Temperature-induced changes in the coenzyme environment of D-amino acid oxidase revealed by the multiple decays of FAD fluorescence

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ABSTRACT A temperature-dependent change in the microenvironment of the coenzyme, FAD, of D-amino acid oxidase was investigated by means of steady-state and picosecond time-resolved fluorescence spectroscopy. Relative emission quantum yields from FAD bound to D-amino acid oxidase revealed the temperature transition when concentration of the enzyme was lowered. The observed fluorescence decay curves were well described with four-exponential decay functions. The amplitude of the shortest lifetime (τ_0), ~25 ps, was always negative, which indicates that the fluorescence of D-amino acid oxidase at ~520 nm appears after a metastable state of the excited isoalloxazine decays. The other components with positive amplitudes were assigned to dimer or associated forms of the enzyme, monomer, and free FAD dissociated from the enzyme. Ethalpy and entropy changes of intermediate states in the quenching processes were evaluated according to the absolute rate theory. The temperature transition was much more pronounced in the monomer than in the dimer or associated forms of the enzyme.

INTRODUCTION

A number of works have focused on the association state of D-amino acid oxidase. The investigation of the association state was initiated by Charlwood et al. (1961). Antonini et al. (1966) demonstrated by light-scattering that the monomer of D-amino acid oxidase of molecular weight 90,000, contains two FAD molecules and that it easily polymerizes as the concentration is increased. Later, a few groups (Yagi et al., 1967; Fonda and Anderson 1968; Henn and Ackers, 1969*a* and *b*; Yagi and Ohishi, 1972) reported that D-amino acid oxidase contains one mole of FAD per monomer with molecular weight of 40,000–50,000 and exists as an equilibrium state between monomer and dimer. Many workers have reported that various physicochemical properties of the enzyme are dependent upon the enzyme concentration, and interpreted their data according to the monomer-dimer equilibrium of the enzyme (Shiga and Shiga, 1972; Shiga et al., 1973; Horiike et al., 1974, 1976, 1977; Yagi et al., 1975, 1983; Tanaka and Yagi, 1979, 1980; Nakashima et al., 1980; Fitzpatrick and Massey, 1982). Recently, Tojo et al. (1985*a* and *b*) have reexamined the self-association modes both of apoenzyme and holoenzyme of D-amino acid oxidase by means of a low-angle laser light scattering and found that the observed data fit well with a model of dimerization of monomer with molecular weight of 40,000 at low concentrations and an isodesmic indefinite self association of the dimer at higher concentration.

A temperature-induced change in the protein structure of D-amino acid oxidase was first observed by Massey et al. (1966). They found that tryptophan fluorescence of D-amino acid oxidase displayed a temperature transition at ~15°C; an Arrhenius plot of the enzyme activity is nonlinear and can be represented as two straight lines with different activation energies intersecting at 14°C. Koster and Veeger (1968) concluded from their study of the enzyme activity that there is a temperature-dependent equilibrium between the high and low temperature states. From the analysis of the enzyme activity, the equilibrium constant of association from monomer to dimer exhibited a discontinuous change at 18°C as temperature was varied (Shiga and Shiga, 1972). Despite these findings, however, a differential scanning microcalorimetric study indicated no evidence for the expected specific heat change at the transition temperature (Sturtevant and Mateo, 1978). The reason for the discrepancy in the results derived by different methods still remains uncertain.

In the present work we report that a temperature-induced transition in the microenvironment surrounding FAD in D-amino acid oxidase is revealed by both the measurements of steady-state and picosecond time-

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resolved fluorescence of FAD, and provide a probable explanation for the discrepancies in the previous data on the temperature transition of D-amino acid oxidase.

EXPERIMENTAL PROCEDURE

Materials

D-Amino acid oxidase was purified from hog kidney according to the method reported (Yagi et al., 1967). FAD was purchased from Nakarai Chemicals Co. Ltd. (Kyoto), and purified by a column chromatography on DEAE-cellulose (Massey and Swoboda, 1963).

Relative fluorescence quantum yields

Fluorescence intensity and polarization anisotropy under steady-state excitation were measured by using a Union Giken Fluorescence Polarization Spectrometer FS-501s. Relative intensities (R_1) of the fluorescence of the enzyme solution to that of the solution containing only FAD were measured changing both the enzyme concentration and solution temperature. The ratio of the relative intensity of the fluorescence of FAD-enzyme complex to that of free FAD (R_2) was determined from polarization anisotropy by using Eq. 1 (Weber, 1952; Rawitch and Weber, 1972),

$$R_2 = (A - A_f)/(A_b - A), \quad (1)$$

where A, A_b, and A_f indicate polarization anisotropies of the enzyme solution, FAD bound to the protein and free FAD, respectively. A_f was measured directly and found to be 0.02–0.04 depending on temperature. The exact value of A_b is not known. It should depend on the degree of motional freedom. The isoalloxazine ring of FAD, however, is considered to be fixed in the protein due to hydrogen-bonding, since it contains one proton-donating site and four proton-accepting sites. Hence, we assumed A_b to be 0.4, the limiting degree of polarization anisotropy. The relative fluorescence quantum yield of FAD bound to the enzyme and to that of free FAD, r, was determined from R_1 and R_2 according to Eq. 2 (Nakashima et al., 1980; Yagi et al., 1983).

