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A MODIFICATION OF D-AMINO ACID OXIDASE APOENZYME BY PYRIDOXAL 5'-PHOSPHATE

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SUMMARY

A modification of D-amino acid oxidase apoenzyme by pyridoxal 5'-phosphate and the mechanism of inhibition of the enzyme by this compound are described. Reduction of D-amino acid oxidase apoenzyme with $NaBH_4$ in the presence of pyridoxal 5'-phosphate resulted in the formation of a reduced Schiff's base between ε-amino group of a lysine residue and pyridoxal 5'-phosphate, and 2 moles of pyridoxal 5'-phosphate were fixed per 50 000 g of protein.

The modified enzyme had about a half enzymic activity of the native enzyme. The affinity of FAD to the modified apoenzyme was about 10-fold less than that of the native apoenzyme, while the Michaelis constant for D-alanine was only 1.5 times larger than that of the native enzyme.

The enzyme was inhibited by pyridoxal 5'-phosphate without reduction by $NaBH_4$. From the kinetic studies on the inhibition, it was also indicated that the interaction of pyridoxal 5'-phosphate with lysine residues of the apoenzyme caused about 10-fold decrease in the affinity of FAD to the apoenzyme and 43% loss of the enzymic activity.

INTRODUCTION

Recently, it has been demonstrated that the sedimentation and diffusion constants of D-amino acid oxidase (D-amino acid: O_2 oxidoreductase, EC 1.4.3.3) apoenzyme[1] were almost the same as those of the holoenzyme[2], while FAD changed the properties of the apoenzyme, probably the structure around the active site[1,3].

In 1965, COFFEY *et al.*[4] have reported that D-amino acid oxidase was modified on treating with $NaBH_4$ and D-alanine. When the reaction was carried out in the presence of D-[^{14}C]alanine or D-[^{14}C]leucine, it was found that 0.9 equiv of ^{14}C label was bound per equiv. of FAD in the holoenzyme. In the subsequent paper, HELLERMAN AND COFFEY[5] have isolated a compound, ε-*N*-(1-carboxyethyl)-L-lysine, from the hydrolysate of the reaction product, and identified that D-[^{14}C]alanine was fixed to the enzyme as a reduced Schiff's base between an ε-amino group of a lysine residue and substrate–intermediate. This evidence posed an interesting problem on the nature

of active site of the enzyme, namely a possibility of involvement of the lysine residue in the enzyme reaction was suggested. A very recent communication reported by MASSEY *et al.*[6], however, has demonstrated no loss of the enzymic activity by the same treatment as reported by COFFEY *et al.*[4]. They also showed characteristic changes in the absorption spectrum of the holoenzyme by reduction with $NaBH_4$ (ref. 6).

The present paper reports on lysine residues of the enzyme. As is well known, pyridoxal phosphate forms a reduced Schiff's base with ε-amino group of lysine residues in some enzymes through the reductive action of $NaBH_4$ (refs. 7–10). The authors have attempted the fixation of pyridoxal phosphate to D-amino acid oxidase apoenzyme to investigate on the role of lysine residues in the structure and function of the enzyme. The results indicated that a very limited number of pyridoxal phosphate was fixed to lysine residues of the apoenzyme with a partial loss of the enzymic activity. The affinity of FAD and D-alanine to the enzyme decreased by the fixation of pyridoxal phosphate. The effect of the fixation of pyridoxal phosphate on the binding of FAD was much more drastic than that of D-alanine. The mechanism of the inhibition of the enzyme by pyridoxal phosphate was also investigated kinetically without fixation, and the possible implication for the effect of the interaction between the lysine residues and pyridoxal phosphate on the active site of the enzyme is discussed.

MATERIALS AND METHODS

D-amino acid oxidase apoenzyme was purified from hog kidneys by the procedure of MIYAKE *et al.*[1]. DL-Alanine, $NaBH_4$, pyridoxal 5′-phosphate, and pyridoxamine hydrochloride were obtained from Nakarai Chemicals Ltd., Kyoto. Pyridoxamine 5′-phosphate and pyridoxal hydrochloride were obtained from Eisai Co., Ltd. Pyridoxine 5′-phosphate was obtained from Tokyo Kasei Co., Ltd., and pyridoxine hydrochloride was obtained from Wako Pure Chemicals Ltd. FAD was obtained from Wakamoto Pharmaceutical Co. All other materials used were of analytical grade.

