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pH dependent dynamic behavior of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) in femtosecond to nanosecond time scale

Abhigyan Sengupta, Rahul V. Khade, Partha Hazra*

Department of Chemistry, Indian Institute of Science Education and Research, Pune 411021, Maharashtra, India

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ABSTRACT

In this study we have tried to explore structure–dynamics relationship of FAD with the help of time resolved fluorescence studies over five orders in time scale from several hundreds of femtosecond to nanosecond. We found that FAD has conformational flexibility, due to which it exhibits several distinct dynamics in the pH range between 0.5 and 13.0. Our lifetime and anisotropy results confirm conformational changes of FAD at pH = 3.0 and pH = 10.0.

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1. Introduction

Light triggered activities in living organisms are mainly controlled by five enzymatic co-factors: Carotinoids, Billins, Chlorophyls, Flavins and Pterins. Out of these five, flavins got greater importance in the last decade, because of its involvement in most of the biological activities in large number of flavoproteins. *DNA-photolyase* (utilized in repairing of UV-induced pyramidine–dimer DNA damage) [1], *phototropins* (controls light induced tropism in low light conditions, known as phototropism) [2], *BLUF proteins* (involved in photophobic responses in *Euglena gracilis* [3], transcriptional regulation in *Rhodobacter sphaeroides* [4] and in *phototaxisin Synechocystis* [5]), and *Cryptocromes* (regulates circadian rhythm in plant and in higher organisms [6]) are the few flavoprotein photoreceptors belonging to flavin family. The interesting behavior of these co-enzymes towards light and photo-induced participation in several biological activities attracts special attention to flavins [7,8].

For several decades the studies are mainly focused on the excited state properties of flavins, namely, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). Comparative study of FAD and FMN shows the existence of two conformers of FAD [9]. One of which is responsible for the quenching of fluorescence due to stacking of adenine moiety and isoalloxazine ring (called 'stack' conformer) [9]. Mainly, the π–π stacking interaction between aromatic rings and intramolecular hydrogen bonds along the phosphate sugar backbone gives the stabilization of this stack conformer [10,11]. Another is 'unstack' conformer, in which lifetime and fluorescence is unaffected [9]. Many spectroscopic techniques have been applied to explore this 'stack–unstack' behavior of FAD [9–19]. Moreover, the pH dependence fluorescence behaviors of flavins are also interesting [12,13]. It was found that fluorescence mainly appears from the neutral form, whereas cationic form is practically non-fluorescent and the anionic form is weakly fluorescent [12,13]. Drossler et al. and Islam et al. investigated pH dependence behavior of riboflavin, FMN and FAD in detail [12,13]. They observed that quantum yield of FAD is maximum around pH = 2.5 and then it decreases sharply until pH = 5.0 [13]. In the pH range of 5.0–9.0, quantum yield almost remains the same and decreases afterwards until it reaches almost zero at pH = 13.0 [13]. However, the quantum yield of riboflavin and FMN is maximum around pH = 4.0 and remains almost constant in the pH range of 4.0–8.0 [12]. They explained their results in terms of two different conformations of FAD. In the pH range between 3.5 and 11.0, stack (closed) conformer of FAD dominates over unstack (open) conformer, whereas at higher and lower pH unstack conformer dominates over stack conformer [13]. Although, they measured lifetime of FAD in all pH ranges with a time resolution of ~400 ps, they are unable to monitor stack conformer due to short lifetime of stack conformer. Until now, a large number of spectroscopic techniques have been used to explore the excited state photochemistry of FAD, but there are few studies in very fast time scale (femtosecond to picosecond time resolution) [14–17]. For the first time, van den Berg et al. monitored the dynamics of FAD with the help of TCSPC technique with an

* Corresponding author. Tel.: +91 20 2590 8077; fax: +91 20 2589 9790.
E-mail address: p.hazra@iiserpune.ac.in (P. Hazra).

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instrument response of 40 ps [14]. They observed that in neutral pH (pH = 7.5) fluorescence intensity decay of FAD is mainly contributed by two components: a dominant 7 ps component contribution that is characteristic of ultrafast fluorescence quenching due to electron transfer from adenine moiety to flavin and a 2.7 ns contribution resulting from moderate quenching [14]. Their molecular dynamics study also concludes the same lifetime with an affirmation about the co-planner stacking between adenine moiety and flavin [14]. As the lifetime of 7 ps is close to the detection limit, the fluorescence upconversion technique has been used to examine the picosecond decay kinetics of FAD [15,16]. It has been observed that stack conformer has a shortened lifetime of around 5–9 ps and this can be attributed to a very efficient quenching route via electron transfer from adenine to light excited flavin [15,16]. Transient absorption studies also provide conclusive evidence that the excited state quenching observed in FAD occurs in less than 5 ps time scale [17]. Fluorescence decay dynamics of FAD in a mixture of alcohol and water has been studied in the femtosecond to nanosecond time range and it has been observed that the population of open conformation increases with the decrease in dielectric constant of the medium [18].

Albeit most of the studies have been focused on the dynamic behavior of FAD [14–18], a very few studies have been devoted to pH dependence dynamic behavior of FAD in femtosecond to nanosecond time scale. For the first time, Li and Glusac extensively studied the pH dependent dynamic behavior of FMN and FAD using transient absorption spectroscopy in femtosecond resolution [19]. From the inspection of their results, they suggested that at low and high pH FAD adopts an unstack conformation and behaves similarly as that of FMN. With the support of transient absorption results they also inferred that conformational changes of FAD take place at pH ∼ 3 (because of adenine protonation) and pH ∼ 10 (because of flavin deprotonation) [19]. Moreover, they observed a dynamics of ∼20 ps in neutral pH range, responsible for the stacking interaction between flavin (or isoalloxazine ring) and adenine moiety of FAD [19]. In this study, we intend to explore the dynamics of stacking interaction of FAD with the help of fluorescence spectroscopic techniques, e.g., fluorescence up-conversion and time correlated single photon counting (TCSPC), in five orders of time span from ∼300 fs to ∼10 ns throughout the pH range of 0.5–13.0. To the best of our knowledge, this work is the first fluorescence up-conversion and ultra-fast anisotropy study on the pH dependence dynamic behaviors of FAD and FMN.

