ketimine with α-ketoglutarate, can be measured by conventional spectroscopy. Suicide substrates also induced slow spectral shifts of the E–PLP form of the enzyme. For the K145N enzyme, exogenous amines affected only the rate of the transaldimination but not the removal of the α-proton of the substrate. These results suggest that in the mutant enzyme some amino acid side chain other than Lys-145 performs this function. In order to identify this site, the K145N mutant enzyme was completely inactivated by the radiolabeled suicide substrate D-serine. Peptide mapping of tryptic digests showed that Lys-267 was the modified site. Therefore, in the mutant enzyme the ε-NH_2 group of Lys-267 may substitute for the ε-NH_2 group of Lys-145 of the wild-type enzyme to provide an alternate base that catalyzes transamination although with a reduced efficiency.

Bacterial D-amino acid transaminase is a target enzyme for the design of novel antimicrobial agents (Soper & Manning, 1985). Transaminases for D- or L-amino acid substrates contain pyridoxal 5′-phsphate (PLP)[1] as cofactor tightly bound to the protein both by noncovalent interactions and by covalent linkage with a lysine residue at the active site (Arnone et al., 1985; Jansonius et al., 1985). This latter linkage, which is referred to as the internal aldimine, mediates the transfer of the coenzyme to the substrate in a transaldimination reaction to form the external aldimine as shown in eq 1. In the next step (eq 2), the ε-NH_2 group of the lysine residue that was formerly attached to the coenzyme PLP may abstract the α-proton of the amino acid substrate to generate the corresponding α-keto acid after passing through several intermediates. In L-aspartate transaminase, Lys-258 participates in the functions described by eqs 1 and 2. Whether there is an analogous function for the corresponding Lys-145 attached to the coenzyme PLP in D-amino acid transaminase has been a subject of study in this laboratory. Equations 1–2 and 3–4 are referred to as the half-reactions of transamination, i.e., the reaction undergone by one of the two substrate pairs where X and Y represent a series of intermediates in the reaction pathway.



In the second half-reaction of transamination, α-ketoglutarate (αKg), the second of the substrate pairs, binds to the E–PMP form of the enzyme to form the ketimine adduct, which is then transformed to external aldimine with D-glutamate (eq 3). In the final step (eq 4), D-glutamate is released from the enzyme and the internal aldimine is regenerated to complete the transamination cycle:



† This work was supported in part by NSF Grant DMB91-06174. It is dedicated to Prof. Doriano Cavallini on the occasion of his 75th birthday.

* To whom correspondence should be addressed.

[1] Abbreviations: PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; Pyr, pyruvate, αKg, α-ketoglutarate; D-Glu, D-glutamic acid; D-Ala, D-alanine; EDTA, ethylenediaminetetraacetate; NAD, *nicotinamide adenine dinucleotide*; LDH, lactate dehydrogenase; K145N, D-amino acid transaminase in which Lys-145 has been changed to asparagine by site-specific mutagenesis; K145Q; D-amino acid transaminase in which Lys-145 has been changed to glutamine by site-specific mutagenesis.

0006-2960/92/0431-11748$03.00/0

Previously, we described the properties of a mutant D-amino acid transaminase from *Bacillus stearothermophilus* in which the active site Lys-145 was replaced by a glutamine residue (K145Q; Futaki et al., 1990). This mutant enzyme catalyzed the slow turnover of substrates, and it was therefore referred to as an *attenuated* enzyme with which we could evaluate some of the factors that influenced the individual steps of the reaction. For example, in the presence of D-alanine, the addition of exogenous amines increased the rate of the transaldimination reaction but had no effect on the abstraction of the α-proton of substrate or the rate of formation of the pyridoxamine form of the enzyme. Therefore, exogenous amines did not substitute for Lys-145 replaced in the mutant K145Q enzyme. Toney and Kirsch (1989) reported that for the active site K258A mutant of L-aspartate transaminase exogenous amines were able to substitute for the missing Lys-258. Nishimura et al. (1991) found that exogenous amines had different effects on the K145R and K145A active site mutants of D-amino acid transaminase; i.e., they affected neither the residual catalytic activity nor the amount of pyruvate formed in the half-reaction with the K145R mutant enzyme. However, a small amount of catalytic activity was bestowed on the K145A mutant enzyme as ascertained by the amount of pyruvate formed. It thus became apparent that exogenous amines behave differently with individual mutant enzymes.

In this communication, we extend the studies on the effect of exogenous amines to the K145N mutant enzyme, which was constructed in order to evaluate the tolerances near the active site Lys residue in the wild-type enzyme, i.e., the size of the replacements that could be made with retention of ability to perform the half-reactions of transamination. The study of transaminase intermediates on the reaction pathway with attenuated enzymes is another goal. We also address the question whether there is an alternate catalytic base that could substitute for Lys-145, which is absent in the K145N mutant enzyme. Such a base may explain the inherent catalytic activity of the active site mutant enzymes K145Q and K145N. A two-base model has been proposed by Shostak and Schirch (1988) for serine transhydroxymethylase to explain its racemase activity. Phillips et al. (1991) have also described a mutant form of tryptophan indole-lyase that has reduced catalytic potency.

METHODS AND MATERIALS

DNA, Phage, Bacterial Strains, Enzymes, Chemicals, and Oligonucleotide for Site-Directed Mutagenesis. These methods and procedures have been described previously (Martinez-Carrion & Jenkins, 1965; Merola et al., 1989; Tanizawa et al., 1989a,b; Martinez del Pozo et al., 1989a). The oligonucleotide for preparation of the K145N mutant enzyme was synthesized at the Rockefeller University Protein Sequencing Facility on an Applied Biosystems DNA 380B Synthesizer using phosphoramidite chemistry. The changed base, which is located approximately in the center of the sequnce, is underlined.