$$r = R_1R_2/(1 + R_2 - R_1). \quad (2)$$

We also examined the dependence of the value of r on A_b by changing A_b from 0.4 to 0.35. The values of r did not change appreciably by the change of A_b.

Measurements of picosecond fluorescence lifetimes

Fluorescence decay curves of FAD of D-amino acid oxidase were measured for different enzyme concentrations and temperatures. Temperature was controlled by circulating water during the measurements. Initially the third harmonic (355 nm) of a passively mode-locked Nd:YAG laser for excitation and a streak camera (model HTV 1000, Hamamatsu Photonics K. K., Hamamatsu City, Japan) (YAG/SC) were used for the measurements of the fluorescence decay. Details of the method are described in the previous works (Nakashima et al., 1980; Yagi et al., 1983). The pulse width of the third harmonic was typically 25 ps when wide slit (50 μm) of the streak camera was used. Each fluorescence decay curve was obtained by numerically accumulating ten decay profiles. Fluorescence lifetimes were determined by minimizing the root of mean square (RMS) between the observed $I_o(t_i)$, and calculated intensities, $I_c(t_i)$, which were obtained by a convolution procedure of an observed excitation pulse with a three-exponential decay function as in the previous works (Nakashima et al., 1980; Yagi et al., 1983). The value of RMS intensity is expressed by Eq. 3.

$$\mathrm{RMS} = \left\{\frac{1}{N}\sum_{i=1}^{n}[I_o(t_i) - I_c(t_i)]^2\right\}^{1/2}. \quad (3)$$

Fluorescence decay curves were also measured with a synchronously pumped, cavity-pumped dye laser and a picosecond time-correlated, single-photon counting apparatus (PS/PC). Details of the instruments are described elsewhere (Yamazaki et al., 1985). Typical time width of the instrumental response function was 30 ps, while current pulse width of the dye laser was <10 ps. The enzyme was excited at 315 nm through a polarizer adjusted to a magic angle (54.7°). The observed decay curves were fit by a sum of exponentials given in Eq. 4 by a nonlinear least-squares iterative convolution method based on the Marquardt algorithm (Boens et al., 1984; Van Den Zegel et al., 1986; Tamai et al., 1988).

$$F(t) = \sum_{i=0}^{N}\alpha_i \exp(-t/\tau_i). \quad (4)$$

Evaluation of the fits were mainly based on the values of a reduced chi-square and Durbin-Watson parameter (O'Connor and Phillips, 1984) as described in the previous work (Tanaka et al., 1989).

RESULTS

Relative quantum yields

The dependencies of r on the enzyme concentration and temperature at pH 8.3 in 0.017 M pyrophosphate buffer are shown in Fig. 1. At all temperatures examined, the value of r increased as the concentration was lowered. The results indicate that the r value increases as the equilib-

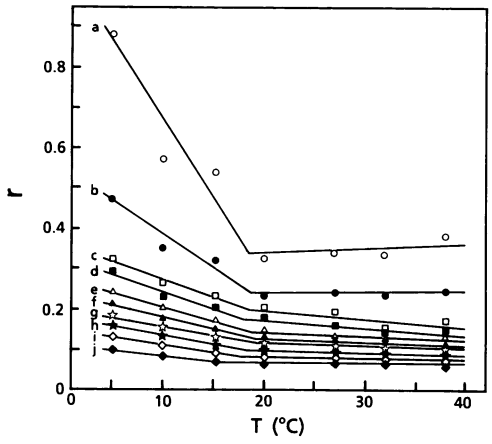

FIGURE 1 Dependence of r on temperature and concentration of D-amino acid oxidase at pH 8.3. The concentrations were as follows: (*a*) 1.0, (*b*) 1.8, (*c*) 3.0, (*d*) 5.0, (*e*) 8.3, (*f*) 14, (*g*) 23, (*h*) 38, (*i*) 64, (*j*) 106, in micromolar. The enzyme was dissolved in 0.017 M pyrophosphate buffer.

rium among monomer, dimer, and higher associated forms shifts toward monomer; in other words, the r value of monomer is larger than that of dimer or higher aggregates. This observation is consistent with the previous measurements (Nakashima et al., 1980) at 20°C. The temperature-dependence of the r value was more pronounced at the lower concentrations than at the higher concentrations. It is also noted that the r value did not change appreciably at any concentration when temperatures were elevated above 20°C. At the lower concentrations the r values profoundly increased as temperature was lowered below 20°C. From Fig. 1 it is evident that a temperature transition takes place at around 18°C. Similar behavior of the r value was also found at pH 7.0 in 0.01 M phosphate buffer as shown in Fig. 2. The transition temperature in this case was slightly lower than that in the solution at pH 8.3. In general the r values obtained at pH 7.0 were lower than those obtained at pH 8.3.