2. Experimental

FAD (HPLC grade, purity ≥95%) was purchased from Sigma–Aldrich and FMN was bought from Fluka (HPLC grade, purity ∼90%). Both of the compounds were used without any further purification. pH of the aqueous solution was set by using sodium phosphate dibasic anhydrous (SRL, India, 99.5% pure) and citric acid (Sigma–Aldrich, purity ≥99.5%) buffer. Fine adjustment of pH was done by drop wise addition of diluted hydrochloric acid (Merck, India, 90% pure) for lower pH range and 0.5 M sodium hydroxide (SRL, India, 98% pure) solution for the higher pH range. pH of the solution was measured by using pH-1500 (Eutech Instruments) and it was further cross checked by silicon micro sensor pocket sized pH meter (ISFETCOM Co. Ltd., Japan). All of the solutions were prepared in Millipore water showing resistance of 18.2 μΩ cm^{-1}. To overcome the effect of hydrolysis of FAD at the very low and high pH, all the measurements were done within 15–20 min after addition of FAD. The concentration of FAD and FMN in all the measurements was ∼3×10^{-5} M.

Absorption spectra of the samples at different pHs were recorded by Evolution 300 UV-Visible spectrophotometer (Thermo Fisher Scientific Evolution 300). Fluorescence was collected in Fluorolog-3 (Horiba Jobin Yvon), with an excitation wavelength of 444 nm. For measurement of the relative quantum yields of the samples, we have used Coumarin153 dissolved in ethanol (Φ_F = 0.53 [20]) as a standard fluorescence probe under the same experimental condition. The detection wavelength range for the quantum yield measurements of both reference and sample was 450–700 nm. During fluorescence quantum yield calculation, we have taken account of the refractive index correction of the solvents.

Fluorescence lifetime and anisotropy were measured by TCSPC instrument (Horiba Jobin Yvon IBH, USA). The experimental setup for TCSPC is described in detail in our earlier publication [21]. Briefly, the sample was excited by 440 nm diode laser (IBH, UK, NanoLED-440L). The fluorescence signal was collected at 535 nm with a magic angle orientation of emission polarizer using MCP-PMT (Hamamatsu, Japan) detector. The instrument response function is ∼120 ps. To avoid the interference of the excitation light, a 495 nm long pass filter was placed before the emission monochromator. The analysis of the data was done using Horiba Jobin Yvon IBH (USA) DAS6 decay analysis software.

We have used femtosecond fluorescence up-conversion setup (FOG100, CDP) to detect faster kinetics (<40 ps). The detailed description of this instrument is mentioned elsewhere [22]. Briefly, the sample was irradiated by a laser beam at 430 nm, obtained from the second harmonic of a mode-locked Ti:Sapphire (Tsunami, Spectra Physics) pumped by a 5 W Millennia (Spectra Physics) DPSS laser. The fundamental 860 nm beam was frequency doubled in a nonlinear BBO crystal. The emitted fluorescence from the sample (collection wavelength was at 530 nm, which was emission maximum of FMN, FAD) was up-converted in a BBO crystal by mixing with the gate pulse, which was taken from the fundamental beam. The upconverted signal was detected by a PMT detector. Cross correlation function was obtained from a Raman scattering of ethanol having a FWHM (full width half maximum) of 300 fs. The femtosecond fluorescence decays were deconvoluted by Igor Pro 6.0 analysis software using a Gaussian shape for the excitation pulse.

3. Results and discussions

3.1. Absorption and fluorescence characteristics of FMN and FAD

Absorption spectral features of FMN and FAD at different pHs are the same as reported by others [13,19]. Characteristic absorption spectra for both FMN and FAD are similar and consist of two peaks. One peak is at ∼375 nm and other is at ∼448 nm. The peak at ∼375 nm corresponds to $S_0 \rightarrow S_2$ transition, whereas the latter peak corresponds to $S_0 \rightarrow S_1$ transition.

The emission spectral features of both FMN and FAD are the same as reported in literature [13,19]. Here, it is better to mention that the spectral shape and peak positions are invariant in the pH range of 1.0–12.0 (Fig. S1 in the Supplementary material). However, there is almost 2 nm blue shift in the emission spectrum of FMN and FAD at pH = 13.0 (Fig. S1 in the Supplementary material). The fluorescence quantum yield features of FMN and FAD at various pHs are shown in Fig. 1(a) and it exhibits almost similar trend as published by an earlier group [13]. The quantum yield of both FMN and FAD is almost zero at very low pH (pH = 0.5). After that it increases with the increase in pH and exhibits maximum quantum yield ($\Phi_{FAD} \approx 0.13$) at pH = 3.0. Then, fluorescence quantum yield of both FMN and FAD drops down sharply and reaches almost zero at pH = 13.0. At biological pH, the quantum yield of FAD is ∼0.035. For FMN, the quantum yield increases with the increase in pH until pH = 4.0 and then it remains almost constant in the pH range of 4.0–10.0. After pH = 10.0, the quantum yield sharply decreases. In this context it