Reduction of the apoenzyme with $NaBH_4$ in the presence of pyridoxal phosphate was performed as follows; 4 ml of the reaction mixture containing about 30 mg of the apoenzyme and 40 μmoles of pyridoxal phosphate in 0.1 M pyrophosphate buffer (pH 8.3) was kept at 30° for 10 min. A few drops of octylalcohol were then added to the solution. After the following addition of solid $NaBH_4$ (10 mg), the reaction mixture was incubated at 30° for 30 min. The reaction product was applied on a Sephadex G-75 column (2.8 cm × 30 cm) equilibrated with 0.1 M pyrophosphate buffer (pH 8.3) and it was eluted from the column with the same buffer. The protein fractions eluted from the column were collected, and the protein was fractionated with $(NH_4)_2SO_4$ (2 g/10 ml). The precipitates were dissolved in 0.1 M pyrophosphate buffer, pH 8.3. The modified apoenzyme solution was then dialysed against 0.1 M pyrophosphate buffer (pH 8.3) overnight at 5°.

Pyridoxal phosphate fixed to the apoenzyme was proved from amino acid analysis to be ε-pyridoxyllysine. After the modified apoenzyme was hydrolysed in 5.7 M HCl at 105° for 24 h, the hydrolysate was applied to a short column (0.9 cm × 15 cm), and eluted with 0.38 M citrate buffer (pH 4.26) and at 50°. The amount of the ε-pyridoxyllysine was estimated by assuming that the ninhydrin color value was

comparable with that of leucine. The amount of ε-pyridoxyl phosphate lysine residue in the modified apoenzyme was also estimated from the absorbance at 325 mμ by using ε_M value for ε-pyridoxyl phosphate lysine of 10 150 $l \cdot mole^{-1} cm^{-1}$ (ref. 11), by assuming that ε-pyridoxyl phosphate lysine residues of the apoenzyme have a comparable absorption. In the latter case, the absorbance due to protein was subtracted from the measured value. The value of 45 000 was used for molecular weight of the enzyme (15–18).

The enzymic activity was measured by the polarographic method by the use of an equipment contrived by HAGIHARA[12]. After the preincubation of the native or the modified apoenzyme with FAD or FAD and pyridoxal phosphate in 0.1 M pyrophosphate buffer (pH 8.3) for 2 min, DL-alanine was added to the enzyme solution. The total volume of the reaction mixture was 2.0 ml. The enzyme reaction was carried out at 38°, and the O_2 consumption in the first 1 min was taken as the velocity of the enzyme reaction.

Protein concentration was determined by the method of GORNALL *et al.*[13], or from the absorbance at 280 mμ by the use of the value of 1.4, which represents the absorbance at 280 mμ of 1 mg of the apoenzyme per ml for 1-cm light path[1]. The concentration of FAD was determined from the absorbancy at 450 mμ using the ε_M value of 11 300 $l \cdot mole^{-1} \cdot cm^{-1}$ (ref. 14).

A Cary spectrophotometer Model 14 or a Shimadzu spectrophotometer MSP L-50 was used for the measurements of absorption spectra, and a Yanaco LC-5S amino acid analyser was used for the determination of ε-pyridoxyllysine.

RESULTS

*The formation of reduced pyridoxal phosphate–apoenzyme**

The effect of some pyridoxal derivatives on the enzymic activity of D-amino acid oxidase shown in Table I. Among the pyridoxal derivatives used, pyridoxal phosphate inhibited the enzymic activity to 64% of the native enzyme under the conditions employed. Pyridoxal also inhibited the enzymic activity, but the extent of the inhibition was less than that by pyridoxal phosphate. Other derivatives used

TABLE I

INHIBITION OF THE ENZYMIC ACTIVITY OF D-AMINO ACID OXIDASE BY PYRIDOXAL DERIVATIVES

The concentration of each pyridoxal derivative was 15 mM. The apoenzyme (20 μg), FAD (20 μM), and pyridoxal derivatives were dissolved in 0.1 M pyrophosphate buffer, pH 8.3. The enzyme reaction was initiated by the addition of DL-alanine (25 μM).

