Fluorescence Decay Dynamics of Flavin Adenine Dinucleotide in a Mixture of Alcohol and Water in the Femtosecond and Nanosecond Time Range

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Fluorescence decays of flavin adenine dinucleotide (FAD) that is a typical autofluorescent species in cells and tissues have been measured in a mixture of alcohol and water in the femtosecond and nanosecond time range. The fluorescence lifetimes of both the stacked conformation between the isoalloxazine and adenine moieties in close proximity and the extended open conformation in water are affected by the addition of alcohol. The nanosecond fluorescence lifetime of the open conformation increases with decreasing dielectric constant of the medium, contributing to the enhancement of the fluorescence intensity of FAD in less dielectric media. The fluorescence lifetime of the open conformation is also affected by medium viscosity, suggesting that the photoexcited open conformation is quenched by the dynamic interaction between the two aromatic rings. The fluorescence component decaying in tens of picoseconds is attributed to the stacked conformation that shows the efficient fluorescence quenching due to the intramolecular electron transfer. The picosecond fluorescence lifetime of the stacked conformation increases with decreasing dielectric constant, suggesting the shift of the distribution of the stacked conformation to a longer intramolecular distance between the two aromatic rings in less dielectric media. The pre-exponential factor of the picosecond decaying component relative to that of the nanosecond one decreases with the increase of the alcohol concentration in the femtosecond time-resolved fluorescence, which demonstrates the increase in the population of the open conformation with the reduction of the dielectric constant. The possibility to evaluate the polar environment in a cell by the fluorescence lifetime of FAD is discussed based on the results obtained.

1. Introduction

Imaging of fluorescence has been extensively used in biochemical research, and a variety of fluorescent dyes and fluorescent proteins have been developed for labeling cells and tissues. In recent years, however, imaging of intrinsic fluorescence rather than fluorescence labeling has received much attention because environmental conditions in biological systems can be maintained without exogeneous fluorescent probes.[1−9] Cells contain endogeneous chromophores that exhibit intrinsic fluorescence called autofluorescence. Nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD) are the important metabolic cofactors exhibiting autofluorescence. Measurements of the fluorescence lifetime of an autofluorescent chromophore can enhance the potential of fluorescence microscopy.[5−9] Fluorescence lifetime is an inherent property of a chromophore and thus is independent of chromophore concentration and photobleaching but dependent on ion concentration or the local environment that affects the nonradiative rate of a chromophore. This makes fluorescence lifetime imaging a powerful tool for quantitative imaging of cellular conditions.[10−13] To clarify cellular environments using the fluorescence lifetime of an autofluorescent chromophore, detailed knowledge about the correlation of the fluorescence lifetime with physiological parameters is necessary.

FAD, which consists of a heterocyclic isoalloxazine ring with ribityl adenine diphosphate (Figure 1), is one of the most important cofactors for electron transportation and photoreceptors in living systems.[14−19] FAD catalyzes a variety of one- or two-electron redox reactions as a component of flavoproteins and also acts as a photoreceptor pigment in photoactive flavoproteins such as DNA photolyases[17,18] that repair pyrimidine dimer damages in DNA and BLUF proteins[19] that mediate a photophobic response of an organism. The isoalloxazine moiety of FAD exhibits the fluorescence that results in autofluorescence in cells. The optical properties of FAD have been the subject of investigation from biological and chemical points of view for a number of years since a marked weakness of the fluorescence of FAD, in comparison with that of free riboflavin, was reported by Weber.[20] The existence of a nonfluorescent intramolecular ground-state complex was proposed to explain the weakness of the fluorescence of FAD in aqueous solution. From the comparison of the fluorescence properties between FAD and flavin mononucleotide (FMN) having no adenine moiety, it was proposed that FAD in aqueous solution exists in two conformations, a nonfluorescent "stacked" conformation, in which the isoalloxazine and adenine moieties are in close proximity, and a fluorescent extended "open" conformation, in

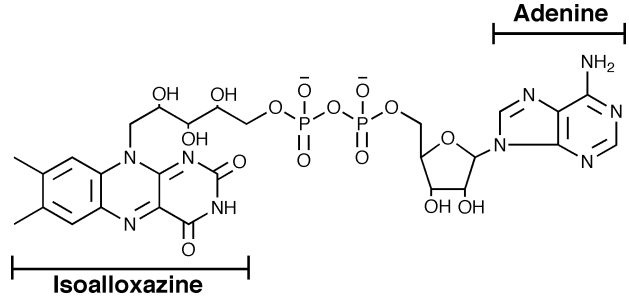

Figure 1. Representation of the chemical structure of FAD.