5′-GTT-CAA-AGA-ATT-AAT-ATC-ACA-TC-3′

The procedures used for preparation of the plasmid for site-directed mutagenesis and for the expression of enzymes using other oligonucleotides have also been described previously (Merola et al., 1989; Tanizawa et al., 1989a,b; Martinez del Pozo et al., 1989a). The presence of the changed base was verified by sequencing the DNA. Sodium cyanoborohydride was obtained from Aldrich and purified before use according to the procedure of Jentoft and Dearborn (1983).

Preparation and Purification of K145N. The procedures used previously for the preparation of the wild-type enzyme and the K145Q mutant enzyme were employed for the K145N enzyme but with the following modifications: the heat treatment was carried out at 51 °C to optimize the yield of enzyme. After the DEAE-Sephadex A-50 step, the enzyme was rechromatographed on the same resin (2 × 20 cm column) equilibrated in 0.01 M potassium phosphate buffer, pH 7.6, containing 50 μM PLP, 0.2 mM EDTA, 0.01% 2-mercaptoethanol, and 0.08 M KCl. The protein was eluted with a linear gradient from 0.08 to 0.13 M KCl. Before use, the enzyme was dialyzed against 50 mM bis-Tris-HCl (pH 7.2) to remove excess PLP. Specific activity is defined as the number of micromoles of pyruvate formed from D-alanine per minute per milligram of enzyme.

Assay for Enzyme Activity in the Presence or Absence of $NaCNBH_3$. Specific activities in the overall reaction with both substrates were measured in a reaction mixture that contained 0.1 M bis-Tris-HCl buffer, pH 7.2, 200 mM D-alanine, 5 mM α-ketoglutarate, 0.2 mM NADH, 8 units of lactate dehydrogenase (LDH, rabbit muscle, Type II), and the appropriate amount of D-amino acid transaminase in a final volume of 1 mL. A Cary Model 2200 spectrophotometer or a Zeiss PMQ2 spectrophotometer, both with cuvettes thermostated at 27 °C, was used for the assay and for measuring the effects of various additives on the rate of transamination.

For measurements of activity vs pH, the reaction mixture (1.0 mL) contained 25 mM each D-alanine and α-ketoglutarate, 1 mM DTT, 0.1 mM NADH, 8 units of lactate dehydrogenase, wild-type enzyme (0.045 μg), or mutant enzyme (K145N, 12 μg; K145Q, 138 μg) in 0.2 M Tris-HCl buffer. The other conditions are the same as described above.

Measurement of Inactivation Rates and Spectral Changes with Suicide Substrates. The effect of various suicide substrates and other inhibitors of the wild-type and K145N mutant enzymes were tested in a reaction mixture (0.5 mL) that contained 5.4 μg of the wild-type enzyme or 500 μg of the K145N mutant enzyme and inhibitors in 0.1 M bis-Tris-HCl, pH 7.2. After appropriate times at 37 °C, a 20-μL portion of the mixture was withdrawn and assayed as described above. For the reaction with 0.1 M D-serine, 0.6 mg of the wild-type enzyme or 2.6 mg of the K145N mutant enzyme in 50 mM bis-Tris-HCl buffer, pH 7.2, was incubated in a final volume of 0.5 mL at 37 °C. At the appropriate times, 50-μL portions of the reaction mixture were withdrawn and diluted 50-fold with 50 mM bis-Tris-HCl (pH 7.2) for assay. The inactivation rate, which is the reciprocal of the half-life of the enzyme inhibitor complex, was calculated from the semilogarithmic plot of remaining activity versus time.

Preparation of PMP Form of the Enzyme with D-Cysteine Sulfinate. The reaction mixture, which contained 0.1 M bis-Tris-HCl (pH 7.2), 20 mM D-cysteine sulfinate (Futaki et al., 1990), 1.5 mM NADH, 1.6 units of LDH, and 5.12 mg (0.16 μmol) of the K145N mutant enzyme, was incubated in 1.0 mL at 37 °C for 17 h. After dialysis against two changes of 50 mM bis-Tris-HCl, pH 7.2, for 8 h at 4 °C, the formation of the PMP form of the enzyme was ascertained by the disappearance of the absorption band around 400 nm and the simultaneous appearance of an absorbance band at 330 nm.

Peptide Mapping of Enzyme Inactivated by D-Serine. The reaction mixture (200 μL) containing 0.1 M bis-Tris-HCl (pH 7.2), 20 mM D-serine, 0.5 mM [3-^{14}C]DL-serine (3.8

μCi), and 5.38 mg of K145N mutant enzyme was incubated by 37 °C for 4 h to achieve nearly complete inactivation. The protein was separated from unreacted D-serine on a Sephadex G-200 column (3 × 40 cm) equilibrated with 50 mM bis-Tris-HCl, pH 7.2. The protein fraction was concentrated to a final volume of 1.8 mL with a Centricon 10 microconcentrator (Amicon) and then reduced with 5 mM $NaCNBH_3$ at 4 °C overnight. After carboxymethylation according to the method of Crestfield et al. (1963) and dialysis against 0.1 M NH_4HCO_3 for 16 h, the protein was concentrated to 0.9 mL as described above and digested with 84 μg (2%) of TPCK-treated trypsin (Sigma type XIII from bovine pancreas) in 0.1 M NH_4HCO_3, pH 8.0, at 37 °C for 18 h. The peptides were separated on Shimadzu LC-6A HPLC equipped with TSK ODS-120A (0.46 × 25 cm) with a linear gradient from 0.1% TFA to 0.1% TFA/50% *n*-propanol (2%–70%) in a volume of 140 mL. The position of the radiolabel was determined by an LKB Model 1218 scintillation counter. Sequence analysis was performed at The Rockfeller University Protein Sequencing Facility.

