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An Anomalous Side Reaction of the Lys303 Mutant Aromatic L-Amino Acid Decarboxylase Unravels the Role of the Residue in Catalysis[1]

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Aromatic L-amino acid decarboxylase (AADC) in which the pyridoxal 5′-phosphate (PLP)-binding residue Lys303 was replaced by an alanine residue is virtually inactive as a catalyst. On reaction of the normal substrate L-dopa with this mutant AADC, the absorption at around 330 nm gradually increased with concomitant decrease of the absorption of the free PLP molecule at 390 nm. Analysis of the 330-nm absorbing species on HPLC and spectrophotometry showed that it is a 1:1 adduct of PLP and dopamine, probably the Pictet-Spengler type adduct formed from the PLP-dopamine Schiff base. The product dopamine was not found outside of the enzyme, showing that the PLP-dopamine Schiff base undergoes adduct formation rather than yields product dopamine. The Pictet-Spengler adduct of PLP with L-dopa was not detected, whereas the corresponding adduct was formed when the carboxylate group of L-dopa was esterified with a methyl group to block the decarboxylation reaction. This suggests that the PLP-L-dopa Schiff base may undergo the Pictet-Spengler reaction, but its rate is much smaller than that of decarboxylation. These results indicate that Lys303 is not essential for the decarboxylation step, but has an important role in the product release, and possibly the transaldimination process.

Key words: aromatic L-amino acid decarboxylase, L-dopa, dopamine, Pictet-Spengler reaction, pyridoxal 5′-phosphate.

Amino acid decarboxylases form a large group in the pyridoxal 5′-phosphate (PLP)-dependent enzymes (*1*). The reaction mechanism of the decarboxylases is unique in that it involves the C(α)-C bond cleavage rather than the C(α)-H bond cleavage (*2*); the latter is the critical reaction of most other PLP enzymes. In many PLP enzymes that involve the C(α)-H bond cleavage, replacement by other amino acid residues of the lysine residue that forms a Schiff base with PLP results in dramatic decrease in the catalytic ability, and it is considered that the ε-amino group of the lysine residue, which is liberated on formation of the PLP-substrate amino acid Schiff base, acts as a general base catalyst that accepts the C(α) proton (*3-7*). In decarboxylases, mutation on the PLP-binding lysine residue also greatly decreased the catalytic ability (*8, 9*). However, chemical considerations tell that the decarboxylation step does not apparently require the activation of α-proton (*9, 10*). Therefore, the precise role of the PLP-binding lysine in decarboxylases has remained obscure. Rat liver aromatic L-amino acid decarboxylase (AADC) has been successfully expressed in *Escherichia coli* (*10*), and several active-site mutant enzymes have been prepared (*9*). Therefore, this enzyme is ready for structural and functional analysis. As a step to clarify the function of the PLP-binding lysine residue in decarboxylases, we analyzed the catalytic reaction of AADC in which the PLP-binding lysine residue was replaced by an alanine residue. During this study, we found an anomalous side reaction in this mutant enzyme, which turned out to have important implications for the role of the lysine residue.

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Abbreviations: AADC, aromatic L-amino acid decarboxylase [EC 4.1.1.28]; Dopa, 3-(3,4-dihydroxyphenyl)-alanine; DopaOMe, Dopa methyl ester; DA, dopamine; K303A AADC, AADC in which the residue Lys303 was replaced by an alanine residue; PLP, pyridoxal 5′-phosphate; PLP<Dopa, Pictet-Spengler adduct of L-dopa with PLP; PLP<DA, Pictet-Spengler adduct of dopamine with PLP; WT AADC, wild-type AADC.

EXPERIMENTAL PROCEDURES

Chemicals—L-Dopa and dopamine hydrochloride were obtained from Nacalai Tesque (Kyoto). L-Dopa methyl ester hydrochloride was from Sigma (St. Lois, MO). All other chemicals were of the highest grade commercially available. The medium used for the bacterial growth contained 0.5% yeast extract (Oriental Yeast, Tokyo), 1% peptone (Nihon Pharmaceutical, Tokyo), and 0.5% NaCl at pH 7.4.

Preparation of the Wild-Type and Mutant AADCs—The K303A AADC was prepared as described previously, using pKKAADCII (pKK233-2 carrying rat AADC cDNA)-*E. coli* MD55 expression system (*9, 10*). The transformed cells were cultivated in 10 liters of the growth medium containing 50 μg/ml ampicillin for 24 h at 37°C. About 30 g of wet weight of cells were harvested. The wild-type (WT) and K303A AADCs were purified as described previously (*9*), and usually 80 mg (WT) and 20 mg (K303A) of the

purified enzyme proteins were obtained.

The K303A AADC preparation just after the final step of purification showed an absorption maximum at 410 nm, probably due to the enzyme-bound Schiff base(s) between PLP and amino acid(s) present in the *E. coli* cells used for the expression of the enzyme. A similar binding of PLP-amino acid Schiff base(s) to the enzyme has been observed with the preparation of aspartate aminotransferase in which the PLP-binding Lys258 had been replaced with alanine (*3*). Therefore, we resolved PLP from K303A AADC by derivation to phenylhydrazone according to the method described previously (*10*), and reconstituted the apoenzyme with 5-fold molar excess of PLP. We used this reconstituted enzyme throughout the study. The reconstituted enzyme showed absorption at 400 nm and at 330 nm (see "RESULTS"). The bound PLP was not resolved from K303A AADC when 1 ml of 10 μM enzyme solution was subjected to gel-filtration (Sephadex G-25, 15×50 mm).