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which the two aromatic rings are separated from each other.[21,22] The stacked conformation is well-stabilized by the $\pi-\pi$ interaction between the two aromatic rings. In neutral aqueous solution, FAD is considered to predominantly exist as the stacked conformation; however, the addition of less polar solvents prevents the intramolecular $\pi-\pi$ interaction in FAD and produces the open conformation.[23−25] The nonfluorescent behavior of the stacked conformation was proposed to result from the efficient fluorescence quenching by the intramolecular electron transfer (IET) between the photoexcited isoalloxazine moiety and the adenine moiety of FAD.

Recently, photoexcitation dynamics of FAD has been extensively investigated using time-resolved spectroscopic techniques.[26−37] Fluorescence decay profiles of FAD in aqueous solution have been measured by subnanosecond time-correlated single-photon counting (TCSPC).[26−30] FAD exhibited a multiexponential fluorescence decay, and two major components whose fluorescence lifetimes are 7 ps and 2.7 ns at 293 K were obtained.[26] The femtosecond fluorescence up-conversion method has been applied to examine the ultrafast fluorescence decays of FAD.[30−32] Three components have been distinguished in aqueous solution from the decay in the time range of 0−30 ps;[31] the shortest-lifetime component (∼1 ps) is assigned to water relaxation around the excited FAD, and the other two components, that is, the 9 ps and nanosecond components are assigned to the stacked conformation exhibiting the efficient IET and the open conformation, respectively. Time-resolved measurements of electronic absorption spectra and vibrational spectra of FAD have also been performed.[33−37] In the transient absorption from the lowest excited singlet state (S_1) of FAD in water, the decaying component having a lifetime of ∼4 ps was observed, which was assigned to the stacked conformation since its absorption intensity was much weaker in FMN than that in FAD.[33] Time-resolved IR spectra of FAD in D_2O have shown that the lifetime of the S_1 state of FAD in the stacked conformation is 1.1 ps in high FAD concentrations.[37] Femtosecond time-resolved absorption spectra of reduced FAD[38] and riboflavin[39,40] have also been recently reported. The next stage of the study is to clarify the factor governing the photoexcitation dynamics of FAD.

In the present study, we have measured fluorescence decays of FAD in the femtosecond and nanosecond time range in a mixture of alcohol and water. Both fluorescence decay profiles of the open conformation having a nanosecond fluorescence lifetime and of the stacked conformation having a fluorescence lifetime of several picoseconds are evaluated by the TCSPC and up-conversion methods, respectively. We have found that the fluorescence lifetimes of both the open conformation and the stacked conformation are affected by the addition of alcohol. The mechanism of the nonradiative processes of the open and stacked conformations and the presence of the conformational equilibrium in alcohol−water mixtures are discussed, based on the results obtained. We have also suggested that the fluorescence lifetime of FAD is applicable to evaluate the polarity of living systems.

2. Experimental Section

Time-resolved fluorescence decay measurements in the picosecond and nanosecond time range were carried out using a TCSPC system (method I).[41,42] The second harmonic of the output from a femtosecond mode-locked Ti:sapphire laser (Tsumami, Spectra Physics) was used for excitation. The repetition rate was reduced to ∼6 MHz with a pulse picker (model 350−160, Conoptics) from the original 81 MHz. Fluorescence from the sample was dispersed by a monochromator (G-250, Nikon) and then detected by a microchannel plate photomultiplier (R3809U-52, Hamamatsu). Fluorescence signals were amplified, discriminated, and then led to a time-to-amplitude converter system. Fluorescence decays were obtained with a multichannel pulse height analyzer (model 7700, SEIKO EG&G). All of the fluorescence decays were collected at a magic angle with respect to the vertically polarized excitation light. The instrumental response function had a full width at half-maximum (FWHM) of ∼60 ps.