The temperature transition observed in the relative fluorescence quantum yield of FAD combined with the enzyme to free FAD may be explained in the following ways. The equilibrium constant of dissociation of dimer into monomer greatly increases below the transition temperature, because the r value of monomer is higher than those of dimer or associated forms of the enzyme. A conformational transition in the vicinity of FAD otherwise occurs more profoundly in monomer than in dimer or associated forms of the enzyme, so that the r value increases more markedly in monomer than in dimer or associated forms of the enzyme below the transition temperature.

Fluorescence lifetimes of FAD of D-amino acid oxidase

Fluorescence lifetimes obtained at various temperatures by the YAG/SC are listed in Table 1. Concentration of the enzyme was 1 μM in 0.017 M pyrophosphate buffer at pH 8.3, so that any associated forms of the enzyme other than dimer is absent (Tojo et al., 1985*b*). The fluorescence lifetimes of τ_3 for free FAD used were reported values (Spencer & Weber, 1972). The fluorescent species with the shortest lifetime, τ_1, and the intermediate lifetime, τ_2, are assigned to be dimer and monomer, respectively, according to the previous work (Nakashima et al., 1980). This is further verified by the more precise investigation of the fluorescence lifetimes by means of the PS/PC as stated below. The fluorescence lifetimes of dimer were 35 ± 5 ps, showing weak dependence on temperature. This value is in good accordance with the value obtained in the previous work (Nakashima et al., 1980). The lifetime of monomer was 160 ps at 20°C which is slightly longer than that obtained at the previous work

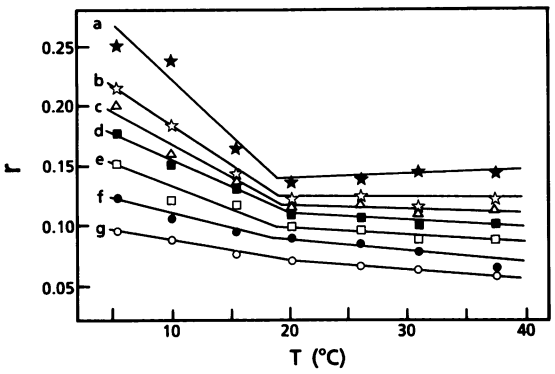

FIGURE 2 Dependence of r on temperature and concentration of D-amino acid oxidase at pH 7.0. The concentrations are as follows (*a*) 1.8, (*b*) 4.9, (*c*) 8.2, (*d*) 23, (*e*) 38, (*f*) 63, (*g*) 105, in micromolar. The enzyme was dissolved in 0.01 M phosphate buffer.

(Nakashima et al., 1980). The lifetime of monomer depended on temperature; 320 ps at 4°C, 280 ps at 10°C, 180 ps at 20°C, 160 ps at 31°C, and 240 ps at 38°C.

Fluorescence lifetimes of FAD bound to D-amino acid oxidase were also systematically examined by changing both temperature from 40 to 10°C and concentration of the enzyme from 100 to 1.6 and 0.78 μM (seven or eight different levels). Fluorescence decay curves of FAD of D-amino acid oxidase obtained at 40°C are shown in Fig. 3 at the concentration of 100 μM. The fluorescence was monitored at 530 nm with a monochromator. The observed decay curves were analyzed with two-, three-, and four-exponential decay functions, the results of which were compared. The best-fit was always obtained using the four-exponential decay functions. Obtained decay parameters for best-fits at the lowest and highest levels of the enzyme and also the averaged lifetimes over all concentrations examined are listed in Table 2. At each temperature from 40 to 10°C the fluorescence lifetimes of τ_1, τ_2, and τ_3 were almost constant within 15%, but

TABLE 1 Fluorescence decay parameters of FAD of D-amino acid oxidase obtained with the mode-locked Nd:YAG laser and streak camera combination

T	τ_1	α_1	τ_2	α_2	τ_3	α_3	RMS*
°C	ps		ps		ns		
4	35	0.71	320	0.18	4.0	0.11	0.0336
10	40	0.71	280	0.17	3.3	0.12	0.0315
20	30	0.65	180	0.22	2.3	0.13	0.0336
31	30	0.63	160	0.21	2.1	0.16	0.0370
38	35	0.66	240	0.16	1.8	0.18	0.0349

The concentration of D-amino acid oxidase was 1 μM in 0.017 M pyrophosphate buffer at pH 8.3. The fluorescent species with the lifetimes of τ_1, τ_2, and τ_3 were assigned to dimer, monomer, and free FAD, respectively. Reported values (Spencer and Weber, 1972) were used for τ_3. *The values of RMS were calculated by Eq. 3.