RESULTS

Activity of the K145N Mutant Enzyme. The K145N mutant enzyme retained some catalytic activity; the specific activity of the purified K145N enzyme was 1.3 units/mg compared to a value of 200 units/mg for the wild-type enzyme and 0.03 units/mg for the K145Q enzyme. In order to exclude the possibility that the activity of the K145N mutant enzyme was due to contamination by traces of endogenous wild-type enzyme, several approaches were taken. First, the specific activities of the K145N mutant enzyme, which was purified to homogeneity from three separate preparations, were practically identical, i.e., 1.20, 1.34, and 1.30 units/mg. Second, the rate of inactivation of the K145N mutant enzyme by five different enzyme inhibitors differed significantly from the inactivation rate of the wild-type enzyme. Third, the mutant enzyme was reduced at a much slower rate than the wild-type enzyme; each reduced enzyme was devoid of activity. Finally, the *complete* spectral shift from 420 nm to 330 nm with a suicide substrate indicated that the mutant enzyme possessed intrinsic activity to catalyze this half-reaction. The experimental details supporting these conclusions are described below.

pH Profile of the Wild-Type, K145Q, and K145N Mutant Enzyme. As shown in Figure 1, the profiles of the pH dependence for the overall transamination reaction in the presence of both substrates (eqs 1–4) catalyzed by the wild-type, the K145Q, and the K145N mutant enzymes were very similar even though their specific enzyme activities differed considerably. These results are consistent with some common reaction mechanism for these three enzymes even though Lys-145 is missing in the mutant enzymes. In order to ensure that buffer effects were not involved in the pH profile, some of the experiments were also performed in potassium phosphate and CHES buffer and gave profiles identical to those in Figure 1.

PLP Content of K145N Mutant Enzyme. The amount of PLP in the K145N mutant was determined in duplicate by the procedure of Wada and Snell (1961); values of 0.84 and 0.85 mol of PLP/mol of enzyme subunit were found.

Spectral Properties of the Active Site Mutant Enzyme. The absorption spectrum of the K145N mutant enzyme where there is no internal aldimine (A_{395}/A_{280} = 0.05; A_{395}/A_{330} = 0.23) was different from that of the wild-type enzyme (Martinez del Pozo et al., 1989), in which Lys-145 forms an

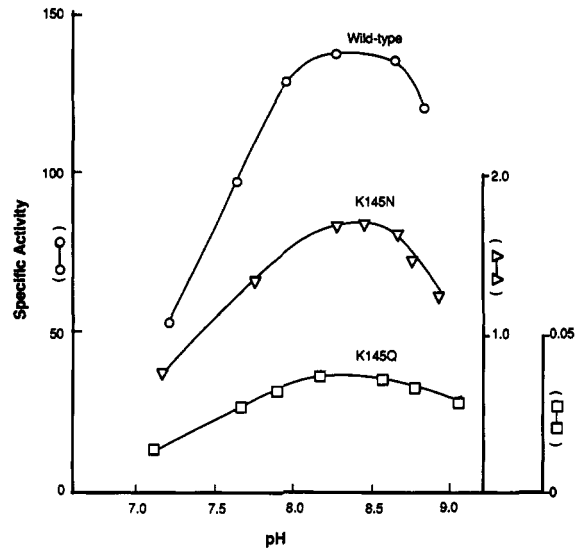


FIGURE 1: pH profiles of the reactions catalyzed by wild-type, K145N, and K145Q mutant enzymes. Each reaction mixture (1 mL) contained the buffer indicated below and 25 mM D-alanine, 25 mM α-ketoglutarate, 1 mM DTT, 0.1 mM NADH, and 8 units of lactate dehydrogenase. The other experimental conditions are described in the text. The buffers employed were 0.2 M potassium phosphate (pH 5.9–7.6), 0.2 M Tris-HCl (pH 7.1–8.9), and 0.2 M CHES (pH 8.2–9.3). Neither the stability of the enzymes nor the efficiency of the lactate dehydrogenase coupling system changed under these conditions.

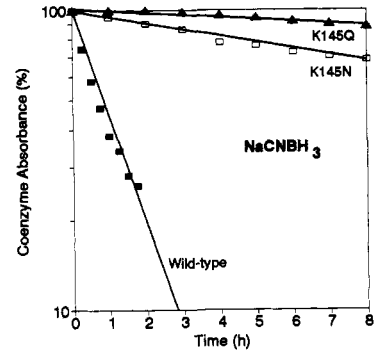


FIGURE 2: Rate of reduction of wild-type K145N and K145Q enzymes. The reactions were carried out in 50 mM bis-Tris, pH 7.2, at 27 °C. For the wild-type and mutant enzymes, a 1-mL reaction mixture contained 1.3 mg of enzyme and 0.25 mM $NaCNBH_3$. For the wild-type enzyme, the decrease at 415 nm was followed, and for the mutant enzymes the decrease at 399 nm was calculated.

internal aldimine with the coenzyme PLP; this structure has an absorption band at 420 nm (A_{420}/A_{280} = 0.26; A_{420}/A_{330} = 2.78).

Effect of $NaCNBH_3$ on the Spectrum of the Wild-Type and K145N Enzymes. The visible spectrum of both the wild-type and the K145N mutant enzymes in the 400–420-nm range was shifted to 300 nm by purified sodium cyanoborohydride to yield the reduced enzyme. However, this shift occurred much faster with the wild-type compared with the mutant enzyme because of the facile reduction of the internal aldimine between Lys-145 and PLP in the wild-type enzyme. For example, in the presence of 0.25 mM $NaCNBH_3$, the wild-type enzyme was reduced with a rate constant of 1.1 h^{-1}, whereas the K145N and K145Q mutant enzymes were reduced at much slower rates (k_{obs} = 0.050 h^{-1} and 0.022 h^{-1}, respectively) (Figure 2). Higher concentrations of $NaCNBH_3$ (5–10 mM) were required to reduce completely the mutant enzymes. This difference demonstrated the intrinsic difference between the wild-type and the mutant enzyme. The completely reduced forms of both wild-type and mutant enzymes were