Spectrophotometric Measurements—Absorption spectra were taken on a Hitachi spectrophotometer U-3300. The buffer solution for the absorption measurements contained 50 mM PIPES-NaOH, pH 7.0.

HPLC Analysis of the Reaction Products—The products formed during the reactions of WT and K303A AADCs with L-dopa and its derivatives were analyzed by HPLC as follows. Liquid chromatography was performed on a Cosmosil $5C_{18}$ column (4.6 mm×250 mm, Nacalai Tesque, Kyoto) with a mobile phase of 50 mM potassium phosphate buffer, pH 2.2, containing 50 mM sodium perchlorate at a flow rate of 0.5 ml/min. The eluate from the column was neutralized (pH 7.0) by mixing with 0.5 M potassium phosphate buffer, pH 7.5, which was supplied at a flow rate of 0.1 ml/min. The mixed flow was monitored for its absorption at 215-400 nm at 2-nm intervals using a Beckman 168 diode array detector.

Protein Concentration—The concentration of the purified AADCs was determined spectrophotometrically using a molar extinction coefficient of $\varepsilon_M = 79{,}000\ M^{-1}\cdot cm^{-1}$ at 280 nm (*9, 10*).

RESULTS

Absorption and Fluorescence Spectra of K303A AADC—The absorption spectrum of K303A AADC at pH 7.0 is shown in Fig. 1. WT AADC has a large absorption band at 335 nm and a small one at 425 nm, which are ascribed to the enolimine and ketoenamine forms, respectively, of the PLP-Lys303 Schiff base (*10*). On the other hand, K303A AADC has absorption bands at around 330 and 400 nm with almost similar intensity. Like the spectrum of WT AADC, no apparent pH dependency was observed for the spectrum of K303A AADC. The absorption spectrum of the apo form of the enzymes, prepared by removing PLP as a phenylhydrazone, is also shown in Fig. 1. The apo enzyme still had a significant absorption at over 300 nm, gradually decreasing with increasing wavelength. The pattern is quite similar to that of the apo form of WT AADC, and some PLP derivative attached to the AADC protein at a site other than the active site is considered to be the origin of this absorption (*10*). The difference spectrum of the holoenzyme and the apoenzyme is shown in the inset in Fig. 1. This spectrum, with absorption peaks at 397 nm and at 328 nm, shows the "true" absorption spectrum of PLP, which binds reversibly to the active site of AADC. Lys303 of AADC has been shown to form a Schiff base with the aldehyde group of PLP, which has an absorption maximum at 335 and 425 nm (*9, 10*). Free PLP has an absorption maximum at 390 nm. Therefore, the 397 nm absorption can reasonably be ascribed to the PLP molecule present in the active site of K303A AADC without forming a Schiff base with the protein lysine residue. This is in accordance with the observation on the other mutant PLP enzymes, such as aspartate aminotransferase (*3*), tryptophan synthase (*4*), and D-amino acid aminotransferase (*5, 6*), and glutamate-*l*-semialdehyde aminotransferase (*7*), in which the PLP-binding lysine residues were replaced by other amino acid residues. On the other hand, the structure of the 328-nm absorbing species is less clear. To clarify the nature of this species, we measured its fluorescence spectra (Fig. 2). On excitation at 328 nm, the holo form of K303A AADC showed an intense and sharp emission band at 383 nm, which was essentially absent in the apoenzyme. This is a strong indication that the 328-nm absorbing species has an sp^3 structure at C4′ of the coenzyme (*10*). WT AADC has broad emission bands at 520 nm on excitation at either 335 nm or at 425 nm, and these are ascribed to the enolimine and ketoenamine structures of the Lys303-PLP Schiff base (*10*). K303A AADC showed no emission band at 520 nm on excitation at 328 nm, and had emission at 480 nm on excitation at 397 nm, which is the fluorescence of the free

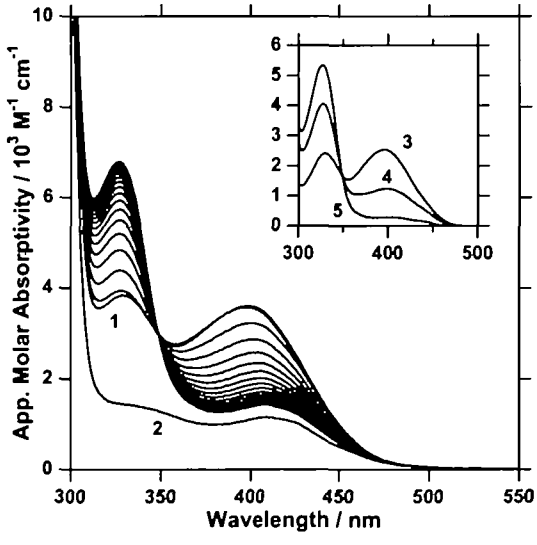


Fig. 1. **Absorption spectra of K303A AADC.** Holoenzyme and apoenzyme, each 10 μM, were prepared as described in "EXPERIMENTAL PROCEDURES." The spectra were taken in 50 mM PIPES-NaOH, pH 7.0, at 25°C. Curve 1: holoenzyme. Curve 2: apoenzyme. To the holoenzyme, Dopa was added to a final concentration of 0.2 mM, and the spectra were taken at 5-min intervals and a scanning rate of 120 nm/min from 550 to 300 nm. The time of the start of measurement was, from bottom to top of the 330-nm absorption, 0 (mixing the solution takes approximately 15 *s*), 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, and 75 min, after the addition of Dopa. The inset shows the difference spectra of the holoenzyme (plus Dopa) and apoenzyme. Curve 3: Subtraction of the spectra of apoenzyme (Curve 2) from the holoenzyme (Curve 1). Curve 4: Subtraction of the spectra of apoenzyme from the spectrum of the holoenzyme plus Dopa 25 min after addition. Curve 5: Subtraction of the spectra of apoenzyme from the spectrum of the holoenzyme plus Dopa 75 min after addition.

