Inhibiting Intramolecular Electron Transfer in Flavin Adenine Dinucleotide by Host−Guest Interaction: A Fluorescence Study

Noufal Kandoth, Sharmistha Dutta Choudhury,* Jyotirmayee Mohanty, Achikanath C. Bhasikuttan, and Haridas Pal*

Radiation & Photochemistry Division, Bhabha Atomic Research Centre, Trombay, Mumbai 400 085, India

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Modulation in the photophysical properties and intramolecular electron transfer behavior of the flavin adenine dinucleotide (FAD) molecule has been investigated in the presence of the macrocyclic hosts, α-, β- and γ-cyclodextrins (CDs), using absorption and steady-state and time-resolved fluorescence measurements. The results demonstrate that only the β-CD host has a suitable cavity dimension to form a weak inclusion complex with FAD by encapsulating the adenine moiety, which is the preferred binding site in the large FAD molecule. Interestingly, in spite of the weak binding interaction, a significant enhancement in the fluorescence intensity of FAD is observed on complexation with β-CD, and this has been attributed mainly to the modulation in the conformational dynamics of FAD in the presence of β-CD. In aqueous solutions, a good fraction of FAD molecules exist in a "closed" conformation with the adenine and isoalloxazine rings stacked on each other, thus leading to very efficient fluorescence quenching due to the ultrafast intramolecular electron transfer from adenine to the isoalloxazine moiety. Complex formation with β-CD inhibits this intramolecular electron transfer by changing the "closed" conformation of FAD to the "open" form, wherein the adenine and isoalloxazine moieties are widely separated, thus prohibiting the fluorescence quenching process. Further evidence for the conformational changes has been obtained by the observation of a long lifetime component in the fluorescence decay of FAD in the presence of β-CD, which corresponds to the decay of the unquenched "open" form of FAD. Fluorescence up-conversion studies also indicate the absence of any ultrafast component in the fluorescence decay arising from the complexed FAD, thus supporting the formation of the "open" form in the presence of β-CD, with no intramolecular electron transfer.

1. Introduction

The photochemistry and photophysics of flavins have drawn considerable research interest due to their essential role in many light-driven biological activities.[1–16] Flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which consist of a heterocyclic isoalloxazine moiety tethered to a ribityl phosphate or ribityl adenine diphosphate chain, respectively, are the most commonly occurring flavins in flavoproteins (Scheme 1a). These flavin cofactors are derivatives of riboflavin (RF), a compound better known as Vitamin B2 (Scheme 1b).[2] Flavin can have three different redox states: oxidized form, one-electron reduced radical semiquinone, and two-electron fully reduced hydroquinone.[3] Because of their chemical versatility, flavoproteins are ubiquitous and participate in a broad spectrum of biological activities.[16,17] Flavoproteins are the ideal systems for studies of intraprotein electron transfer and conformational dynamics of biomacromolecules, not only because the flavin moiety is a redox-active group suitably located in the heart of the active site, but also because it has a fluorescent chromophoric group, thus making it amenable for fluorescence studies.[6,15,18,19]

The fluorescence spectral characteristics as well as the fluorescence quantum yield of flavins strongly depend on the environmental factors such as refractive index and solvent polarity.[1,12,20] Although RF and FMN have reasonably high fluorescence quantum yields ($Q = 0.26$) in aqueous solutions, FAD is very weakly fluorescent ($Q = 0.03$).[11] The remarkably low fluorescence yield of FAD compared to RF or FMN was first reported by Weber and was proposed to be due to the formation of an intramolecular ground-state complex between the isoalloxazine ring and the adenine moiety of FAD.[11] It is now well understood that the reduction in the quantum yield of FAD results from both static and dynamic quenching of the flavin fluorescence due to photoinduced electron transfer from the adenine moiety to the isoalloxazine moiety.[4,9,13,14,21–23] On enzymatic digestion of the diphosphate bridge of the FAD molecule, the fluorescence intensity increases substantially to match with that of the free FMN. Based on these studies, it was proposed that, in solution, FAD exists in two conformations: an extended or "open" form in which the isoalloxazine and the adenine moieties are largely separated from each other, and a "closed" conformation in which the two aromatic rings are in close proximity.[4,9,13,14,21–23] The "closed" conformation is stabilized by the combined effect of the π−π interaction between the isoalloxazine ring and the adenine moiety and the intramolecular hydrogen bonding interactions along the phosphate sugar backbone.[4] Support for the presence of stacked conformation has been obtained from circular dichroism,[24] NMR studies,[25] ultraviolet resonance Raman spectroscopy[26] and molecular dynamics (MD) simulations,[13] leading to different structural models for the interactions between the flavin and adenine moieties. Sequence−structure relationships of several FAD binding proteins have revealed that, in most of these proteins, the FAD cofactor is bound in an extended manner except in some members of the ferredoxin reductase family and in the DNA photolyase enzyme, where it adopts a bent conformation.[27]

* Corresponding author. E-mail: sharmidc@barc.gov.in (S.D.C.); hpal@barc.gov.in (H.P.). Fax: 91-22-25505151/25519613.