Pyridoxal derivatives	*Residual activity (%)*
—	100
Pyridoxal	81
Pyridoxamine	100
Pyridoxine	94
Pyridoxal 5′-phosphate	64
Pyridoxamine 5′-phosphate	95
Pyridoxine 5′-phosphate	95

* Nomenclature: reduced pyridoxal phosphate–apoenzyme, D-amino acid oxidase apoenzyme which fixed pyridoxal phosphate as a form of reduced Schiff's base.

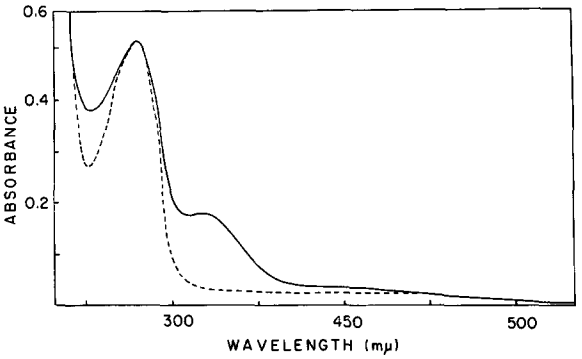


Fig. 1. The absorption spectra of the native apoenzyme and reduced pyridoxal phosphate–apoenzyme. The native apoenzyme and reduced pyridoxal phosphate–apoenzyme were dissolved in 0.1 M pyrophosphate buffer (pH 8.3) respectively. The concentration of the apoenzymes was 0.33 mg protein per ml: ———, reduced pyridoxal phosphate–apoenzyme; — — —, the native apoenzyme.

showed almost no effect on the enzymic activity. As this result was indicative of some interaction between pyridoxal phosphate and the apoenzyme, the fixation of pyridoxal phosphate to the apoenzyme was attempted. Under the conditions defined in MATERIALS AND METHODS, reduction of the apoenzyme with $NaBH_4$ at pH 8.3 and 30° in the presence of pyridoxal phosphate resulted in the formation of reduced pyridoxal phosphate apoenzyme. The absorption spectrum of the reduced pyridoxal phosphate–apoenzyme showed a broad band at around 320 mμ in addition to the absorption band at 280 mμ (Fig. 1). The spectral pattern of the reduced pyridoxal phosphate–apoenzyme was essentially the same as those of other reduced pyridoxal phosphate enzymes. Amino acid analysis of the reduced pyridoxal phosphate–apoenzyme was then performed to confirm the fixation of pyridoxal phosphate to the apoenzyme. A peak appeared as well-separated peak after arginine peak on a short column (0.9 cm × 15 cm) with 0.38 M citrate buffer (pH 4.26) as the eluent, and 50°. The elution volume of the peak was the same as that of ε-pyridoxyllysine peak. These results apparently showed that pyridoxal phosphate formed a reduced Schiff's base with ε-amino group of lysine residue of the apoenzyme.

Estimation of ε-pyridoxyl phosphate lysine residue in reduced pyridoxal phosphate–apoenzyme

Pyridoxal phosphate fixed to the apoenzyme was estimated by the comparison

TABLE II

ESTIMATION OF ε-PYRIDOXYL PHOSPHATE LYSINE RESIDUE IN REDUCED PYRIDOXAL PHOSPHATE–APOENZYME

Pyridoxal phosphate Concn.(mM)*	$A_{280m\mu}/A_{325m\mu}$	*ε-Pyridoxyl phosphate lysine residue (moles / 5·10⁴ g protein)*	
		Spectrophotometric	*Amino acid analysis*
0	24.00	—	—
5	2.86	2.1	—
10	2.70	2.2	2.0

* The concentration of pyridoxal phosphate added to the apoenzyme solution for the reduction with $NaBH_4$.