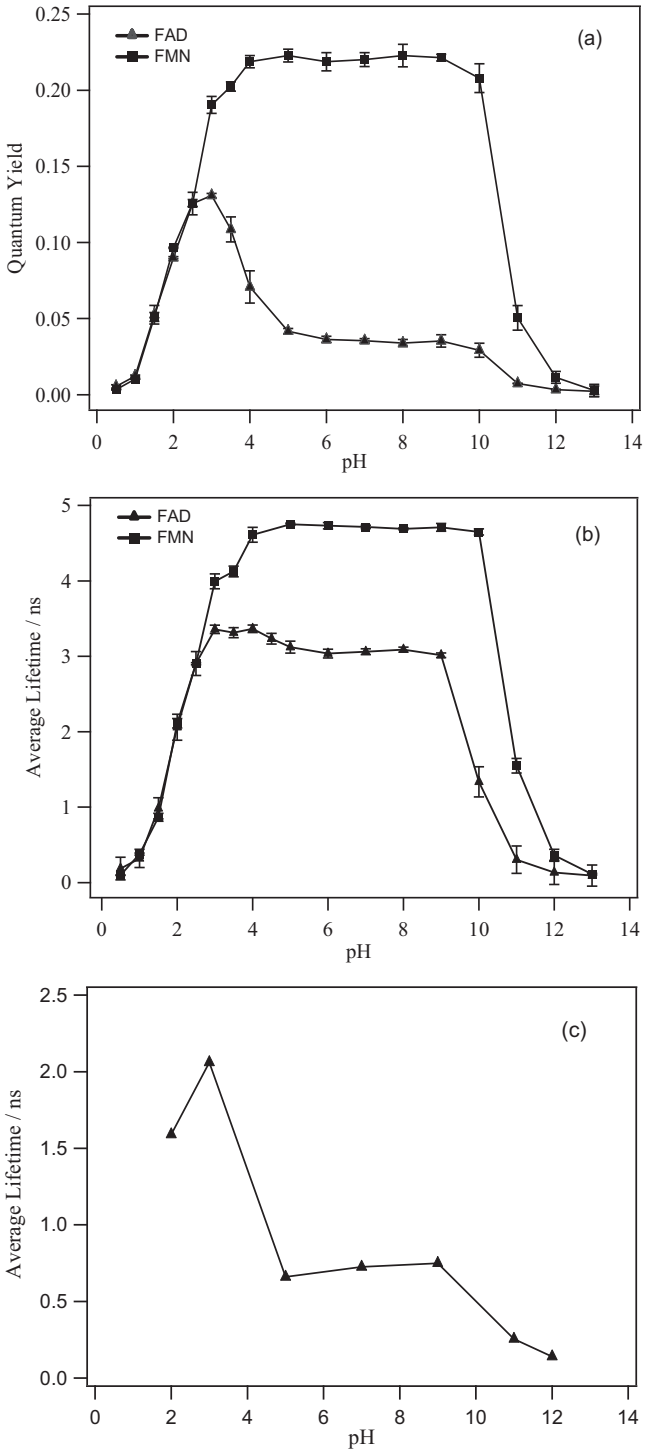
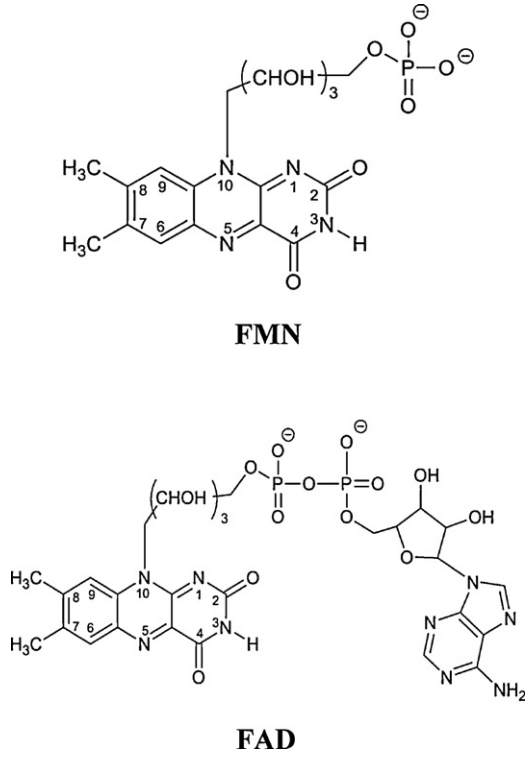


Fig. 1. (a) Relative quantum yield of FAD and FMN at various pHs. (b) Average lifetime of FAD and FMN at various pHs using TCSPC results (Tables 1 and 2). Error bars are indicated. (c) Average lifetime against pH plot of FAD considering up-conversion fitting results (Table 3).



Scheme 1. Representation of the structures of FMN and FAD in the oxidized state.

should be mentioned that flavin is non fluorescent in the cationic form (FLH^+), weakly fluorescent in anionic form (FL^-) and is fluorescent in neutral form (FL) [12]. In the lower pH range (pH = 0.5 to pH = 1.0), there is a reasonable amount of FLH^+ as pK_a of N1 protonation of flavin (Scheme 1) is 0.5 [12], and this explains the observed low fluorescence quantum yield in this pH range. The calculations of ground and excited state π electron densities suggest that protonation site of flavin ring in the ground state is at N1 position, whereas in the excited state it is at N5 position (Scheme 1) [23]. Moreover, it was previously observed that acidity constant of the flavin derivatives in the excited state is ~2.5 [12]. This suggests that excited state protonation at N5 position of neutral flavin ring is the likely reason for relatively low fluorescence quantum yield of FMN and FAD in the pH range of 1.0 to ≤2.5. The difference in fluorescence quantum yield between FMN and FAD in the pH range of 3.0–10.0 arises due to the existence of adenine moiety in FAD, which is absent in case of FMN. Adenine favors the formation of stack conformer in which flavin moiety forms intramolecular complex with adenine and this intramolecular complex is almost non-fluorescent due to excited state electron transfer between adenine and flavin moiety of FAD [14–19]. Presence of this stack conformer reduces the quantum yield of FAD compared to FMN in the above mentioned pH range. Above pH = 10.0, deprotonation of N3 nitrogen (Scheme 1) in flavin ring (the reported pK_a of this process is 9.75 [19]) lowers the quantum yield of both FMN and FAD. Although the fluorescence quantum yield behavior predicts the existence of stack conformer in FAD, it cannot give detailed understandings of the behaviors of stack and unstack conformers unless we monitor the dynamics of these conformers. To get insight about the dynamics, we have carried out fluorescence lifetime measurements in the whole pH range of 0.5–13.0.