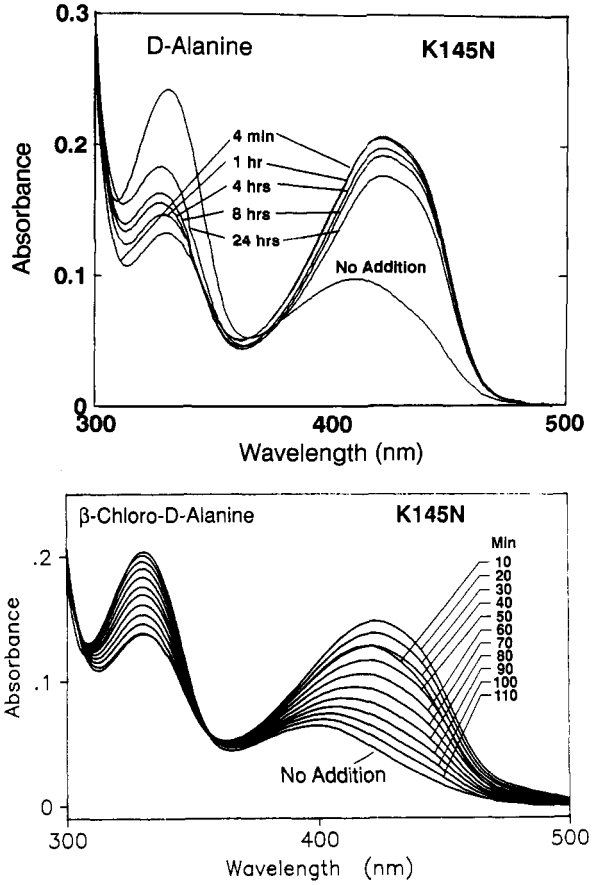


FIGURE 3: (A, top) Effect of D-alanine on the half-reaction with the K145N enzyme. The reaction mixture (final volume of 1 mL) contained 1.3 mg of the K145N mutant enzyme in 50 mM bis-Tris-HCl (pH 7.2) at 27 °C. The reaction was initiated by addition of 25 μL of 1 M D-alanine. The absorption spectra were taken at the indicated times. (B, bottom) Effect of β-chloro-D-alanine on the half-reaction with the K145N enzyme. The reaction mixture contained 50 mM bis-Tris-HCl (pH 7.2), 1.0 mg of K145N enzyme, and 10 mM β-chloro-D-alanine in a final volume of 1.0 mL at 27 °C. The spectra were recorded at the indicated times.

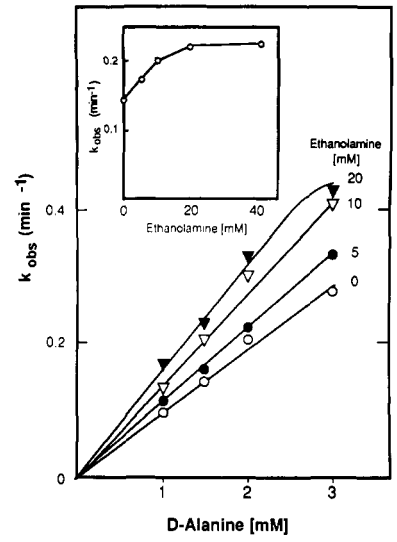


FIGURE 4: Rate of transaldimination as a function of D-alanine and ethanolamine concentrations. Each reaction mixture contained 50 mM bis-Tris-HCl (pH 7.2), 1.3 mg of K145N enzyme, and varying concentrations of ethanolamine in a final volume of 1.0 mL at 27 °C. After a plateau was reached at 408 nm (in about 5 min), the reaction was initiated by addition of D-alanine. The pseudo-first-order rate constant, k_{obs}, was obtained from the rate of the increase of the absorbance at 420 nm. The inset shows the change of k_{obs} against varying concentrations of ethanolamine in the presence of 1.5 mM D-alanine.

inactive. It has been reported (Morino et al., 1990) that the K258R mutant of L-aspartate transaminase was not reduced by $NaCNBH_3$ under conditions where the wild-type enzyme was completely reduced. Hence, the mutant forms of each transaminase are distinctly different in their susceptibility to reduction.

Effects of D-*Alanine* and *β-Chloro-*D-*alanine on the Spectral Properties of the K145N Mutant* D-*Amino Acid Transaminase.* Previous studies have shown that for the wild-type enzyme the rate of formation of the external aldimine with D-alanine is practically instantaneous (Martinez del Pozo et al., 1989) because the transaldimination process is very efficient (eq 1). As shown in Figure 3A, upon addition of D-alanine to the K145N mutant enzyme the absorption band at 395 nm was first shifted to 420 nm due to formation of an external aldimine. Subsequently, this absorbance slowly decreases concomitant with an increase in the absorbance at 330 nm due to formation of the ketimine form of the enzyme.

β-Chloro-D-alanine is an enzyme-activated inhibitor that has been previously studied with D-amino acid transaminase (Soper & Manning, 1985). The slow formation of the external aldimine was readily observed with β-chloro-D-alanine (Figure 3B) by the shift in absorbance from 400 nm to 420 nm in the presence of the suicide substrate. During the next 90 min there was a nearly complete transformation of the 420-nm absorbance band to one at 330 nm with a sharp isosbestic point, consistent with the transformation of one species into another. When this transition is complete, there was about 95% inactivation of the enzyme. These results with normal or suicide substrates (Figure 3A,B) demonstrate the ability of the attenuated mutant enzyme to undergo nearly complete spectral transitions.

Effect of Ethanolamine on the Formation of Some Transamination Intermediates. The slow kinetics of the attenuated K145N enzyme permitted the transaldimination reaction with D-alanine and the subsequent transformation to E–PMP to be studied separately. In the presence of simple amines (not amino acids), a spectral shift from 400 nm to about 408 nm occurred due to formation of an aldimine between the exogenous amine and the coenzyme. Formation of this aldimine enhanced the rate of subsequent transaldimination with D-alanine. With the K145N mutant enzyme, the latter transformation was also slow enough that it can be measured accurately at room temperature on a conventional spectrophotometer; its rate was studied at three different concentrations of ethanolamine, i.e., 5, 10, and 20 mM (Figure 4). Increasing concentration of such amines increased the rate of transaldimination with D-alanine.