PLP molecule. These results are also consistent with the notion that PLP does not form a Schiff base in the active site of K303A AADC.

The content of PLP in the holo form of K303A AADC was determined by the phenylhydrazine method (*11*) to be 0.80 mol/mol subunit. The amount of the aldehyde form of PLP in K303A AADC was estimated from the apparent molar absorptivity at 397 nm of K303A AADC (subtracted for the "apo" absorption, see Fig. 1 inset) and the molar absorptivity of PLP at pH 7.0 (4,900 $M^{-1} \cdot cm^{-1}$) to be 0.52 mol/mol subunit. Therefore, the percentage of PLP in the aldehyde form in K303A AADC was calculated to be 65%.

Reaction of K303A AADC with L-Dopa and Its Derivatives—On reaction of K303A AADC with 3-(3,4-dihydroxyphenyl)-alanine (Dopa), the absorption at 328 nm gradually increased with concomitant decrease in absorption at 397 nm (Fig. 1). After the spectral change was over, the solution was subjected to centrifugal ultrafiltration

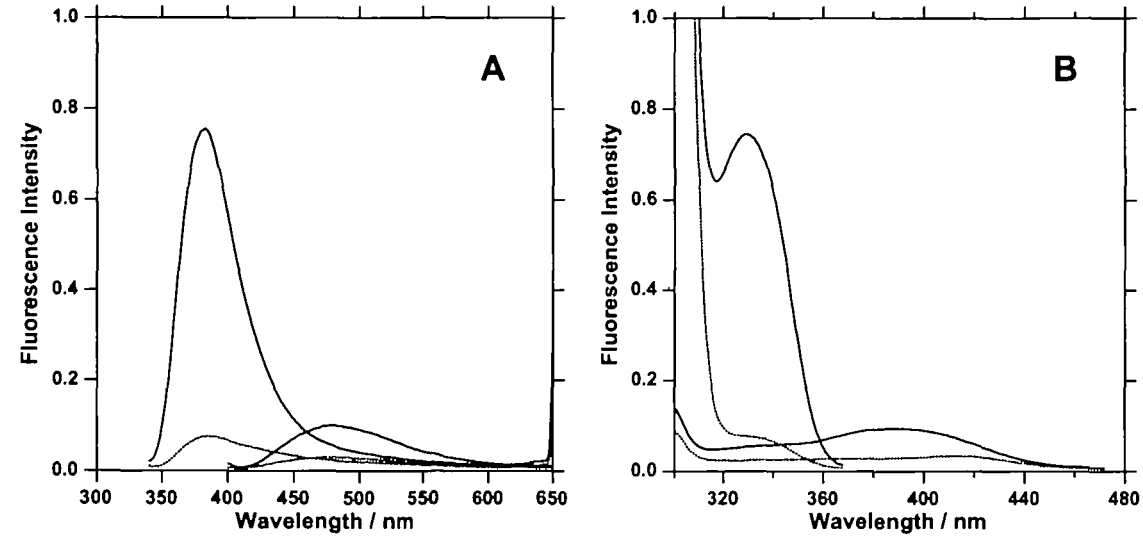


Fig. 2. **Fluorescence spectra of K303A AADC.** The conditions used in the spectral measurement were the same as in Fig. 1. Solid line: holoenzyme. Dashed line: apoenzyme. (A) Fluorescence emission spectra. The left two spectra are those obtained by excitation at 328 nm, and the right two spectra are those excited at 397 nm. (B) Fluorescence excitation spectra. The left two spectra are those monitored at 383 nm, and the right two spectra are at 480 nm. Fluorescence intensity is expressed in arbitrary units.

