Effect of Substitution of a Lysyl Residue That Binds Pyridoxal Phosphate in Thermostable D-Amino Acid Aminotransferase by Arginine and Alanine

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ABSTRACT: Lys-145 of the thermostable D-amino acid aminotransferase, which binds pyridoxal phosphate, was replaced by Ala or Arg by site-directed mutagenesis. Both mutant enzymes were purified to homogeneity; their absorption spectra indicated that both mutant enzymes contained pyridoxal phosphate bound noncovalently. Even though the standard assay method did not indicate any activity with either mutant, addition of an amino donor, D-alanine, to the Arg-145 mutant enzyme led to a slow decrease in absorption at 392 nm with a concomitant increase in absorption at 333 nm. This result suggests that the enzyme was converted into the pyridoxamine phosphate form. The amount of pyruvate formed was almost equivalent to that of the reactive pyridoxal phosphate in the mutant enzyme. Thus, the Arg-145 mutant enzyme is able to catalyze slowly the half-reaction of transamination. Exogenous amines, such as methylamine, had no effect on the half-reaction with the Arg-145 mutant enzyme. In contrast, the Ala-145 mutant enzyme neither underwent the spectral change by addition of D-alanine nor catalyzed pyruvate formation, in the absence of added amine. However, the Ala-145 mutant enzyme catalyzed the half-reaction significantly in the presence of added amine. These findings suggest that a basic amino acid residue, such as lysine or arginine, is required at position 145 for catalysis of the half-reaction. The role of the exogenous amines differs with various active-site mutant enzymes.

D-Amino acid aminotransferase (D-AAT, EC 2.6.1.21)[1] catalyzes transamination of various D-amino acids. The enzyme participates in the metabolism of D-amino acids, several of which are important as constituents of the peptidoglycan layer of bacterial cell walls, peptide antibiotics, and some other biologically active compounds (Meister, 1965). The enzyme has been a target of the antibiotics acting as suicide substrates (Soper et al., 1977a,b; Soper & Manning, 1978). It is essential to elucidate the detailed reaction mechanism and the active-site structure of the enzyme to design and develop specific inhibitors for D-AAT.

Recently, we have isolated a thermophilic *Bacillus* species that produced the thermostable D-AAT abundantly; the enzyme was purified and characterized (Tanizawa et al., 1989b). The gene encoding the thermostable D-AAT has also been cloned to *Escherichia coli* and expressed efficiently, and the primary structure of the enzyme was determined from the nucleotide sequence (Tanizawa et al., 1989a).

The availability of the gene and the large amount of purified enzyme have allowed us to study the relationship between the enzyme function and the structure of the active-site (Merola et al., 1989; Martinez del Pozo et al., 1989a–c). The present paper deals with site-directed mutagenesis studies of the active-site lysyl residue (Lys-145), to which the cofactor PLP is covalently bound (Tanizawa et al., 1989b). The PLP-binding Lys (Lys-258) in aspartate aminotransferase (AspAT) has been postulated to be the α-hydrogen-withdrawing base during catalysis (Snell, 1962, 1985; Dunathan, 1971), a role supported by recent X-ray diffraction results (Arnone et al., 1985). In the present study, we have changed Lys-145 of D-AAT to Arg or Ala, and we have studied the properties of the mutant enzymes in the presence or absence of added amines.

Experimental Procedures

Materials. The plasmid pICT113 carrying the D-AAT gene was prepared as described previously (Tanizawa et al., 1989a). Phage vector M13mp19, restriction endonucleases, T4 DNA ligase, and the Klenow fragment of DNA polymerase I were obtained from Takara Shuzo, Kyoto, Japan. The oligonucleotide-directed in vitro mutagenesis system, consisting of the Klenow fragment, DNA polymerase I, T4 DNA ligase, restriction endonuclease *Nci*I, exonuclease III, a mixture of deoxynucleotides, a deoxynucleotide mixture containing phosphorothioate-modified dCTP instead of dCTP, and *Escherichia coli* strain TG1, was purchased from Amersham. Fluorescent dye labeled M13 primers for automated DNA sequencing, (4′,4′-dimethoxytrityl)deoxynucleotide triphosphoramidites, solid-phase resins, and other chemicals for oligonucleotide synthesis were from Applied Biosystems. Deoxy- and dideoxynucleotide triphosphates were obtained from Boehringer Mannheim. All other chemicals were of the highest purity commercially available.

