Picosecond-Resolved Fluorescence Spectra of D-Amino-Acid Oxidase. A New Fluorescent Species of the Coenzyme

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ABSTRACT: Protein dynamics of D-amino-acid oxidase in the picosecond region was investigated by measuring time-resolved fluorescence of the bound coenzyme, FAD. The observed nonexponential fluorescence decay curves were analyzed with four-exponential decay functions. The fluorescence lifetimes at the best fit were 26.6 ± 0.7 ps, 44.0 ± 4.2 ps, 177 ± 11 ps, and 2.28 ± 0.21 ns at 20 °C and 25.2 ± 3.0 ps, 50.3 ± 8.7 ps, 228 ± 27 ps, and 2.75 ± 0.33 ns at 5 °C. Component fractions with the shortest lifetime, ca. 26 ps, were always negative and close to −1. The other fluorescent components of the lifetimes, ca. 47 ps, 200 ps, and 2.6 ns, with positive fractions were assigned to different forms of the enzyme including the dimer, the monomer, and free FAD dissociated from the enzyme. Measurements of the time-resolved fluorescence spectra revealed that the maximum wavelengths of the spectra shifted toward shorter wavelength by 65 nm at 20 °C and 36 nm at 5 °C within 100 ps after pulsed excitation. The remarkable blue shift was not observed in free FAD. The first spectra immediately after the excitation of the enzyme exhibited maximum wavelengths of 584 nm at 20 °C and 557 nm at 5 °C. The fluorescence spectra obtained at times later than 100 ps are in good agreement with the one obtained under steady-state excitation of D-amino-acid oxidase. These results indicate that the electronic state of the excited flavin drastically changes immediately after the pulsed excitation, which is coupled with the protein dynamics of D-amino-acid oxidase in the picosecond region.

D-Amino-acid oxidase is a flavoenzyme with molecular weight of 40 000 per monomer and contains FAD as coenzyme. The enzyme is in an equilibrium state among monomer, dimer, and higher associated forms depending on its concentration (Antonini et al., 1966; Tojo et al., 1985a,b). A number of works have revealed that variuos physicochemical properties of D-amino-acid oxidase (Shiga et al., 1973; Horiike et al., 1977a,b; Yagi et al., 1975; Tanaka & Yagi, 1979) including the enzyme activity (Shiga & Shiga, 1972; Fitzpatrick & Massey, 1982) are dependent on the enzyme concentration. The binding process of FAD displays positive cooperativity (Tanaka & Yagi, 1979, 1980).

The fluorescence lifetimes of FAD of D-amino-acid oxidase in the picosecond region (Nakashima et al., 1980; Yagi et al., 1983) suggest that the microenvironment surrounding FAD in the monomeric enzyme is quite different from that in the dimer or oligomer. It has been also shown that the remarkable quenching of fluorescence of FAD in the protein is due to an electron transfer from tryptophan to the excited FAD (Karen et al., 1983, 1987).

The fluorescence spectrum of FAD of D-amino-acid oxidase displays a maximum wavelength at ca. 520 nm corresponding to the absorption band at 450 nm, which is similar to the emission maxima of free flavins. In the present work we demonstrate that the fluorescence spectrum of FAD of D-amino-acid oxidase undergoes a remarkable blue shift, as a metastable fluorescent state decays within 100 ps after a pulsed excitation.

Materials and Methods

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

Measurements and Analyses. The fluorescence decay curves and time-resolved fluorescence spectra were measured with a synchronously pumped, cavity-dumped dye laser and a picosecond time-correlated, single-photon counting apparatus (Yamazaki et al., 1985). Temperature of the samples was controlled with circulating water. Typical time width of the instrumental response function was 30 ps. The observed decay curves were analyzed with multiexponential decay functions

$$F(t) = \sum_i \alpha_i \exp(-t/\tau_i) \quad (1)$$

where α_i and τ_i are a fraction and a lifetime of ith fluorescent species. These decay parameters were determined by a nonlinear least-squares iterative convolution method based on the Marquardt algorithm according to the method of Boens et al. (1984) and Van Den Zegel et al. (1986) with some modifications (Tamai et al., 1988). The evaluations of success of the fit between the observed and calculated decay curves were mainly judged from both reduced χ^2 and the Durbin–Watson parameter, DW (O'Connor & Phillips, 1984). Time-resolved fluorescence spectra were constructed from the observed decay curves measured at 104 different wavelengths from 470 to 626 nm.