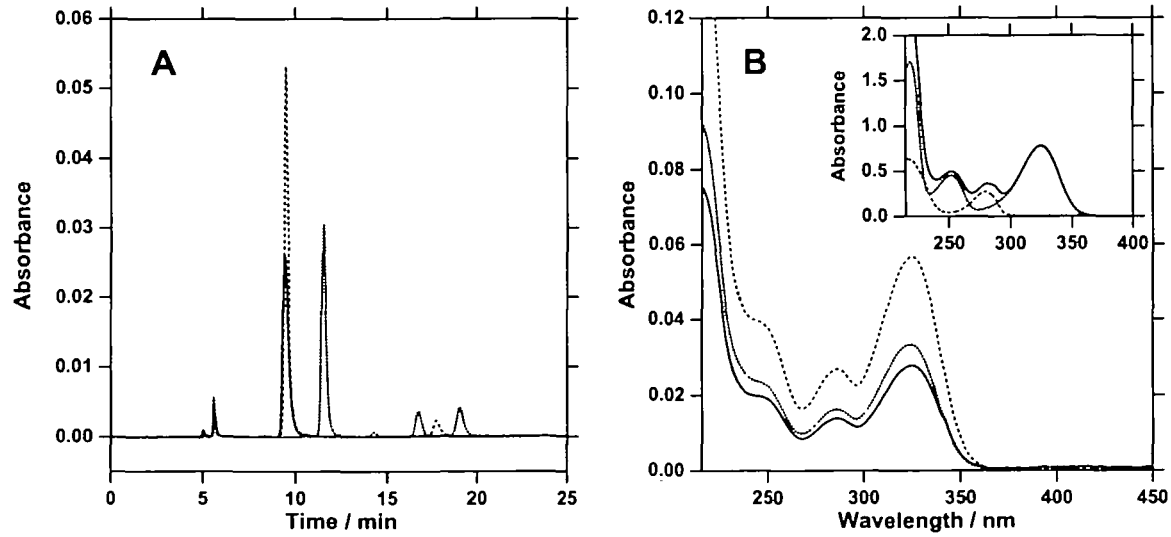
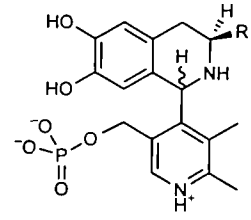


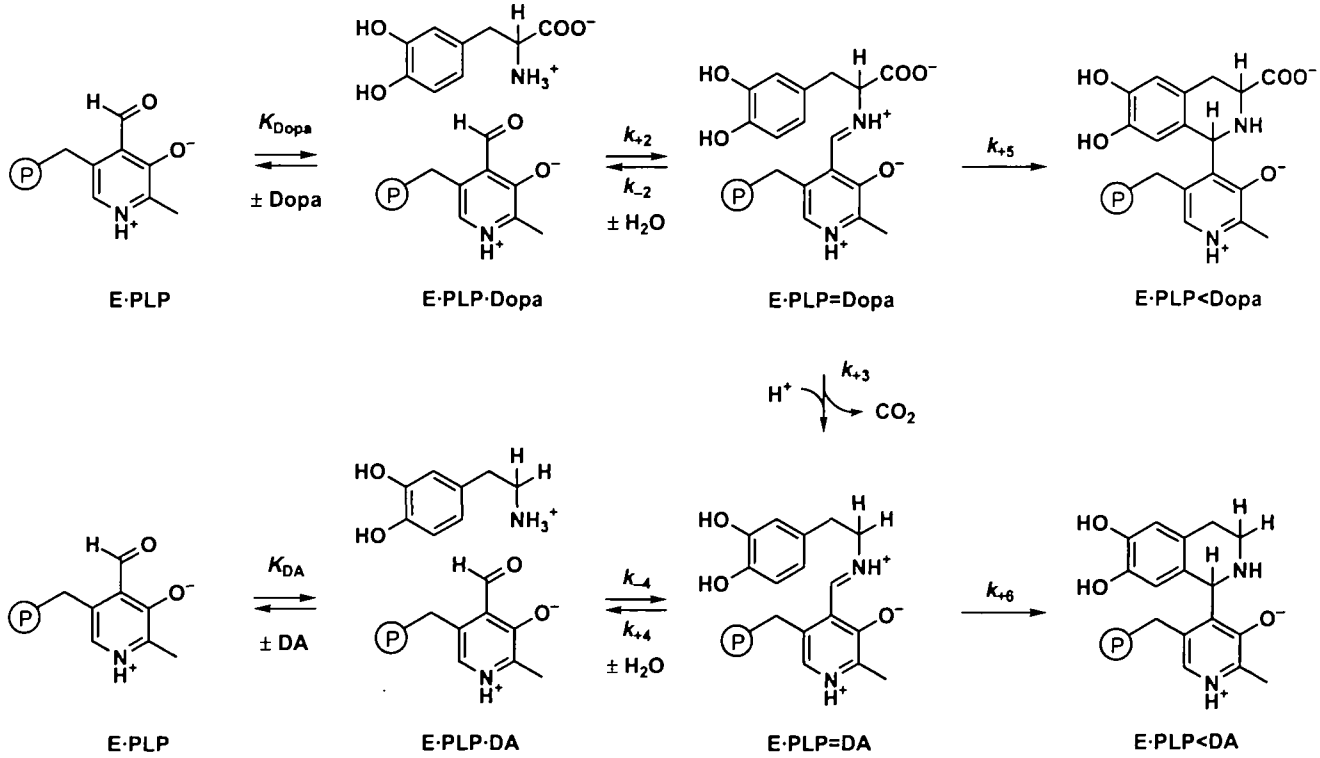
Fig. 3. **HPLC analyses of the reaction products and PLP < Dopa and PLP < DA.** HPLC analyses were performed as described in "MATERIALS AND METHODS." Eluates were monitored for their absorbance using a Beckman model 168 photodiode-array detector (215-450 nm). (A) HPLC profiles monitored at 330 nm. Solid line: 100 μl of the reaction mixture of 10 μM K303A and 0.2 mM Dopa, incubated for 75 min. The reaction mixture was ultrafiltered to remove the AADC protein before being applied to HPLC. The peaks at 5 to 6 min are injection noises. Dashed line: 10 μl of 0.1 mM PLP < DA, prepared by incubating 0.1 mM PLP with 1 mM DA in 50 mM K-P_i for 4 h. Dotted line: 10 μl of 0.1 mM PLP < Dopa, prepared by incubating 0.1 mM PLP with 1 mM Dopa in 50 mM K-P_i for 4 h. (B) Absorption spectra of the peaks. Absorbance values at 2-nm intervals between 216 and 450 nm and at the highest position of the 330-nm peaks were plotted against the wavelength. Line styles are the same as in panel (A). Inset shows the absorption spectra of 0.1 mM DA (dashed line), 0.1 mM pyridoxamine 5'-phosphate (dotted line), and the sum of the two spectra (solid line).