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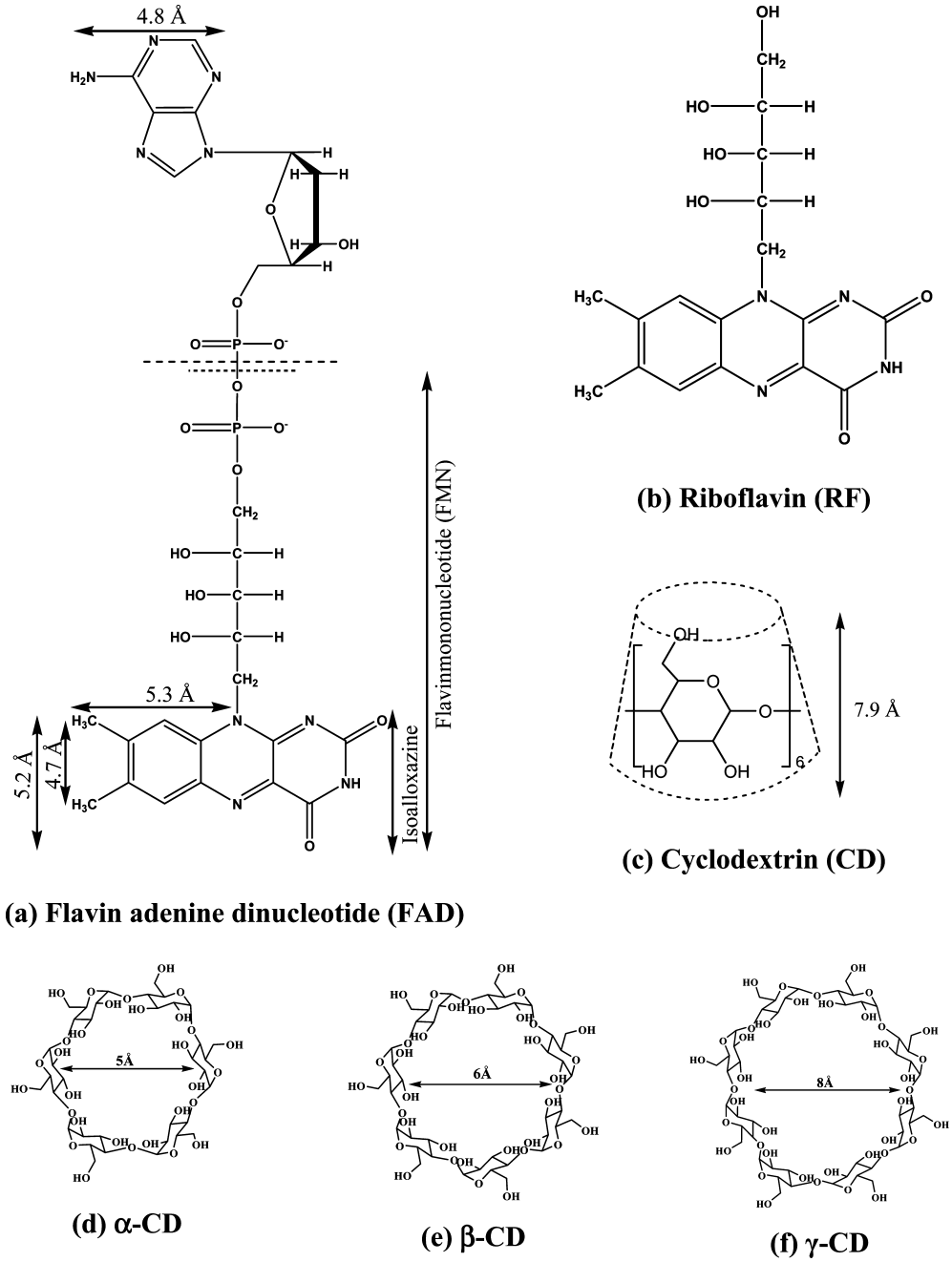
SCHEME 1: Chemical Structures of FAD, RF, and α-, *β*- and *γ*-CD[a]



[a] Major dimensions of interest for these systems are also indicated.

Although there is no explicit correlation between the conformation of FAD and the activity of these proteins, there have been some indications of a functional implication of the FAD conformation.[8,28] Quantum chemical calculations on the FAD cofactor in DNA photolyase, which is an enzyme that catalyzes photorepair of UV-damaged DNA by an electron transfer mechanism, suggest that, as a result of the bent conformation of FAD, the electron transfer between flavin and the thymine bases takes place indirectly, with the adenine moiety acting as an intermediate.[28] The calculated electronic structure of the $FADH^{\bullet}$ radical in this enzyme also provides evidence for a superexchange-mediated electron transfer from the thymine dimer lesion to the isoalloxazine moiety of the flavin cofactor via the adenine moiety.[29] From a comparative transient absorption study of different flavoproteins, Pan et al. observed a distinctly different spectral feature of excited $FADH^{\bullet}$ in DNA photolyase compared to the other flavoproteins and attributed this to a charge transfer interaction between the adenine and the isoalloxazine moieties due to the unique bent conformation of FAD in DNA photolyase.[8] This redistribution of electron density between the isoalloxazine and the adenine rings is thought to be favorable for extracting an electron from a neighboring tryptophan moiety in photolyase and also in preventing the back reaction between oxidized tryptophan and the reduced FAD.[8] Hence, it is quite interesting to study the conformational changes of FAD as well as the modulation in its photophysical properties and intramolecular electron transfer behavior in the presence of biomimetic binding pockets of macrocyclic hosts.

Among the different types of available hosts, cyclodextrins (CDs) have been one of the most extensively investigated molecules because of their higher solubility in aqueous medium.

CDs are cyclic oligosaccharides composed of D-glucopyranose units joined by ether linkages (Scheme 1c).[30–32] Depending upon the number of monomer units, CD homologues of different sizes are obtained, the most common members of this family being α-, β-, and γ-CDs, which are made up of six, seven, and eight D-glucopyranose units, respectively (Scheme 1d−f). The structure of the CDs is that of a truncated cone, having an inner hydrophobic cavity and outer hydrophilic edges consisting of hydroxyl groups. Solute molecules of suitable dimensions can interact and bind to the CD cavity by inclusion complex formation.[33–35] The reduced polarity and restricted microenvironment provided by the CD cavity can markedly and advantageously influence a number of properties of the included solute.[33–37] In this respect, the complexation of fluorescent molecules by macrocyclic hosts is of considerable interest because the host molecules can tune the photophysical characteristics, often by enhancing the fluorescence quantum yields, fluorescence lifetimes, and photostability of the molecules.[37–39] In this article, we present a systematic investigation on the interaction of FAD with α-, β- and γ-CD hosts, using absorption, steady-state fluorescence, and time-resolved fluorescence measurements. To understand the mode of interaction and to identify the binding sites of FAD, we have also studied the interaction of the parent molecule, RF, with these CD hosts. The chemical structures of FAD, RF, and the CDs used in this study are shown in Scheme 1 along with their relative dimensions.

2. Experimental Section

RF was obtained from Aldrich and used as received. FAD was obtained from Sigma and used after purification by ion exchange chromatography for the removal of the possible degradation products (FMN and RF) in the sample. Thus, the FAD sample from Sigma (purity 96%) was eluted at ion strength gradient between 1 to 10 mM phosphate buffer (pH 7.0) using a diethylaminoethyl (DEAE) Sephacil column (Sigma). The concentrations of RF and FAD were calculated from their molar extinction coefficients ($\varepsilon_{450,RF}$ = 12 200 M^{-1} cm^{-1}, $\varepsilon_{450,FAD}$ = 11 300 M^{-1} cm^{-1})[40] and were maintained in the range of 5−6 × 10^{-6} M in all the experimental solutions except for the fluorescence up-conversion studies. For the latter case, the concentration of FAD was kept at about 1.5 × 10^{-3} M in order to have better signal-to-noise ratio. The α-, β-, and γ-CDs were purchased from TCI Mark, Tokyo, and were used as received without further purification. The inclusion behavior of RF or FAD with α-, β-, and γ-CDs was studied by adding different weighed amounts of the corresponding CDs to the respective solutions. All studies with FAD were performed in 10 mM phosphate buffer in Nanopure water (Millipore Elix3/A10 water purification system; conductivity of <0.1 μS cm^{-1}) at ambient temperature.