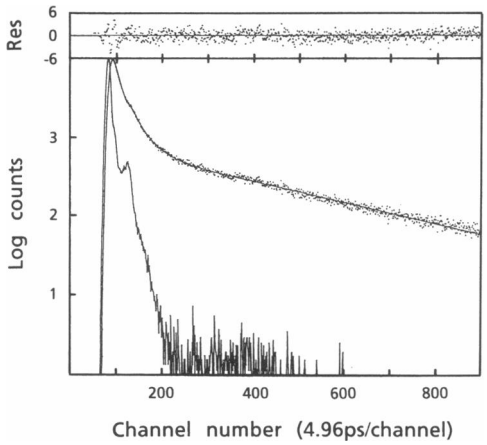

FIGURE 3 Fluorescence decay curve of FAD of 100 μM D-amino acid oxidase. The fluorescence decay curve was measured at 40°C monitoring at 530 nm with a monochromator by means of the synchronously pumped, cavity-dumped dye laser and single-photon counting system. The enzyme was dissolved in 0.017 M pyrophosphate buffer at pH 8.3. The observed intensities are shown with dots. The decay curve was analyzed with a four-exponential decay function, using nonlinear least squares method based on Marquardt algorithm. Calculated decay curve for the best fit is illustrated with a solid curve in the dots. The excitating pulse profile (315 nm) is also shown with another solid curve. Response function of the system was ~30 ps. *Res* indicates weighed residuals between the observed and calculated fluorescence intensities. Obtained decay parameters are listed in Table 2.

component fractions of α_1, α_2, and α_3 significantly changed upon changing the concentration from 100 to 0.78 μM. These results reveal that the concentration fractions of the fluorescent species with lifetimes of τ_1, τ_2, and τ_3 change as the equilibria for the association state of D-amino acid oxidase and also for the binding of free FAD shift upon changing the enzyme concentration. The fluorescent species with the lifetimes of τ_1, τ_2, and τ_3 are assigned to dimer or associated forms of the enzyme, monomer and free FAD dissociated from the enzyme, respectively, because α_1 decreases while α_2 and α_3 increase, when the concentration was lowered. Furthermore the values of τ_3 are in good accordance with the reported lifetimes of free FAD (Spencer and Weber, 1972; Weber et al., 1974; Wahl et al., 1974). More recently Visser (1984) found by means of subnanosecond pulse fluorometry that fluorescence of free FAD shows two lifetime components, 3 ns and 200 ps. He concluded that an intramolecular complex between the isoalloxazine and adenine moieties is weakly fluorescent, which was believed to be nonfluorescent by the earlier workers. The contribution of the fluorescence of free FAD with the shorter lifetime to that of FAD bound to the enzyme, however, should be negligible in the enzyme solution, judging from the values of the amplitude α_3 and the dissociation constant of FAD–D-amino acid oxidase complex (Tanaka and Yagi, 1979, 1980). At concentrations <6.3 μM, where the higher associated states of the enzyme are absent and only dimer, monomer, and free FAD are present in the system (Tojo et al., 1985*b*), the values of τ_1 are slightly longer than those at the higher concentration. Accordingly, the fluorescence lifetime of dimer may be slightly longer than those of the higher aggregates. At any rate it is considered that the lifetime of dimer is not much different from those of higher aggregates. The values of τ_1 and τ_2 are also in good agreement with those obtained with YAG/SC as stated above.

