Cooperative Binding of Coenzyme in D-Amino Acid Oxidase†

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ABSTRACT: The binding property of the coenzyme flavin adenine dinucleotide (FAD) in D-amino acid oxidase (D-amino acid:oxygen oxidoreductase (deaminating), EC 1.4.3.3) was investigated in relation to the equilibrium state of the enzyme between monomer and dimer. The apparent equilibrium constant of FAD dissociation, K, was determined under various conditions, by means of measurements of fluorescence intensity and of polarization of the enzyme solution. The value of K at pH 8.3 and 20 °C was dependent on the concentration of the enzyme, increasing at low concentration and decreasing at high concentration. The temperature dependence of K was also examined at pH 8.3. At least three phases in enthalpy change were observed in the Arrhenius plot of K. Two of three components showed negative changes in enthalpy at lower temperature, while the third component showed positive changes at higher temperature. Temperature transitions were obtained at around 30 and 18 °C, and the latter could be attributed to the conformational change reported. The K value also depended on the pH of the solution: above pH 9.7, the values were about 2.5 times larger than at pH 9.4. This could be ascribed to the ionization of the 3-imino group of the isoalloxazine nucleus of FAD. Hill coefficients of FAD binding obtained when we varied the concentration of the holoenzyme were close to 1 at lower concentration and increased at higher concentration, indicating that the binding process exhibits positive cooperativity. Considering that D-amino acid oxidase is in a state of equilibrium between monomer and dimer, we determined all of the equilibrium constants for FAD dissociation by computer simulation. From the data thus obtained and the equilibrium constant for the dissociation of the apoenzyme dimer into monomers evaluated from sedimentation coefficients, both the dissociation constant for the symmetric (holoenzyme) dimer and the dissociation constant for the asymmetric (holoenzyme–apoenzyme) dimer were calculated at 20 °C and pH 8.3. The former value was in good agreement with that presumed from the sedimentation coefficient of the holoenzyme. On the basis of these data, it could be concluded that the asymmetric dimer is very unstable.

The concentration-dependent dissociation and association of D-amino acid oxidase (D-amino acid:O_2 oxidoreductase (deaminating), EC 1.4.3.3) have been widely investigated. Massey et al. (1961), Charlwood et al. (1961), and Antonini et al. (1966) reported a concentration-dependent polymerization of the enzyme. However, Yagi and co-workers (Yagi et al., 1967; Yagi & Ohishi, 1972) reported that this enzyme exists in a dimeric state, especially when the enzyme is combined with benzoate, and that polymers do not exist at ordinary concentration of the enzyme. They also found that the apoenzyme exists as a monomer but dimerizes when it binds the coenzyme flavin adenine dinucleotide (FAD).[1] This view was confirmed by Fonda & Anderson (1968).

A concentration-dependent dimerization of the apoenzyme was also previously demonstrated by Yagi et al. (1968) and further emphasized by Henn & Ackers (1969). Presently, most authors seem to agree on this point, i.e., that, at the usual concentrations used for physico–chemical investigations, the enzyme is only involved in monomer–dimer equilibrium. Recently, it was further established that the dissociation constant for the coenzyme is smaller for the dimeric than for the monomeric form of this enzyme (Yagi et al., 1975).

The above results led us to study the fine mechanism of binding of the coenzyme in D-amino acid oxidase. In our previous work (Yagi et al., 1975), the relative quantum yield of FAD bound to the enzyme to free FAD was assumed to be independent of protein concentration. When we considered that the protein conformation surrounding FAD may differ between monomer and dimer, a more rigorous analysis appeared to be required for a detailed description of the mechanism of coenzyme binding.

Such a study was considered to be worthwhile since differences in enzymatic activities for the monomer and dimer forms of the enzyme were reported. The molecular activity of the monomer was found to be larger than that of the dimer (Shiga & Shiga, 1971, 1972; Yagi et al., 1972a, 1973). On the other hand, the rate of formation of the purple intermediate was larger in the dimer than in the monomer. Accordingly, the dissociation of the product from the enzyme was suspected to be larger in the monomer than in the dimer (Yagi et al., 1972b).

Materials and Methods

Chemicals. D-Amino acid oxidase was purified from hog kidney according to the method reported previously (Yagi et al., 1967). The apoenzyme was prepared by dialysis against 1 M KBr according to Massey & Curti (1966). FAD was prepared by splitting it from the purified holoenzyme (Yagi et al., 1975).

Fluorescence Measurements. Fluorescence spectra were recorded on a Shimadzu corrected recording spectrofluorometer, RF-502. The instrument was fitted with Polacoat ultraviolet polarizing filters for the measurements of polarization degree. The temperature-controlled sample was excited at 450 nm (slit width 15 nm) and monitored at 530 nm (slit width 7.5 nm). The depolarization effect by the instrument was corrected according to the method described elsewhere (Yagi et al., 1975). Polarization anisotropy, g, was calculated from polarization degree, P, as

$$g = \frac{2}{3}\left(\frac{1}{P} - \frac{1}{3}\right)^{-1} \quad (1)$$

Determination of the Apparent Equilibrium Constant of FAD Dissociation, K. By measurement of the fluorescence intensity of the enzyme solution (I) and that of the solution containing an equimolar amount of free FAD (I_0), the value for R_1 can be obtained. R_1 represents the relative intensity

$$R_1 = \frac{I}{I_0} \quad (2)$$

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[1] Abbreviation used: FAD, flavin adenine dinucleotide.