Absorption spectra were recorded with a Shimadzu UV−vis spectrophotometer (model UV-160A), and steady-state fluorescence spectra were recorded with a Hitachi spectrofluorimeter (F-4010). The time-resolved fluorescence measurements were carried out with a time-correlated single photon counting (TCSPC) spectrometer (Horiba Jobin Yvon IBH, UK). A 374 nm diode laser (~100 ps, 1 MHz repetition rate) was used as the excitation source, and a microchannel plate photomultiplier tube (MCP-PMT)-based detection module was used for the measurement of the fluorescence decays. Except for anisotropy measurements, all other fluorescence decays were collected at magic angle (54.7°) with respect to the vertically polarized excitation light, to avoid the effect of rotational depolarization of the dye on the measured fluorescence lifetimes.[41,42] The DAS-6 software from IBH was used for the deconvolution analysis of the observed decays, considering either monoexponential or biexponential decay functions. The quality of the fits and, consequently, the mono- and biexponential natures of the decays were judged by the reduced χ^2 values and the distribution of the weighted residuals among the data channels. For a good fit, the χ^2 value was close to unity, and the weighted residuals were distributed randomly among the data channels.[41,42] For fluorescence anisotropy measurements, the polarized fluorescence decays for the parallel ($I_{||}(t)$) and perpendicular ($I_{\perp}(t)$) emission polarizations with respect to the vertical excitation polarization were first collected. Using these $I_{||}(t)$ and $I_{\perp}(t)$ decays, the anisotropy decay function, $r(t)$, was constructed as

$$r(t) = \frac{I_{||}(t) - GI_{\perp}(t)}{I_{||}(t) + 2GI_{\perp}(t)} \quad (1)$$

where G is a correction factor for the polarization bias of the detection set up.[41,42] The G factor was obtained independently by measuring the two perpendicularly polarized fluorescence decays and using horizontally polarized light for sample excitation.

Fluorescence decays faster than the time resolution of the above TCSPC instrument were measured using a femtosecond fluorescence up-conversion setup (FOG 100, CDP Corp., Russia). Briefly, the sample was excited by the second harmonic pulses (~400 nm, 50 fs, 88 MHz) of a Ti-Sapphire laser. Fluorescence from the sample was collected and focused onto a 0.5 mm thick, Type-1 β-BaB_2O_4 (BBO) crystal to overlap with the residual fundamental laser beam (~800 nm), used as the gate pulse, after passing through an optical delay rail. The up-converted light obtained as a result of the mixing of the fluorescence and the gate light in the BBO crystal was dispersed in a double monochromator after passing through a bandpass filter to avoid the light from the excitation and the gate pulses. The instrument response function (IRF) was independently measured through the cross correlation of the solvent Raman scattering and the fundamental laser pulse. The IRF was found to have a Gaussian intensity profile with a full width at half-maximum (fwhm) of 215 fs. The samples were taken in a rotating cell with an optical path length of 0.4 mm to avoid photodecomposition. For each of the decays, at least two repetitive scans were taken to average the data points and also to check their reproducibility. The measured decay traces were fitted to a multiexponential function, following convolution to the IRF and using an iterative nonlinear least-squares fitting method.

3. Results and Discussion

In aqueous solution, FAD has absorption bands with maxima around 450 and 370 nm, corresponding to the S_0-S_1 and S_0-S_2 transitions, respectively.[1] The changes in these absorption bands of FAD in the presence of varying β-CD concentrations are shown in Figure 1. On addition of β-CD to the FAD solution, a small blue shift (~3−4 nm) along with a slight increase in absorbance is observed for the longer wavelength absorption band. The 370 nm absorption band shows somewhat larger increase than the 450 nm band, which is probably due to the overlapping absorption of β-CD in the UV region (Figure 1, inset I). As can be seen from inset I, the absorption spectrum of FAD in the presence of β-CD is only slightly different from that obtained by the addition of the individual absorption spectra of FAD and β-CD. So these changes cannot be used for any

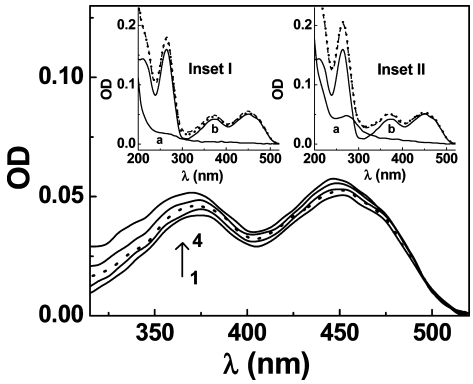


Figure 1. Representative absorption spectra of FAD (5 μM) in 10 mM phosphate buffer at pH 7 with different β-CD concentrations. [β-CD]/mM: (1) 0.0, (2) 7.5, (3) 14, and (4) 18. The dotted line shows the spectrum obtained by the addition of the individual spectra of FAD (5 μM) and β-CD (14 mM), for comparison. Inset I shows the absorption spectra of β-CD (14 mM) (a), FAD (5 μM) (b), β-CD·FAD (-----), and β-CD + FAD (•••••). Inset II shows the absorption spectra of α-CD (19 mM) (a), FAD (5 μM) (b), α-CD·FAD (-----), and α-CD + FAD (•••••). The spectra for α-CD·FAD (-----) and α-CD + FAD (•••••) are indistinguishable from each other.

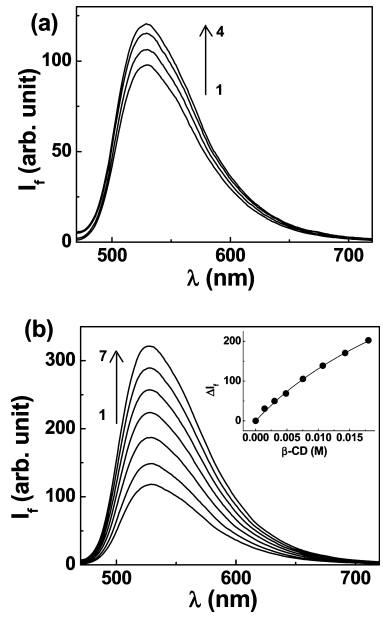


Figure 2. Steady-state fluorescence spectra of FAD (5 μM) in 10 mM phosphate buffer (pH 7) at (a) different α-CD concentrations, [α-CD]/mM: (1) 0.0, (2) 11, (3) 40, and (4) 50 and (b) different β-CD concentrations, [β-CD]/mM: (1) 0.0, (2) 1.5, (3) 5, (4) 7.5, (5) 11, (6) 14, and (7) 18. Excitation wavelength was 450 nm. Inset of Figure 2b shows the binding isotherm for β-CD·FAD with the fitted curve according to eq 5. Since the changes are quite small for α-CD·FAD, no reasonable binding isotherm could be constructed for any satisfactory quantitative analysis.

meaningful quantitative analysis and have not been considered further in this study. In the presence of α- or γ-CDs, however, the changes observed in the absorption spectrum of FAD are simply due to the overlapping absorptions of the corresponding CDs, which qualitatively indicates some difference in the interaction of FAD with the β-CD host compared to α- or γ-CDs. Representative spectra for FAD, α-CD, α-CD·FAD and that obtained by the addition of the individual absorption spectra of FAD and α-CD are presented in Figure 1, inset II.