Results

Fluorescence Lifetimes. Fluorescence decay curves of FAD in D-amino-acid oxidase were measured at 20 and 5 °C at various levels of the enzyme from 100 to 0.78 μM. The decay curves monitored at 530 nm were all nonexponential and were analyzed with two-, three-, and four-exponential decay functions. Figure 1 shows a decay curve obtained at 20 °C (0.78 μM enzyme in 0.017 M pyrophosphate buffer at pH 8.3). The decay parameters and the reduced χ^2 and Durbin–Watson parameter at the best fit with both three- and four-exponential decay functions are listed in Table I. An improved fitting should be reflected by a decreased value of the reduced χ^2 and

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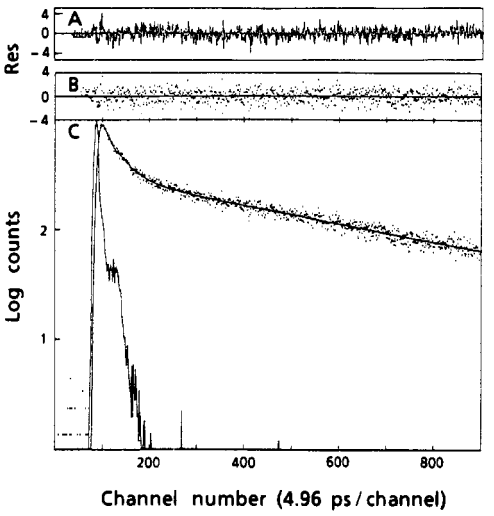

FIGURE 1: Fluorescence decay of FAD of D-amino-acid oxidase. Fluorescence intensities at 20 °C are shown with dots in (C). The emission was monitored at 530 nm upon excitation at 315 nm. The enzyme (concentration 0.78 μM) was dissolved in 0.017 M pyrophosphate buffer, pH 8.3. The calculated four-exponential decay function is indicated with a solid curve. The excitation pulse also is shown with a solid curve. Typical response function of the exciting pulse was 30 ps, while the pulse width at half-maximum intensity was 6 ps. Weighted residuals resulting from fits to simulated three-exponential and four-exponential decay functions are shown in (A) and B), respectively. Decay parameters at the best fit are listed in Table I.

by an increased value of the Durbin–Watson parameter (O'Conner & Phillips, 1984). Judging from these values in Table I, the best fits were always obtained when the four-exponential decay functions were used for the analyses. The values of the lifetimes were almost constant within 15% upon changing the concentration, although the lifetimes, τ_1 and τ_2 became a little longer as the enzyme was diluted. Averaged values of the lifetimes were $\tau_0 = 26.6 \pm 0.7$ ps, $\tau_1 = 44.0 \pm 4.2$ ps, $\tau_2 = 177 \pm 11$ ps, and $\tau_3 = 2.28 \pm 0.21$ ns at 20 °C and $\tau_0 = 25.2 \pm 3.0$ ps, $\tau_1 = 50.3 \pm 8.7$ ps, $\tau_2 = 228 \pm 27$ ps, and $\tau_3 = 2.75 \pm 0.33$ ns at 5 °C. The fluorescent components with the shortest lifetime, τ_0, were not obtained when we used the three-exponential decay functions. The component fractions of the shortest lifetimes (α_0) were -0.998 ± 0.029 at 20 °C and -0.949 ± 0.084 at 5 °C, over all concentrations. They were always negative and close to -1 within experimental accuracy. The fact suggests that the normal fluorescent state of FAD at around 520 nm forms as a metastable state of the excited isoalloxazine decays. The rise-up component with a negative amplitude was not observed with free FAD. The fluorescent species of τ_1 and τ_2 can be assigned to associated forms of the enzyme including dimer and monomer, respectively, since the component fraction, α_1 (with the lifetime of τ_1) decreases as the concentration is lowered, while α_2 (with the lifetime of τ_2) increases. The fluorescent species with lifetime of τ_3 is assigned to free FAD dissociated from the enzyme (Spencer et al., 1972; Weber et al., 1974; Wahl et al., 1974).