0006-2960/79/0418-1531$01.00/0

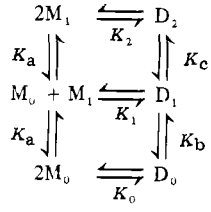
of FAD fluorescence of the enzyme solution (FAD fluorescence bound to the enzyme plus dissociated free FAD) to that of free FAD. On the other hand, from the polarization anisotropy of the enzyme solution, the relative intensity of FAD fluorescence bound to the enzyme to that of free FAD in the enzyme solution, R_2, can be calculated as

$$R_2 = \frac{g - g_f}{g_b - g} \tag{3}$$

where g_f, g_b, and g indicate polarization anisotropies of free FAD, of FAD bound to the enzyme, and of the enzyme solution observed, respectively. Equation 3 is based on the additivity law of polarization anisotropy derived by Weber (1952) and Rawitch & Weber (1972). g_f is obtained as 0.027, but g_b is not known. Then, considering that rotational relaxation times of the monomer and the dimer are much longer than the lifetime of FAD bound to the protein (Yagi et al., 1975, 1977) and that the coenzyme is fixed on the apoenzyme, we assumed that g_b is equal to 0.4. According to Weber (1950), the limiting polarization degree of flavins is 0.5 when the first absorption band is excited. Sum et al. (1972) supported this result by measuring the polarization spectra of flavins at low temperature, although the value obtained by them was slightly lower than 0.5. Thus, the K value can be obtained by R_1, R_2, total protein concentration ($[P]_0$), and total FAD concentration ($[F]_0$).

$$K = \frac{R_1}{1 + R_2 - R_1}\left([P]_0 - [F]_0 + \frac{R_1}{1 + R_2}[F]_0 \right) \tag{4}$$

Reaction Scheme of FAD Binding. Sedimentation coefficients of D-amino acid oxidase reported previously are summarized in Table I. For the holoenzyme, $s_{20,w}$ did not change appreciably in the range of enzyme concentration from 5×10^{-5} to 3.8×10^{-4} M. By contrast, $s_{20,w}$ for the apoenzyme in this range changes as the concentration is varied. The values of $s_{20,w}$ are 4.3 S at 5.1×10^{-5} M and 6.3 S at 3.2×10^{-4} M. These results indicate that the holoenzyme exists exclusively as a dimer while the apoenzyme is in a state of equilibrium between monomer and dimer at the above concentrations. The reaction scheme is



where M and D indicate the species for the monomer and the dimer, respectively. The number of coenzyme molecules bound to the protein moiety is shown by the suffix for M or D. K_a, K_b, and K_c represent the equilibrium constants of FAD dissociation corresponding to the processes indicated. K_2, K_1, and K_0 denote the equilibrium constants for the dissociation of the protein dimers into the corresponding monomers.

Calculation of the Equilibrium Constants. In order to analyze the reaction mechanism, an attempt was made to determine all of the equilibrium constants. The total concentration of FAD ($[F]_0$) and that of the protein ($[P]_0$) are represented by eq 5 and 6. Then, K_0 can be expressed in terms

$$[F]_0 = [F] + [M_1] + [D_1] + 2[D_2] \tag{5}$$

$$[P]_0 = [M_0] + [M_1] + 2[D_0] + 2[D_1] + 2[D_2] \tag{6}$$

$$K_0 = \frac{K_b\left[\frac{2([F]_0 - [F])}{[F]}\left(1 + \frac{[F]}{K_b} + \frac{[F]^2}{K_b K_c} \right) - \frac{[P]_0}{K_b}\left(1 + \frac{2[F]}{K_c} \right) \right]^2}{\left[1 + \frac{2[F]}{K_c} - \frac{K_b}{K_a}\left(2 + \frac{[F]}{K_b} \right) \right]\left[\frac{[F]_0 - [F]}{[F]}\left(1 + \frac{[F]}{K_a} \right) - \frac{[P]_0}{K_a} \right]} \tag{7}$$

of K_a, K_b, K_c, and the concentration of free FAD, [F], as in eq 7. Among the equilibrium constants, the following relations hold:

$$\frac{K_b}{K_a} = \frac{K_1}{K_0} \tag{8}$$

$$\frac{K_c}{K_a} = \frac{K_2}{K_1} \tag{9}$$

Therefore, only K_a, K_b, and K_c can be independent variables. $K_c = 1 \times 10^{-8}$ M was used for the calculation (Yagi et al., 1975). K_a was determined so as to minimize a relative standard deviation for K_0 in eq 7 by use of hypothetical value for K_b, since the relative standard deviation was not sensitive to K_b but was remarkably dependent on K_a. K_b was determined from K_c, K_a, $[F]_0$, $[P]_0$, and [F] so as to fit the value of K_0 estimated by the sedimentation coefficients. The concentration of free FAD was obtained from R_1 and R_2. According to eq 8 and 9, K_2 and K_1 were calculated from K_a, K_b, K_c, and K_0 thus obtained.