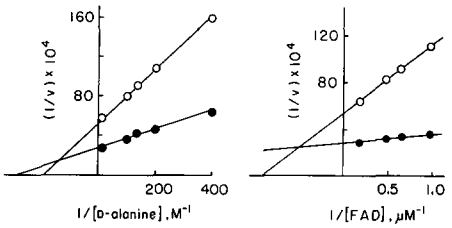


Fig. 2. Double reciprocal plots for D-alanine (left) and FAD (right) with respect to the native and reduced pyridoxal phosphate–apoenzyme. In the plot for D-alanine, 25 μM FAD was used, and in that for FAD, 50 mM DL-alanine was used. 15 μg apoenzyme (●———●) and 15 μg reduced pyridoxal phosphate–apoenzyme (○———○) were used, respectively. Initial velocity (v) is expressed as μl O_2 consumed per min per mg protein under the assay conditions defined in MATERIALS AND METHODS.

of the color value of the ε-pyridoxyllysine peak with that of leucine peak in the elution pattern of amino acid analysis. As shown in Table II, 2 moles of pyridoxal phosphate per 50 000 g of protein were found to be fixed. This value nearly corresponds to 2 moles of pyridoxal phosphate per subunit molecular weight of the enzyme[15–18]. The amount of pyridoxal phosphate fixed to the apoenzyme was also estimated from absorbance at 325 mμ of reduced pyridoxal phosphate–apoenzyme. 2 moles of ε-pyridoxyl phosphate lysine were detected per 50 000 g of protein (Table II). No additional incorporation of pyridoxal phosphate to the apoenzyme was observed by increasing the concentration of pyridoxal phosphate up to 20 mM.

Kinetic constants of reduced pyridoxal phosphate–apoenzyme

The apparent dissociation constant for FAD–reduced pyridoxal phosphate–apoenzyme complex was estimated kinetically. As shown in Fig. 2, $1/v$ *versus* 1/[FAD] was plotted according to the equation of WARBURG AND CHRISTIAN[19]. From the intercept on the abscissa, a value of $1.1 \cdot 10^{-6}$ M was obtained for the dissociation constant. The value was about 10-fold greater than that of the dissociation constant for FAD–native apoenzyme complex (Table III). In fact, the dissociation constant for FAD–native apoenzyme complex could not be obtained from the concentration range of FAD employed in the experiment shown in Fig. 2. The Michaelis constant for D-alanine with respect to reduced pyridoxal phosphate–apoenzyme was also estimated from the Lineweaver–Burk plot of $1/v$ *versus* 1/[D-alanine] (Fig. 2). From the intercept on the abscissa, a value of $5.4 \cdot 10^{-3}$ M was obtained for the Michaelis constant. As a value of $3.8 \cdot 10^{-3}$ M was obtained for the Michaelis constant of D-alanine with

TABLE III

KINETIC CONSTANTS OF REDUCED PYRIDOXAL PHOSPHATE–APOENZYME

Kinetic constant	*Native apoenzyme*	*Reduced pyridoxal phosphate–apoenzyme*
Michaelis constant for D-alanine (mM)	3.8	5.4
Dissociation constant for FAD (μM)	0.1 *	1.1

* The value was obtained from the data in Fig. 3.

respect to the native apoenzyme, the change in the Michaelis constant for D-alanine by the fixation of pyridoxal phosphate to the apoenzyme was small (Table III). It was also obtained that the maximum velocity of the modified enzyme was about a half that of the native enzyme. As the apoenzyme treated with $NaBH_4$ in the absence of pyridoxal phosphate resulted in no loss of the enzymic activity, nor changes in the kinetic constants of the enzyme reaction, the changes in the kinetic constants and the reaction velocity by the modification were due to the fixation of pyridoxal phosphate. Therefore, the results in Fig. 2 showed that reduced pyridoxal phosphate–apoenzyme still combined FAD and exhibited a partial enzymic activity, though the affinity of FAD greatly decreased.