The fluorescence spectrum of FAD in aqueous solution is broad with maximum at ~530 nm. On addition of β-CD, the fluorescence intensity is found to increase substantially, although the nature of the spectra remains effectively unchanged. In the presence of α-CD, the fluorescence enhancement is very small, and in the presence of γ-CD no changes could be observed in the fluorescence spectrum of FAD. The steady-state fluorescence spectra of FAD in the presence of varying concentrations of α- and β-CDs are shown in Figure 2a,b, respectively. The fluorescence spectra have been corrected for the optical density (OD) changes (relative to the initial OD) occurring at the excitation wavelength to nullify the effect of these OD changes on the fluorescence intensity. Since the OD at the excitation wavelength is quite low (λ_{ex} 450 nm, OD ~ 0.05), reabsorption effects on the fluorescence spectra can be neglected.

The differences in the interactions of FAD with α-, β-, and γ-CDs can be interpreted by a comparison of the relative cavity sizes of the CDs and the dimension of the FAD molecule. As mentioned earlier, FAD consists of an isoalloxazine ring attached to an adenine moiety through a ribityl diphosphate chain. So there can be two potential sites for interaction with the CDs; the isoalloxazine ring or the adenine moiety. Further, because of the presence of the ribityl side chain, the isoalloxazine moiety can only be partially encapsulated inside the CD cavities and thus should show less binding interaction. The isoalloxazine ring has a width of ~5.2 Å, whereas the adenine moiety has a width of ~4.8 Å (cf. Scheme 1). So for α-CD (cavity dimension ~ 5 Å), encapsulation of either the isoalloxazine ring or the adenine moiety of FAD is expected to be difficult. The slight increase in the fluorescence intensity of FAD in the presence of α-CD is possibly due to a very weak binding of α-CD with the relatively smaller adenine moiety of FAD. The cavity size of γ-CD (~8 Å) is too large to encapsulate and hold tightly either the isoalloxazine ring or the adenine moiety. Thus, no binding interaction is evident in this case. The β-CD cavity (~6 Å), on the other hand, has a suitable dimension to interact with either of the two potential binding sites of FAD. As a result, significant binding is observed only in the case of β-CD host. However, it is likely that among the two potential binding sites, the adenine moiety is the preferred site of interaction, since, besides the hydrophobic interaction inside the host cavity; hydrogen bonding interaction between the hydroxyl groups of CD and the adenine moiety can provide additional stability to the complex. Moreover, the partial inclusion of isoalloxazine ring will give much less stability for the host−guest complex than that with the almost complete inclusion of the adenine moiety.

To have a better idea about the actual binding site of FAD, the interactions of α, β-, and γ-CDs have also been investigated with the parent molecule RF, which consists only of the isoalloxazine ring attached to a ribityl chain (cf. Scheme 1b). The absorption and emission spectral characteristics of RF are almost similar to that of FAD.[1] A slight increase in the absorbance of RF was observed in the presence of α, β-, or γ-CDs, which is attributed to the overlapping absorptions of the respective CDs (cf. Figure 1). Interestingly, no increase was observed in the fluorescence intensity of RF in the presence of either of the CD hosts. This gives clear support to our proposition that β-CD does not interact with the isoalloxazine ring of FAD, but interacts via the inclusion of the adenine moiety of FAD into the β-CD cavity. This also explains the small changes in the absorption spectra of FAD (cf. Figure 1) in the presence of β-CD, since the 450 and 370 nm absorption bands mainly correspond to the transitions of the isoalloxazine ring, which is the basic chromophoric unit of FAD. The adenine moiety absorbs in the UV region but absorption changes in this

region are difficult to investigate because of the overlapping absorption of the β-CD hosts (cf. Figure 1, inset I), and also because of the higher energy transitions of the isoalloxazine chromophore.[20,24] The fluorescence spectral characteristic of FAD, which also corresponds to the emission from the isoalloxazine chromophore, accordingly remains unchanged, although, interestingly, the fluorescence intensity is considerably enhanced in the presence of β-CD, the reason for which will be discussed later.

^{1}H NMR studies on FAD in the presence of β-CD have been carried out with a view to identify the binding sites (Figure S1, Supporting Information). Although the changes in the NMR signals are quite small (Note S1, Supporting Information), a downfield shift is clearly observed for the adenine C2 proton of FAD with no shift for the C8 proton. Interestingly, downfield shifts are also observed for the isoalloxazine protons, which is attributed to the conformational change (discussed later) of FAD in the presence of β-CD. So in conjunction with the other evidence obtained in the present study, the NMR results can be interpreted to indicate that the β-CD host binds to FAD through the six-membered ring of the adenine moiety, thus leaving the C8 proton unaffected (Note S1, Supporting Information).

Since the fluorescence changes of FAD in the presence of α-CD were very nominal, no quantitative analysis was attempted to determine the binding constant for the α-CD·FAD complex using the fluorescence results. For the β-CD·FAD system, however, the changes in the fluorescence intensity were quite substantial, so the binding constants (K) for the β-CD·FAD complex, was determined by using the fluorescence titration method, considering 1:1 complexation between the host (β-CD) and the guest (FAD) (eq 2).[37]

$$\mathrm{FAD} + \mathrm{CD} \overset{K}{\rightleftharpoons} \mathrm{CD{\cdot}FAD} \tag{2}$$

Taking $[\mathrm{Dye}]_0$ and $[\mathrm{CD}]_0$ as the total concentrations of FAD and CD, respectively, eq 3 applies for the concentration of the free (uncomplexed) FAD in equilibrium with the β-CD·FAD complex.

$$[\mathrm{Dye}]_{eq} = \{K[\mathrm{Dye}]_0 - K[\mathrm{CD}]_0 - 1 + \sqrt{(K[\mathrm{Dye}]_0 + K[\mathrm{CD}]_0 + 1)^2 - 4K^2[\mathrm{Dye}]_0[\mathrm{CD}]_0}\}/2K \tag{3}$$

Exchange of the dye during its excited-state lifetime (~2.4−4.5 ns, see below), i.e., the conversion of the uncomplexed dye to the complexed one and vice versa, can be excluded since the corresponding guest exchange rate constants are very small for CD (occurs in microseconds).[38] The fluorescence intensity can therefore be understood as a composite of the fluorescence intensity contributions from the complexed and uncomplexed forms of the dye and should be expressed as eq 4:

$$I_f = I^0_{\mathrm{Dye}}\frac{[\mathrm{Dye}]_{eq}}{[\mathrm{Dye}]_0} + I^{\infty}_{\mathrm{CD{\cdot}Dye}}\frac{[\mathrm{CD{\cdot}Dye}]_{eq}}{[\mathrm{Dye}]_0} \tag{4}$$

where I^0_{Dye} is the initial fluorescence intensity in the absence of CD and $I^{\infty}_{\mathrm{CD{\cdot}Dye}}$ corresponds to the fluorescence intensity if all the dye molecules in the solution were complexed with CD. The relation for the changes in the fluorescence intensity (ΔI^{λ}_f) in the presence of β-CD can therefore be obtained by the rearrangement of eq 4 and is expressed as[37,38]

$$\Delta I^{\lambda}_f = \left(1 - \frac{[\mathrm{Dye}]_{eq}}{[\mathrm{Dye}]_0}\right)(I^{\infty}_{\mathrm{CD{\cdot}Dye}} - I^0_{\mathrm{Dye}}) \tag{5}$$