Table I: Sedimentation Coefficients of D-Amino Acid Oxidase[a]

	protein concn (M)	$s_{20,w}$ (S)
holoenzyme[b]	38×10^{-5}	7.3
	5	7.1
apoenzyme[c]	32×10^{-5}	6.3
	20	6.0
	8.2	4.9
	7.2	4.7
	5.1	4.3

[a] pH 8.3, 0.017 M pyrophosphate buffer. [b] Data from Yagi et al. (1967). [c] Data from Yagi et al. (1977).

Results

Concentration Dependence of K. g and R_1 were measured at various concentrations of the enzyme and of free FAD as shown in Figure 1. When we lowered the concentration, g decreased while R_1 increased. From g and R_1, K was determined at pH 8.3 and 20 °C according to eq 4. K depended on the concentration of the protein. As shown in Figure 2A, K increased as the concentration of the holoenzyme was lowered. From this result, it is obvious that K_c is smaller than K_a. Free FAD ($[F]_0 = 3.6 \times 10^{-6}$ M) was titrated with the apoenzyme (Figure 2B). K increased in the range of $[P]_0$ higher than 2×10^{-5} M. In the case of the apoenzyme, addition of an excess of the apoenzyme to the solution should accelerate a formation of D_0, as judged from the result shown in Table I. Therefore K_b is considered to be quite larger than K_a.

Temperature Dependence of K. Both g and R_1 are dependent on solution temperature as can be seen from Figure 3. The value of g reached its maximum at 19 °C and de-

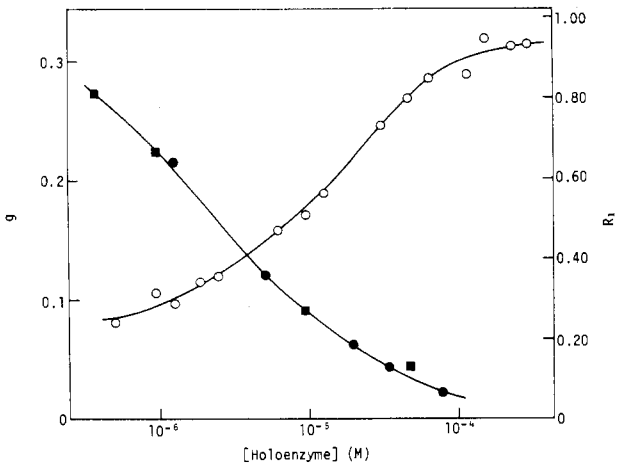


FIGURE 1: Concentration dependences of g and R_1. Measurements were made at pH 8.3 and 20 °C. The open circle indicates the g value. The filled circle indicates the R_1 value calculated from the fluorescence intensities obtained from the spectrum heights at the maximum wavelength, and the filled square indicates the R_1 value calculated from the fluorescence intensities evaluated from the area of the corrected spectra.

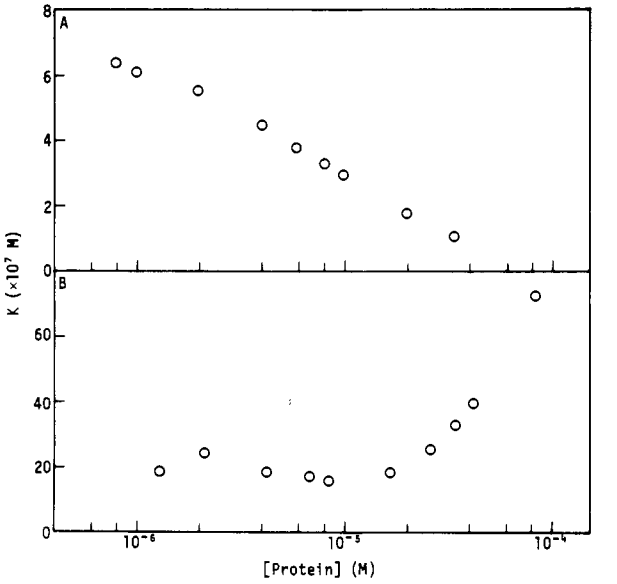


FIGURE 2: Concentration dependences of K. Measurements were made at pH 8.3 and 20 °C. In (A), a high concentration of the holoenzyme (approximately 4×10^{-4} M) was diluted stepwise. In (B), FAD (3.6×10^{-6} M) was titrated with the apoenzyme.