3.2. Time resolved fluorescence features of FMN and FAD measured by TCSPC set-up

The excited state fluorescence lifetimes of FAD and FMN are measured in the whole pH range with the help of TCSPC set-up with a time resolution of ~40 ps. Lifetime data is analyzed by deconvolution method using a minimum number of exponentials. The characteristics of fluorescence lifetime of FAD and FMN are tabulated in Tables 1 and 2, respectively. The representative decay profiles of FAD and FMN at different pHs are shown in Fig. 2. Inspection of the results indicates that FAD decay exhibits single exponential feature in the pH range of 0.5–2.5 with the increase in

Table 1
Fluorescence lifetime parameters of FAD at different pHs measured at 535 nm in TCSPC set-up.

Sample name	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	a_1	a_2	a_3	τ_{av}[a] (ns)	χ^2[b]
FAD at pH 0.5	0.18	–	–	1	–	–	0.18	1.17
FAD at pH 1.0	0.38	–	–	1	–	–	0.38	1.15
FAD at pH 1.5	0.77	–	–	1	–	–	0.77	0.82
FAD at pH 2.0	1.85	–	–	1	–	–	1.85	0.98
FAD at pH 2.5	2.76	–	–	1	–	–	2.76	1.06
FAD at pH 3.0	3.52	2.11	–	0.89	0.11	–	3.32	1.07
FAD at pH 3.5	3.74	2.16	–	0.78	0.22	–	3.39	1.05
FAD at pH 4.0	3.92	2.25	–	0.67	0.33	–	3.36	0.92
FAD at pH 4.5	4.08	2.22	–	0.54	0.46	–	3.22	0.96
FAD at pH 5.0	4.31	2.24	–	0.43	0.57	–	3.12	1.08
FAD at pH 6.0	4.42	2.27	–	0.37	0.63	–	3.06	1.07
FAD at pH 7.0	4.44	2.27	–	0.36	0.64	–	3.06	0.96
FAD at pH 8.0	4.46	2.27	–	0.36	0.64	–	3.06	1.01
FAD at pH 9.0	4.39	2.28	–	0.37	0.63	–	3.06	1.08
FAD at pH 10.0	4.44	2.28	0.10	0.19	0.28	0.54	1.50	0.97
FAD at pH 11.0	3.60	–	0.08	0.06	–	0.94	0.30	0.99
FAD at pH 12.0	3.52	–	0.08	0.02	–	0.98	0.15	1.07
FAD at pH 13.0	–	–	0.08	–	–	1.00	0.08	1.08

[a] $\tau_{av} = (a_1\tau_1 + a_2\tau_2 + a_3\tau_3)$.
[b] χ^2 defined the goodness of fit and for good fit the value of χ^2 should be close to 1.

lifetime from 0.18 ns to 2.76 ns. As the pK_a value for the protonated flavin (FLH^+) is 0.25 [13,19], hence, it is expected that flavin moiety of FAD exists as neutral flavin (FL) in the above mentioned pH range. Thus, the lower value of lifetime in this pH range might arise due to dynamic quenching of flavin by adenine moiety. However, FMN, devoid of adenine moiety, exhibits almost equally reduced lifetime in this pH range. Moreover, Islam et al. observed that the fluorescence lifetime of both riboflavin and FAD in this pH range is shortened due to excited state protonation of neutral flavin [13]. Therefore, we can rule out the possibility of dynamic quenching for the reduced lifetime of FAD, and the excited state protonation of flavin ring is considered to be the prime reason for the shortened lifetime of FMN and FAD in this pH range.

FAD decays exhibit bi-exponential feature in the pH range of 3.0–9.0 (Table 1, Fig. 2). One component has a lifetime of ~2.2 ns and another component has a lifetime between ~3.5 ns and ~4.0 ns (Table 1). The slow component may represent the lifetime of unstack conformer of FAD, as it is reported that free flavin has a lifetime of 4.7 ns [9,12]. For affirmation, we have also collected the lifetime of FMN in the same pH range and we have observed that all these decays consist of single component having lifetime between 4.1 ns and 4.7 ns (Table 2). This confirms that slow component is responsible for the lifetime of unstack conformer of FAD. The slight difference in lifetime between the FMN and unstack conformer of FAD might arise due to presence of extra phosphate group and adenine moiety in FAD, which slightly modifies the excited state character of FAD. The observed ~2.2 ns component might reflect the presence of stack conformer of FAD, as FMN is devoid of ~2.2 ns component. However, stack conformer has a shortened lifetime of 5–9 ps due to the excited state intramolecular electron transfer from adenine to flavin moiety [14–19]. Thus, the ~2.2 ns component is not responsible for the stack conformer of FAD, instead, it represents lifetime of a new conformer of FAD in which flavin does not stack but interacts with the other parts of the molecule, resulting in less efficient quenching (hereafter we will call this conformer as 'partially stack' conformer of FAD).

FAD decay exhibits tri-exponential feature at pH = 10.0 (Table 1). Here, in addition to the above mentioned components, a third component with a lifetime of ~90 ps arises. As the pK_a value for the deprotonation of N3 nitrogen (Scheme 1) of the flavin ring is ~9.75 [19], we believe that this ~90 ps component is the lifetime of deprotonated FAD. Islam et al. also pointed out the presence of ~100 ps component from the simulation of their observed data [13]. The percentage of this component increases with the increase in pH and the contribution of this component reaches almost 100% at pH > 12.0 (Table 1). Unlike FAD, this ~90 ps component starts

Table 2
Fluorescence lifetime parameters of FMN at different pHs measured at 535 nm in TCSPC set-up.