Time-Resolved Spectra. Time-resolved fluorescence spectra of FAD of D-amino-acid oxidase were measured at 20 and 5 °C at a concentration of 100 μM. The spectra at 20 °C are shown in Figure 2. A gated tme is expressed by measuring from the peak of the exciting pulse as zero time and indicated beside each spectrum. The first spectrum obtained by accumulating from −20 ps to 0 ps has the maximum at 584 nm. Successively, the emission spectra obtained by accumulating over successive 20-ps intervals shifted to shorter wavelengths.

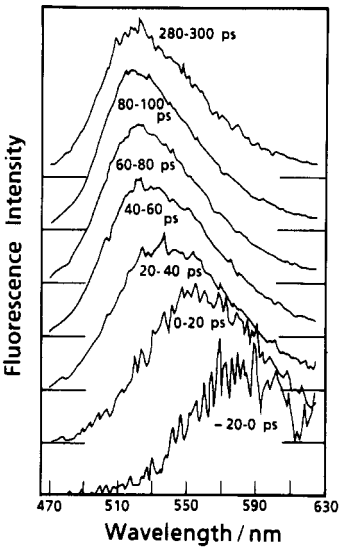

FIGURE 2: Time-resolved fluorescence spectra of FAD of D-amino-acid oxidase at 20 °C. The spectra were accumulated for the indicated time intervals relative to the excitation pulse at 315 nm. The maximum wavelength of the first spectrum (−20 to 0 ps) was 584 nm. Subsequent maxima were 556 nm at 0–20 ps, 539 nm at 20–40 ps, 526 nm at 40–60 ps, 522 nm at 60–80 ps, and 520 nm at later than 100 ps. The enzyme (100 μM) was dissolved in 0.017 M pyrophosphate buffer, pH 8.3. The spectra were not corrected for the wavelength-dependent sensitivity of the detector system.

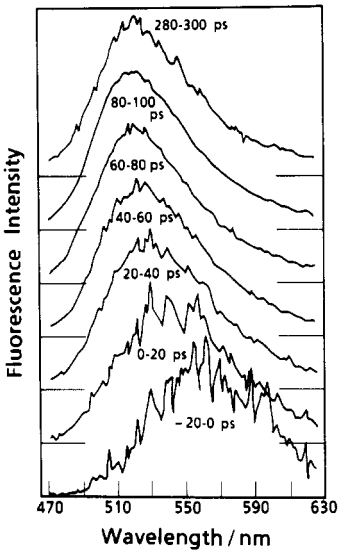

FIGURE 3: Time-resolved fluorescence spectra of FAD of D-amino-acid oxidase at 5 °C. Time-resolved fluorescence spectra are shown at various gated times. The maximum wavelengths of the spectra were 557 nm at −20 to 0 ps of the gated time, 543 nm at 0–20 ps, 527 nm at 20–40 ps, 524 nm at 40–60 ps, and 520 nm at 60–80 ps. These were 520 nm at the gated times for the accumulation later than 80 ps. Concentration of the enzyme was 100 μM in 0.017 M pyrophosphate buffer, pH 8.3. The spectra were not corrected for the wavelength-dependent sensitivity of the detector system.

The spectra became steady at 100 ps after the pulsed excitation. The maximum then is at 520 nm, which is in good agreement with the spectrum obtained by steady-state excitation. Figure 3 shows the time-resolved spectra of D-amino-acid oxidase obtained at 5 °C. The first spectrum obtained immediately after the excitation displays the maximum at 557 nm, which was shorter than the value obtained at 20 °C. Subsequently, it shifted toward 520 nm. The spectrum became steady at times later than 80 ps. The time-resolved spectra of free FAD were also examined. It is suggested that the metastable state shows the spectrum at longer wavelengths at 20 than at 5 °C. This may be ascribed