Measurements of femtosecond time-resolved fluorescence decay profiles were carried out using an up-conversion method (method II). A femtosecond mode-locked Ti:sapphire laser was used as a light source. The duration and the repetition rate of the laser pulse were ∼80 fs and 81 MHz, respectively. The output from the mode-locked Ti:sapphire laser was frequency-doubled by a 2 mm thick LiB_3O_5 (LBO) crystal to obtain the pump pulse for photoexcitation. The remainder of the output was used as the gate pulse for the up-conversion process. The pump pulse was focused by a 50 mm focal length lens into a sample cell, and the emitted fluorescence in the forward direction was collected by a 30 mm focal length lens. The sample solution was flowed through a quartz cell with 1 mm optical path length. The fluorescence and the gate pulse were focused into a 1 mm thick β-BaB_2O_4 (BBO) crystal. The generated up-converted signal at the sum frequency was spatially and spectrally separated by a combination of optical filters and a monochromator (TRIAX 190, Jovin Yvon-SPEX) and detected by a photomultiplier tube (R585S, Hamamatsu). The signal was discriminated by a photon-counting unit (C3866, Hamamatsu) and then led to a counting board (M8784, Hamamatsu). Fluorescence decays were obtained by varying the optical path length for the gate pulse. All of the measurements were performed under the magic angle condition by rotating the pump polarization with a half-wave plate. The instrumental response function, which was estimated to be a Gaussian function with a FWHM of ∼380 fs, was determined by a sum frequency mixing between the pump and gate pulses or a temporal rise of the up-conversion signal of fluorescent dyes.

The steady-state absorption and fluorescence spectra were recorded with an absorption spectrometer (Hitachi, U-3500) and a fluorescence spectrometer (FP-777, JASCO), respectively. FAD was purchased from Wako Pure Chemicals and was used without further purification. Glycerol of specially prepared reagent (Nakarai Chemicals), ethanol of special grade (Wako Pure Chemicals), and phosphate-buffered saline (PBS) buffer without calcium and magnesium ions (Wako Pure Chemicals) were used as received. The concentration of FAD was $\sim 1 \times 10^{-4}$ mol dm^{-3}. All of the experiments were carried out at room temperature.

3. Results and Discussion

3.1. Absorption and Fluorescence Spectra of FAD in a Mixture of Alcohol and Water. The steady-state absorption and fluorescence spectra of FAD in a mixture of water and alcohol, that is, glycerol or ethanol at different weight percents (wt %), are shown in Figure 2a and b, respectively. FAD exhibits two absorption bands with peaks at 450 and 375 nm, which are assigned to the $S_1 \leftarrow S_0$ and $S_2 \leftarrow S_0$ transitions, respectively. These transitions are ascribed to the $\pi-\pi^*$ transition of the isoalloxazine moiety of FAD since FMN exhibits almost the same absorption spectrum.[32,33,43] The fluorescence spectrum of FAD shows a peak at around 530 nm, which also comes from the isoalloxazine moiety.

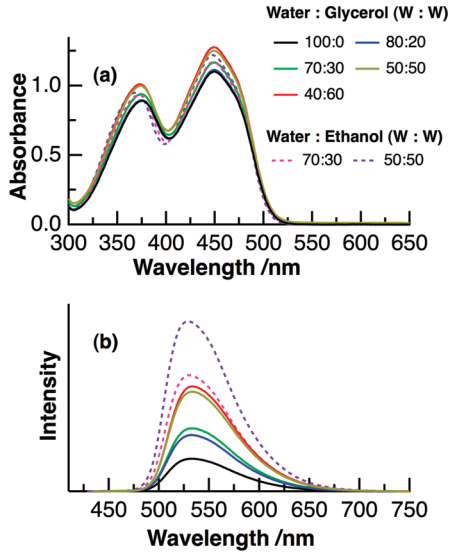

Figure 2. Absorption (a) and fluorescence (b) spectra of FAD in a mixture of water and alcohol at different alcohol weight percents. The excitation wavelength was 410 nm.

It is shown in Figure 2b that the fluorescence intensity is enhanced with increasing alcohol weight percent. The magnitude of the enhancement of the fluorescence is much higher than that of the increase in the absorption intensity, as shown in Figure 2a. It is suggested from circular dichroism,[23] NMR,[24,25] and transient absorption spectra[33,37] that the addition of less polar solvents breaks the $\pi-\pi$ interaction in the stacked conformation and produces the open conformation having a long fluorescence lifetime. Then, the observed enhancement of the fluorescence intensity is ascribed to the increase in population of the open conformation with the reduction of the dielectric constant of the medium. The peak wavelengths of both the $S_1 \leftarrow S_0$ absorption band and the fluorescence spectrum remain unchanged with the addition of glycerol; the electronic structure of the S_1 state is not affected by glycerol. It is noted that a small blue shift of ~4 nm is observed both in the absorption and in the fluorescence at 50 wt % ethanol.