Sample name	τ_1 (ns)	τ_2 (ns)	a_1	a_2	τ_{av}[a] (ns)	χ^2[b]
FMN at pH 0.50	0.10	–	1	–	0.10	1.10
FMN at pH 1.0	0.34	–	1	–	0.34	0.85
FMN at pH 1.5	0.88	–	1	–	0.88	0.89
FMN at pH 2.0	2.00	–	1	–	2.00	1.10
FMN at pH 2.5	2.93	–	1	–	2.93	0.99
FMN at pH 3.0	4.14	–	1	–	4.14	0.98
FMN at pH 3.5	4.14	–	1	–	4.14	1.03
FMN at pH 4.0	4.68	–	1	–	4.68	0.97
FMN at pH 5.0	4.76	–	1	–	4.76	0.99
FMN at pH 6.0	4.70	–	1	–	4.70	0.97
FMN at pH 7.0	4.70	–	1	–	4.70	0.99
FMN at pH 8.0	4.68	–	1	–	4.68	1.05
FMN at pH 9.0	4.71	–	1	–	4.71	1.03
FMN at pH 10.0	4.62	–	1	–	4.62	1.07
FMN at pH 11.0	4.51	0.107	0.32	0.68	1.51	0.96
FMN at pH 12.0	3.77	0.093	0.06	0.94	0.31	1.04
FMN at pH 13.0	1.70	0.093	0.02	0.98	0.13	0.96

[a] $\tau_{av} = (a_1\tau_1 + a_2\tau_2)$.
[b] χ^2 defined the goodness of fit and for good fit the value of χ^2 should be close to 1.

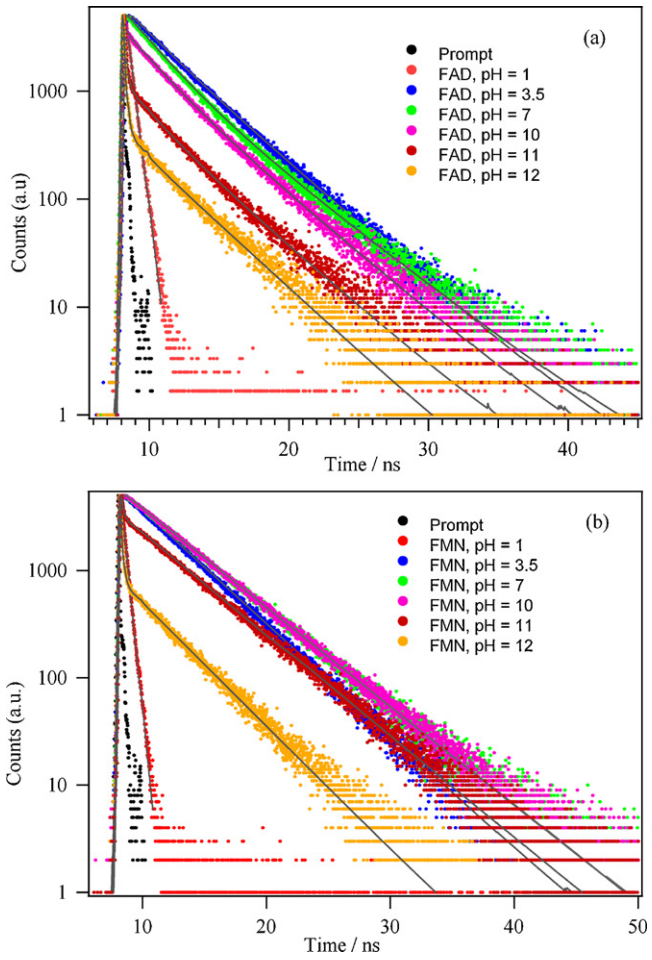


Fig. 2. TCSPC fluorescence decays of FAD and FMN at different pHs monitored at 535 nm. (a) and (b) represent FAD and FMN, respectively. The solid gray lines denote the line of best fit.

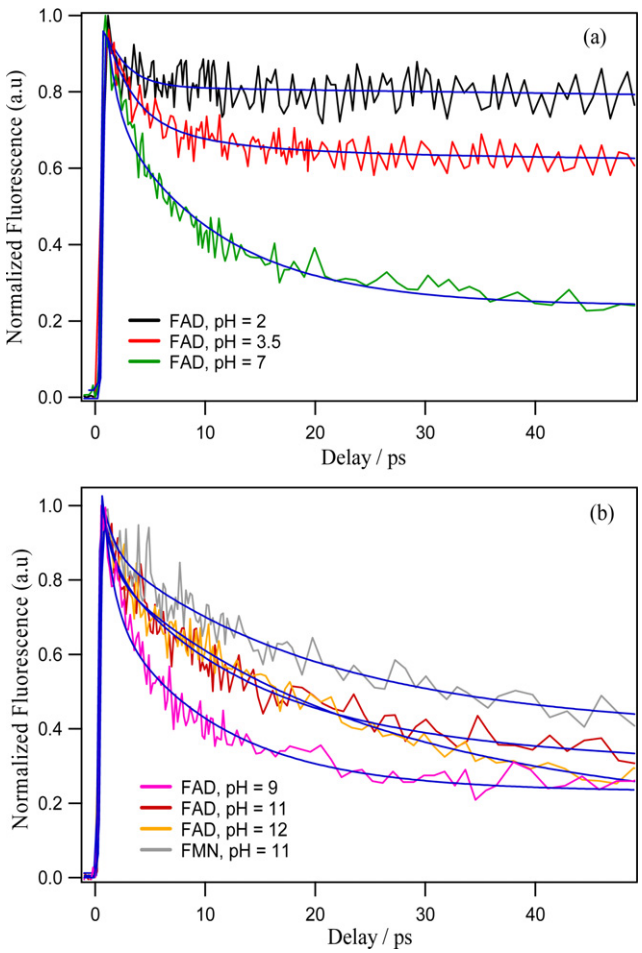


Fig. 3. Femtosecond fluorescence decays of FAD and FMN at different pHs monitored at 535 nm. (a) Represents decay profiles of FAD at pH = 2, pH = 3.5 and pH = 7.0. (b) Represents decay profiles of FAD at pH = 9.0, pH = 11.0 and pH = 12.0. Gray color line in (b) represents decay profile of FMN at pH = 11.0. The solid blue lines denote the line of best fit. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

appearing from slightly higher pH (pH > 10.0) for FMN (Table 2). This suggests that pK_a value for the deprotonation of N3 nitrogen of the flavin ring of FMN is slightly higher compared to FAD. This is reasonable as the electron density of flavin in FAD is less compared to FMN due to the presence of one more phosphate group, which draws more electron cloud from the flavin moiety of FAD. Around pH ≥ 11.0, almost all the FAD molecules are deprotonated (Table 1). Hence, there is less possibility of existence of stack conformer due to electrostatic repulsion between the negatively charged flavin ring and phosphate groups of FAD. Moreover, deprotonated flavin can be better solvated than the neutral flavin ring and the solvents around the flavin ring might prevent FAD from stacking.