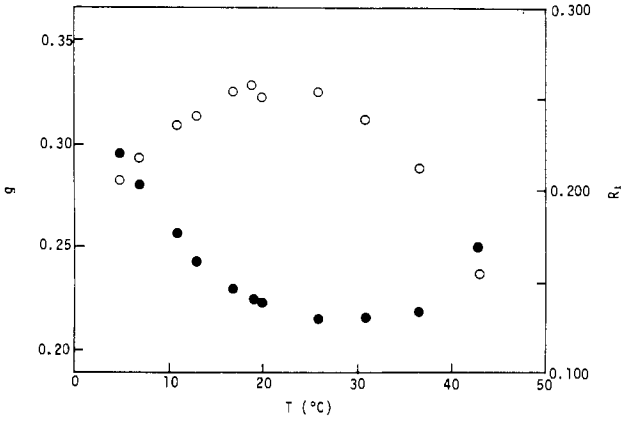


FIGURE 3: Temperature dependences of g and R_1. The enzyme concentration, $[P]_0$, was 3.4×10^{-5} M. Measurements were made at pH 8.3. The open circle indicates the g value and the filled circle indicates the R_1 value.

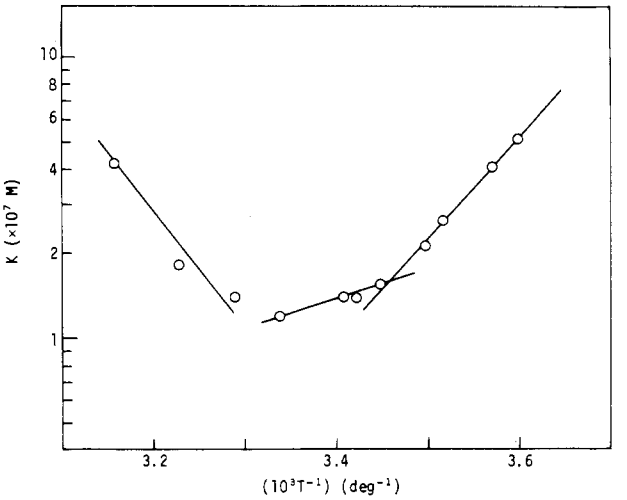


FIGURE 4: Arrhenius plot of K. The K value at the respective temperature was obtained from the data in Figure 3 as described in the text.

Table II: Thermodynamic Parameters of FAD Binding in D-Amino Acid Oxidase[a]

temp range (°C)	$\Delta H°$ (kcal mol^{-1})	$\Delta S°$ (kcal mol^{-1} deg^{-1})	$\Delta G°$ [b] (kcal mol^{-1})
5-17	−1.7	−0.037	9.0 (15 °C)
17-26	−0.48	−0.033	9.2 (20 °C)
26-43	1.9	−0.028	10.6 (37 °C)

[a] Calculated from van't Hoff's equation $\Delta H° - T\Delta S° = -RT \ln K = \Delta G°$. [b] $\Delta G°$ was calculated at the temperature indicated in parentheses.

Table III: Dependence of FAD Binding in D-Amino Acid Oxidase on pH[a]

pH[b]	K (×10⁷ M)	pH[b]	K (×10⁷ M)
7.3	0.66	9.7	3.9
7.9	1.1	10.1	3.2
8.3	1.4	10.6	3.4
9.0	1.2	11.0	3.0
9.4	1.4		

[a] $[P]_0 = 3.4 \times 10^{-5}$ M and the temperature is 20 °C. [b] Phosphate buffer (0.067 M) was used for the pH range 7.3-7.9, pyrophosphate buffer (0.017 M) was used for pH 8.3, and carbonate buffer (0.067 M) was used for the pH range 9.0-11.0.

creased either at lower or at higher temperature. On the other hand, R_1 reached its minimum at around 26 °C and increased either at lower or higher temperature. An Arrhenius plot of K determined by g and R_1 is shown in Figure 4. At least three phases seem to exist. $\Delta H°$ was negative below 30 °C and positive above 30 °C. Judging from $\Delta H°$, we found the temperature transitions at around 30 and at 18 °C. According to Massey & Curti (1966), Koster & Veeger (1968), and Shiga & Shiga (1972), D-amino acid oxidase shows a temperature transition between 14 and 18 °C. This was also found for K. Thermodynamic parameters are given in Table II. At temperatures higher than 30 °C, dissociation of FAD from the protein moiety was enhanced. According to Shiga & Shiga (1972), K_2 itself displayed the temperature transition and the concentration of M_1 increased above the transition temperature. Therefore it seems reasonable to consider that the monomer possesses a larger K value than the dimer; $K_c < K_a$.

pH Dependence of K. The pH dependence of K was examined in the pH range from 7.3 to 11.0 (Table III). The K value was rather constant from pH 7.9 to 9.4. Above pH