3.2. Fluorescence Decays of FAD in a Mixture of Alcohol and Water in the Picosecond and Nanosecond Time Range. Figure 3 shows fluorescence decays of FAD in the picosecond and nanosecond time range in a mixture of alcohol and water at different alcohol weight percents. In all of the decay profiles, the maximum intensity is normalized to unity. The average fluorescence lifetime as well as the fluorescence intensity increases with the addition of alcohol (see Figure 3). The fluorescence decay profiles obtained with method I exhibit a multiexponential intensity decay in every case. The fluorescence decay profiles were fitted by assuming a tetra-exponential decay, that is, $\sum_i A_i \exp(-t/\tau_i)$, where A_i and τ_i denote the pre-exponential factor and the fluorescence lifetime of component i, respectively, and the obtained pre-exponential factors and the fluorescence lifetimes are shown in Table 1. The fastest decaying component, that is, the component 1, can be assigned to the stacked conformation, which shows the efficient fluorescence quenching due to the IET. The time resolution of method I however was not enough to determine the precise decay profile of the component 1, that is, τ_1, and the up-conversion method (method II) was used to obtain the detailed profile of the fast-decaying components, as will be discussed in the next subsection.

All of the decay profiles obtained with method I exhibit the decaying component whose lifetime is in the subnanosecond range, that is, the component 2, in agreement with the previous report.[26] The component 2 probably comes from the IET of stacked conformations having very weak intramolecular interactions between the isoalloxazine and adenine moieties since the IET rate depends on the distance between the two aromatic rings. The components 3 and 4, which give the nanosecond fluorescence lifetime, that is, τ_3 and τ_4, are ascribed to the open conformation, suggesting that different structures of the open conformation exist in solutions.

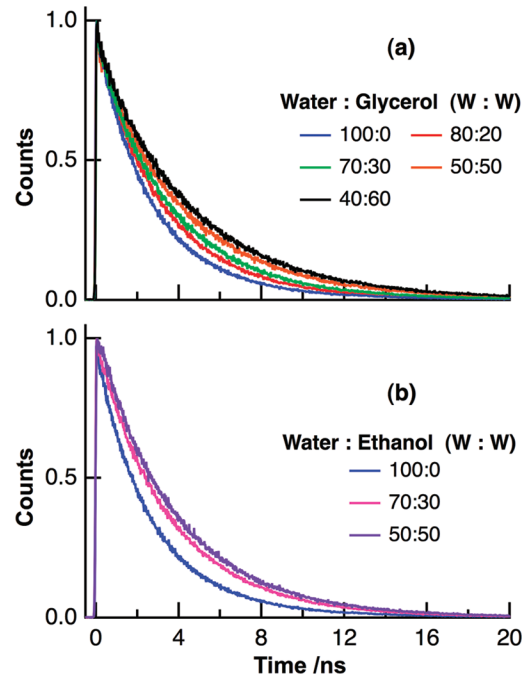

Figure 3. Fluorescence decay profiles of FAD obtained with method I in mixtures of glycerol and water (a) and of ethanol and water (b) at different alcohol weight percents. The intensities are plotted on a linear scale. The excitation and fluorescence wavelengths were 410 and 580 nm, respectively.

TABLE 1: Fitting Parameters of Fluorescence Decays of FAD in the Picosecond and Nanosecond Time Range in a Mixture of Alcohol and Water[a]

alcohol	alcohol wt%	τ_1 (ps)[b]	τ_2 (ps)	τ_3 (ns)	τ_4 (ns)
glycerol	0	7 (0.65)	220 (0.04)	2.15 (0.20)	3.92 (0.11)
	20	18 (0.47)	170 (0.06)	2.38 (0.21)	3.88 (0.26)
	30	21 (0.34)	190 (0.10)	2.56 (0.21)	4.19 (0.35)
	50	28 (0.22)	120 (0.14)	1.42 (0.07)	4.40 (0.57)
	60	34 (0.33)	190 (0.09)	1.14 (0.03)	4.67 (0.55)
ethanol	30	44 (0.32)	170 (0.08)	1.78 (0.07)	3.78 (0.53)
	50	61 (0.03)	200 (0.16)	2.18 (0.07)	3.98 (0.74)

[a] The pre-exponential factor of each component is given in parentheses. [b] See the text for the evaluation of τ_1.

The nanosecond fluorescence lifetime of the open conformation can be quantitatively evaluated in Figure 3. Plots of the average fluorescence lifetime (τ_{ave}) of the open conformation given by $\sum A_i\tau_i$ ($i = 3, 4$) against the dielectric constant of the medium[45] are shown in Figure 4. The τ_{ave} increases with the reduction of the dielectric constant, contributing to the enhancement of the fluorescence intensity in less dielectric media. This result indicates that consideration of the increase in the fluorescence lifetime is necessary to evaluate the population of the open conformation by fluorescence intensity measurements. Since FMN that does not have the adenine moiety exhibits a fluorescence lifetime of 4.67 ns,[44] the excited state of the open conformation having τ_{ave} of ~2.8 ns in water is considered to be quenched by the dynamic interaction between the excited