The effect of exogenous amines on the formation of E–PMP (eq 2), i.e., on the removal of the α-proton of substrate in the formation of E–PMP, was also evaluated. In the presence of 20 mM ethanolamine, the rate constant for this transformation catalyzed by K145N mutant enzyme was calculated to be 0.215 h^{-1}, similar to the corresponding value of 0.223 h^{-1} in the absence of exogenous amines. Thus, added ethanolamine does not affect the rate of E–PMP formation from the external aldimine, as determined by spectroscopic means.

When the K145N mutant enzyme was assayed with the lactate dehydrogenase-coupled assay system for the *overall* reaction at three concentrations (1.5, 20, and 200 mM) of D-alanine, the rates of pyruvate formation at a given D-alanine concentration did not vary in the presence (20 mM) or absence of ethanolamine. Thus, added ethanolamine affected neither the rate of the complete first half-reaction nor the overall

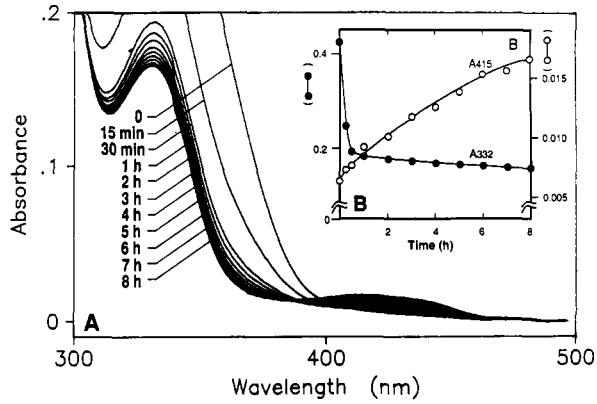


FIGURE 5: Rates of the second half-reactions catalyzed by K145N mutant enzyme. The reaction mixture contained 1.3 mg of PMP form of K145N mutant enzyme in 50 mM bis-Tris-HCl (pH 7.2) in a final volume of 1 mL at 27 °C. The reaction was initiated by addition of 5 μL of 1 M α-ketoglutarate to both the sample and reference cuvettes. The absorption spectra were recorded at the indicated times (A). The changes of the absorbance at 332 nm and 420 nm were plotted against time (B).

Table I: Effect of Suicide Substrates on the Wild-Type and K145N Mutant Enzymes

inhibitors	concn (mM)	inactivation rate (min^{-1}) wild type	K145N
gabaculine	0.02	0.137	0.116
methyl acetyl phosphate	1.0	0.009	0.046
O-carbamyl-D-serine	10.0	0.165	0.131
O-phospho-D-serine	10.0	0.021	0
β-chloro-DL-α-aminobutyric acid	10.0	0	0
DL-2-amino-4-phosphobutyric acid	10.0	0	0
DL-4-fluoroglutamic acid	10.0	0	0
D-serine	100.0	0.046	0.018

reaction catalyzed by the K145N mutant enzyme.

Second Half-reaction Catalyzed by K145N Mutant Enzyme. The slow turnover of substrates with the K145N enzyme also presented the opportunity to examine the overall second half-reaction (eqs 3 and 4). For the wild-type and the K145N enzymes, both the ketimine of α-keto acid linked to E–PMP and the free PMP form of the enzyme (eq 3) absorb near 330 nm. An advantage with the mutant enzyme compared to the wild-type enzyme was that the possible release of the amino acid in the second half-reaction could be ascertained; i.e., it was possible to distinguish between eqs 3 and 4 since the free aldehyde form of the mutant enzyme absorbs at 400 nm, whereas the external aldimine absorbs at 420 nm. In wild-type transaminases, these reactions cannot be studied separately not only because they are so rapid but also because the external and the internal aldimine absorb at the same wavelength.

With the K145N mutant enzyme, the kinetics of these two steps showed that the overall reaction profile was biphasic (Figure 5). The initial, more rapid decrease was complete within 1 h, and the second phase took place much more slowly; after 14 h the overall conversion was 86% complete. Since there was not an increase in A_{420} corresponding to the initial large decrease in A_{330} (Figure 5A), aldimine formation did not occur during this period. Therefore, it is likely that this first phase represented the formation of the ketimine form of the enzyme from E–PMP and ketoglutarate, which probably occurred rapidly because of the high affinity of α-keto acids for the enzyme (Bhatia et al., unpublished results), analogous to the corresponding high affinity of keto acids for the E–PMP form of L-aspartate transaminase. There was the isosbestic