Site-Directed Mutagenesis. To remove the *Acc*I site in the multiple cloning site of pICT113 (Tanizawa et al., 1989a), which is unfavorable for the site-directed mutagenesis, the plasmid pICT113p was derived from pICT113 by the *Pst*I digestion and religation by T4 DNA ligase (Figure 1). Two oligonucleotides of mutually complementary 13-mers (5′-

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[1] Abbreviations: D-AAT, D-amino acid aminotransferase; PLP, pyridoxal phosphate; PMP, pyridoxamine phosphate; AspAT, L-aspartate aminotransferase; Tris, tris(hydroxymethyl)aminomethane; kbp, kilobase pair(s); ssDNA, single-stranded DNA; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CD, circular dichroism.

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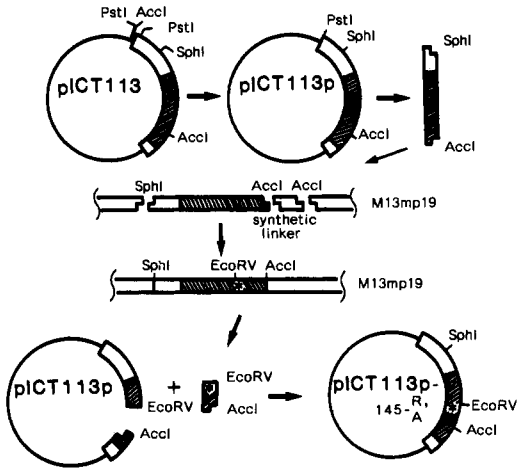

FIGURE 1: Scheme for construction of mutant plasmids, pICT113p-145R and pICT113p-145A. The hatched area and asterisks represent the structural gene of D-AAT and an approximate position of the mutated site, respectively.

ATACTGCAGCGTA-3′ and 5′-CGTACGCTGCAGT-3′) synthesized with an Applied Biosystems 381A DNA synthesizer were used as the linker to join the M13mp19 RF DNA and the 0.95 kbp *Sph*I–*Acc*I fragment of pICT113p. After ligation, the resultant M13mp19 RF DNA containing the *Sph*I–*Acc*I fragment was used to transform *E. coli* TG1. About 0.2 mg of the phage ssDNA was prepared from a 50-mL cuture of the transformant and used as the template for site-directed mutagenesis. Two oligonucleotides were synthesized for mutagenesis: 5′-GTTCAAAGATC*G*-AATATCACATCGT-3′ for replacement of Lys-145 (the underlined codon is the complementary sequence) by Arg and 5′-AGTTCAAAGAT(A*G*C*T)C*AATATCACAT-3′ for replacement of Lys-145 by Ala, Gly, Glu, or Val, where asterisks indicate mismatched bases. Site-directed mutagenesis was carried out by the method of Taylor and Eckstein (1985) using a commercial kit.

Each ssDNA of the mutated phage was prepared and sequenced by the dideoxy chain termination method (Sanger et al., 1977) with an Applied Biosystems 370A automated DNA sequencer (Connell et al., 1987). The mutant M13mp19 DNAs which had the codons in the complementary sequence for Arg (TCG), Ala (TGC), Glu (TTC), Gly (TCC), and Val (TAC) at the position corresponding to Lys-145 (TTT, wild type) were obtained.

The 0.16 kbp *Eco*RV–*Acc*I fragment was excised from each of the mutated phage DNAs and inserted between the *Eco*RV and *Acc*I sites of the plasmid pICT113p (Figure 1). The five mutant plasmids thus obtained were designated as pICT113p-145R, pICT113p-145A, pICT113p-145E, pICT113p-145G, and pICT113p-145V, using the one-letter code for each substituted amino acid residue. Their constructs were confirmed by restriction mapping.