The fluorescence titration curve obtained for FAD in the presence of varying β-CD concentration is shown in the inset of Figure 2b. The binding constant value was estimated to be 28 (±7) M^{-1} by fitting the titration curve (ΔI^{λ}_f vs $[\beta\text{-CD}]_0$) following eq 5 where $[\mathrm{Dye}]_{eq}$ is obtained from eq 3. This is certainly a low binding constant, lower than the binding constant value of ~90 M^{-1} reported for the interaction of adenosine with β-CD.[43] The low binding constant value in the present case is probably due to the inclusion of only a small part (the adenine moiety) of the large FAD molecule inside the β-CD cavity. From the fitting of the titration curve using eq 5, the value of $I^{\infty}_{\mathrm{CD{\cdot}Dye}}$ is determined to be 582 (relative to the arbitrary unit used for the fluorescence intensity measurement), where the initial intensity corresponding to I^0_{Dye} is 118. Thus, complex formation of FAD with β-CD leads to an enhancement in the fluorescence intensity by a factor of 4.9.

Evidence for complex formation in the β-CD·FAD system has also been obtained from time-resolved anisotropy measurements. Since, the anisotropy decay rate depends upon the effective size of the fluorescent molecule and the microviscosity of its local environment, it is expected that the rotational diffusion rate of FAD should decrease upon complexation with the β-CD host due to an increase in the size of the fluorescent moiety (dye + host).[33] The fluorescence anisotropy decay for free FAD in aqueous solution is found to be single-exponential in nature with a rotational correlation time of ~200 ps. In the presence of β-CD, the anisotropy decay of FAD could not be fitted with a single-exponential function, but a reasonable fit is possible following a biexponential function, giving a shorter 230 ps component (32%) possibly corresponding to the fraction of the free FAD molecules in the solution and a longer 770 ps component (68%) suggested to be due to the complexed FAD with β-CD. Using the binding constant (K) value of 28 M^{-1}, the concentrations of free FAD and β-CD·FAD complex in the solution having 5 μM FAD and 18 mM β-CD are estimated as 3.32 μM and 1.68 μM, respectively, using eq 3. However, these concentrations of free and complexed FAD cannot be used directly to correlate the relative contributions ($A_i = a_i\tau_{ri}/\sum_i a_i\tau_{ri}$) of the two rotational time constants, as will be discussed in the latter part of this section. Nevertheless, since the β-CD·FAD complex has a higher rotational correlation time and also the fluorescence yield and lifetime (discussed later) of the complex is much higher than that of the free dye, an apparently larger contribution (68%) observed for the rotational time constant of the β-CD•FAD complex is quite expected, even though the binding interaction is very weak. The anisotropy decay traces for FAD in the absence and in the presence of β-CD are shown in Figure 3, and the decay parameters are listed in Table 1. Although a quantitative analysis for the effective increase in the size of FAD upon complexation with β-CD is difficult due to the existence of different structural conformers of FAD in aqueous solution and also due to the partial inclusion of FAD in β-CD cavity, the slow anisotropy decay in the presence of β-CD definitely provides a conclusive proof for the complex formation in spite of the weak binding interaction in the present system. The anisotropy decay trace of the model compound RF was also determined in the presence of β-CD, but no significant changes were observed, which rules out the possibility of any appreciable interaction of β-CD with the isoalloxazine moiety or with the ribityl side chain (Figure S2, Supporting Information).

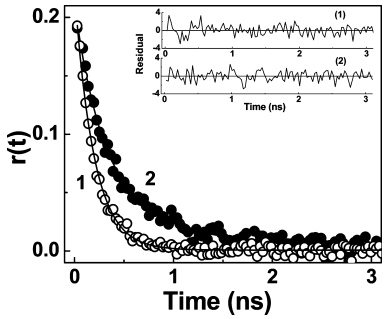


Figure 3. Fluorescence anisotropy decays, $r(t)$, for FAD (5 μM) in 10 mM phosphate buffer (pH 7) in the absence (1) and in the presence (2) of β-CD (18 mM). The smooth lines show the best fit curves. Inset shows the residual distribution of the fitted curves.

TABLE 1: Fluorescence Anisotropy Decay Parameters[a] for FAD (5 μM) in the Absence and in Presence of 18 mM β-CD[b]

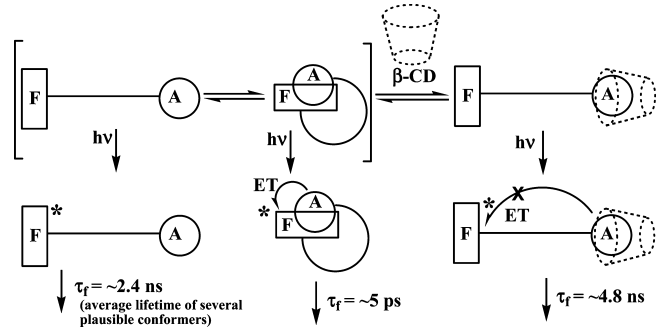
β-CD (mM)	a_1	A_1 (%)	τ_{r1} (ps)	a_2	A_2 (%)	τ_{r2} (ps)	χ^2
0	0.19	100	207				1.12
18	0.11	32	230	0.07	68	770	1.14

[a] The anisotropy decays were fitted according to, $r(t) = \sum_i a_i \exp(-t/\tau_{ri})$, for single and biexponential decays. [b] The absolute pre-exponential factors are listed as a_1 and a_2, while A_1 and A_2 correspond to the relative contributions[c] of the two rotational correlation times, τ_{r1} and τ_{r2}. [c] The relative contributions were estimated as $A_i = a_i\tau_{ri}/\sum_i a_i\tau_{ri}$.

The very low binding constant for the β-CD·FAD system is apparently in contradiction with the large enhancement in the fluorescence intensity of FAD in the presence of β-CD, which is much higher than normally anticipated for such a weak host−guest binding. In general, the fluorescence enhancement of dye molecules upon complexation with macrocyclic hosts results from the restricted vibrational and rotational motions inside the host cavity and the consequent reduction in the nonradiative decay rates of the excited dyes. In the present case, the rigidity imposed by the host molecule on FAD is quite less because the complex is formed mainly via the inclusion of the adenine moiety inside the host cavity, which is only a small part of the large FAD molecule. So the exceedingly large fluorescence enhancement in the β-CD·FAD system is definitely due to some other reason than the simple inclusion effect on the nonradiative decay channel of the excited dye.