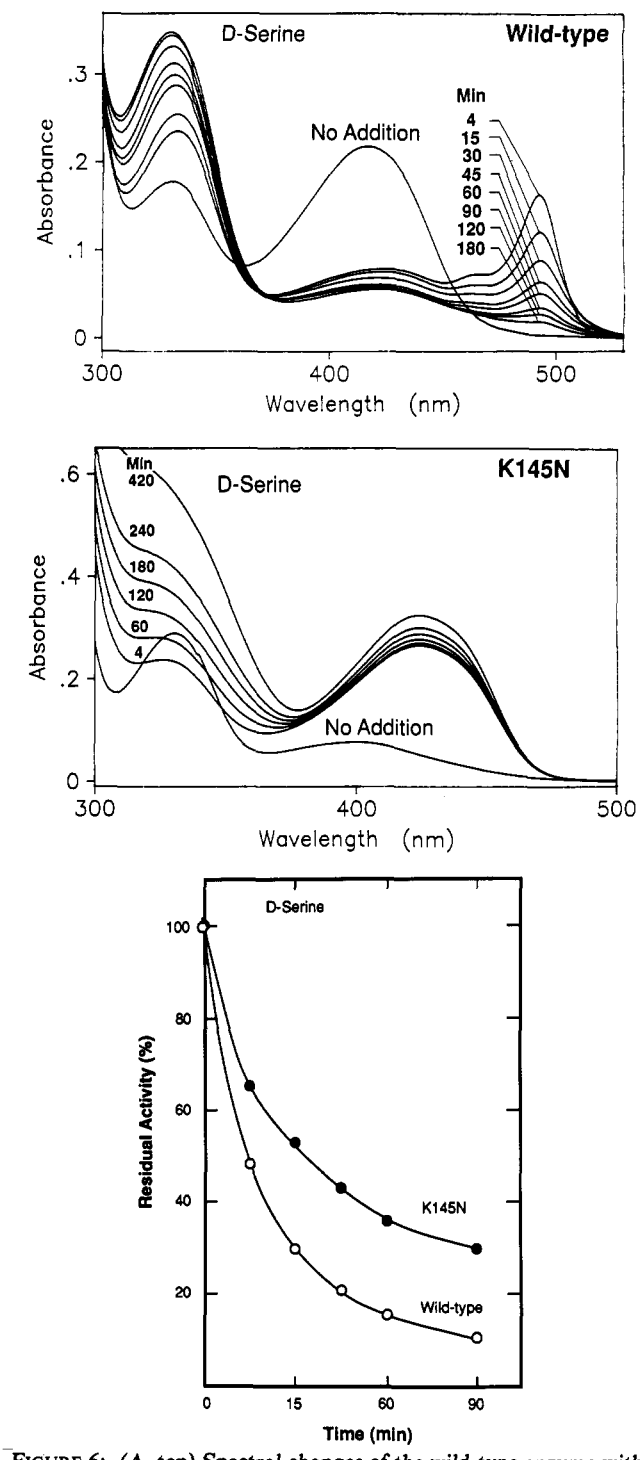


FIGURE 6: (A, top) Spectral changes of the wild-type enzyme with D-serine. The enzyme concentration was 1.35 mg/mL in 50 mM bis-Tris-HCl (pH 7.2) in a 1-mL cuvette thermostated at 27 °C. After addition of D-serine (final concentration, 0.1 M), the spectra were recorded at the indicated times. (B, middle) Spectral changes of the K145 enzyme with D-serine. The experimental conditions are as for part A. (C, bottom) Inactivation of the wild-type and K145N mutant enzyme by D-serine. The experimental details are described in the text.

point that was present only during the second slower phase of the reaction, which was accompanied by a corresponding increase at 420 nm characteristic of the external aldimine (eq 3). This intermediate did not detectably hydrolyze to E–PLP and D-Glu (eq 4) because there was no detectable subsequent spectral shift to the 400-nm absorbance band characteristic of the free PLP form of the K145N enzyme. A possible reaction for this behavior is discussed below.

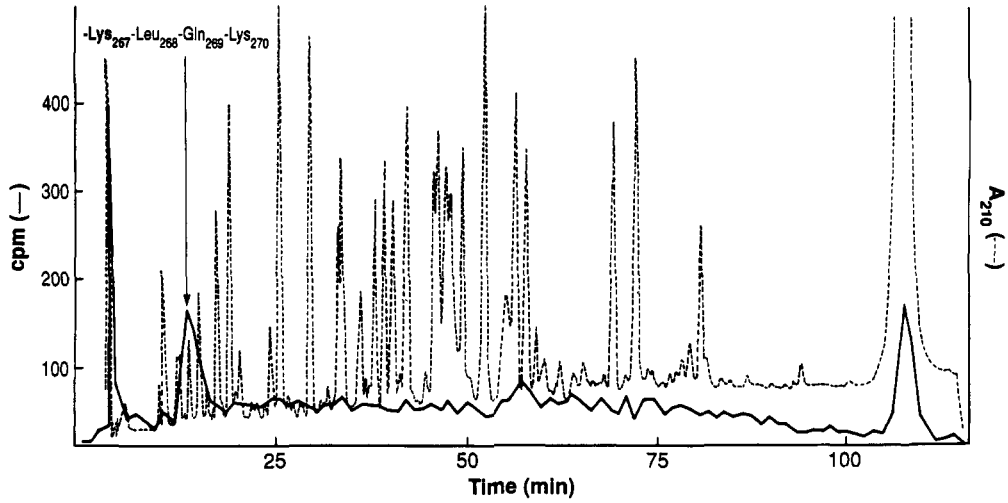


FIGURE 7: Peptide mapping of [^{14}C]D-serine modified K145N mutant enzyme. The flow rate was 1.0 mL/min, and fractions were collected each minute. A 400-μL aliquot of each fraction was subjected to the radioactivity determination. After purification as described in the text, the radiolabeled peptide was subjected to N-terminal sequencing.

Effect of Exogenous Amines on the Second Half-Reaction. For the second half-reaction (eqs 3 and 4) catalyzed by the wild-type enzyme, the rate of change of absorption from 330 to 420 nm was also too rapid to be measured with a conventional spectrophotometer. However, for the K145N mutant enzyme in the presence or absence of 20 mM ethanolamine, the values calculated according to the method of Swinbourne (1960) were 0.171 h^{-1} and 0.181 h^{-1}, respectively (data not shown). Thus, exogenous ethanolamine had no effect on the second half-reaction. Therefore, exogenous amines, which substitute for the active site Lys-258 residue to catalyze the 1,3-prototropic shift with the K258A mutant of L-aspartate transaminase (Toney & Kirsch, 1989, 1992), do not have an analogous role with the K145N active site mutant of D-amino acid transaminase.