Kinetics of the inhibition of the enzyme with pyridoxal phosphate

The effect of pyridoxal phosphate on the enzyme reaction was investigated kinetically without fixation of pyridoxal phosphate to the apoenzyme, and the results were compared with those obtained from Fig. 2. Double reciprocal plots of $1/v$ *versus* $1/[\text{FAD}]$ and $1/v$ *versus* $1/[\text{D-alanine}]$ in the presence or in the absence of pyridoxal phosphate are shown in Fig. 3. The two lines in each figure intersected at the second quadrant. v_i/v_0 *versus* [PALP] was also plotted at different concentrations of FAD, where v_i and v_0 are the velocity of the enzyme reaction in the presence and in the absence of pyridoxal phosphate, respectively. As shown in Fig. 4, v_i/v_0 decreased by

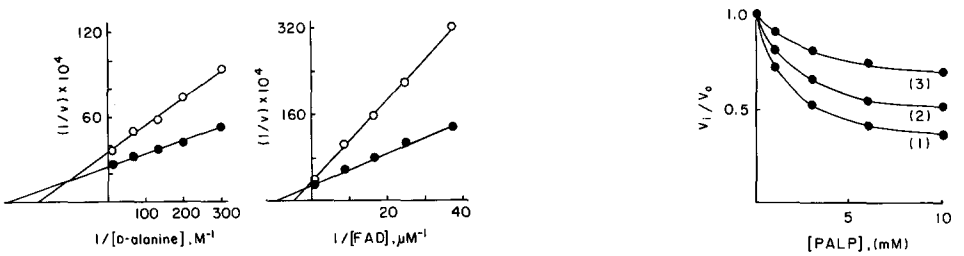


Fig. 3. Double reciprocal plots for D-alanine (left) and FAD (right) in the presence or absence of pyridoxal phosphate. In the plot for D-alanine, 25 μM FAD was used, and in that for FAD, 50 mM DL-alanine was used. The concentrations of the apoenzyme and pyridoxal phosphate were 15 μg and 2 mM, respectively. Initial velocity (v) is expressed as μl O_2 consumed per min per mg protein under the assay conditions defined in MATERIALS AND METHODS. ●———●, in the absence of pyridoxal phosphate; ○———○, in the presence of pyridoxal phosphate.

Fig. 4. Plot of v_i/v_0 *versus* pyridoxal phosphate ([PALP]) at several concentrations of FAD. The concentrations of the apoenzyme and DL-alanine were 20 μg and 50 mM, respectively. The concentration of FAD was (1) 0.67 μM, (2) 1.82 μM, and (3) 20 μM, respectively.

increasing the concentration of pyridoxal phosphate, but the extent of the inhibition was dependent on the concentration of FAD. It was also observed that v_i/v_0 seemed to reach a constant value by increasing the concentration of pyridoxal phosphate at each concentration of FAD. These results suggested that the inhibition was of a mixed type as was described by DIXON AND WEBB[20], and pyridoxal phosphate influenced the binding of FAD and D-alanine to the apoenzyme. A mechanism was therefore assumed for the inhibition of the enzyme reaction by pyridoxal phosphate (Mechanism I). From the mechanism, the following equation is obtained

TABLE IV

ESTIMATION OF THE VALUE OF $K_1(1/f + 1/K_4)/K_2(1 + K_1/f)$ AND $k'K_1/kK_2K_4$ IN Eqn. 1

FAD (*μM*)	$A = \frac{K_1(1/f + 1/K_4)}{K_2(1 + K_1/f)}$ * (M^{-1})	$B = \frac{k'K_1}{kK_2K_4}$ * (M^{-1})	$\frac{B}{A} = \frac{v_{i\infty}}{v_0}$ **
0.67	600	150	0.25
1.82	405	150	0.37
20.00	270	150	0.56

* By applying the values of v_i/v_0 and [pyridoxal phosphate] in Fig. 4 to Eqn. 1, four equations were obtained at a given concentration of FAD. Then the values in Table IV were obtained by solving simultaneous equation from different combinations of two equations among the four equations.