Purification of Mutant Enzymes. Of the five mutant plasmids, pICT113p-145R and pICT113p-145A were used to transform *E. coli* HB101. Both mutant enzymes produced in the recombinant cells were purified according to published procedures (Stoddard et al., 1987) with a minor modification; the gel filtration was carried out with two tandemly connected columns of Superose 12 (Pharmacia) or G3000SW-XL (Tosoh, Tokyo, Japan) instead of Asahi Pack GS-520 column. Each purified enzyme was shown to be homogeneous by SDS–PAGE. Protein concentrations of the wild-type and mutant enzymes were determined with the molar extinction coefficient of 61 000 M^{-1} cm^{-1} at 279 nm, which had been determined previously by amino acid analysis after acid hydrolysis (Tanizawa et al., 1989b).

Spectrophotometric Measurements. Absorption spectra were taken with a Shimadzu UV3000 or MPS2000 recording spectrophotometer, or with a Beckman DU-50 recording spectrophotometer. PLP (10 μM final concentration) was added to both sample and reference cuvettes; PLP bound to the protein absorbs at 333 nm and at 392–415 nm (see below). Unbound PLP was subtracted by the presence of PLP in the reference cuvette.

Assay of Pyruvate Formation. The formation of pyruvate from D-alanine by the wild-type and mutant enzymes was assayed by the lactate dehydrogenase coupled method. The reaction mixture, 0.894 mL in a cuvette, contained 100 mM Tris-HCl buffer (pH 8.1), 25 mM D-alanine, and a 15 μM sample of each enzyme. Then mixture was incubated at 55 °C for 8 min, and then NADH solution was added to a final concentration of 50 μM. After further incubation at 55 °C for 4 min, lactate dehydrogenase (4 units) was added to the cuvette, and the decrease in absorbance at 340 nm was monitored. The amount of pyruvate produced was estimated from the total decrease in the amount of NADH.

RESULTS

Purification and Specific Activity of Mutant Enzymes. SDS–PAGE of extract from the transformant cells harboring plasmids pICT113p–145R or pICT113p-145A showed a predominant protein band with a mobility identical with that of the wild-type D-AAT (32 kDa). Both mutant proteins were purified to near-homogeneity (>95%) on the basis of SDS–PAGE. The amount of enzyme purified from 10 g (wet weight) of cells was usually about 15 mg. The purified K145R and K145A enzymes were immunochemically indistinguishable from the wild-type enzyme when examined by Ouchterlony double-diffusion analysis. Their circular dichroism spectra in the far-UV region were also identical with that of the wild-type enzyme (data not shown). These results demonstrate that essentially no change in the overall conformation of the enzyme protein was elicited by mutation of Lys-145 to Arg or Ala. Any subtle changes are assumed to be confined to the region immediately surrounding the site of the mutation by analogy with the X-ray crystallographic studies of the K258A mutant of *E. coli* AspAT (Smith et al., 1989).

Both K145R and K145A mutants of D-AAT were substantially inactive (1.2×10^{-5} and $<10^{-5}$ of the specific activity of the wild-type enzyme, respectively) under the standard assay conditions, in which a k_{cat} value for the wild-type enzyme was about 180 s^{-1} at 55 °C (Tanizawa et al., 1989b). In contrast, in the presence of the D-amino acid substrate alone (half-reaction of transamination), some activity was found as described below.

Absorption Spectra and PLP Content. Figure 2 shows absorption spectra of the wild-type and K145R and K145A mutant enzymes in the wavelength region of 300–500 nm. The spectra of both the K145R and K145A mutant enzymes indicated that they contain PLP, although the 415-nm peak characteristic of wild-type enzyme is shifted to a shorter wavelength (about 400 nm) in the spectra of mutant enzymes. In addition, the 333-nm peak in the spectra of mutant enzymes was considerably higher than that in the 400-nm region as compared to that of the wild-type enzyme. The 415- and 400-nm region absorbance bands of the wild-type and mutant enzymes, respectively, are considered to be due to the internal aldimine in the wild-type enzyme and the free aldehyde form in the mutant enzymes (see below). The 333-nm absorbance band for these enzymes in the absence of substrate increases