We have also plotted average lifetime of FMN and FAD against pH as some of the decays are non-exponential in nature (Tables 1 and 2 and Fig. 1(b)). Interestingly, average lifetime plot of FAD (Fig. 1(b)) deviates significantly from fluorescence quantum yield plot (Fig. 1(a)), particularly in the pH range from 3.0 to 10.0. In contrast, features of the quantum yield and average lifetime plots of FMN almost remain the same (Fig. 1). This is because quantum yield (or fluorescence intensity) reflects average fluorescence property of all types of conformers of FAD (stack, unstack, partially stack conformers) present in the solution, whereas lifetime data measured by TCSPC are not able to detect stack conformer due to short lifetime (~9 ps) of stack conformer of FAD. Thus, the mismatch of the features between average lifetime and quantum yield plots of FAD give an indirect hint for the existence of stack conformer of FAD, which could not be detected by our TCSPC set-up.

FAD exists in different conformers and redox states depending on the pH of the medium. Moreover, the radiative rates of these redox states and conformers are different. Hence, it is not easy to make a simple discussion based on fluorescence lifetime at a single wavelength, unless we check the fluorescence transients are not dependent on collection wavelengths. Therefore, we have collected decay profiles of FAD and FMN at three different wavelengths of the emission spectra at pH = 2.0, pH = 7.0 and pH = 11.0 (Fig. S2 in the Supplementary material). However, it was observed that the decay profiles are independent on the collection wavelengths within the time resolution of TCSPC instrument.

3.3. Time resolved fluorescence features of FMN and FAD measured by femtosecond up-conversion set-up

In order to probe the dynamics of stack conformer, we have measured the lifetime of FAD using femtosecond fluorescence up-conversion set-up with a time resolution of ~300 fs. The representative femtosecond fluorescence transients are shown in Fig. 3 and the results are tabulated in Table 3. Unless noted otherwise, during the analysis of decay profiles of FAD in the pH range of 2.0–9.0 the long component (detected by TCSPC set-up) is kept fixed. It was noticed that at pH = 2.0, the decay profile of FAD consist of ~1 ps component with a relative contribution of 14% and the remaining decay is contributed by a long component (~2 ns),

Table 3
Fitting results of fluorescence up-conversion decays (monitored at 535 nm) of FAD and FMN at different pHs.

(a) FAD in the pH range between 2.0 and 9.0

Name of the sample	τ_1 (ps)	a_1	τ_2 (ps)	a_2	τ_3[a] (ps)	a_3
FAD, pH = 2.0	1(±0.2)	0.14(±0.02)	–	–	1850	0.86(±0.04)
FAD, pH = 3.0	1(±0.2)	0.26(±0.02)	10(±1)	0.12(±0.02)	3320	0.62(±0.04)
FAD, pH = 5.0	1(±0.2)	0.35(±0.02)	10(±1)	0.44(±0.03)	3120	0.21(±0.02)
FAD, pH = 7.0	1(±0.2)	0.31(±0.02)	10(±1)	0.45(±0.02)	3000	0.24(±0.02)
FAD, pH = 9.0	1(±0.2)	0.27(±0.02)	10(±1)	0.48(±0.03)	3000	0.25(±0.02)

(b) FAD and FMN at pH = 11.0 and pH = 12.0

Name of the sample	τ_1 (ps)	a_1	τ_2 (ps)	a_2	τ_3[b] (ps)	a_3	a_4[c]
FAD, pH = 11.0	1.2	0.15(±0.02)	10(±2)	0.36(±0.03)	80	0.44(±0.02)	0.06
FAD, pH = 12.0	1.2	0.18(±0.02)	12(±2)	0.41(±0.02)	80	0.38(±0.02)	0.03
FMN, pH = 11.0	1.2	0.11(±0.02)	13(±2)	0.27(±0.03)	100	0.56(±0.04)	0.06
FMN, pH = 12.0	1.2	0.14(±0.02)	10(±2)	0.34(±0.03)	90	0.50(±0.03)	0.02

[a] Taken from TCSPC measurement and it was fixed during analysis.
[b] Taken from TCSPC studies and fixed during analysis.
[c] Nanosecond background.