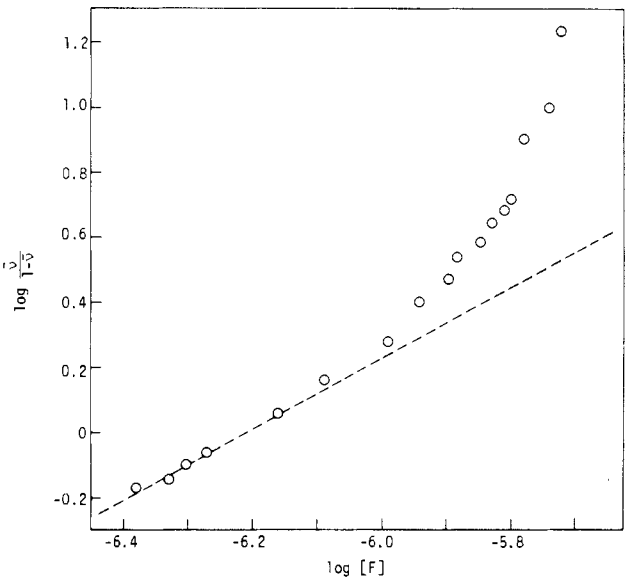


FIGURE 5: Hill plot of FAD binding in D-amino acid oxidase. Measurements were made at pH 8.3 and 20 °C. A binding fraction of FAD, $\bar{\nu}$, was determined by R_1, R_2, $[F]_0$, and $[P]_0$. The dashed line indicates a straight line with the Hill coefficient equal to 1.

9.7, K values were about 2.5 times larger than those obtained at pH 9.4. Massey & Ganther (1965) reported that the pK_a for the 3-imino group of the isoalloxazine ring is 9.4 in the coenzyme bound to D-amino acid oxidase. Accordingly, FAD tends to dissociate from the protein moiety when the hydrogen atom of the 3-imino group is ionized.

Hill Plot of FAD Binding. The binding fraction of FAD ($\bar{\nu}$), defined as a ratio of concentration of FAD bound to the protein to that of total protein, was determined at various values of $[P]_0$ by measurement of g and R_1 at pH 8.3 and 20 °C. The value of $\bar{\nu}$ was calculated from R_1, R_2, $[F]_0$, and $[P]_0$ as

$$\bar{\nu} = \frac{[F]_b}{[P]_0} = \frac{[F]_0}{[P]_0}\left(1 - \frac{R_1}{1 + R_2}\right) \qquad (10)$$

where $[F]_b$ represents the concentration of FAD bound to the protein. The Hill plot obtained is shown in Figure 5. A Hill coefficient equal to 1 is indicated by the straight, dashed line. The Hill coefficient was close to 1 at lower concentration of the holoenzyme and increased at higher concentration. The result clearly indicates that the binding process displays positive cooperativity.

Calculated K Values. The apparent equilibrium constant of FAD dissociation, K, can be expressed in terms of K_a, K_b, and K_c. K was calculated from K_a, K_b, and K_c by eq 11. K_0

$$K = \frac{K_a[M_1] + 2K_b[D_1] + K_c[D_2]}{[M_1] + [D_1] + 2[D_2]} \qquad (11)$$

was highly sensitive to the choice of the parameters. Accordingly, K_a and K_b were determined so as to give the experimental value of K_0. By use of K_a, K_b, K_c, and K_0, concentrations of the species M_1, D_1, and D_2 and the value of K were calculated. Taking the values of $K_a = 7.4 \times 10^{-7}$ M, $K_b = 1.5 \times 10^{-6}$ M, $K_c = 1.0 \times 10^{-8}$ M, and $K_0 = 1.4 \times 10^{-4}$ M, we obtained theoretical values of K as shown by the curve in Figure 6. The data are in fairly good agreement with the observed values. From the above parameters, $K_1 = 2.8 \times 10^{-4}$ M and $K_2 = 3.8 \times 10^{-6}$ M were obtained by eq 8 and 9.

Weight fractions of various species of D-amino acid oxidase were calculated as a function of $[P]_0$ (Figure 7). $[D_2]$ is the main species in the range of $[P]_0$ higher than 10^{-5} M and

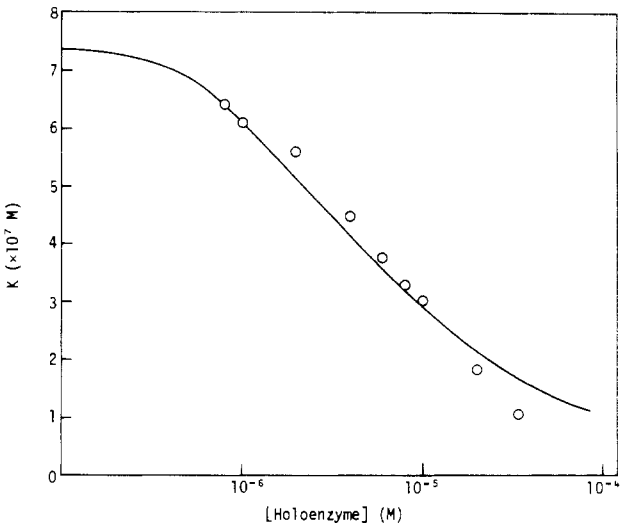


FIGURE 6: Dependence of the calculated K value on concentration of the holoenzyme. The curve indicates the calculated K value as a function of $[P]_0$. Parameters used for the calculation were as follows: $K_a = 7.4 \times 10^{-7}$ M, $K_b = 1.5 \times 10^{-6}$ M, and $K_c = 1 \times 10^{-8}$ M. The K_c used was the experimental value obtained by equilibrium dialysis. K_a was determined so as to obtain the minimum value in relative standard deviation of K_0, SD/K_0. K_b was determined so as to fit the K_0 value evaluated from the sedimentation coefficients of the apoenzyme. Open circles indicate the observed K (redrawn from Figure 2A).