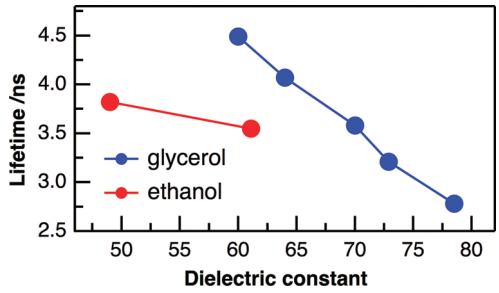

Figure 4. Plots of the average fluorescence lifetime of FAD in the nanosecond region against the dielectric constant of the medium. The dielectric constants were used at 298 K.[45] The blue and red markers are the values in glycerol−water and ethanol−water mixtures, respectively.

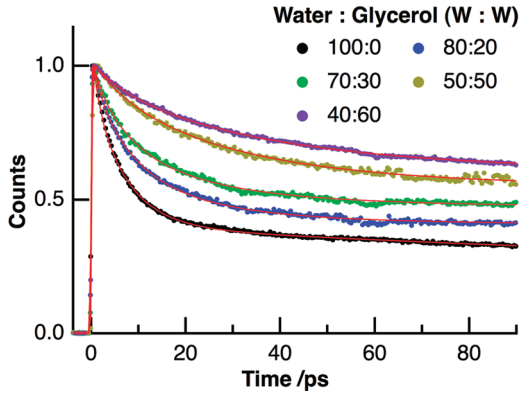

Figure 5. Normalized femtosecond time-resolved fluorescence decays of FAD in a mixture of water and glycerol at different glycerol weight percents. The intensities are plotted on a linear scale. The excitation and fluorescence wavelengths were 410 and 580 nm, respectively. The glycerol weight percents were 0 (black), 20 (blue), 30 (green), 50 (yellow), and 60 (purple). The simulated curve is shown for each by a red line.

isoalloxazine and adenine moieties within the radiative decay constant.[26] Thus, the present increase in the fluorescence lifetime with decreasing dielectric constant suggests that less dielectric media interfere with the interaction between the isoalloxazine and adenine moieties. As shown in Figure 4, further, the magnitude of τ_{ave} is different between glycerol and ethanol, suggesting that viscosity as well as dielectricity should be considered to obtain the medium parameter using the nanosecond fluorescence lifetime of FAD. This result is consistent with the model of the quenching interaction between the two aromatic rings because geometrical changes in the photoexcited open conformation are necessary to interact between these two rings.

3.3. Femtosecond Time-Resolved Fluorescence Decays of FAD in a Mixture of Alcohol and Water. The photoexcitation dynamics of the stacked conformation having a lifetime of several picoseconds can be evaluated by the femtosecond time-resolved fluorescence. Figures 5 and 6 show the representative femtosecond time-resolved fluorescence decays of FAD obtained with method II in glycerol−water and ethanol−water mixtures, respectively, at different alcohol weight percents. The maximum intensity at each decay curve is normalized to unity. The excitation wavelength is 410 nm, where the S_1 state is preferentially populated. It is noted that the internal conversion from the S_2 to S_1 states of FAD was reported to occur in less than ~100 fs.[33] It is clearly shown in Figures 5 and 6 that the average fluorescence lifetime of the picosecond decaying components increases with increasing alcohol weight percent. The residual background having a long decay time also increases with the increase of alcohol concentration.

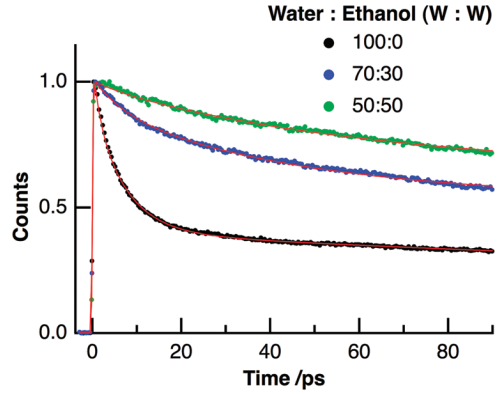

Figure 6. Normalized femtosecond time-resolved fluorescence decays of FAD in a mixture of water and ethanol at different ethanol weight percents. The intensities are plotted on a linear scale. The excitation and fluorescence wavelengths were 410 and 580 nm, respectively. The ethanol weight percents were 0 (black), 30 (blue), and 50 (green). The simulated curve is shown for each by a red line.