Rate constant for formation of the normal fluorescent state of FAD in D-amino acid oxidase from a metastable fluorescent state

The exponential rise of τ_0 were not observed by the measurements with YAG/SC combination. The values were also nearly constant upon changing the concentration. The component fraction, α_0, with the lifetime of τ_0

TABLE 2 Fluorescence decay parameters of FAD of D-amino acid oxidase obtained with the synchronously pumped, cavity-dumped dye laser and single-photon counting system

T	Conc	τ_0	α_0	τ_1	α_1	τ_2	α_2	τ_3	α_3	χ^{2*}
°C	μM	ps		ps		ps		ns		
40	1.6	27.9	−0.877	43.7	0.850	191	0.079	1.91	0.071	1.028
	100	26.5	−0.962	36.2	0.945	162	0.047	1.61	0.008	1.362
	Av.‡	25.9		40.0		169		1.76		
35	0.78	26.2	−1.030	45.4	0.834	202	0.084	2.10	0.081	0.993
	100	23.5	−0.903	39.9	0.910	161	0.080	1.68	0.010	1.516
	Av.	25.6		41.8		170		1.91		
30	1.6	26.7	−0.989	48.3	0.824	182	0.116	2.23	0.060	1.007
	100	26.7	−1.024	37.7	0.956	169	0.044	1.76	0.005	1.479
	Av.	26.2		43.2		177		2.02		
25	0.78	28.6	−0.842	54.7	0.822	245	0.080	2.47	0.098	1.148
	100	23.5	−0.977	41.0	0.906	165	0.086	1.83	0.008	1.559
	Av.	26.0		43.7		184		2.20		
15	0.78	29.5	−0.841	46.6	0.822	214	0.110	2.78	0.068	1.134
	100	23.5	−1.003	42.9	0.892	179	0.099	1.95	0.009	1.324
	Av.	26.1		45.2		190		2.44		
10	0.78	27.6	−0.980	47.1	0.807	228	0.121	2.93	0.072	1.016
	100	25.3	−0.979	44.2	0.899	194	0.092	2.04	0.009	1.366
	Av.	25.1		48.5		208		2.61		

D-Amino acid oxidase was dissolved in 0.017 M pyrophosphate buffer at pH 8.3. The decay parameters were determined by a nonlinear least-squares method based on the Marquardt algorithm (Tamai et al., 1988). The fluorescent species with the lifetimes, τ_1, τ_2, τ_3 were assigned to be dimer or associated forms of the enzyme, monomer, and free FAD, respectively. *χ^2 means a reduced chi-square distribution. ‡Av. indicates the averaged lifetimes over seven or eight different levels of the enzyme ranging from 100 to 1.6 or 0.78 μM.

was negative at all concentrations and temperatures. The averaged values of $\alpha_0/(\alpha_1 + \alpha_2)$ ratio were -0.985 at 40°C, -1.032 at 35°C, -1.042 at 30°C, -0.992 at 25°C, -1.028 at 20°C, -1.023 at 15°C, -1.033 at 10°C, and -0.993 at 5°C, that is nearly equal to -1. The data at 20 and 5°C were taken from the previous work (Tanaka et al., 1989). These observations indicate that the normal fluorescence of D-amino acid oxidase with its spectrum at ~520 nm which is obtained by steady-state excitation, appears after a metastable state decays. In the previous work (Tanaka et al., 1989) we have demonstrated by the measurements of the picosecond time-resolved fluorescence spectra of D-amino acid oxidase that the metastable state fluoresces at ~580 nm of the emission peak. The transient fluorescence spectra at times later than 100 ps after pulsed excitation were similar to the one obtained by steady-state excitation, and did not change any longer. Hence, the inverse of τ_0 is rate of formation (k_{form}) of the normal fluorescent state of D-amino acid oxidase from the metastable fluorescent state.

Temperature dependence of the averaged lifetimes

Temperature dependence of the averaged fluorescence lifetimes over all concentrations is shown in Fig. 4. The values of τ_1, τ_2, and τ_3 increased as temperature was lowered, whereas that of τ_0 slightly decreased. Temperature dependence of τ_1 and τ_2 were rather small above 20°C.

Temperature dependence of the ratio of component fraction of monomer to that of dimer or associated forms of the enzyme

As can be seen from Table 2, α_2 increases as temperature is lowered at a certain concentration, while α_1 decreases, although these are rather qualitative. Fig. 5 shows the values of ratio, α_2/α_1, at every temperature. The vertical bars indicate the range of the values of the ratio as the concentration was changed. The averaged values of α_2/α_1 over all concentrations are denoted by circles. The ratio, namely, the concentration fraction of monomer to dimer or associated forms of the enzyme, increases as temperature was lowered.