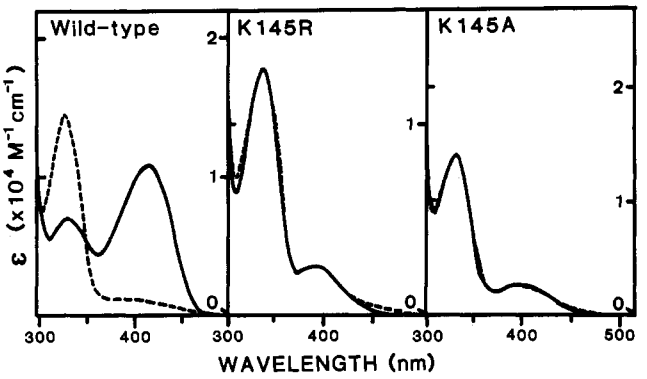

FIGURE 2: Absorption spectra of the wild-type D-AAT and K145R and K145A mutant enzymes before (—) and after (---) reduction with 2 mM $NaCNBH_3$. The protein concentrations (as determined by amino acid analysis after acid hydrolysis for the wild-type enzyme and by the 279-nm absorbance reading for the mutant enzymes) were 2.2 (wild type), 1.9 (K145R), and 0.82 mg/mL (K145A) in 50 mM potassium phosphate buffer, pH 7.5, containing 10 μM PLP (in both sample and reference cuvettes). The reduction was carried out at 0 °C for 12 h.

Table I: Spectrophotometric Properties and PLP Content of Wild-Type and Mutant Enzymes

			PLP content (per subunit)		
enzyme	absorption maximum (nm)	ε (M^{-1} cm^{-1} $dimer^{-1}$)	before phenylhydrazine treatment (*A*)	after phenylhydrazine treatment[a] (*B*)	*A* − *B*
wild type	279	61000			
	333	5700	0.93	0.13	0.80
	415	11500			
K145R	279	61000			
	333	13000	0.98	0.47	0.51
	392	3700			
K145A	279	61000			
	333	14000	0.43	0.16	0.27
	398	4800			

[a] The enzyme (0.2 mg of each, determined as described under Experimental Procedures) was incubated with 10 mM phenylhydrazine in 50 mM potassium phosphate buffer, pH 7.5, containing 0.005% 2-mercaptoethanol in a final volume of 0.1 mL at 37 °C for 90 min and dialyzed against 2000 volumes of 50 mM potassium phosphate buffer, pH 7.5, at 4 °C for 21 h.

with the age of the preparation and is considered to be due to an unknown complex at the active site that renders it resistant to derivatization with phenylhydrazine (see below). The formation of this absorbance band may correspond with the low activity of the enzyme. This complex may be analogous to the inactive γ-subform of L-aspartate aminotransferase (Martinez-Carrion et al., 1967). However, the structure of the inactive form of 333-nm PLP cannot be a substituted aldamine, carbinolamine, or nonionic hydrogen-bonded Schiff base proposed for the semiapo form of L-lysine-α-ketoglutarate ε-aminotransferase (Misono & Soda, 1977) since the ε-amino group of Lys-145 is absent in K145R or K145A mutant of D-AAT. The molar extinction coefficients of these enzymes are summarized in Table I. The wild-type enzyme shows a negative CD band at 418 nm due to the bound PLP (Tanizawa et al., 1989b). However, in the CD spectra of mutant enzymes, no Cotton effects were observed in the 400-nm region (data not shown). This finding suggests that the mutant enzymes differ somewhat from the wild-type enzyme in the structure of the PLP-binding site. The absorption peak at 333 nm was also slightly optically active and showed a negative CD band in the spectra of wild-type (Tanizawa et al., 1989b) and mutant enzymes.

When the wild-type enzyme was treated with $NaCNBH_3$, which does not readily reduce a free aldehyde group but does reduce a Schiff base (Lane, 1975), the absorption at 415 nm was diminished in parallel with an increase in the absorption at about 333 nm. This result is consistent with reduction of the internal Schiff base formed between the aldehyde group of PLP and the ε-amino group of Lys-145 in the wild-type enzyme. In contrast, treatment with $NaCNBH_3$ had no effect on the spectra of K145R and K145A enzymes (Figure 2). This result is consistent with the idea that PLP is bound as the free aldehyde (about 400-nm form) in the K145R and K145A mutant proteins.