Table I: Fluorescence Decay Parameters of FAD of D-Amino-Acid Oxidase[a]

concn (μM)	T (°C)	τ_0 (α_0)[b] (ps)	τ_1 (α_1) (ps)	τ_2 (α_2) (ps)	τ_3 (α_3) (ns)	χ^2[c]	DW[d]
0.78	20	26.5 (−1.013)	50.0 (0.797)	197 (0.127)	2.58 (0.077)	0.982	1.876
		13.2 (−0.929)		114 (0.816)	2.48 (0.184)	1.046	1.759
	5	23.3 (−0.982)	60.3 (0.759)	281 (0.132)	3.16 (0.109)	1.069	1.961
		12.1 (−1.013)		131 (0.790)	2.90 (0.210)	1.211	1.728
1.6	20	26.8 (−0.966)	47.1 (0.817)	180 (0.132)	2.46 (0.051)	1.009	1.927
		12.4 (−0.601)		112 (0.862)	2.35 (0.138)	1.134	1.777
	5	21.9 (−0.733)	69.0 (0.716)	268 (0.165)	3.10 (0.119)	1.046	1.914
			92.0 (0.687)	328 (0.150)	3.14 (0.163)	1.221	1.645
3.1	20	26.5 (−1.062)	47.7 (0.821)	175 (0.143)	2.46 (0.035)	1.011	1.952
			112 (0.848)	351 (0.048)	2.50 (0.104)	1.238	1.602
	5	27.4 (−0.986)	43.1 (0.829)	212 (0.132)	3.01 (0.039)	1.078	1.824
			117 (0.746)	350 (0.125)	3.12 (0.128)	1.412	1.420
6.3	20	26.5 (−0.983)	43.3 (0.853)	171 (0.123)	2.38 (0.024)	1.137	1.876
			94.8 (0.842)	294 (0.086)	2.45 (0.073)	1.526	1.414
	5	27.1 (−0.959)	46.6 (0.818)	215 (0.149)	2.90 (0.033)	1.082	1.612
			108 (0.758)	324 (0.152)	3.03 (0.090)	1.664	1.087
13	20	26.4 (−0.980)	46.4 (0.883)	191 (0.099)	2.35 (0.018)	1.216	1.535
			89.2 (0.863)	309 (0.085)	2.45 (0.051)	2.110	0.916
	5	25.2 (−0.946)	44.2 (0.840)	217 (0.137)	2.72 (0.024)	1.631	1.256
			95.4 (0.782)	324 (0.157)	2.93 (0.061)	2.590	0.845
25	20	27.9 (−0.993)	38.2 (0.922)	166 (0.070)	2.15 (0.008)	1.306	1.613
			83.5 (0.860)	257 (0.100)	2.22 (0.040)	2.076	1.045
	5	25.5 (−0.986)	45.9 (0.840)	211 (0.141)	2.60 (0.020)	1.579	1.212
		98.4 (−0.941)		131 (0.934)	2.24 (0.066)	3.887	0.510
50	20	25.1 (−0.971)	41.5 (0.892)	168 (0.098)	2.02 (0.010)	1.426	1.391
			80.3 (0.875)	257 (0.095)	2.11 (0.030)	2.315	0.890
	5	26.0 (−0.995)	47.7 (0.854)	208 (0.131)	2.36 (0.015)	1.879	1.192
			107 (0.870)	395 (0.092)	2.70 (0.039)	3.663	0.668
100	20	26.9 (−1.012)	37.8 (0.936)	166 (0.059)	1.86 (0.005)	1.505	1.399
			75.1 (0.882)	248 (0.094)	1.96 (0.024)	2.429	0.900
	5	25.4 (−1.001)	45.3 (0.881)	208 (0.108)	2.18 (0.011)	1.582	1.228
			96.4 (0.868)	349 (0.103)	2.47 (0.029)	3.206	0.664

[a] The enzyme dissolved in 0.017 M pyrophosphate buffer at pH 8.3. [b] α_0 was obtained by normalizing a sum of α_1, α_2, and α_3 to unity. [c] χ^2 denotes a reduced χ^2 distribution between the observed and calculated fluorescence intensities (O'Connor & Phillips, 1984). [d] DW is the Durbin–Watson parameter (O'Connor & Phillips, 1984).

to a temperature-induced change of the conformation of D-amino-acid oxidase observed by Massey et al. (1966). In free FAD the spectrum did not shift at all.