Enthalpy and entropy changes of activation of the various rate constants of the excited states of FAD

The values of fluorescence quenching constants of k_q^A (s = A, i = 1 in Eq. 5) for dimer or associated forms of

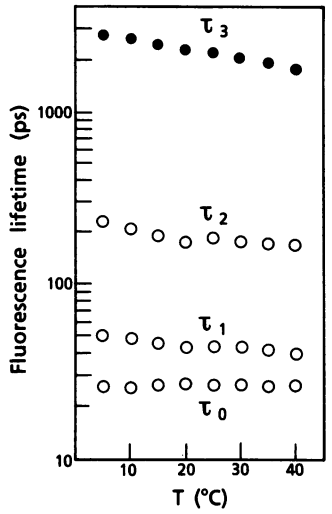

FIGURE 4 Temperature dependence of the averaged fluorescence lifetimes of FAD. The averaged fluorescence lifetimes over all concentrations of D-amino acid oxidase were obtained from Table 2 and the data of the previous work (Tanaka et al., 1989).

the enzyme, k_q^M (s = M, i = 2) for monomer and k_q^F (s = F, i = 3) for free FAD were evaluted from Eq. 5,

$$k_q^s = -1/\tau_i - 1/\tau_{FMN}, \quad (5)$$

where τ_{FMN} denotes fluorescence lifetime of FMN. The reported values for τ_{FMN} were used (Spencer and Weber, 1972). The rate constants of k_{form}, k_q^A, k_q^M, and k_q^F are expressed as Eq. 6, according to the absolute rate theory,

$$k_i = \frac{k_B T}{h} \exp\left(\frac{\Delta S^*}{R}\right) \exp\left(-\frac{\Delta H^*}{RT}\right), \quad (6)$$

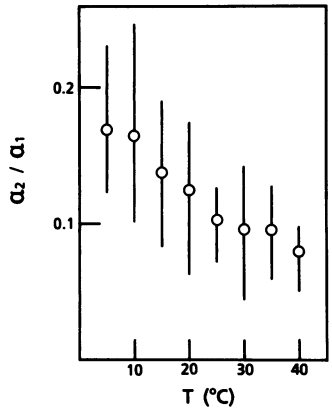

FIGURE 5 Temperature dependence of α_2/α_1. The values of α_2/α_1 represent the ratios of component fraction of monomer to dimer or associated forms of the enzyme. The ratios are from Table 2 and the previous work (Tanaka et al., 1989). Vertical bars indicate range of variation with change in concentration. Circles show averaged values of α_2/α_1.

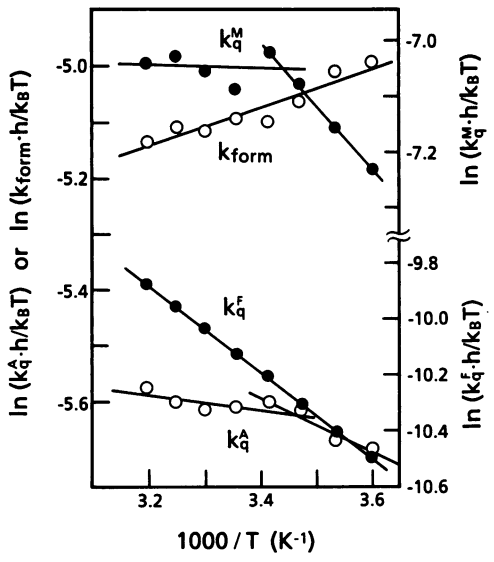

FIGURE 6 Modified Arrhenius plot of the rate constants of the excited state of FAD according to the absolute rate theory. The rate constants of the formation of the normal fluorescent state from a metastable fluorescent state of FAD of D-amino acid oxidase, k_{form}, were obtained from the inverse of τ_0 at various temperatures. The fluorescence quenching rate constants of FAD of dimer or associated forms and monomer of the enzyme, k_q^A and k_q^M, respectively, and that of free FAD, k_q^F, were evaluated from Eq. 5. Logarithm of the various rate constants of the excited state of FAD multiplied by h/k_BT was plotted against $1/T$, according to the absolute rate theory, Eq. 6. Obtained enthalpy and entropy changes of activation in these processes are listed in Table 3.

where k_i represents above rate constants. ΔH^* and ΔS^* denote enthalpy and entropy changes of activation for the various rate constants. k_B and h are Boltzmann constant and Planck's constant, respectively. To obtain ΔH^* and ΔS^* logarithm of k_ih/k_BT was plotted against $1/T$ in Fig. 6. From Fig. 6 it is evident that k_{form} and k_q^F can be expressed with single free energy changes of activation. However, k_q^A and k_q^M cannot be represented with single free energy changes of activation. Temperature transitions in k_q^A and k_q^M revealed at 16 and 18°C, respectively. Obtained enthalpy and entropy changes of activation for the rate constants of the excited FAD are listed in Table 3. The enthalpy change of activation of k_{form} was negative, whereas the other ones were all positive. This means that k_{form} increases as temperature is lowered, which is consistent with the results obtained by the time-resolved fluorescence spectra in the previous work (Tanaka et al., 1989). The enthalpy change of activation for free FAD was 3.1 kcal/mol. This was greatest among the rate constants. It is well known that the fluorescence of isoalloxazine in FAD is strongly quenched by adenine moiety due to the formation of an intramolecular complex (Spencer and Weber, 1972; Weber et al., 1974; Wahl et al., 1974; Visser, 1984). The enthalpy change of activation for monomer were 2.3 kcal/mol in the low-temperature state and 0.02 kcal/mol in the high-temperature state. The enthalpy change of activation in the low-temperature state is greater by ~10 times than that in the high-temperature state. On the other hand, the enthalpy changes of activation for dimer or associated forms of the enzyme were 0.98 kcal/mol in the low-temperature state and 0.25 kcal/mol in the high-temperature state. Difference between the two values is not so much as for monomer. This was also true for the entropy changes of activation. These results indicate that the conformational transition of dimer or associated forms of the enzyme is not so pronounced as that of monomer, which is consistent with the relative quantum yield data.