It is suggested that, in aqueous solution, a large fraction of the FAD molecules in the ground state exists in the "closed" conformation, where the isoalloxazine ring and the adenine moiety are stacked on each other. It is further reported that the formation of this "closed" conformation is a dynamic process, with stacking and unstacking occurring in the time scale of about 19 ns.[22,44] The stacking of the isoalloxazine ring with the adenine moiety results in efficient fluorescence quenching due to an ultrafast photoinduced intramolecular electron transfer from the adenine moiety to the isoalloxazine moiety.[4,9,13,22,44] On complexation of the adenine moiety of FAD with β-CD, it is expected that the stacking interaction will be prohibited as a result of the steric repulsion between the β-CD complexed adenine moiety and the isoalloxazine ring (Scheme 2). So it is likely that in the presence of β-CD there will be a large change in the conformational dynamics of FAD, causing the stacking−unstacking equilibrium to shift toward the "open" conformation. This conformational change will thus significantly reduce the propensity of the photoinduced electron transfer from the adenine moiety to the isoalloxazine ring due to an increase in the distance between the electron donor and acceptor groups, and accordingly will reduce the inherent fluorescence quenching observed in the FAD molecule. So, the change over from the "closed" to the "open" conformation, and the concomitant reduction in the intramolecular electron transfer process possibly leads to the substantial increase in the fluorescence intensity of FAD in the presence of β-CD, even though the binding constant for the present host−guest system is not that high.

SCHEME 2: Schematic Representation of the Conformational Dynamics of FAD in the Presence of β-CD and Its Effect on the Fluorescence Lifetime of the Molecule in Aqueous Solution



To have a better idea about the binding mechanism and the inhibition of the intramolecular electron transfer process in FAD on binding to β-CD, the fluorescence decays of FAD in aqueous solution were also investigated using TCSPC measurements in the presence of different β-CD concentrations. Some representative decay traces in the presence and in absence of β-CD are shown in Figure 4. The fluorescence decay of FAD in aqueous solution is found to be biexponential in nature, with a major (~97%) long lifetime component of ~2.4 ns and a small (~3%) short lifetime component of ~0.3 ns. In the presence of β-CD, the fluorescence decay kinetics of FAD becomes effectively slower. The decays can be fitted to a biexponential function with one of the decay times in the range of ~2.4 ns and the other in the range of ~4.2−4.8 ns. Table 2 lists the fluorescence decay parameters of FAD in aqueous solution in the presence of different β-CD concentrations. As indicated from Table 2, with increasing β-CD concentration, the contribution of the longer lifetime component increases gradually with a corresponding decrease in the contribution of the ~2.4 ns component. It may be mentioned here that the fluorescence decay of RF shows no significant changes in the presence of the β-CD host.

The ultrafast decays of FAD were also evaluated in the absence and in the presence of β-CD by using fluorescence up-

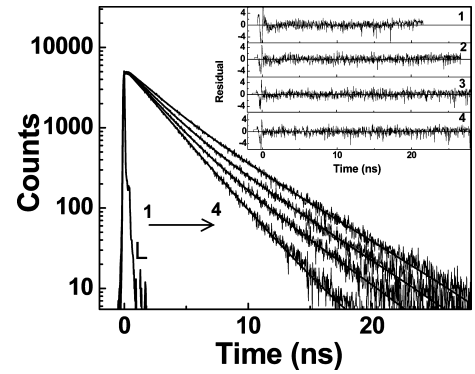


Figure 4. Fluorescence decay traces for FAD (5 μM) monitored at 530 nm in the presence of β-CD; [β-CD]/mM: (1) 0.0, (2) 4, (3) 8, and (4) 18. L is the lamp profile. Inset shows the residual distribution of the fitted curves.

TABLE 2: Fluorescence Decay Parameters for FAD (5 μM) in the Absence[b] and in the Presence of β-CD As Determined from TCSPC Measurements[a]

β-CD (mM)	A_1 (%)	τ_1 (ns)	A_2 (%)	τ_2 (ns)	χ^2
0	97[b]	2.4			1.21
4	65	2.2	35	4.2	1.19
8	51	2.3	49	4.5	1.14
14	44	2.4	56	4.7	1.17
18	40 (0.57)[c]	2.4	60 (0.43)[c]	4.8	1.20

[a] A_1 and A_2 correspond to the relative contributions (cf. footnote Table 1) of the two lifetimes, τ_1 and τ_2. [b] A small contribution (3%) of a 0.3 ns component is also observed in the absence of β-CD. This short component is, however, not detectable in the presence of β-CD using TCSPC measurement. [c] The values in parentheses correspond to the relative absolute pre-exponential factors ($a_i/\sum a_i$).

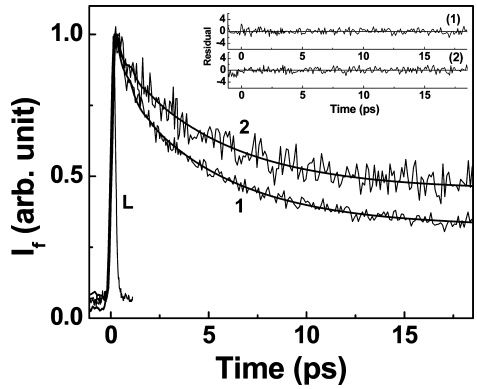


Figure 5. Fluorescence decay traces along with the triexponential fitted curves for FAD (1.5 mM) monitored at 530 nm by fluorescence up-conversion measurements in the absence (1) and in the presence of 18 mM β-CD (2). L is the IRF. Inset shows the residual distribution of the fitted curves.

conversion measurements. As mentioned in the experimental section, to get a good up-conversion signal, the FAD concentration was required to be kept very high (1.5 mM) compared to that used in other fluorescence studies (5−6 μM). Since the binding constant for the β-CD·FAD complex is quite low ($K = 28\ M^{-1}$), in these experimental solutions only about 2% of the dye was complexed with β-CD (18 mM). The decay traces obtained in the present cases both in the absence and presence of β-CD are shown in Figure 5. As expected, the fluorescence decay is not complete within the experimental time window (~20 ps); however, considerable reduction in the decay rate of FAD is clearly observed in the presence of the host molecule. The experimental decay traces could be nicely fitted to a triexponential decay function keeping the long decay time fixed at 2.3 ns for FAD in the absence of β-CD and 3.8 ns in the presence of β-CD, in accordance with the average lifetime values obtained from the TCSPC measurements. The other two decay components (~0.3 ps and ~5 ps) were found to be almost in a similar range, both in the presence and in the absence of β-CD. Interestingly, comparing the relative contributions of the 0.3 and 5 ps components (excluding that of the long decay component), it is seen that the two contributions remain effectively similar irrespective of the presence of β-CD, although the decay apparently shows a higher intensity tail at the longer delays in the presence of β-CD due to the higher contribution of the long lifetime (3.8 ns) component. In comparing the contributions of the 0.3 and 5 ps components in the absence and presence of β-CD, the contributions of the long nanosecond components in the respective cases have been excluded because the percentage contributions of these long components to the total fluorescence are so high (>99.9%) that, without excluding their contributions, it is difficult to express and compare the contributions of the ultrafast decay components, as their numbers become unusually low. Thus the fitted parameters for the femtosecond decay traces are listed in Table 3 only with the relative contributions of the 0.3 and 5 ps components for comparison, excluding the contribution of the respective nanosecond components. For the completeness of the data presentation, however, we also list in Table 3 the relative absolute pre-exponential factors ($a_i/\sum a_i$) of different lifetime components of the ultrafast decays of FAD both in the absence and in the presence of β-CD.