The total amounts of PLP bound to each enzyme species absorbing at both 333 nm and 392–415 nm were measured by the KCN–fluorophotometric method (Bonavita, 1960) (Table I). To analyze the amount of PLP bound in a reactive form, this measurement was done with enzyme samples before and after treatment with phenylhydrazine (Table I). Treatment of the wild-type holoenzyme with phenylhydrazine has been shown to resolve it into the inactive "semiapo" form, which absorbs at 333 nm [Tanizawa et al., 1989b; cf. Yonaha et al. (1975)]. PLP bound in an active state (415-nm absorption band) is released by phenylhydrazine. The phenylhydrazine-treated K145R and K145A mutant enzymes showed little absorption at about 400 nm and about 30% lower absorbance at 333 nm than that in the native enzyme. The mutant enzymes treated with phenylhydrazine resemble the semiapo form of the wild-type enzyme. The difference between the total PLP content before and after phenylhydrazine treatment is considered to represent the PLP bound in an active mode. These estimates range from 80% for the wild-type enzyme to 51% for the K145R mutant enzyme and 27% for the K145A mutant enzyme (Table I).

Spectral Change upon Addition of D-*Alanine.* Figure 3 shows spectrophotometric changes caused by addition of D-alanine to the wild-type, K145R, and K145A enzymes. The rate of decrease of the absorption of wild-type enzyme at 415 nm and the rate of the increase at 333 nm were too rapid to measure their rate constants with an ordinary spectrophotometer. This spectral shift suggests that the PLP form of enzyme was converted into the PMP form by the half-reaction of transamination. The addition of D-alanine to the K145R mutant enzyme also resulted in a similar spectral change, i.e., a decrease in absorbance at 392 nm and an increase at 333 nm. However, the spectral shift is very slow since it occurs over a period of minutes; the apparent rate constant estimated from an increase in absorbance at 333 nm by a Guggenheim plot is 3.3×10^{-3} s^{-1} (Table II). During the shift from 392 to 333 nm, we observed a transient formation of a small absorption peak in the 500-nm region, which disappeared after a period of several minutes (Figure 3b). This 500-nm absorption is very similar to that observed for the reaction of wild-type D-AAT with D-alanine under the conditions where the enzyme concentration is relatively high (Martinez del Pozo et al., 1989b), and has been assigned to the quinonoid intermediate formed during conversion of the PLP form to the PMP form. When the K145A mutant enzyme was used, no spectral change indicative of PMP formation occurred even over a longer period than that used for the K145R mutant enzyme (Figure 3c). The shift of the absorption peak from 398 to 420 nm is probably due to the formation of an external Schiff base with D-alanine. In the absence of enzymes, we could not detect any spectral changes with or without amines (Table II).

Formation of Pyruvate. The spectral change of the PLP bound to the K145R mutant enzyme upon addition of D-ala-

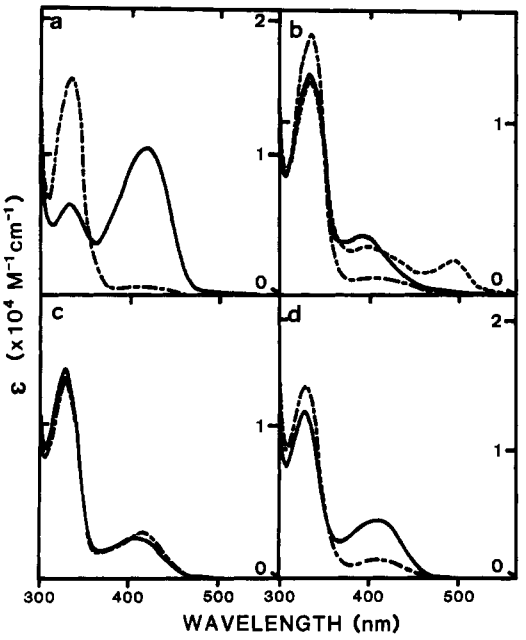

FIGURE 3: Spectral changes of the wild-type D-AAT and K145R and K145A mutant enzymes upon addition of D-alanine. The spectra were taken in 50 mM potassium phosphate buffer, pH 7.5, containing 10 μM PLP (in both sample and reference cuvettes). (a) Spectra of the wild-type enzyme (4.2 mg/mL) (—) before addition of D-alanine and (-·-) 5 min after addition of 20 mM (final) D-alanine. (b) Spectra of K145R mutant enzyme (2.9 mg/mL) (—) before addition of D-alanine, (---) immediately after addition of 20 mM (final) D-alanine, and (-·-) 10 min after addition. (c) Spectra of K145A mutant enzyme (4.2 mg/mL) (—) before addition of D-alanine and (-·-) 1 h after addition of 25 mM (final) D-alanine. (d) Spectra of K145A mutant enzyme (1.2 mg/mL) in the presence of 30 mM ethylamine (—) before addition of D-alanine and (-·-) 90 min after addition. The protein concentrations used to determine the molar extinction coefficients were measured as described in the legend in Figure 2.