** As Eqn. 1 becomes $v_{i\infty}/v_0 = (k'K_1/kK_2K_4) / (K_1(1/f + 1/K_4)/K_2(1 + K_1/f)]$ at infinite concentration of pyridoxal phosphate, $v_{i\infty}/v_0$ is obtained from A and B.

$$v_i/v_0 = \frac{1 + [k'K_1i/kK_2K_4]}{1 + [K_1(1/f + 1/K_4)i/K_2(1 + K_1/f)]} \quad (1)$$

where i and f are the concentrations of pyridoxal phosphate and FAD, respectively. The results shown in Fig. 4 were then analysed by the use of Eqn. 1. As shown in Tables IV and V, each term in Eqn. 1 was constant at a given concentration of FAD, and v_i/v_0 gave a value at infinite concentration of pyridoxal phosphate and at a given concentration of FAD. On the other hand, the equation which was obtained by assuming that AIF was inactive, did not satisfy the curves in Fig. 4 (Table V). As the

TABLE V

ESTIMATION OF $K_1(1/f + 1/K_4)/K_2(1 + K_1/f)$

A. Values calculated from Eqn. 1, Values expressed as M^{-1}. For the calculations a constant value of 150 M^{-1} was used for $k'K_1/kK_2K_4$.

Pyridoxal phosphate (*mM*)	*0.67 μM FAD*	*1.82 μM FAD*	*20.0 μM FAD*
1	597	402	264
3	596	410	263
6	605	409	261
10	594	400	262

B. When it is assumed that AIF is inactive, the following equation is obtained from Eqn. 1: $v_i/v_0 = 1/\{1 + [K_1(1/f + 1/K_4)i/K_2(1 + K_1/f)]\}$. From this equation the values (expressed as M^{-1}) were calculated.

Pyridoxal phosphate (*mM*)	*0.67 μM FAD*	*1.82 μM FAD*	*20.0 μM FAD*
1	389	219	99
3	307	179	78
6	239	136	59
10	177	100	45

following equation was obtained from Eqn. 1 at infinite concentration of pyridoxal phosphate, k'/k and K_4 were estimated.

$$k'/k = v_{i\infty}(f + K_4)/v_0(f + K_1) \quad (2)$$

where $v_{i\infty}$ is the velocity of the enzyme reaction at infinite concentration of pyridoxal phosphate. The two values, k'/k and K_4, were shown in Table VI. It was thus confirmed from these results that the mechanism of the inhibition of the enzyme by pyridoxal phosphate was as represented in Mechanism I, in which pyridoxal phosphate apparently influenced the binding of FAD to the apoenzyme, and the ternary

TABLE VI

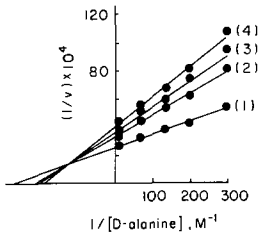
SUMMARY OF KINETIC CONSTANTS OF D-AMINO ACID OXIDASE AND ITS MODIFIED FORM

	Native apoenzyme (M)	*Reduced pyridoxal phosphate–apoenzyme (M)*
Dissociation constant for FAD (K_1)	$0.1 \cdot 10^{-6}$	$1.1 \cdot 10^{-6}$
Dissociation constant for FAD in the presence of pyridoxal phosphate (K_4)	$1.1 \cdot 10^{-6}$	—
Michaelis constant for D-alanine in the presence of FAD	$3.8 \cdot 10^{-3}$	$5.4 \cdot 10^{-3}$
Michaelis constant for D-alanine in the presence of FAD and pyridoxal phosphate	$5.4 \cdot 10^{-3}$	—
k'/k	0.57*	0.52

* By applying the values of $v_{i\infty}/v_0$ and [FAD] in Table IV to Eqn. 2, three equations were obtained. Then the value of k'/k was obtained by solving simultaneous equation from different combinations of two equations among the three equations.

complex (AIF) had 57% enzymic activity of the native holoenzyme. It is of interest to note that the enzymic activity of the ternary complex and the apparent dissociation constant of FAD with respect to the ternary complex were almost the same as those obtained with reduced pyridoxal phosphate–apoenzyme (Table VI). These findings strongly suggest that the mechanism of the inhibition by pyridoxal phosphate is essentially identical irrespective of the type of linkage of pyridoxal phosphate to the apoenzyme (aldimine or reduced aldimine).