(exclusion molecular size 10,000; Ultrafree MC NMWL-10000, Millipore, Bedford, MA). The 330-nm absorbing species was recovered in the filtrate, and the filtrate was analyzed on an HPLC column equipped with a photodiode array detector (Fig. 3A). When being monitored at 330 nm, a peak was observed at r.t. = 9.5 min. The absorption spectrum of this peak (Fig. 3B) showed maxima at 325 nm and at 280 nm. The pattern of the spectrum of the compound, named **I**, was in good agreement with that of the sum of the spectra of pyridoxamine 5′-phosphate and dopamine (DA) (Fig. 3B, inset). This strongly suggests that the compound has a catechol ring and a hydroxypyridine ring derived from the coenzyme, and has an sp^3 structure at C4′. It has been known that Dopa, DA, and its derivatives easily undergo condensation with aldehydes, called the Pictet-Spengler reaction (*12*). The Pictet-Spengler adduct of Dopa and PLP (PLP<Dopa, Chart 1), and that of DA and PLP (PLP<DA, Chart 1) were prepared and subjected to HPLC (Fig. 3A; for preparation of the adducts, see the legend). The retention time was 11.5 min for PLP<Dopa and 9.5 min for PLP<DA. The peak of both adducts showed a spectral pattern identical to that of **I** (Fig. 3B). The retention time of **I** is identical to that of PLP<DA, and no peak corresponding to PLP<Dopa was observed on the chromatogram of the reaction mixture of K303A AADC and Dopa. These results strongly indicate that **I** is PLP<DA. The amount of PLP<DA in the reaction mixture was determined by comparison of the area of the peaks shown in Fig. 3A to be 7.5 μM. This value is close to the content of PLP, 8.0 μM, present in 10 μM K303A AADC. Therefore, we can conclude that almost all of the PLP molecules in the active site of K303A AADC are converted to PLP<DA upon reaction of the holoenzyme with Dopa. By using this HPLC system, Dopa (r.t. = 35.4 min) and DA (r.t. = 29.3 min) could also be measured. The amount of the product DA in the reaction mixture of 10 μM K303A AADC and 0.2 mM Dopa was below the detection limit of 0.1 μM. We tested whether the catalytic reaction of WT AADC with Dopa produces PLP<DA. WT AADC (5 μM) was incubated with 7.5 mM Dopa for 6 h at 25°C. Under these conditions, PLP of WT AADC is known to undergo abortive transamination to form pyridoxamine 5′-phosphate (*12, 13*), but the amount of PLP<DA was below the detection limit of 0.05 μM on analysis of the reaction mixture on HPLC. This indicates that the anomalous side reaction does not occur in the catalytic reaction of WT AADC.



R = COO^- (L-dopa)
H (dopamine)

Chart I. **Chemical structure of the Pictet-Spengler condensation product of PLP and Dopa (DA).**



Scheme 1. **Mechanism of the reaction of K303A AADC with Dopa.** Chemical structures are shown together with the symbols used in the text. In WT AADC, PLP forms a Schiff base with the ε-amino group of Lys303. Interconversion of the Lys303-PLP Schiff base with the substrate (product)-PLP Schiff base proceeds without release or incorporation of a water molecule (transaldimination).

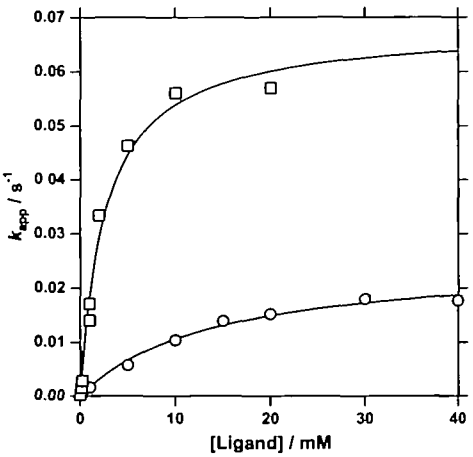


Fig. 4. **Dependency of the apparent rate constant for the spectral change on the ligand concentration.** The absorbance of K303A AADC at 330 nm increased and that at 390 nm decreased, in an exponential manner on the reaction with DA and DopaOMe. The apparent rate constants for the exponential time course were plotted against the ligand concentration. Open circles: DA. Open squares: DopaOMe. The value of apparent dissociation constant and the maximum value of rate constant are 14 mM and 0.025 s^{-1} for the reaction with DA and 2.6 mM and 0.068 s^{-1} for the reaction with DopaOMe.

Dopa methyl ester (DopaOMe) forms an aldimine with PLP but is unable to undergo decarboxylation in WT AADC because of the presence of a methyl group attached to the carboxylate group (*10*). When K303A AADC was reacted with DopaOMe, the enzyme underwent spectral changes essentially identical to those shown in Fig. 1, and analysis of the 330-nm absorbing species on HPLC showed it to be the Pictet-Spengler adduct of PLP and DopaOMe (data not shown). This indicates that, although formation of PLP< Dopa was not observed in the reaction of K303A AADC with Dopa, this pathway can be detected when the decarboxylation reaction was blocked by methylating the carboxylate group of Dopa. Also, the spectrum of K303A AADC changed in the same way as above when it was incubated with DA, and the 330-nm absorbing species was identified on HPLC as PLP<DA (data not shown).