TABLE 2: Fitting Parameters of Femtosecond Time-Resolved Fluorescence Decays of FAD in a Mixture of Alcohol and Water[a−c]

alcohol	alcohol wt%	A'_1	A'_2	A'_3	A'_4	τ'_3 (ps)
glycerol	0	0.15	0.48	0.19[d]	0.18	220[d]
	20	0.10	0.26	0.24	0.40	18
	30	0.06	0.23	0.24	0.47	21
	50	0.00	0.09	0.35	0.56	28
	60	0.00	0.07	0.31	0.62	34
ethanol	30	0.00	0.12	0.34	0.54	44
	50	0.00	0.02	0.31	0.67	61

[a] τ'_1 and τ'_2 were fixed to 1.4 and 7.0 ps, respectively. [b] τ'_4 was assumed to be τ_{ave} in each decay profile. [c] Experimental error of ~30%. [d] The subnanosecond component of 220 ps (τ_2 in Table 1) was used to fit the decay.

The femtosecond time-resolved fluorescence exhibits a multiexponential decay, in agreement with the previous reports,[30−32] and a tri- or tetra-exponential decay, that is, $\sum_i A'_i \exp(-t/\tau'_i)$, where A'_i and τ'_i denote the pre-exponential factor and the fluorescence lifetime of component i, respectively, was needed to reproduce the decay profiles. From the fitting of the decay at 0 wt % alcohol, the two picosecond decaying components having the lifetimes of 1.4 and 7.0 ps, that is, the components 1′ and 2′, respectively, were obtained. These lifetimes are denoted by τ'_1 and τ'_2, respectively. In the present study, all of the decay profiles observed with method II could be fitted with the assumption that the same fast-decaying components having the lifetime of 1.4 and 7.0 ps exist in every case. The component 4′, having a long decay time, is confirmed, and it is assignable to the open conformation. The fluorescence lifetime τ'_4 of the component 4′ was therefore fixed to the τ_{ave} of the open conformation obtained by method I in each decay profile (see Figure 4). Another picosecond decaying component, that is, the component 3′, whose lifetime changes with the solvent, is confirmed, and its fluorescence lifetime (τ'_3) and all of the pre-exponential factors ($A'_1 - A'_4$) were optimized as variable parameters to fit the decays (see Figures 5 and 6). Note that τ'_3 is much shorter than τ'_4. The obtained parameters are shown in Table 2. Actually, the subnanosecond lifetime component that corresponds to the component 2 confirmed in the decay profile observed with method I was added for fitting the decay profile at 0 wt % alcohol. As shown in Figures 5 and 6, the other decay profiles obtained with method II could be reasonably fitted without consideration of the component 2. In the present study, the τ_1 of the component 1 in the decay profile obtained with

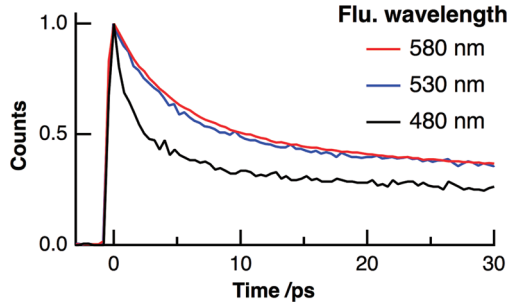

Figure 7. Normalized femtosecond time-resolved fluorescence decays of FAD in PBS buffer at different fluorescence wavelengths. The intensities are plotted on a linear scale. The excitation wavelength was 410 nm. The monitored fluorescence wavelengths were 480 (black line), 530 (blue line), and 580 nm (red line).

method I was fixed to τ'_3 (mixtures) or τ'_2 (0 wt % alcohol) in each decay profile (see Table 1).

The component 1′, having the lifetime of 1.4 ps, is ascribed to the relaxation of FAD, where solvation dynamics is not completed. As was previously reported,[31,32] the component 1′ becomes significant in the blue edge of the fluorescence spectrum (at 480 nm in Figure 7), and such a fast-decaying component in the blue side of the fluorescence was observed in several proteins, which is ascribed to hydration of a protein surface.[46,47] The components 2′ and 3′ correspond to the stacked conformation in which the efficient quenching occurs due to the close proximity of the isoalloxazine and the adenine moiety.[26,29−33,35−37] From the oxidation potential of adenine (E_{ox} = 1.5 eV),[48] the ground-state reduction potential of flavin (E_{red} = −0.24 eV),[49] and the $S_1 \leftarrow S_0$ (0−0) transition at 480 nm (2.58 eV),[32] the quenching on the picosecond time scale could be ascribed to the IET from the adenine to the photoexcited isoalloxazine. It is noted that such a decay in the picosecond time range was not observed in FMN that lacks the adenine moiety.[32] The IET depends on the distance between the two aromatic rings; therefore, the existence of fast- and slowly-decaying components in the IET, that is, the components 2′ and 3′, suggests that different structures of the stacked conformation exist in alcohol−water mixtures. The component 4′ is ascribed to the open conformation, as mentioned above. No rise component was observed within our experimental uncertainty, which is consistent with the previous studies.[30−32] In the present study, we discuss the populations of the open and stacked conformations and the dynamics and the structure of the stacked conformation in alcohol−water mixtures, based on the pre-exponential factors of A'_1−A'_4 and τ'_3 in Table 2.