The effect of pyridoxal phosphate on the Michaelis constant for D-alanine was also estimated. Double reciprocal plot of $1/v$ *versus* 1/[D-alanine] at different concentrations of pyridoxal phosphate was shown in Fig. 5. The lines intersected at the second quadrant, and the values of the lines at the intercept to the abscissa increased by increasing the concentration of pyridoxal phosphate. The value became almost constant at about 10 mM pyridoxal phosphate (Fig. 6). The Michaelis constant for D-alanine with respect to AIF was approximated from the constant value by assuming that almost all the holoenzyme were in pyridoxal phosphate-bound form at about 10 mM pyridoxal phosphate. The value of $5.4 \cdot 10^{-3}$ M was obtained for the Michaelis



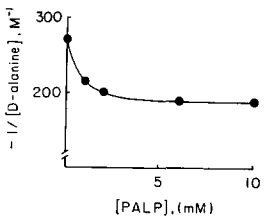


Fig. 5. Double reciprocal plot for D-alanine at several concentrations of pyridoxal phosphate. The concentrations of pyridoxal phosphate were (1), 0 mM; (2), 1 mM; (3), 2 mM; and (4), 6 mM. The concentration of the apoenzyme and FAD used were 15 μg and 7 μM, respectively. Initial velocity (v) is expressed as μl O_2 consumed per min per mg protein under the assay conditions defined in MATERIALS AND METHODS.

Fig. 6. Plot of $-1/$[D-alanine] *versus* [pyridoxal phosphate] ([PALP]). The values were obtained from Fig. 5. The values of $-1/$[D-alanine] were those at the intercept of the lines on the abscissa in Fig. 5. The value at 10 mM pyridoxal phosphate was obtained by doing the experiment under the same conditions employed in Fig. 5.

constant, and it compared well with that of the Michaelis constant for D-alanine in reduced pyridoxal phosphate-apoenzyme system (Table VI).

Effect of benzoate on FAD-bound reduced pyridoxal phosphate–apoenzyme

The effect of benzoate on the absorption spectra of FAD-bound reduced pyridoxal phosphate–apoenzyme is shown in Fig. 7. In the presence of 8.5 μM FAD and 2 mM benzoate, 17 μM native apoenzyme showed a characteristic absorption spectrum of the ternary complex of the apoenzyme–FAD–benzoate as has been reported by YAGI AND OZAWA[21], while 17 μM reduced pyridoxal phosphate–apoenzyme did not show a typical absorption spectrum of the ternary complex in the presence of the same concentrations of FAD and benzoate that was used for the native apoenzyme. The typical ternary complex type spectrum appeared by increasing the concentration

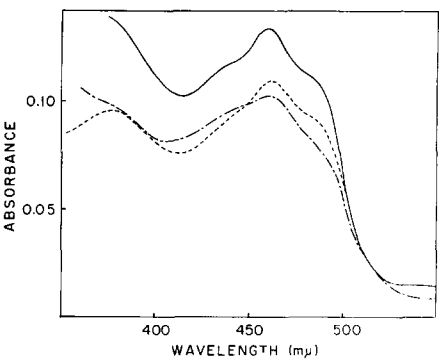


Fig. 7. The absorption spectra of the native apoenzyme and reduced pyridoxal phosphate–apoenzyme in the presence of FAD and benzoate. Each apoenzyme was dissolved in 0.1 M pyrophosphate buffer, pH 8.3. The concentrations of FAD and benzoate were 8.5 μM and 2 mM, respectively. The concentrations of the native apoenzyme and reduced pyridoxal phosphate–apoenzyme were: — — —, the native apoenzyme (17 μM); — · —, the reduced pyridoxal phosphate–apoenzyme (17 μM); ———, the reduced pyridoxal phosphate–apoenzyme (50 μM), respectively.