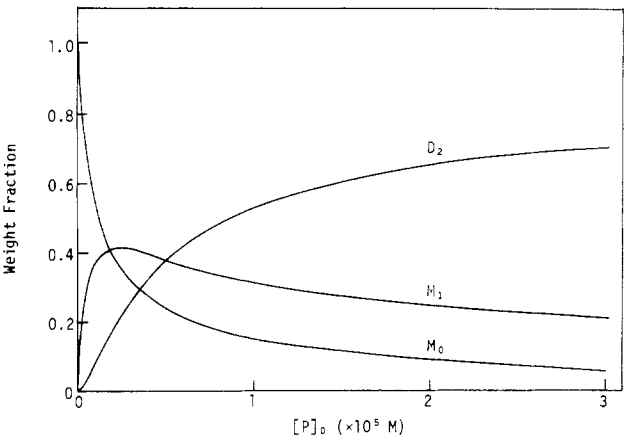


FIGURE 7: Weight fraction of various species of D-amino acid oxidase. M_0, M_1, and D_2 indicate the weight fractions of the respective species. Weight fractions of D_1 and D_0 were negligible in the entire range of $[P]_0$ examined. The K_2, K_1, and K_0 value used for the calculation of weight fractions were 3.8×10^{-6}, 2.8×10^{-4}, and 1.4×10^{-4} M, respectively.

rapidly decreased at $[P]_0$ below 5×10^{-6} M. On the other hand, the fraction of M_0 is small in the range of $[P]_0$ higher than 5×10^{-6} M. The fraction of M_1 (monomer of the holoenzyme) increased as $[P]_0$ was lowered and attained a maximum at $[P]_0$ of 2×10^{-6} M. Weight fractions for D_1 and D_0 were negligible in the whole range of $[P]_0$ examined.

Discussion

The equilibrium state among various species of D-amino acid oxidase involved in monomer–dimer equilibrium was quantitatively investigated. Precise analysis was made possible through determination of the apparent equilibrium constant of FAD dissociation based on the measurements of fluorescence intensity and of polarization anisotropy of the coenzyme. The quaternary structure of D-amino acid oxidase can be examined by analysis of the apparent equilibrium constant. The present method is useful for such a study when a small

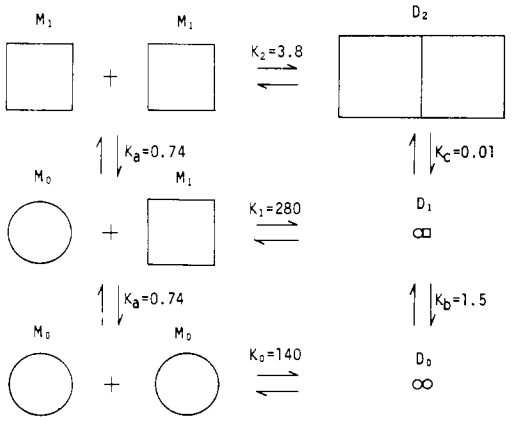


FIGURE 8: Relative concentrations of various species of D-amino acid oxidase. The concentration of the enzyme was 5×10^{-6} M. The values of the various equilibrium constants were indicated in micromolar units. The square and the circle show the species of the holoenzyme and the apoenzyme. Relative concentrations of the species are represented as area.

ligand is fluorescent both in its free and associated forms.

It was demonstrated that the binding process of FAD exhibits positive cooperativity. The reaction mechanism is easily delineated from the relative concentrations of various species of the enzyme. Figure 8 illustrates the equilibrium state at $[P]_0$ of 5×10^{-6} M. The holoenzyme and the apoenzyme are represented by a square and a circle, respectively. Relative concentrations of these species are shown by their size. From Figure 8 we can conclude the following: (a) both D_0 and D_1 are unstable, (b) K_b is much larger than K_a, as judged from the result obtained with the apoenzyme (see Figure 2B), (c) K_a is much larger than K_c, which is consistent with our previous result (Yagi et al., 1975), (d) the dissociation constant for holoenzyme dimers is much smaller than that for apoenzyme dimers, while that for asymmetric dimers is greater than that obtained for dimers of the apoenzyme; $K_2 \ll K_0 < K_1$. This is in good agreement with the result obtained by sedimentation analysis (Table I). The K_2 value is several times larger than that obtained by enzyme assay (Shiga & Shiga, 1972). The difference can be ascribed to an effect of substrate binding to the holoenzyme under these experimental conditions.

It should be noted that in the present experiment all dissociation constants were determined on the basis of experimental data. In most of the treatments in the theoretical consideration so far made on the cooperativity, K_c was assumed to be equal to K_b except for a statistical factor (Monod et al., 1965; Nichol et al., 1967). However, our results indicate that this assumption is not always valid. It seems significant to point out that the asymmetric dimer, D_1, is unstable. In relation to this finding, the allosteric model proposed by Monod et al. (1965) should be recalled. The model is based on the assumption that both protomers in a dimer must be in the same conformational state so that the symmetry is conserved. The greater stability of D_2 as compared to D_1 supports this assumption.