DISCUSSION

To investigate the temperature-induced change in the conformation of the surroundings of FAD of D-amino acid oxidase, the relative fluorescence quantum yield of FAD bound to the enzyme to free FAD and the fluorescence lifetime of FAD were systematically measured by changing the concentration of the enzyme and temperature. The relative quantum yield increased as concentration and temperature were lowered. These results were consistent with fluorescence decay parameters. The fluores-

TABLE 3 Enthalpy and entropy changes of activation for the rate constants of the excited states of FAD

Rate Constant	T_c*	Enthalpy change of activation			Entropy change of activation		
		ΔH_l*‡	ΔH_h*§	ΔH_s*‖	ΔS_l*‡	ΔS_h*§	ΔS_s*‖
	°C	kcal/mol	kcal/mol	kcal/mol	cal/K/mol	cal/K/mol	cal/K/mol
k_{form}	—	—	—	−0.72	—	—	−13
k_q^A	16	0.98	0.25	—	−7.9	−10	—
k_q^M	18	2.3	0.02	—	−6.2	−14	—
k_q^F	—	—	—	3.1	—	—	−9.8

The enthalpy and entropy changes of activation of the rate constants were determined by the absolute rate theory, according to a least-square procedure. *Transition temperature. ‡Subscript l indicates low-temperature state. §Subscript h indicates high-temperature state. ‖Subscript s indicates single state where ΔH^* and ΔS^* can be represented with single values.

cence lifetime of the monomer was ~200 ps, whereas for the dimer or associated forms of the enzyme it was ~45 ps. When the concentration is decreased, the equlibrium shifts from associated forms of D-amino acid oxidase to a monomeric one, and hence the relative quantum yield increases. When the temperature was varied, discontinuity in the relative quantum yield was observed at 18°C. This was especially clear at the lower concentrations. From the measurement of fluorescence lifetimes of FAD of D-amino acid oxidase it was found that the temperature transition occurs in the fluorescence quenching rate constants of FAD. Assuming that the rate constants are expressed with the absolute rate theory, the enthalpy and entropy changes of activation for the fluorescence quenching processes were evaluated (see Table 3). From these parameters it was found that the conformational transition in the microenvironment of FAD occurs at 18°C in the monomer and at 16°C in the dimer or associated forms of the enzyme, and it is much more evident in the monomer than in the dimer or associated forms. It was also found that the concentration fraction of monomer relative to dimer or associated forms of the enzyme increased as temperature was lowered, in accordance with the results by Massey et al. (1966) and also Shiga and Shiga (1972).

According to Massey et al. (1966), a temperature-dependent conformational change in this enzyme was observed in the sedimentation coefficients, absorption spectra of aromatic amino acid residues and FAD, fluorescence of tryptophanyl residues, and the enzyme activity. The values of the sedimentation coefficient increased slightly as temperature was elevated. They interpreted the results to mean that the equilibrium of the association state shifts toward a higher association state, which is coupled with a conformational change in the protein. According to Koster and Veeger (1968), there exists an equilibrium between high-temperature and low-temperature conformations, which was concluded from the measurements of the enzyme activity. Shiga and Shiga (1972) analyzed the V_{max} values of D-amino acid oxidase which were obtained under systematic variations of the concentration, temperature, and pH value, assuming a monomer-dimer equilibrium as well as a high-temperature and low-temperature equilibrium. They concluded that the temperature transition observed by Massey et al. (1966) is due to a sharp transition at 18°C in the equilibrium constant between monomer and dimer.

Despite these findings, however, Sturtevant and Mateo (1978) could not detect any change in the specific heat of D-amino acid oxidase at the concentrations of 125 and 25 μM, by means of differential scanning microcalorimetry. They concluded that failure to detect the transition even at 25 μM could not be attributed to the occurrence of the transition only in the monomeric form of the enzyme. As we can see from Figs. 1 and 2, however, the concentration of 25 μM (0.98 mg/ml) is still too high to observe a clear transition. Our results reveal that the conformational transition is more pronounced in monomer than in dimer or associated forms of the enzyme. According to the recent results of the equilibrium constants (Tojo et al., 1985*b*), at 25 μM, 25°C and pH 8.3, the concentration fraction of monomer is ~10% of the total concentration, which is considerably less than the value used for the calculation of the specific heat of the enzyme (Sturtevant and Mateo, 1978).