$$\begin{array}{ccc} & & \overset{K_1}{} \\ A & + & F \rightleftharpoons AF \\ + & & + \\ I & & I \\ \Updownarrow K_2 & & \Updownarrow K_3 \\ AI & + & F \rightleftharpoons AIF \\ & & \underset{K_4}{} \end{array}$$

$$v = k[AF] + k'[AIF]$$

Mechanism I. Mechanism of the interaction of the apoenzyme with FAD and pyridoxal phosphate. A, F, I, AF, AI, and AIF represent the native apoenzyme, FAD, pyridoxal phosphate, FAD-bound apoenzyme, pyridoxal phosphate-bound apoenzyme, and ternary complex of apoenzyme–FAD–pyridoxal phosphate, respectively. v is the velocity of the enzyme reaction, and k and k' are the rate constants of the reaction of AF and AIF, respectively. K_1, K_2, K_3, and K_4 represent dissociation constants, *i. e.* $K_1 = [A][F]/[AF]$, $K_2 = [A][I]/[AI]$, $K_3 = [AF][I]/[AIF]$, and $K_4 = [AI][F]/[AIF]$.

of reduced pyridoxal phosphate–apoenzyme up to 50 μM. Though an increase in absorbance to shorter wavelength due to the tail end of the absorption of the fixed reduced pyridoxal phosphate was observed, the spectrum has a peak and a shoulder at 465 mμ and 495 mμ, respectively. These observations again indicated that the affinity of FAD to the apoenzyme decreased by the fixation of pyridoxal phosphate. In addition, it was shown that there was no essential difference in the effect of benzoate on the absorption spectrum of enzyme-bound FAD between the native apoenzyme and reduced pyridoxal phosphate–apoenzyme.

DISCUSSION

Recent studies on the modification of D-amino acid oxidase have been performed to get informations on the properties of the active site of the enzyme. Trinitrobenzene sulfonate[22], glyoxal[23], and succinic anhydride[15] have been used for this purpose. These compounds were incorporated in the enzyme, and the enzymic activity was greatly reduced by these modifications with a concomitant change in the absorption spectrum of enzyme-bound FAD. Benzoate showed a protective effect on these modifications. These evidences suggest that some amino acid residue, probably either a lysine or an arginine residue, is involved in the active site of the enzyme. However, the lack of the specificity in the modifications by these reagents has hampered the definite conclusion leading to the understanding of the nature of the active site.

In this respect, the evidences reported by COFFEY *et al.*[4], and HELLERMAN AND COFFEY[5] strongly suggested that a lysine residue was involved in the active site, though a very recent communication reported by MASSEY *et al.*[6] did not support the idea of direct participation of the lysine residue in the enzyme reaction. FONDA AND ANDERSON[24] also indicated that 3 moles of maleimide were bound per 50 000 g of D-amino acid oxidase. It attacked sulfhydryl groups selectively, and the one specific sulfhydryl group was concerned with FAD-binding site.

Several lines of evidences in the present results indicated that 2 moles of pyridoxal phosphate per 50 000 g of protein were fixed to lysine residues as a reduced Schiff's base by the reductive action of $NaBH_4$. The affinity of FAD for the modified apoenzyme was about 10-fold less than that of the native apoenzyme, while the Michaelis constant for D-alanine was not so much influenced by the modification.

Furthermore, the modified apoenzyme was partially active. These results suggested that pyridoxal phosphate reacted with the lysine residue, which is located near the FAD-binding site but not directly involved in it. Kinetics on the inhibition of the enzyme by pyridoxal phosphate without reduction by $NaBH_4$ confirmed that the lysine residues which reacted with pyridoxal phosphate closely related with the FAD-binding site.

It was noted that there was almost no difference in the apparent dissociation constants for FAD, in the Michaelis constant for D-alanine, and in the enzymic activity between the reduced pyridoxal phosphate–apoenzyme and the pyridoxal phosphate–apoenzyme. This fact indicates that the type of bonding between pyridoxal phosphate and lysine residues of the apoenzyme does not affect the kinetics of enzyme. Accordingly, a steric effect due to the pyridoxal phosphate bound near the active site might be one of the main factors for the inhibition rather than the modification of the active site *per se*. In connection with this problem, it is also interesting to consider whether the lysine residue which reacts with substrate-intermediate[4,5] is identical with one of the lysine residues which react with pyridoxal phosphate or not. Though the present paper could not confirm this problem, it seems very important for the elucidation of the structure of the active site and the role of lysine residues in the enzyme reaction.

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