Comparison of the Effects of Enzyme-Activated Inhibitors (Suicide Substrates) on Wild-Type and K145N Mutant Enzyme. The possibility that the activity of the K145N mutant enzyme was due to a small amount of contamination by endogenous wild-type enzyme was also examined by comparative studies with several inhibitors of the wild-type enzyme and K145N mutant enzymes. The rationale was that if the activity of the K145N mutant enzyme was due to such contamination, then inactivators should behave in the same fashion with both the wild-type and the mutant enzymes. However, as described next, this was found not to be the case (Table I).

Gabaculine, an efficient suicide substrate that inactivates by coenzyme immobilization (Soper & Manning, 1985), rapidly inactivated both the wild-type and K145N mutant enzyme at different rates (Table I). Methyl acetyl phosphate, an acetylating agent for anion binding regions in proteins (Ueno & Manning, 1989) inactivated the wild-type enzyme at a much slower rate than the mutant enzyme, most likely because acetylation of an important amino group (Lys-145) in the wild-type enzyme was hindered by its internal aldimine structure. In the mutant enzyme, this structure is absent. The inactivation caused by *O*-carbamyl-D-serine was also reversible for the K145N mutant enzyme but irreversible for the wild-type enzyme. With the enzyme-activated inhibitor *O*-phospho-D-serine, the wild-type enzyme was inactivated at a considerably faster rate under conditions whereas the K145N mutant showed barely detectable loss in activity (Table I). On the other hand, with β-chloro-α-aminobutyric acid there was no inactivation of either the wild-type or the K145N mutant enzyme. However, both enzymes were able to catalyze turnover of this amino acid analog to form α-ketobutyrate and ammonia. Neither DL-2-amino-4-phosphobutyric acid nor DL-4-fluoroglutamate caused inactivation of either the wild-type or the K145N enzymes. D-Serine also inactivated both enzymes at significantly different rates (described more fully below), consistent with the intrinsic activity of the K145N mutant enzyme.

Inactivation of K145N Mutant Enzyme with D-Alanine. Recently, we reported that the wild-type enzyme was slowly inactivated by one of its best substrates, D-alanine (Martinez del Pozo et al., 1992). We now find that the K145N mutant was also slowly inactivated by the natural substrate D-alanine. Thus, after about 20 h in the presence of 0.1 M D-alanine in bis-Tris-HCl, pH 7.2, there was about 30%–40% activity remaining with both the wild-type and K145N mutant enzyme.

Inactivation of K145N Mutant by D-Serine and Identification of the Labeled Site. Earlier studies had shown that D-serine is an inactivator of the wild-type enzyme (Martinez del Pozo et al., 1989b); the effects of D-serine on the spectral properties of the wild-type enzyme are shown in Figure 6A. These spectral changes were much different than those found with D-serine and the K145N mutant enzyme (Figure 6B), consistent with the intrinsic differences between these two enzymes. Thus, treatment of the K145N mutant enzyme with D-serine did not give rise to the absorbance band at 493 nm observed with the wild-type enzyme (Figure 6A), but it did give rise to an increased absorbance around 330 nm (Figure 6B) probably due to the formation of hydroxypyruvate formed during suicide inactivation. The rate constant for the inactivation of the wild-type enzyme by D-serine is 0.046 (Table I). On the other hand, the rate constant for the inactivation of the K145N mutant enzyme under the same conditions is 0.018 min. The higher amount of product absorbing at 330 nm (probably hydroxypyruvate) with the mutant enzyme is consistent with its slower inactivation (Figure 6C), which suggests that the partition ratio (rate of conversion to products to rate of inactivation) is higher in the mutant enzyme.

Since D-serine caused 82% inactivation of the mutant enzyme, the question arose as to the nature of the site modified by D-serine. This site was identified by peptide mapping of a tryptic digest of the enzyme inactivated by radiolabeled D-serine. The inactive enzyme contained 1.3 mol of label from [^{14}C]D-serine/mol of carboxymethylated subunit. The peptide maps (Figure 7) indicated that a major labeled peptide

was eluted from the column in addition to the labeled undigested core material that eluted later in the column. This peptide was further purified by rechromatography on the same column but with a gradient from 0.1% TFA to 0.1% TFA/50% *n*-propanol (2%–50%). The identity of this labeled peptide (700 pmol) was established by N-terminal sequencing using the Edman degradation method. The results indicated that the N-terminal residue of this tryptic peptide was modified because it eluted in a position different than that for any of the known PTH-amino acids. In addition, this PTH-amino acid contained most of the radioactivity. The sequence of this tryptic peptide fits the sequence Lys_{267}-Leu_{268}-Gln_{269}-Lys_{270} of D-amino acid transaminase. The lack of cleavage at Lys_{267} by trypsin is further evidence of its modification. Taken together, the data indicate that Lys-267 is the site whose modification by D-serine caused the complete inactivation of K145N mutant enzyme.

DISCUSSION

In the K145N mutant enzyme where there is no internal aldimine between coenzyme and protein, the full complement of coenzyme per subunit was found. Thus, the pyridoxal 5′-phosphate coenzyme was still anchored by noncovalent bonds with sufficient strength that it did not readily dissociate from the enzyme under the conditions used in these studies. The two spectral bands for the K145N mutant enzyme at 408 nm and 330 nm may arise from the free aldehyde form of enzyme-bound PLP and its hydrated form or some other type of adduct, respectively. These forms are likely to be in equlibrium since addition of D-alanine promoted a spectral shift from 330 nm to a corresponding increase in intensity at 420 nm.

Exogenous amines, such as ethanolamine, were able to substitute for Lys-145 in the K145N mutant enzyme to the extent that they form an external aldimine with the coenzyme to accelerate the transaldimination reaction with substrates. However, exogenous ethanolamine does not substitute for Lys-145 as a base for the abstraction of the α-proton of substrates. Exogenous amines were apparently able to abstract the α-proton with other mutant active site enzymes as shown in previous studies with K145A mutant enzymes (Nishimura et al., 1991) as well as in the K258A mutant of L-aspartate transaminase (Toney & Kirsch, 1989, 1992). Thus the exogenous amines act in different ways with mutant enzymes.