which is measured by TCSPC set-up. The ~1 ps dynamics arises from the non-relaxed excited state of flavin of FAD for which solvation dynamics is not yet completed [15,18]. The decay profile of FAD at pH = 2.0 lacks ~10 ps component, which is lifetime of the stack conformer of FAD [15–17]. This suggests that the stack conformer does not exist at pH = 2.0. ~10 ps component starts appearing around pH = 3.0 with a relative contribution of 12%, along with the ~1 ps component (with a relative contribution of 26%) and a long component of ~3.0 ns (with a relative contribution of 62%). At pH = 3.0, the relative contribution of long component (contributed by unstack or partially stack conformer of FAD) is more compared to stack component. Hence, FAD exhibits higher quantum yield at pH = 3.0. At pH = 5.0, the relative contribution of this ~10 ps component is 44%; at biological pH = 7.0, the same has a contribution of 45%; at pH = 9.0, it has a contribution of almost 48% of the total decay. This indicates that the relative contribution of the stack conformer remains almost constant in the pH range of 5.0–9.0. This is consistent with the quantum yield results, where we also found that quantum yield remains unaltered in the pH range between 5.0 and 9.0 (Fig. 1(a)). Moreover, in the pH range of 3.0–9.0, the relative contribution of long component is less compared to stack component. Therefore, FAD exhibits lower quantum yield in this pH range (see Fig. 1(a)). The flavin ring of FAD contains negative charge at pH ≥ 11.0 as the pK_a value for the deprotonation of N3 nitrogen of the flavin ring is 9.75 [19]. So, one could expect that decay profile of FAD should be devoid of ~10 ps component, due to electrostatic repulsion between negatively charged flavin and phosphate group. A different approach has been adopted for the analysis of FAD at pH ≥ 11.0. As shown in Tables 1 and 2, the decaying component with a lifetime of 80–90 ps is dominant in the picosecond decay obtained from TCSPC set-up at and above pH = 11.0. Hence, it is expected that this picosecond component also contributes significantly in the femtosecond time-resolved fluorescence decay in 0–50 ps range. Therefore, femtosecond time resolved decays have been analyzed using triexponential function and a constant that reflects the nanosecond background in this time-scale. Moreover, during analysis we kept fixed τ_1 and τ_3 as 1.2 ps and 80–90 ps, respectively; whereas, all other parameters remain as free variables (Table 3(b)). It has been noticed that ~10–13 ps dynamics is present even in the decay profiles of FAD at pH ≥ 11.0. To understand this dynamics, we have also collected the characteristic decay features of FMN at pH = 11.0 and pH = 12.0 (Fig. 3(b)) and we have analyzed these decay profiles using the above mentioned procedure. Surprisingly, we have noticed that the decay profile of FMN also consists of ~10–13 ps component and, hence, it cannot reflect the lifetime of stack conformer. The appearance of the ~10–13 ps component seems to be quite interesting, however, the origin of this newly observed dynamics is hard to predict in absence of any cogent evidence.

Now, it would be interesting to see the average lifetime against pH plot of FAD considering all the components obtained from the up-conversion and TCSPC studies. We have rebuilt the plot using the fitting results in Table 3 and it is shown in Fig. 1(c), and, as expected now the average lifetime plot of FAD resembles to that of quantum yield plot (Fig. 1(a) and (c)).

3.4. Time-resolved fluorescence anisotropy features of FMN and FAD measured by TCSPC set-up

Fluorescence anisotropy decay measures the rotational dynamics of molecule and this could be affected by the conformational changes of the molecule. Therefore, we used time resolved fluorescence anisotropy to detect conformational changes of FAD while switching from stack to unstack or vice versa. The fluorescence anisotropy decay ($r(t)$) is given by

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

where $I_{||}(t)$ and $I_{\perp}(t)$ are fluorescence decays polarized parallel and perpendicular to the polarization of the excitation light, respectively. G is the correction factor for detector sensitivity to the polarization direction of the emission. From the anisotropy decay

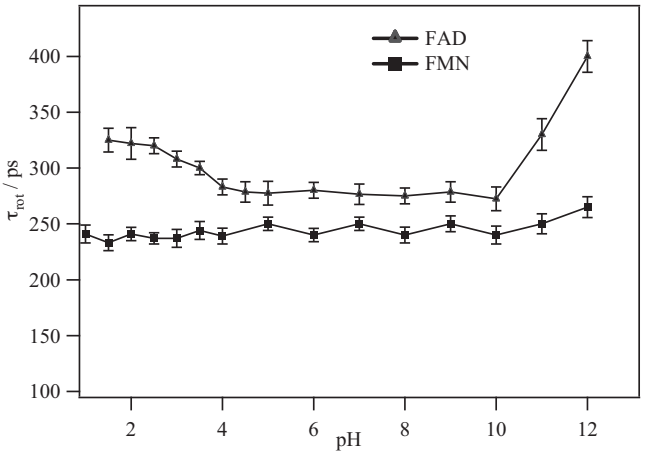
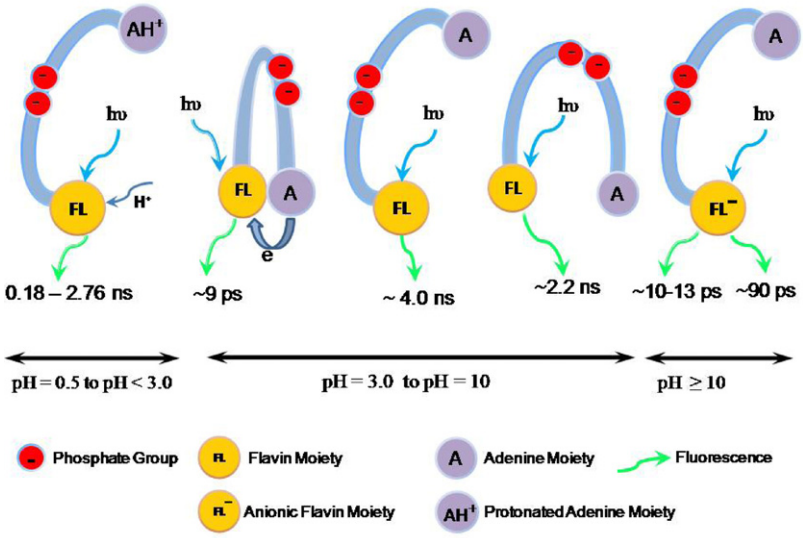


Fig. 4. Rotational correlation time (measured in TCSPC set-up) of FAD and FMN at various pHs monitored at 535 nm. Error bars are indicated.