Discussion

Protein dynamics in the surroundings of FAD of D-amino-acid oxidase was investigated by means of the picosecond-resolved fluorometry. Fluorescence decay curves of FAD were well described with four-exponential decay functions. Fluorescence species with the lifetimes of τ_2 and τ_1 (Table I) are assigned to monomer and associated forms of the enzyme, respectively. This is in good agreement with previous results obtained by assuming a monomer–dimer equilibrium of the enzyme (Nakashima et al., 1980; Yagi et al., 1983). The lifetimes of the higher associates of D-amino-acid oxidase may be somewhat shorter than that of dimer, since the value of τ_1 becomes shorter as the concentration is increased (see Table I).

Visser (1984) demonstrated with a subnanosecond pulse fluorometry that fluorescence of free FAD decays with two-exponential decay functions with lifetimes of 3 ns (70–50%) and 0.2 ns (30–50%), although it was believed in the earlier works (Spencer & Weber, 1972; Weber et al., 1974; Wahl et al., 1974) that it decays with single-exponential decay functions with the lifetime of 2.5 ns. Visser concluded that an intramolecular complex between isoalloxazine ring and adenine moiety is weakly fluorescent. Since the fraction of dissociated FAD is small in the enzyme solution (less than 10%), the fluorescent species with the shorter lifetime of free FAD does not contribute significantly to the fluorescence of D-amino-acid oxidase.

The results obtained by the measurements of the time-resolved fluorescence spectra of D-amino-acid oxidase are consistent with the following reaction scheme of the excited FAD in the protein:

FAD → FAD**	excitation
FAD** → FAD	emission at around 580 nm; lifetime of FAD**, τ_m
FAD** → FAD*	rate constant, $1/\tau_0$
FAD* → FAD	emission at around 520 nm; fluorescence lifetime of monomer, τ_2; fluorescence lifetime of dimer or associated form of the enzyme, τ_1

In this scheme FAD** and FAD* denote fluorescent species of FAD with emission peaks around 580 and 520 nm, respectively. Fluorescence decay functions of the species FAD** and FAD* may be represented by

$$F_m(t) = \exp(-t/\tau_m) \quad (2)$$

$$F(t) = \alpha_1 \exp(-t/\tau_1) + \alpha_2 \exp(-t/\tau_2) - \alpha_0 \exp(-t/\tau_0) \quad (3)$$

where τ_m is assumed to be the same in monomer and dimer or associated form of D-amino-acid oxidase. The fluorescence decay function obtained by monitoring at 530 nm in the present work is $F(t)$. The lifetime of τ_m is not obtained.

The remarkable blue shifts in the fluorescence spectra of the coenzyme of D-amino-acid oxidase are consistent with the observation of a rise-up component with a negative value of α_0 in the decay curves. If the fluorescence spectrum at extremely long wavelength that appears immediately after the pulsed excitation were due to a fluorescent contaminant, then the rise-up component with a negative amplitude (α_0) should not be obtained. The averaged values of ratio, $\alpha_0/(\alpha_1 + \alpha_2)$, over all concentrations were −1.028 at 20 °C and −0.993 at 5 °C, which are very close to −1. Since they were not observed in free FAD, these phenomena are considered to take place

only in the protein. Such an ultrafast change in the spectra suggests that the electronic state of the excited flavin changes coupled with the dynamics of the protein structure. A blue shift in the time-resolved fluorescence spectra is very rare in any systems. Free flavins in aqueous solution always exhibit fluorescence maxima around 520 nm (530 nm in the corrected spectra), corresponding to the absorption bands of ca. 450 nm. It is signficant that a fluorescent species with a much longer emission band is seen immediately after the pulsed excitation. The metastable fluorescent state may be related with one form of various types of hydrogen bondings (Nishimoto et al., 1978) or with proton-transfer complexes between isoalloxazine, which contains four proton acceptors and one proton donor, and nearby amino acid residues.

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