Kinetic Studies—The above findings are consistent with the reaction mechanism shown in Scheme 1. In WT AADC, PLP is bound to the ε-amino group of Lys303, and on binding of substrate amino acid, the α-amino group of the substrate displaces the ε-amino group. However, there is no amino group at the position 303 in K303A AADC, and the substrate α-amino group forms a Schiff base with PLP *via* a dehydration reaction. Correspondingly, the product release proceeds *via* hydrolysis, not transaldimination, in K303A AADC. In addition, there are pathways for the formation of the Pictet-Spengler adduct from the PLP Schiff bases in K303A AADC. On the reaction of K303A AADC with DA, the spectral change proceeded in a single exponential curve (data not shown), and plots of apparent rate constant (k_{app}) against the concentration of DA showed a hyperbolic curve (Fig. 4). As this process involves three steps (E·PLP+DA⇄E·PLP·DA⇄E·PLP=DA→E·PLP<DA), one of which is a rapid association/dissociation of DA to/from the enzyme (E·PLP+DA⇄E·PLP·DA), the spectral change is described as follows.

$$A_{I}\exp\left(-\frac{[DA]}{K_{DA}+[DA]}k_{-4}t\right)+A_{II}\exp(-k_{+6}t) \qquad (1)$$

where A_I and A_{II} denote the amplitudes of spectral changes (see "APPENDIX"). We used the condition of $k_{+4} \ll k_{+6}$. This is because DA was not formed, whereas PLP<DA was nearly stoichiometrically formed from PLP=DA during the reaction of K303A AADC with Dopa. That the k_{app} value showed dependency on the DA concentration in a hyperbolic manner (Fig. 4) indicates that the exponential spectral change is largely expressed by the first term of Eq. 1. Accordingly, k_{-4} is considered to have a value of 0.025 s^{-1} (see the legend to Fig. 4).

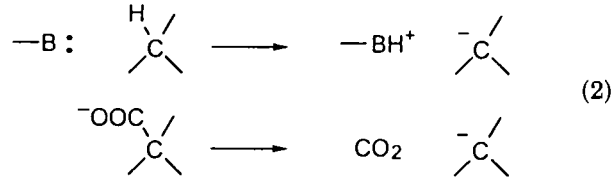
The spectral changes on the reactions of K303A AADC with DopaOMe and Dopa proceeded in the same way as above. Since we have no experimental support for considering $k_{-2} \ll k_{+5}$, which is a condition analogous to $k_{+4} \ll k_{+6}$ used above, we can perform less extensive analysis for these two cases. As shown above, PLP<DA accumulates up to 8.5 μM, whereas PLP<Dopa is lower than the detection limit of 0.05 μM, when 10 μM K303A AADC was reacted with 0.2 mM Dopa (Fig. 3). This indicates that k_{+3} is at least 170 times as large as k_{+5}. The maximal value of the apparent rate constant for the spectral change on the reaction of K303A AADC with DopaOMe was 0.068 s-1 (Fig. 4). This must be the lower limit of the k_{+5} value for DopaOMe. Assuming that this is also the lower limit of the k_{+5} value for Dopa, we can estimate that the k_{+3} value for Dopa is more than 11.8 s^{-1}. The corresponding value for WT AADC is 11.2 s^{-1} (*10*). Therefore, we can conclude that the decarboxylation step is essentially unaffected by the [Lys303→Ala] mutation.

DISCUSSION

The absorption spectrum of K303A AADC after gel filtration on Sephadex G-25 (Fig. 1) showed that this enzyme, although it does not have a lysine ε-amino group to fix PLP through an imino bond, can bind PLP. Determination of the PLP content of K303A AADC using the phenylhydrazine method indicated that the binding is nearly stoichiometric (0.8 mol/mol subunit). These results imply that there are interactions other than the imine bond between AADC and PLP, probably hydrogen-bonds and/or ionic interactions with the phosphate group of PLP, as has been shown in the other PLP enzymes (*3*). PLP exists in K303A AADC in two forms, the free aldehyde form absorbing at 397 nm and an unidentified form that has an sp^3 structure at C4′ and absorbs at 328 nm. One possible structure for the latter is a hydrated form of PLP. Model studies using dioxane and *N,N′*-dimethylformamide as water-miscible apolar solvents showed, however, that PLP in the zwitterionic form tends to be less hydrated in an apolar environment like the active site of AADC (*14*). Therefore, although the 328-nm absorbing species of K303A AADC is considered to be the adduct of PLP with a nucleophile, the precise structure of the nucleophile awaits further characterization. For bacterial D-amino acid aminotransferase, the mutant enzyme in which the PLP-binding lysine was replaced by an alanine residue showed intense absorption at 333 nm (*6*). The species responsible for this absorption was resistant to phenylhydrazine treatment and is considered to be a stable complex having a sp^3 structure at C4′. On the other hand,

the reaction of K303A AADC with Dopa and its derivatives gave stoichiometric formation of adducts of PLP with catechol amino compounds. Therefore, the 328-nm absorbing species of K303A AADC can participate in the reaction, probably by being converted to the free aldehyde form. This indicates that the above nucleophile reversibly forms an adduct with PLP.