Table II: Half-Reaction Catalyzed by Wild-Type and Mutant Enzymes

enzyme	pyruvate formation[a] (mol/mol of subunit)	k_{app}[b] ($\times 10^{-3}$ s^{-1})
wild type	0.65	nd[c]
K145R	0.24	3.3
K145A	0.0	0.0
	0.12[d]	9.8[d]
none	nd[c]	0.0[e]
	nd[c]	0.0[f]

[a] Determined with lactate dehydrogenase (see Experimental Procedures). [b] The apparent rate constant determined from an increase in absorption at 333 nm by a Guggenheim plot (Guggenheim, 1926). The reaction mixture (0.4 mL) contained 40 μmol of Tris-HCl buffer, pH 8.0, 4 nmol of PLP, and 0.4 mg of enzyme. The reaction was started by addition of 1.2 μmol of D-alanine. [c] Not determined. [d] Determined in the presence of 200 mM ethylamine. [e] The reaction mixture (0.1 mL) contained 10 μmol of potassium phosphate buffer (pH 7.5) and 5 nmol of PLP. The reaction was started by addition of 5 μmol of D-alanine and performed at 25 °C. The other conditions were the same as described for *b*. [f] Determined in the presence of 100 mM ethylamine or methylamine.

nine indicates that K145R catalyzes the half-reaction of transamination. This suggestion was confirmed by measuring the formation of pyruvate from D-alanine with lactate dehydrogenase. As summarized in Table II, the wild-type and K145R mutant enzymes produced 0.65 and 0.24 mol of pyruvate/mol of subunit, respectively. The K145A mutant enzyme produced no pyruvate, in agreement with the absence of a spectral change on addition of D-alanine as described above. The amounts of pyruvate produced by the wild-type and K145R mutant enzymes correspond to about 80 and 50%,

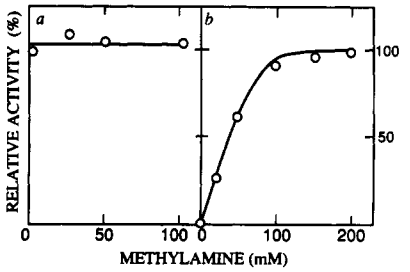

FIGURE 4: Effect of methylamine on the half-reaction of K145R (a) and K145A (b) mutant enzymes. The apparent rate constants were determined as described in the legend to Table II. The relative activity was derived from the apparent rate constant. The rate constant in the presence of 0 or 200 mM methylamine was used as 100% of activity of K145R or K145A mutant enzyme, respectively. The reaction mixture (0.07 mL) contained 100 mM potassium phosphate buffer, pH 7.5, 0.68 mg/mL enzyme, and methylamine (0, 20, 50, or 100 mM K145R; 0, 20, 50, 100, 150, or 200 mM K145A). The reaction was started by addition of D-alanine (8 mM, final, K145R; 2 mM, final, K145A).

respectively, of the content of the phenylhydrazine-reactive PLP in each enzyme (cf. Table I). Considering that the amounts of pyruvate formed may be underestimated by the coupled assay method with a limited concentration of NADH, it is conceivable that the half-reaction proceeded stoichiometrically with regard to the amount of reactive PLP bound to each enzyme. Alternatively, some of the pyruvate may not be readily released from the ketimine form of the mutant enzymes. The results suggest that a basic amino acid residue, such as lysine or arginine, at position 145 is required for the enzyme to catalyze the half-reaction.