The viscosity of a mixture at 60 wt % glycerol is over twice as large as that of a mixture at 50 wt % ethanol at around room temperature.[50] However, as shown in Table 2, both the τ'_3 and A'_4 values are larger at 50 wt % ethanol than those at 60 wt % glycerol, suggesting that the change in viscosity caused by the addition of alcohol is not a significant factor for the change in the decay profile obtained by method II with the solvent. It is also noted that the photoexcitation dynamics of FAD in the picosecond region remains almost unchanged with pH in the pH range of 5−10.[27,36] As mentioned in subsection 3.2, the τ_{ave} of the open conformation is influenced by the dielectric constant. Thus, the obtained A'_1−A'_4 and τ'_3 are plotted against the dielectric constant of the medium,[45] which is shown in Figure 8. It is clearly shown in Figure 8a that the A'_1 representing the magnitude of the solvation relaxation of FAD decreases with decreasing dielectric constant and becomes negligible for the dielectric constant less than 64. On the other hand, the

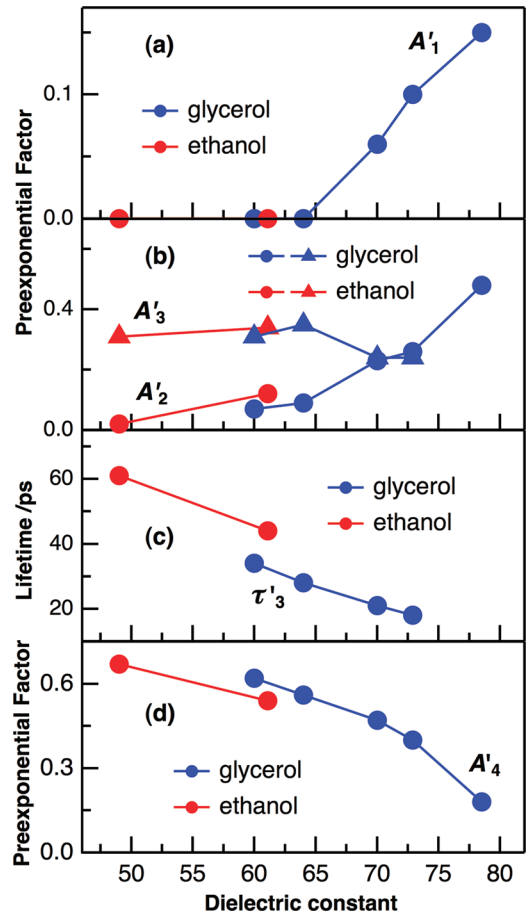

Figure 8. Plots of the pre-exponential factors (A'_1−A'_4) and the lifetime (τ'_3) shown in Table 2 against the dielectric constant of the medium. The dielectric constants were used at 298 K.[45] (a) A'_1, (b) A'_2 (circle) and A'_3 (triangle), (c) τ'_3, (d) A'_4. The blue and red markers are the values in glycerol−water and ethanol−water mixtures, respectively.

correlation between A'_1 and the medium viscosity is not observed in glycerol−water and ethanol−water mixtures. The clear relationship of A'_1 with the dielectric constant in Figure 8a indicates that the fast solvation process occurring in 1 ps is affected by the dielectric constant of the medium. It is conceivable that the solute−solvent interaction becomes weak with decreasing dielectric constant, which results in the small magnitude of A'_1 in less dielectric media. Hydrogen-bonding displacements as well as internal response of the solvent should also be considered to fully explain the solvation of FAD in protic solvents.