K303A AADC is essentially inactive as a catalyst, for 10 nmol of the enzyme produces less than 0.1 nmol of DA over a period of 75 min (calculated from the detection limit, see "RESULTS"). However, analysis of the reaction product showed that decarboxylation of Dopa takes place in the enzyme, and the resulting DA undergoes Pictet-Spengler reaction to form a cyclized compound absorbing at 330 nm (PLP < DA, Chart 1). Therefore, although DA is not formed by K303A AADC, this enzyme still retains the ability to remove the carboxylate group of Dopa. The rate of decarboxylation of PLP=Dopa to PLP=DA in K303A AADC is considered to be close to that in WT AADC, based on the assumption that the rate of the Pictet-Spengler reaction of PLP=Dopa is of the same order as that of PLP=DopaOMe in K303A AADC (see RESULTS "*Kinetic Studies*"). These results indicate that Lys303 is not essential for the decarboxylation step, and are in contrast with the findings on several PLP enzymes that the PLP-binding lysine residue is essential for the deprotonation step (*3, 4, 7*). Both deprotonation and decarboxylation produce a carbanion, which is stabilized by adjacent electron-accepting modules, such as the protonated pyridine ring of PLP (*2*). However, deprotonation requires a base that accepts the proton, whereas decarboxylation does not require such a base (Eq. 2).



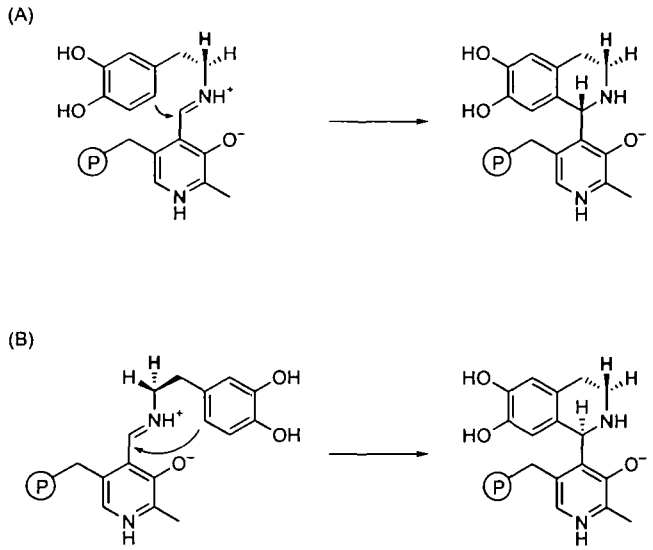
(2)

Therefore, the present results are consistent with the chemistry of deprotonation and decarboxylation, and it can be anticipated that decarboxylases generally does not require the ε-amino group for the decarboxylation step. The bacterial D-amino acid aminotransferase in which the PLP-binding lysine was replaced by asparagine retained significant catalytic ability (1-2%), but this residual activity has been ascribed to a base other than the lysine residue in the active site (*5*), again consistent with the above discussion. Reprotonation at C(α) after decarboxylation is necessary for the generation of PLP-DA. A role of the PLP-binding lysine residue as a proton donor in this reprotonation step of decarboxylases has been suggested (*15*). However, no accumulation of the quinonoid intermediate, which shows absorption at around 500 nm, was observed for the reaction of K303A AADC with Dopa. This indicates that the reprotonation of the quinonoid intermediate is faster than the rate of decarboxylation (forming the quinonoid intermediate) in the reaction of K303A AADC with Dopa, and suggests that Lys303 of AADC may not function as a proton donor in the reprotonation step.

Whereas PLP < DA is formed from PLP=DA, DA was not detected in the solution. This is due to either the slow rate of hydrolysis of PLP-DA as compared to the rate of PLP < DA formation, or the equilibrium shift of the step of E·PLP=DA + H_2O ⇄ E·PLP·DA toward the formation of the Schiff base. In either case, the situation is unfavorable for the formation of DA. Therefore, we can conclude that the ε-amino group of Lys303 has important roles in the step of product release. It is considered that the ε-amino group of the lysine residue displaces the amino group of DA by nucleophilic attack on the imine bond of PLP=DA (transaldimination). This promotes the rate of cleavage of the imine bond of PLP=DA and also prevents the unfavorable equilibrium shift to the formation of PLP=DA. This is in accordance with the observations on the other PLP enzymes in which the PLP-binding lysine residue is replaced by other amino acid residues; in these mutant enzymes, products (substrates) usually form stable Schiff bases with PLP and are not easily released from the enzyme (*3-6*). The rate of the formation of E·PLP=DA from E·PLP·DA is 0.025 s^{-1} and the dissociation constant for DA is 14 mM (see "RESULTS"). This small rate constant for the Schiff base formation indicates the possibility that Lys303 may also participate in accelerating Schiff base formation between PLP and amino group of substrates/products, for aldimines (*i.e.*, Schiff bases) are more easily attacked by nucleophiles than free aldehydes.