Effect of Added Amine on the Half-Reaction of Transamination. Addition of ethylamine to the K145A mutant enzyme had marked effects on both the spectral change by D-alanine (Figure 3d) and the formation of pyruvate (Table II). The absorption maximum of the K145A mutant enzyme at 392 nm was changed to 412 nm by the addition of ethylamine. The observed decrease in absorption at 412 nm by the addition of D-alanine, with a concomitant increase in absorption at 333 nm and formation of a significant amount of pyruvate, approximates the results observed for the K145R mutant enzyme in the absence of exogenous ethylamine. Such activation by exogenous amines was also reported in the half-reaction catalyzed by the inactive K258A mutant enzyme of *E. coli* AspAT (Toney & Kirsch, 1989). A study of the dependence of methylamine concentration (Figure 4b) indicates that increasing concentrations up to 100 mM accelerate the rate of the half-reaction with the K145A mutant enzyme. A study of the effect of added amine of the formation of the external aldimine (Figure 5b) clearly shows both the spectral shift with methylamine alone (due to formation of an external aldimine) and the formation of an external aldimine complex with 2-methylalanine (2-amino-2-methylpropanoic acid). In this case, however, a fairly large concentration of the poor pseudosubstrate is required, and the acceleration by methylamine is effective at a lower concentration (Figure 5d). The addition of methylamine to the K145R mutant enzyme has no effect on either the rate of the half-reaction (Figure 4a) or the rate of formation of the external aldimine with the pseudosubstrate 2-methylalanine (Figure 5c). These results indicate that the exogenous amines function differently with the two mutant enzymes.

Discussion

In all the PLP-dependent enzymes so far studied, the cofactor is bound to an active-site lysyl residue through a Schiff

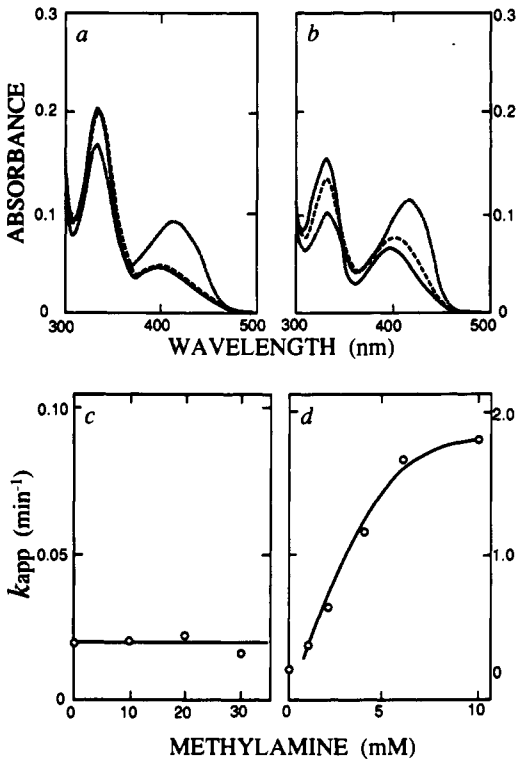

FIGURE 5: Spectral changes of K145R and K145A mutant enzymes upon addition of 2-methylalanine in the presence of methylamine (a, b) and effect of methylamine on the formation of external aldimine between K145R or K145A mutant enzyme and 2-methylalanine (c, d). Spectra of the mutant enzymes (0.90 mg/mL) were taken in 100 mM potassium phosphate buffer, pH 7.5. (a) Spectra of K145R mutant enzymes (—) before addition of methylamine, (- - -) 6 min after addition of 20 mM (final) methylamine, and (—·—) 40 min after of addition of 50 mM (final) 2-methylalanine. (b) Spectra of K145A mutant enzymes (—) before addition of methylamine, (- - -) 3 min after addition of 20 mM (final) methylamine, and (—·—) 3 min after of addition of 50 mM (final) 2-methylalanine. The apparent rate constant was determined from an increase in absorption at 425 nm by a Swinbourne plot (Swinbourne, 1960). The reaction mixture (0.055 mL) contained 100 mM potassium phosphate buffer, pH 7.5, 0.90 mg/mL enzyme, and methylamine [(c) 0, 10, 20, or 30 mM K145R; (d) 0, 1, 2, 4, 6, or 10 mM K145A]. The reaction was started by addition of 50 mM (final) 2-methylalanine.