In the previous work (Tanaka et al., 1989) we have demonstrated that the normal fluorescence of D-amino acid oxidase with maximum wavelength of the spectrum at ~520 nm, which is similar with those of free FAD and other flavins in aqueous solutions, appears after a metastable fluorescent state decays. The fluorescence maximum of the metastable state is at 584 nm at 20°C and 557 nm at 5°C at the instant of the pulsed excitation, and it clearly shifts toward shorter wavelength to 520 nm in 100 ps after the excitation at both temperatures. It was found that the enthalpy change of activation of k_{form} in this process is negative, whereas those of the other rate constants of fluorescence quenching including the one of free FAD show positive values. This may be related to the remarkable blue shift of the fluorescence spectrum. Dynamics of the interaction between the excited isoalloxazine nucleus and amino acid residues existing nearby in D-amino acid oxidase are illustrated in Fig. 7. The metastable state with the fluorescence spectrum at ~584 nm transfers to the normal fluorescent state with the rate constant of k_{form} ($\sim 4 \times 10^{10}\ s^{-1}$). The normal fluorescent state of D-amino acid oxidase decays with the quenching rate constants of k_q^A ($\sim 2 \times 10^{10}\ s^{-1}$) for dimer or associated forms of the enzyme and of k_q^M ($\sim 5 \times 10^9\ s^{-1}$) for monomer. It was shown (Karen et al., 1983, 1987) that the fluorescence of this state is quenched by electron transfer interaction from tryptophan to the excited isoalloxazine to form a charge transfer (CT) state ($Trp^+ \cdots Fl^-$) between the cation radical of tryptophan and anion radical of the flavin. The CT state decays in several nanoseconds without emission (Karen et al., 1983, 1987). It is suggested that the ground state of G_1 is higher than the ground state of G_2. At the present stage of investigation it is not known what induces the difference between the states of G_1 and G_2, and also why the state of G_2 transfers to the state of G_1 with the higher energy after the fluorescence at ~520 nm decays. In this regard, the recent NMR experiments by Miura and Miyake (1987) may be especially significant. According to them, a chemical shift of the ^{13}C resonance at the 10a position in the isoalloxazine ring relative to free FMN shifted extensively upfield only in D-amino acid oxidase, whereas it shifted downfield in seven other flavoproteins. They claim

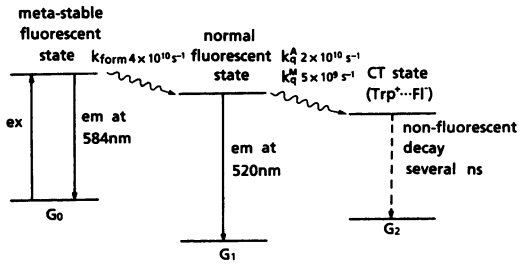

FIGURE 7 Dynamics of the excited states of FAD of D-amino acid oxidase. Dynamics of the excited states of FAD of D-amino acid oxidase is illustrated. At the instant of the pulsed irradiation, the ground state of isoalloxazine of FAD of the protein, G_0, is excited to the metastable fluorescent state with the spectrum at ~580 nm (Tanaka et al., 1989). This state transfers to the normal fluorescent state with the spectrum at ~520 nm (530 nm in the corrected spectrum) which is common in flavins of oxidized form, accompanied by the remarkable blue shift. The rate constant of the process is $\sim 4 \times 10^{10}\ s^{-1}$. The fluorescence at ~520 nm is quenched with the rate constants of k_q^A, $\sim 2 \times 10^{10}\ s^{-1}$, in dimer or associated forms of the enzyme and of k_q^M, $\sim 5 \times 10^9\ s^{-1}$, in monomer to form a charge transfer (CT) state of an ion pair between tryptophan cation and isoalloxazine anion radicals produced by electron transfer from tryptophanyl residue to the excited FAD (Karen et al., 1984, 1987). The CT state decays without emission in several nanoseconds after pulsed excitation.

that the extraordinary chemical shift is ascribed to enriched electron density at the 10a-carbon of the aromatic ring in D-amino acid oxidase. At any rate, hydrogen bonding interactions (Nishimoto et al., 1978, 1984; Yagi et al., 1980), or proton transfer interactions between the isoalloxazine nucleus in the ground or excited state, and amino acid residues could play an important role in the dynamic behavior of D-amino acid oxidase.

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