PLP < DA dissociates easily from the enzyme protein, as indicated by the finding that it exists in the ultrafiltered solution in a stoichiometric amount (7.5 nmol recovered from 8 nmol of PLP present in 10 nmol of enzyme). This low affinity of PLP < DA to K303A AADC indicates that the three-dimensional structure of this compound causes strain in the active site of K303A AADC. As the Pictet-Spengler reaction is an irreversible process, the energy released by this reaction is considered to be utilized to cause the strain. According to the theory of Dunathan (*16*), the substrate carboxylate group must be fixed perpendicular to the plane of PLP pyridine ring. This stereochemical requirement in amino acid decarboxylase gives two possible conformations for the structure of E·PLP=DA (Scheme 2). For conformation A, the ring C(6) is considered to attack from the *si* face of the Schiff base, and the resultant PLP < DA will have an *S* configuration. On the other hand, conformation B will give an *R* configuration. Therefore, if we can determine the absolute configuration of E·PLP < DA released from the enzyme, we can estimate the conformation of PLP=DA in K303A AADC, and accordingly the mode of recognition of the substrate side chain by AADC. At present, this is hampered by the limited amount of K303A AADC protein obtained by expression in *E. coli* cells, and further improvement in the expression system of this mutant enzyme is required.

Another important issue to be addressed is the complete determination of the kinetic parameters shown in Scheme 1. If these values are obtained together with the corresponding values of WT AADC, the rate of transaldimination, decarboxylation, and product release can be directly compared. Then, the role of Lys303 in decarboxylation and product release will be quantitatively described, and we will also be able to discuss the role of the residue in transaldimination. The incomplete determination of these values in this study is due to the fact that E·PLP=Dopa and E·PLP=DA were not detected spectrophotometrically. This is because either these species are accumulated in low amount during the reaction of K303A AADC, or they have



Scheme 2. **Two possible routes to the Pictet-Spengler adduct of PLP with DA from the PLP-DA Schiff base.** Hydrogen atoms shown in bold letters indicate the position originally occupied by the carboxylate group in the PLP-Dopa Schiff base and must be fixed perpendicular to the pyridine plane according to the theory of Dunathan (*16*).

similar absorption spectra to PLP. The latter possibility cannot be ruled out, because E·PLP=Dopa in WT AADC is considered to have an absorption band at 380 nm (*10*). In this regard, it would be of great help to study the ^{13}C and ^{15}N isotope effects, as has been done with the wild-type histidine decarboxylase (*17*), on the reactions of WT and K303A AADCs with L-dopa and its derivatives.

The Pictet-Spengler reaction also occurs when PLP is incubated with *m*-tyrosine (*12*). The hydroxy group at *ring* C(3) assists the nucleophilic attack of the catechol ring to the imine carbon atom, by increasing the electron density at *ring* C(6) (Scheme 2). Therefore, amino acids with a 3-hydroxyphenyl group at C(β) can undergo the above adduct formation. Histidine and histamine are known to form a cyclized compound similar to the Pictet-Spengler adduct on the reaction with PLP (*18*). In this regard, studies on the reaction of histidine with histidine decarboxylase in which the PLP-binding lysine is replaced by other residues are of great importance to know whether the anomalous side reaction described in this study is a general phenomenon in the catalytic reactions of amino acid decarboxylases.

In conclusion, the present study indicated that Lys303, the PLP-binding lysine residue, of AADC is not essential for the decarboxylation step, but is important for the product release and possibly for increasing the rate of the substrate–PLP aldimine formation.

APPENDIX

For the following reaction scheme,

$$\text{E·PLP}+\text{DA} \overset{K_{DA}}{\rightleftharpoons} \text{E·PLP·DA} \overset{k_{+4}}{\underset{k_{-4}}{\rightleftharpoons}}$$

$$\text{E·PLP=DA} \xrightarrow{k_{+6}} \text{E·PLP<DA} \qquad (1a)$$

two rate equations are obtained.

$$-\frac{d([\text{E·PLP}]+[\text{E·PLP·DA}])}{dt}=$$

$$\frac{[\text{DA}]}{K_{DA}+[\text{DA}]}k_{-4}([\text{E·PLP}]+[\text{E·PLP·DA}])$$

$$-k_{+4}[\text{E·PLP=DA}] \qquad (2a)$$

$$-\frac{d[\text{E·PLP=DA}]}{dt}=$$

$$-\frac{[\text{DA}]}{K_{DA}+[\text{DA}]}k_{-4}([\text{E·PLP}]+[\text{E·PLP·DA}])$$

$$+(k_{+4}+k_{+6})[\text{E·PLP=DA}] \qquad (3a)$$

Solving the above simultaneous differential equations, we find that [E·PLP] + [E·PLP·DA] conform to the following equation.

$$A_I\exp(-\lambda_1)+A_{II}\exp(-\lambda_2) \qquad (4a)$$

where A_I and A_{II} are amplitudes of the spectral change, and λ_1 and λ_2 are the solutions to the equation

$$\begin{vmatrix} \frac{[\text{DA}]}{K_{DA}+[\text{DA}]}k_{-4}-\lambda & -k_{+4} \\ -\frac{[\text{DA}]}{K_{DA}+[\text{DA}]}k_{-4} & k_{+6}+k_{+4}-\lambda \end{vmatrix}=0 \qquad (5a)$$

Assuming that $k_{+4} \ll k_{+6}$ (see "RESULTS"), we obtain the values of λ to be:

$$\lambda_1=\frac{[\text{DA}]}{K_{DA}+[\text{DA}]}k_{-4}$$

$$\lambda_2=k_{+6}$$

In this case, A_I corresponds to the spectral shift from the mixture of E·PLP and E·PLP·DA to E·PLP=DA, and A_{II} to that from E·PLP=DA to E·PLP<DA.

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