base linkage [Fisher et al., 1958; Hughes et al., 1962; Dunathan, 1971; see also Morino and Nagashima (1984)]. It has been believed that the ε-amino group of the lysyl residue is indispensable not only for the covalent binding of PLP but also for enhancement of the reactivity of the 4′-aldehyde group of PLP by forming the Schiff base; the formation of an external Schiff base with a substrate by transaldimination is several orders of magnitude faster than the direct formation of that from the free aldehyde by condensation (Cordes & Jencks, 1962). Furthermore, the liberated ε-amino group of a PLP-binding lysyl residue has been postulated to act in the subsequent reaction as a catalytic base withdrawing an α-hydrogen from the substrate moiety of the external Schiff base (Snell, 1962, 1985; Dunathan, 1971; Arnone et al., 1985). The essentiality of the PLP-binding Lys-258 of AspAT was later established by site-directed mutagenesis studies (Malcolm & Kirsch, 1985; Kuramitsu et al., 1987; Ziak et al., 1990). In the present study, the effects of replacement of the PLP-binding Lys-145 of D-AAT by Arg and Ala are in general agreement with the finding for AspAT.

We could detect no activity of either the K145R and the K145A mutant enzymes in the overall reaction when assayed by the standard method in the presence of the both substrates, but considerable activity of the K145R mutant enzyme for the half-reaction with D-alanine was found from the spectral change and the account of pyruvate formation. Since we have not performed stopped-flow kinetic studies to determine the rate constant for the half-reaction by the wild-type enzyme, we cannot compare the rate of half-reaction catalyzed by the K145R mutant enzyme with that by the wild-type enzyme. The catalytic efficiency of the K145R mutant enzyme (3.3×10^{-3} s^{-1}) is 10^5-fold lower than that of *E. coli* AspAT (530 s^{-1}; Inoue et al., 1989) whose rate constant for the overall reaction (220 s^{-1}; Inoue et al., 1989) is comparable with that of the wild-type D-AAT (180 s^{-1}).

The 415-nm absorption peak of the wild-type enzyme derived from the internal Schiff base with Lys-145 was shifted to a slightly shorter wavelength (about 400 nm) by replacement of Lys-145 by Arg or Ala. Since the spectra of the mutant enzymes were not affected by $NaCNBH_3$ reduction, the internal Schiff base is not present in the mutant enzymes. This is probably the reason for the low catalytic efficiency of the K145R mutant enzyme. The interal Schiff base presumably facilitates the transaldimination step as postulated by Sizer and Jenkins (1963), Snell (1985), and Cordes and Jencks (1962) and later shown by Arnone et al. (1985) on the basis of X-ray structures of porcine heart AspAT. In addition, the accounts of reactive PLP in the mutant enzymes are lower than in the wild-type enzyme (Table I).

The K145A mutant enzyme catalyzed the half-reaction only in the presence of exogenous amines. Such a finding was first demonstrated for the mutant enzyme in which an alanyl residue was substituted for PLP-binding Lys-258 of *E. coli* AspAT and considered as a chemical rescue of an inactive mutant protein of aminotransferase (Toney & Kirsch, 1989). It has been suggested that the free amino group of the lysyl residue liberated by transaldimination probably acts as a catalytic base abstracting the α-hydrogen from the external Schiff base (Snell, 1962, 1985; Dunathan, 1971; Arnone, 1985). At present, we have no information how amines function to abstract the α-hydrogen of the substrate in the presence of the K145A mutant enzyme. However, we observed that the absorption maximum of the spectrum of the K145A mutant enzyme at 398 nm shifts to 412 nm both in the presence of 200 mM ethylamine (Figure 3d) and in the presence of 20 mM methylamine (Figure 5b). This finding suggests that a Schiff base between the added amines and the enzyme-bound PLP is formed. In addition, the exogenous amines added to the K145Q mutant enzyme of D-AAT affect the rate of formation of the external aldimine with D-alanine, but not the rate of the half-reaction of transamination (Futaki et al., 1990). Thus, the amines facilitate the half-reaction for some active-site mutant enzyme through external aldimine formation and transaldimination, but for other mutant enzymes (i.e., K145R) they have no effect. Thus, function studies are required to elucidate this mode of action.

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