Previous studies on time-resolved fluorescence measurements using nanosecond time resolution have revealed a significantly shorter and nonexponential excited-state decay of FAD as compared to its analogue FMN.[22] These findings are consistent with the quenching of FAD fluorescence due to intramolecular electron transfer in its "closed" conformer. While the decay analysis of FMN revealed a fluorescence lifetime of 4.7 ns, FAD yielded two lifetime components of 2.8 and 0.3 ns.[22] A recent study with sub-nanosecond time resolution has shown that FAD displays a 7 ps component that is characteristic of ultrafast fluorescence quenching and a 2.6 ns contribution resulting from less efficient quenching.[13] A 9 ps component arising from the intramolecular complex formation between flavin and adenine has also been reported from a fluorescence up-conversion study.[14] It is also known that a slightly less polar solvent, such as formamide, prevents the π-stacked complex formation, leading predominantly to the "open" conformer, and in fact a recent transient absorption study has reported a long excited state lifetime of FAD in formamide, comparable to that of FMN.[9] In the present study, we have recorded the decay traces for FAD and RF in a relatively less polar and highly viscous solvent, glycerol (Figure 6) and observed similar long decay times of ~5.2 ns for both the molecules in addition to a small contribution (~5%) of 0.5 ns for FAD, possibly arising from a small population of the stacked conformation. So, in essence, it is indicated that the short 5−10 ps decay components arise as a result of the efficient excited state quenching by intramolecular electron transfer in the stacked conformation of FAD, and the long ~2.4 ns component in water probably corresponds to the average lifetime of several plausible intermediate conformations of FAD excluding the stacked one. Recent MD simulation studies have in fact confirmed the existence of several partially folded conformations of FAD within its excited state lifetime.[13] The observation of the 5.2 ns lifetime component of FAD in glycerol suggests that the long 4.8 ns component observed in the presence of 18 mM β-CD could be due to the extended "open" conformation of FAD in which no intramolecular electron transfer is possible and thus should have a lifetime quite similar to that of its analogue, FMN (lifetime ~4.7 ns). The present results in glycerol and those reported recently in formamide, also suggest that the fluorescence quenching in FAD in aqueous solution is mainly due to the stacking interactions of the isoalloxazine ring with the adenine moiety and not due to the electronic coupling between the adenine and the isoalloxazine moieties through the ribityl chain.

So the appearance of a fluorescence lifetime component of ~4.3−4.8 ns for FAD in the presence of β-CD clearly supports the formation of the extended "open" conformer of FAD due to complexation of the molecule with β-CD host that prevents intramolecular interaction between the isoalloxazine ring and the adenine moiety. It is interesting to note that only two discrete lifetimes of ~2.4 ns and ~4.3−4.8 ns are observed throughout

TABLE 3: Fluorescence Decay Parameters for FAD (1.5 mM) in the Absence and in the Presence of 18 mM β-CD As Determined from Fluorescence Up-Conversion Measurements[a]

β-CD (mM)	$a_1/\sum a_i$	A_1 (%)	τ_1 (ps)	$a_2/\sum a_i$	A_2 (%)	τ_2 (ps)	$a_3/\sum a_i$	τ_3 (ps)	χ^2
0	0.48	8	0.28	0.32	92	5.1	0.20	2300	1.12
18	0.47	10	0.37	0.29	90	5.2	0.24	3800	1.17

[a] A_1 and A_2 correspond to the relative contributions (cf. footnote Table 1) of the two ultrafast lifetime components, τ_1 and τ_2. The τ_3 component was kept fixed according to the average lifetime values obtained from the TCSPC studies (vide infra). The relative values of the absolute pre-exponential factors ($a_i/\sum a_i$) are listed for all the decay components.

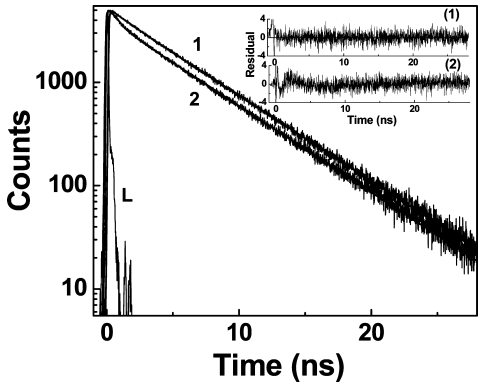


Figure 6. Fluorescence decay traces for (1) RF and (2) FAD monitored at 530 nm in glycerol solvent. L is the lamp profile. The decay trace for RF was fitted to a single exponential function with $\tau = 5.2$ ns, and that for FAD was fitted to a biexponential function with $\tau_1 = 0.5$ ns (5%) and $\tau_2 = 5.2$ ns (95%). The χ^2 values were close to unity in each case. Inset shows the residual distribution of the fitted curves.

the β-CD concentrations used, with only the relative contribution of the longer lifetime component increasing with increasing host concentrations. Normally in bimolecular fluorescence quenching, the lifetime of the fluorophore gradually decreases with increasing quencher concentration, or, conversely, the fluorescence lifetime gradually increases with a decrease in the quencher concentration.[41] So the nearly constant lifetime values observed in the present case can be suitably explained by considering Scheme 2. In the presence of β-CD host, a dynamic equilibrium is established between the free and the β-CD bound FAD molecules in the ground state. Since the formation and dissociation of the host−guest complexes occur only in the microsecond time scale, within the excited state lifetime of FAD (a few nanoseconds) there is hardly any change in the equilibrium for the free and bound dye. Accordingly, the free FAD molecules, following their independent excitation, will decay predominantly with ∼2.4 ns decay time as also observed for FAD in aqueous solution in the absence of β-CD. Similarly, the bound FAD molecules having an "open" conformation will decay predominantly with a lifetime in the range of ∼4.2−4.8 ns, following their independent excitation. As the concentration of the host molecule increases in the solution, the population of the FAD molecules bound to β-CD also increases, leading to a gradual increase in the 4.2−4.8 ns lifetime component. Since the binding of β-CD to FAD is quite weak, a significant amount of the free dye also remains in the solution even at the highest possible concentration of β-CD used (solubility limit of β-CD ∼16−18 mM),[30–32] leading to the reasonable contribution of the 2.4 ns component. The small increase in the longer lifetime component of FAD (from 4.2 to 4.8 ns) with increasing β-CD concentrations is possibly due to the combined effect of the slight differences in the data parameters obtained by fitting the observed decay to a biexponential function, which effectively leads to a slightly lower decay time (∼4.2 ns) at low β-CD concentrations due to the smaller contribution of this component and the associated small changes in the solvent characteristics at the higher β-CD concentrations. As the results indicate, it is reasonable to assume that the β-CD complexed FAD has an effective fluorescence lifetime of ∼4.8 ns.