Plots of A'_2 and A'_3 against the dielectric constant are shown in Figure 8b. Both the A'_2 and A'_3 values correspond to the population of the stacked conformation existing in solution. The component 2′ having the lifetime of 7 ps suggests a stronger intramolecular interaction between the adenine and the isoalloxazine moiety than that suggested by the component 3′, whose lifetime is much longer than 7 ps, because the kinetics of the IET is determined by the intramolecular interaction. As shown in Figure 8b, A'_2 decreases with decreasing dielectric constant, indicating the reduction of the population of the stacked conformation having a strong intramolecular interaction in less dielectric media. On the other hand, A'_3 does not exhibit a marked change with different dielectric constants. These results suggest the shift of the distribution of the structure of the stacked conformation to a longer intramolecular distance between the two aromatic rings with decreasing dielectric constant. Recently, Radoszkowicz et al. have theoretically evaluated the distribution

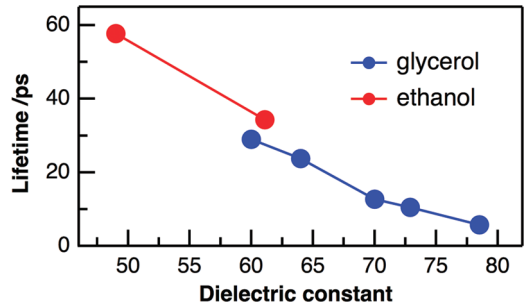

Figure 9. Plots of the average fluorescence lifetime of FAD in the range of tens of picoseconds against the dielectric constant of the medium. The dielectric constants were used at 298 K.[45] The blue and red markers are the values in glycerol−water and ethanol−water mixtures, respectively.

of the distance between the two aromatic rings of FAD in methanol−water mixtures,[29] and the shift to a longer intramolecular distance with the addition of methanol was suggested by molecular dynamics simulations. In low-temperature conditions, such as iced conditions, the increase in the lifetime of the picosecond decaying component of FAD with a 5% methanol mole fraction has been detected with a response function of ∼35 ps because the nonradiative rate of FAD becomes much smaller at low temperatures. In the present study, the decrease in the population of the stacked conformation having a very short intramolecular distance has been observed in the solution state at room temperature using femtosecond time-resolved up-conversion fluorescence.

As shown in Figure 8c, the τ'_3 increases with decreasing dielectric constant, meaning that the IET of the stacked conformation exhibiting the component 3′ becomes slow in less dielectric media. It is conceivable that the decrease in the IET rate of the component 3′ arises from the increase in the length of the intramolecular distance between the two aromatic rings with the reduction of the dielectric constant. Figure 9 shows plots of the average fluorescence lifetime (τ'_{ave}) in the range of tens of picoseconds against the dielectric constant of the medium.[45] The τ'_{ave} was given by $\sum A'_i\tau'_i$, where $i = 1-3$ for the mixtures and $i = 1$ and 2 for 0 wt % alcohol. The τ'_{ave} is well-correlated with the dielectric constant; τ'_{ave} almost monotonically increases with decreasing dielectric constant. This result suggests that the polar environment around FAD can be evaluated by the fluorescence lifetime of FAD in the range of tens of picoseconds.[51] Finally, the A'_4 value increases with decreasing dielectric constant (see Figure 8d). This result leads us to a conclusion that the population of the open conformation increases with the reduction of the dielectric constant.

4. Conclusions

The photoexcitation dynamics of the open and the stacked conformation of FAD in a mixture of alcohol and water has been studied by measuring the fluorescence decays in the femtosecond and nanosecond time range. The average nanosecond fluorescence lifetime of the open conformation increases with decreasing dielectric constant, resulting in the enhancement of the fluorescence in less dielectric media. The nanosecond lifetime is also affected by the medium viscosity, suggesting that the open conformation is effectively quenched by the dynamic interaction between the two aromatic rings. The IET in the stacked conformation occurs in tens of picoseconds, and the existence of the two picosecond decaying components suggests that at least two structures of the stacked conformation exist in alcohol−water mixtures. The IET becomes slower with the reduction of the dielectric constant, suggesting the generation of the stacked conformation having a longer intramolecular distance between the two aromatic rings in less dielectric media. The reduction of the intensity of the picosecond decaying component relative to that of the nanosecond one in less dielectric media indicates the population increase of the open conformation with decreasing dielectric constant.

The present study suggests that the polar environment around FAD can be analyzed using the fluorescence lifetime of FAD in the range of tens of picoseconds. The medium viscosity around FAD may be considered to evaluate the polar environment using the nanosecond fluorescence lifetime. These results suggest that the fluorescence lifetime of FAD is capable of noninvasive detection of polar environment in living systems, although additional factors may be considered in some cases because of the complexity of cells and tissues. Finally, the present study demonstrates that the detailed analysis of the population of the conformation of FAD can be performed using the femtosecond time-resolved fluorescence. Combinations of this method with theoretical calculations will give new scope for elucidating photophysics and thermodynamics of FAD and flavoproteins.

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