The results in the present investigation of the K145N mutant of D-amino acid transaminase as well as the previous studies on the K145Q mutant of this enzyme (Futaki et al., 1990) bring into question the exclusive role attributed by Lys-145 attached to PLP in the catalytic function of the enzyme. This side chain plays an important role not only in the binding of coenzyme, analogous to that of Lys-258 in L-aspartate transaminase. However, as demonstrated in the present study, it also accelerates the process of transaldimination in the wild-type enzyme (eq 1) and subsequently in re-forming the internal aldimine and thereby releasing the amino acid product in the second half-reaction (eq 4). In addition to these functions, the ϵ-NH_2 group of the lysyl residue that binds PLP in L-aspartate transaminase is considered to act as a catalytic base in abstracting an α-hydrogen from the amino acid substrate. However, the K145N mutant enzyme does not contain an active site lysine residue, yet is capable of performing some partial reactions of transamination. Since the shape of the pH dependence curves of the activity of the wild-type, K145Q, and K145N mutant enzymes are nearly the same, some amino acid residue common to these three enzymes acts as a base, although not a particularly efficient one, in these attenuated mutant enzymes. The results of the peptide mapping studies of the K145N mutant enzyme inactivated by [^{14}C]D-serine indicate that Lys-267 plays such a role. Whether ϵ-NH_2 residue of Lys-267 has a function in the wild-type enzyme is not known, but it is possible that it plays an auxiliary role in catalysis. In the absence of Lys-145 in the mutant enzyme, it may become the sole, although less efficient, catalytic residue. Similar studies with other attenuated mutant transaminase enzymes may answer this question.

ACKNOWLEDGMENT

We are grateful to Adelaide Acquaviva for preparing the manuscript and the Maria Pospischil for assistance in some of these studies.

REFERENCES

Arnone, A., Rogers, P. H., Hyde, C. C., Briley, P. O., Metzler, C. M. & Metzler, D. E. (1985) in *Transaminases* (Christen, P., & Metzler, D. E., Eds.) p 138, Wiley, New York.

Crestfield, A. M., Stein, W. H., & Moore, S. (1963) *J. Biol. Chem. 238*, 622–627.

Futaki, S., Ueno, H., Martinez del Pozo, A., Pospischil, M. A., Manning, J. M., Ringe, D., Stoddard, B., Tanizawa, K., Yoshimura, T., & Soda, K. (1990) *J. Biol. Chem. 265*, 22306–22312.

Jansonius, J. N., Eichele, G., Ford, G. C., Picot, D., Thaller, C., & Vincent, M. G. (1985) in *Transaminases* (Christen, P., & Metzler, D. E., Eds.) p 110, John Wiley & Sons, New York.

Jentoft, N., & Dearborn, D. J. (1983) *Methods Enzymol. 91*, 570–579.

Martinez-Carrion, M., & Jenkins, W. T. (1965) *J. Biol. Chem. 240*, 3538–3546.

Martinez del Pozo, A., Merola, M., Ueno, H., Manning, J. M., Tanizawa, K., Nishimura, K., Asano, S., Tanaka, H., Soda, K., Ringe, D., & Petsko, G. A. (1989a) *Biochemistry, 28*, 510–516.

Martinez del Pozo, A., Pospischil, M. A., Ueno, H., Manning, J. M., Tanizawa, K., Nishimura, K., Soda, K., Ringe, D., Stoddard, B., & Petsko, G. A. (1989b) *Biochemistry 28*, 8798–8803.

Martinez del Pozo, A., Yoshimura, T., Bhatia, M. B., Futaki, S., Manning, J. M., Ringe, D., & Soda, K. (1992) *Biochemistry 31*, 6018–6023.

Merola, M., Martinez del Pozo, A., Ueno, H., Recsei, P., Di Donato, A., Manning, J. M., Tanizawa, K., Masu, Y., Asano, S., Tanaka, H., Soda, K., Ringe, D., & Petsko, G. A. (1989) *Biochemistry 28*, 505–509.

Morino, Y., Shimada, K., & Kagamiyama, H. (1990) *Ann. N.Y. Acad. Sci. 585*, 32–47.

Nishimura, K., Tanizawa, K., Yoshimura, T., Esaki, N., Futaki, S., Manning, J. M., & Soda, K. (1991) *Biochemistry 30*, 4072–4077.

Phillips, R. S., Richter, I., Gollnick, P., Brzovic, P., & Dunn, M. F. (1991) *J. Biol. Chem. 266*, 18642–18648.

Shostak, K., & Schirch, V. (1988) *Biochemistry 27*, 8007–8014.

Soper, T. S., & Manning, J. M. (1985) in *Transaminases* (Christen, P., & Metzler, D. E., Eds.) p 266, Wiley, New York.

Swinbourne, E. S. (1960) *J. Chem. Soc. II*, 2371–2372.

Tanizawa, K., Asano, S., Masu, Y., Kuramitsu, K., Kagamiyama, N., Tanaka, H., & Soda, K. (1989a) *J. Biol. Chem. 262*, 2450–2454.

Tanizawa, K., Masu, Y., Asano, S., Tanaka, H., & Soda, K. (1989b) *J. Biol. Chem. 264*, 2445–2449.

Toney, M. D. & Kirsch, J. F. (1989) *Science 24*, 1485–1488.

Toney, M. D. & Kirsch, J. F. (1992) *Protein Science 1*, 107–119.

Ueno, H., & Manning, J. M. (1989) *J. Biol. Chem. 264*, 12344–12351.

Wada, H., & Snell, E. E. (1961) *J. Biol. Chem. 236*, 2089–2095.

Registry No. Asn, 70-47-3; Lys, 56-87-1; PLP, 54-47-7; D-amino acid transaminase, 50864-42-1; ethanolamine, 141-43-5.