At this point it is interesting to re-examine the observed time-resolved anisotropy results of the β-CD·FAD system, especially in relation to the relative contributions of the two rotational time constants and their correlation with the fluorescence decay parameters. Considering the contributions of both free and complexed dye to the observed time-dependent fluorescence intensity, the anisotropy decay function, $r(t)$ for FAD in the presence of β-CD, can be expressed as (cf. eq 1),

$$r(t) = r^{\text{free}}(t)\left(\frac{I^{\text{free}}(t)}{I^{\text{total}}(t)}\right) + r^{\text{cmplx}}(t)\left(\frac{I^{\text{cmplx}}(t)}{I^{\text{total}}(t)}\right) \qquad (6)$$

where $I^{\text{total}}(t) = I^{\text{free}}(t) + I^{\text{cmplx}}(t)$. The above expression indicates that $r(t)$ in the present case is a complex function of time, although for simplicity we have analyzed the anisotropy decay as a biexponential function (cf. Table 1). It is, however, evident that immediately after excitation (at $t = 0$), the fluorescence intensity ratios for the free and complexed FAD should be equal to the relative values of their absolute pre-exponential factors ($a_i/\sum a_i$) in the observed fluorescence decay, which are found to be 0.57 and 0.43, respectively, for the 2.4 ns (free FAD) and 4.8 ns (β-CD·FAD complex) lifetime components (cf. Table 2) in the solution containing 5 mM FAD and 18 mM β-CD. Observing that the total initial anisotropy in the present case is about 0.19 (cf. Figure 3), it is expected that the initial anisotropy for the free FAD should be about 0.11 and that of the β-CD·FAD complex should be about 0.08. The experimentally observed initial anisotropy values for the two rotational time constants (the absolute pre-exponential factors, a_1 and a_2, cf. Table 1) are in fact found to be 0.11 and 0.07, respectively. Considering the complexity involved in the calculation and analysis of the anisotropy decay (cf. eq 1 and 6), such a correlation seems to be quite reasonable.

The up-conversion measurements also qualitatively support our proposition that the free and the β-CD-bound FAD molecules follow their independent excitation and fluorescence decay dynamics (cf. Scheme 2). In the up-conversion results, the ultrafast decay times (0.3 and 5 ps) and their relative contributions (10% and 90%, respectively) are observed to remain almost similar, irrespective of the presence or absence of β-CD, although the contribution of the long decay component (2.3 or 3.8 ns average lifetime in the absence and presence of β-CD) increases in the presence of β-CD, and thus the decay profile visually appears to be slower. The very short fluorescence lifetime component, <1 ps, observed in the up-conversion measurement is believed to be due to the effect of solvent relaxation dynamics.[14] The ∼5 ps component is attributed to the fast electron transfer in the "closed" conformation of free FAD in aqueous solution. The 5 ps component matches reasonably well with that reported earlier from transient absorption studies.[9] Since the time constants of the two ultrafast

components remain unchanged even in the presence of β-CD, it is proposed that the ultrafast decays arise from the free FAD molecules present in the solution, while the FAD molecules bound to the β-CD host do not display any ultrafast component and contribute only to the long nanosecond decay. In the up-conversion experiment, as the population of the free FAD is very high (because only 2% of the dye is complexed with β-CD), the contribution of the ultrafast decay components is quite significant, even in the presence of the highest concentration of β-CD used (18 mM). However, a comparison of the relative values of the absolute pre-exponential factors of all the decay components shows that the value corresponding to the 5 ps component (attributed to the "closed" conformer) reduces from 0.32 to 0.29, and that corresponding to the long nanosecond component (attributed to the "open" conformer and β-CD complexed dye) increases from 0.20 to 0.24 in the presence of 18 mM β-CD. Considering the errors involved in the pre-exponential values, very small percentage of FAD·β-CD complex present in the solution and that there is an additional decay component (0.3 ps) related to the solvation process, the above changes in the absolute pre-exponential factors seems to be quite justified. Overall, the present results, however, indicate that the complex formation of FAD with β-CD leads to a change in the conformation of the dye from the "closed" to the "open" forms and thus inhibits the characteristic ultrafast intramolecular electron transfer process operative in the "closed" conformer of the FAD molecule.

4. Conclusions

The results obtained from ground-state absorption and steady-state as well as time-resolved fluorescence studies indicate that FAD forms inclusion complex with β-CD but not with α- or γ-CDs. This is because, the β-CD cavity has a suitable cavity dimension for encapsulating the adenine moiety, which is the preferred binding site of FAD. That the adenine moiety of FAD is the actual binding site has been confirmed by comparative binding studies with the parent molecule RF, which consists only of the isoalloxazine ring attached to a ribityl side chain. No interaction is observed for RF with either of the CDs used. Complex formation of FAD with β-CD is supported by an increase in the rotational correlation time of FAD in the presence of the host and from ^{1}H NMR studies. The large increase in the fluorescence intensity of FAD upon binding to β-CD is proposed to be mainly due to the modulation in the conformational dynamics and the consequent reduction in the electron transfer process in FAD in its stacked or "closed" conformation. In aqueous solution, a large fraction of FAD molecules exist in the "closed" conformation where the adenine and the isoalloxazine ring stack on each other, leading to an efficient fluorescence quenching due to ultrafast electron transfer from the adenine to the isoalloxazine moiety. In the presence of β-CD, due to the encapsulation of the adenine moiety within the host cavity, the "open" conformation is favored where the electron donor and acceptor moieties are widely separated. This hinders the fluorescence quenching by electron transfer and leads to a large increase in the fluorescence intensity. The conformational changes are also supported by the fluorescence decay analyses. Complex formation leads to the appearance of a long lifetime component of ~4.8 ns, which corresponds to the unquenched, extended form of FAD. Fluorescence up-conversion studies also indicate that the β-CD-bound FAD does not have any ultrafast decay component; rather the fast decay component arises mainly from the fraction of the free FAD molecules remaining in the solution. Present results indicate that the macrocyclic host molecules can affect the intramolecular electron transfer phenomenon in FAD simply by changing the conformational dynamics of the molecule. These results could aid in the studies and interpretation of the conformational dynamics and activity of FAD binding proteins, which are based on the measurement of the intrinsic fluorescence of the flavin moiety.

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Supporting Information Available: Figures S1−S2 and Note S1 as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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