It is evident that the dissociation of FAD induces monomerization. The mechanism of the positive cooperativity coincides essentially with that proposed by Nichol et al. (1967) and Nichol & Winzor (1976). Ligand-induced polymerization of a protein was considered as an alternative model to explain the allosteric effect. Horiike et al. (1977a) also examined in detail the ligand-induced modifications of a monomer–dimer equilibrium state.

Cooperativity for *p*-aminobenzoate binding to D-amino acid oxidase could not be demonstrated in the pH range of 6.5–10.0 (Shiga et al., 1973; Horiike et al., 1974, 1976, 1977a,b). According to their mechanism, *p*-aminobenzoate association to one of the subunits of the dimer did not affect further binding of the ligand. By contrast, binding of 1 molecule of FAD to one of the subunits of the apoenzyme dimer greatly affects further binding to the second subunit ($K_c \ll K_b$). Although *p*-aminobenzoate binding is noncooperative, it induces dimerization of the protein (Horiike et al., 1977a,b).

Upon formation of a multiple ligand complex, a cooperative effect has been observed for a large number of enzymes. Kolb & Weber (1975) demonstrated cooperativity in binding of both oxalate and NADH to lactate dehydrogenase. Cooperative binding of the coenzyme was also reported in some NAD enzymes. The binding of NAD to glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle exhibits negative cooperativity (Conway & Koshland, 1968; De Vijlder & Slater, 1968). Pyridoxal 5′-phosphate binds with positive cooperativity to the apo-β_2 but with noncooperativity to the α_2–apo-β_2 complex of tryptophan synthase from *Escherichia coli* (Bartholmes et al., 1976). On the basis of the above data, D-amino acid oxidase displays similar characteristics. Precise information has been provided on the cooperative binding of FAD accompanied by a modification in the quaternary structure of the enzyme.

References

Antonini, E., Brunori, M., Bruzzesi, M. R., Chiancone, E., & Massey, V. (1966) *J. Biol. Chem. 241*, 2358.

Bartholmes, P., Kirschner, K., & Gschwind, H.-P. (1976) *Biochemistry 15*, 4712.

Charlwood, P. A., Palmer, G., & Bennett, R. (1961) *Biochim. Biophys. Acta 50*, 17.

Conway, A., & Koshland, D. E., Jr. (1968) *Biochemistry 7*, 4011.

De Vijlder, J. J. M., & Slater, E. C. (1968) *Biochim. Biophys. Acta 167*, 23.

Fonda, M. L., & Anderson, B. M. (1968) *J. Biol. Chem. 243*, 5635.

Henn, S. W., & Ackers, G. K. (1969) *Biochemistry 8*, 3829.

Horiike, K., Shiga, K., Isomoto, A., & Yamano, T. (1974) *J. Biochem. (Tokyo) 75*, 925.

Horiike, K., Shiga, K., Isomoto, A., & Yamano, T. (1976) *J. Biochem. (Tokyo) 80*, 1073.

Horiike, K., Shiga, K., Isomoto, A., & Yamano, T. (1977a) *J. Biochem. (Tokyo) 81*, 179.

Horiike, K., Shiga, K., Nishina, Y., Isomoto, A., & Yamano, T. (1977b) *J. Biochem. (Tokyo) 82*, 1247.

Kolb, D. A., & Weber, G. (1975) *Biochemistry 14*, 4471.

Koster, J. F., & Veeger, C. (1968) *Biochim. Biophys. Acta 167*, 48.

Massey, V., & Ganther, H. (1965) *Biochemistry 4*, 1161.

Massey, V., & Curti, B. (1966) *J. Biol. Chem. 241*, 3417.

Massey, V., Palmer, G., & Bennett, R. (1961) *Biochim. Biophys. Acta 48*, 1.

Monod, J., Wyman, J., & Changeux, J.-P. (1965) *J. Mol. Biol. 12*, 88.

Nichol, L. W., & Winzor, D. J. (1976) *Biochemistry 15*, 3015.

Nichol, L. W., Jackson, W. J. H., & Winzor, D. J. (1967) *Biochemistry 6*, 2449.

Rawitch, A. B., & Weber, G. (1972) *J. Biol. Chem. 247*, 680.

Shiga, K., & Shiga, T. (1971) *Arch. Biochem. Biophys. 145*, 701.

Shiga, K., & Shiga, T. (1972) *Biochim. Biophys. Acta 263*, 294.

Shiga, K., Isomoto, A., Horiike, K., & Yamano, T. (1973) *J. Biochem. (Tokyo) 74*, 481.