Scheme 2. Schematic representations for various structures and dynamics of FAD at different pHs.

profiles, we have determined rotational relaxation time (τ_r) of FAD throughout the pH range of 1.0–12.0 and the plot of rotational relaxation time (τ_r) against pH is shown in Fig. 4. The gradient of the rotational correlation time with different pH values seem to change at around pH of 3.0 and 10.0 (Fig. 4). All the solutions are homogeneous in nature and devoid of any rigid environment. Therefore, observed changes in the rotational correlation time is most likely due to the conformational changes of FAD around above mentioned pH. However, one obvious question may arise that the change of viscosity of the medium may be also responsible for the observed change in anisotropy. To verify that we have also collected anisotropy decays of FMN in the whole pH range (Fig. 4). However, it was noticed that rotational correlation time of FMN is almost unchanged throughout the pH range of 1.0–10.0, but spanned in different time regions. After pH = 10.0, there is slight increase in rotational correlation time of FMN up to pH = 12.0. This slight increase in the rotational correlation time of FMN after pH = 10.0 might arise due to the fact that in this pH range we have used dilute NaOH solution, which slightly increases the viscosity of the medium. However, there is steady and sharp increase in the rotational correlation time of FAD after pH = 10.0. Therefore, we believe that the change of rotational dynamics after pH = 10.0 is due to conformational change of FAD rather than viscosity change of the medium. Our anisotropy results nicely reconcile the earlier transient absorption results, which nicely explained the conformation change of FAD at pH ~ 3 and pH ~ 10 [19]. Another important feature observed from our anisotropy study is that in both acidic and basic pH regions rotational relaxation time of FAD increases, which is attributable to unstack conformer of FAD that rotates at slower rate than the stack conformer. This is because unstack conformer occupies larger van der Waals volume compared to stack conformer.

3.5. Conformational dynamics of FAD

FAD has pH dependent conformational flexibility. In the low pH range (pH < 3.0), excited state protonation of N5 nitrogen of flavin ring prevents intramolecular π complex formation between flavin and adenine moiety. Moreover, pK_a of protonated adenine moiety is ~3.5 [24] and the protonated adenine cannot participate in π complex formation with flavin moiety. This is supported by our femtosecond results, which indicate that stack conformer of FAD starts appearing around pH ~ 3. Moreover, there is a change of rotational dynamics of FAD around this pH. Therefore, we conclude that a deprotonation of adenine moiety induces conformational change of FAD around this pH. From pH = 3.0 onwards, we have observed that FAD exists in three distinct conformations: one is stack conformer, another is unstack conformer and third one is the partially stack conformer, in which flavin ring does not stack but interacts with the other parts of the molecule. After pH = 10.0, introduction of negative charge in flavin induces the conformational change from stack to unstack as phosphate group and negatively charged flavin moiety repels each other. As a result, stack to unstack transition takes place just after pH = 10.0. All the structure and dynamics related to conformational flexibility of FAD is shown in Scheme 2. Molecular dynamics simulation studies have been used to study the conformational dynamics of FAD [14]. The simulation studies confirmed the transition from unstack to stack conformer in which flavin and adenine ring stack coplanarly [14]. Simulations also characterized many intermediates in going from unstack to stack conformer and some of the conformations are long lived [14]. We believe that partially stack conformer of FAD is one of the long lived intermediate during transition from unstack to stack conformer.

The relative contribution of stack conformer of FAD dominates over other two conformers in a wide range of pH (from pH = 5.0 to pH = 10.0). This means that stack conformer gains extra stability compared to unstack conformer and this stabilization energy comes from the π–π interaction between flavin and adenine moiety of FAD. That is why FAD adopts stack conformer in many flavoproteins [25,26]. Interestingly, FAD also pursues unstack conformer in many flavoproteins in spite of the stability of stack conformer compared to unstack [27–30]. This is probably due to the fact that stack conformer cannot participate in electron transfer reaction with surrounding amino acids, as flavin moiety already involved in intramolecular electron transfer reaction with adenine. Therefore, in order for the electron transfer to take place, which is key step for the functioning of flavoproteins, FAD adopts unstack conformer in many flavoproteins. But the little insight has been given on the structure–function relationship of FAD. We hope that the observed distinct dynamics controlled by the flavin ring flexibility as well as oxidation state of flavin, will help to elucidate the

structure–function relationship of FAD in various biological systems.

4. Conclusion

pH dependent conformational dynamics of FAD is monitored with the help of time resolved fluorescence techniques from femtosecond to nanosecond time scale. We have observed that in the low pH range (pH < 3.0), only unstack conformer of FAD is favored as either adenine or flavin moiety gets protonated in this pH range and the excited state protonation of flavin moiety results in low fluorescence quantum yield and short lifetime of both FMN and FAD in this pH range. Stack conformer of FAD starts appearing from pH = 3.0 and relative population of stack conformer is significantly smaller than the unstack conformer at this pH. Therefore, FAD exhibits relatively higher quantum yield at pH = 3.0. The relative population of stack conformer increases with the increase in pH, and in the pH range of 5.0 to <10.0, relative contribution of stack conformer dominates over unstack conformer. Hence, FAD exhibits low fluorescence quantum yield between pH = 5.0 and pH = 10.0. Interestingly, in the pH range of pH = 3.0 to pH < 10.0, we have observed three distinct dynamics of FAD; one is ∼10 ps dynamics (responsible for stack conformer), another is ∼3.5 to ∼4.5 ns dynamics (responsible for unstack conformer) and third one is ∼2.2 ns component (responsible for the partial quenching of flavin by adenine moiety of FAD in the excited state lifetime of FAD). Around pH ≥ 10.0, the population of unstack conformer dominates over stack conformer as the deprotonated flavin ring and phosphate group repels each other. Moreover, we have detected a new dynamics of 10–13 ps both for FMN and FAD at pH ≥ 11.0. However, the origin of this newly observed dynamics is not clearly understood at the present moment. We also found change of rotational motion of FAD around pH ∼ 3 and pH ∼ 10 corresponding to conformational from their transient absorption results changes of FAD at these two pHs. Moreover, our time resolved fluorescence anisotropy study indicates that the rotational motion of unstack conformer is slower than the stack conformer of FAD, which can be ascribed to the larger van der Waals volume of unstack conformer than that of stack conformer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jphotochem.2011.04.033.

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