Sum, M., Moore, T. A., & Song, P.-S. (1972) *J. Am. Chem. Soc. 94*, 1730.
Weber, G. (1950) *Biochem. J. 47*, 114.
Weber, G. (1952) *Biochem. J. 51*, 145.
Yagi, K., & Ohishi, N. (1972) *J. Biochem. (Tokyo) 71*, 993.
Yagi, K., Naoi, M., Harada, M., Okamura, K., Hidaka, H., Ozawa, T., & Kotaki, A. (1967) *J. Biochem. (Tokyo) 61*, 580.
Yagi, K., Ozawa, T., & Ohishi, N. (1968) *J. Biochem. (Tokyo) 64*, 567.
Yagi, K., Sugiura, N., & Ōhama, H. (1972a) *J. Biochem. (Tokyo) 72*, 215.
Yagi, K., Nishikimi, M., & Ohishi, N. (1972b) *J. Biochem. (Tokyo) 72*, 1369.
Yagi, K., Sugiura, N., Ōhama, H., & Ohishi, N. (1973) *J. Biochem. (Tokyo) 73*, 909.
Yagi, K., Tanaka, F., & Ohishi, N. (1975) *J. Biochem. (Tokyo) 77*, 463.
Yagi, K., Tanaka, F., Ohishi, N., & Morita, M. (1977) *Biochim. Biophys. Acta 492*, 112.

Phosphorus-31 Nuclear Magnetic Resonance Study of D-Serine Dehydratase: Pyridoxal Phosphate Binding Site†

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ABSTRACT: The pyridoxal phosphate dependent enzyme D-serine dehydratase has been investigated using ^{31}P nuclear magnetic resonance (NMR) at 72.86 MHz. In the native enzyme, the pyridoxal phosphate ^{31}P chemical shift is pH dependent with $pK_a = 6.4$, indicating exposure of the phosphate group to solvent. Binding of the competitive inhibitor isoserine results in the formation of the isoserine–pyridoxal phosphate complex. This transaldimination complex is fixed to the enzyme via the phosphate group of the cofactor as the dianion, independent of pH. At pH 6.6 the dissociation constant K_D for isoserine determined by NMR is 0.43 mM. Reconstitution of the apoenzyme with pyridoxal phosphate monomethyl ester produces an inactive enzyme. NMR and fluorescence measurements show that this enzyme does not form the transaldimination complex, indicating that the fixation of the dianionic phosphate (probably via a salt bridge with an arginine residue) observed in the native enzyme is required for the transaldimination step of the catalytic mechanism.

Crystalline D-serine dehydratase (EC 4.2.1.14) from *Escherichia coli* is a monomeric enzyme containing a single polypeptide chain (M_r = 45 500) and has one catalytically essential pyridoxal-P[1] per molecule (Dowhan & Snell, 1970a). From reconstitution studies of D-serine apodehydratase with various pyridoxal-P analogues, it was concluded that substitutions at positions 2 and 6 of pyridoxal-P are not critical for catalytic activity (Dowhan & Snell, 1970b). On the other hand, a phosphate dianion at the 5′ position of the coenzyme is essential for catalysis but not required for the binding of the cofactor (Groman et al., 1972; Kazarinoff & Snell, 1976).

Reconstitution experiments monitored by UV and visible absorption spectroscopy indicate an extension of the π-electron system of the pyridine chromophore consistent with the formation of a Schiff base between cofactor and an ϵ-amino group of a lysyl residue of the protein. This technique, however, provides no information about the environment of the essential phosphate group of the cofactor. ^{31}P nuclear magnetic resonance spectroscopy has been shown to be a powerful tool to obtain direct evidence on the environment of the phosphate group of pyridoxal-P bound to enzymes (Feldmann & Hull, 1977; Martinez-Carrion, 1975). We have therefore utilized this NMR method to gather specific knowledge about the ionization state of pyridoxal-P bound to D-serine dehydratase and its interactions with the protein during catalysis.

Experimental Procedures

Materials. Isoserine was purchased from ICN Pharmaceuticals Inc. (Cleveland, OH). 2-(*N*-Morpholino)ethanesulfonic acid (Mes), 3-(*N*-morpholino)propanesulfonic acid (Mops), and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes) were obtained from Serva (Heidelberg, West Germany). Pyridoxal-P monomethyl ester was prepared according to the method of Pfeuffer et al. (1972). All other chemicals were of analytical reagent grade from Merck (Darmstadt, West Germany).

Enzymes. D-Serine dehydratase was isolated from *Escherichia coli* K 12 mutant C 6 as described previously (Schiltz & Schnackerz, 1976). Enzymatic activity and protein concentrations were determined according to published procedures (Schiltz & Schnackerz, 1976). D-Serine apodehydratase was prepared by using the resolution procedure of Dowhan & Snell (1970a). The apoenzyme had a residual activity of 1.9 units/mg of protein. The specific activity of pyridoxal-P reconstituted dehydratase was 100. Reconstitution of apodehydratase with pyridoxal-P monomethyl ester was achieved by incubating apoenzyme (350 μM) with a fivefold excess of cofactor analogue for 1 h at 25 °C in the dark. The excess

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[1] Abbreviations used: NMR, nuclear magnetic resonance; UV, ultraviolet; AMP, 5′-adenosine monophosphate; pyridoxal-P, pyridoxal phosphate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

0006-2960/79/0418-1536$01.00/0