20

Anisotropic excited states relaxation of FAD bound with bacterial diaphorase

© I.A. Gorbunova, D.V. Yashkov, D.A. Volkov, M.E. Sasin, O.S. Vasyutinskii

Ioffe Institute, St. Petersburg, Russia

e-mail: i.gorbunova@mail.ioffe.ru

Received March 20, 2023
Revised May 28, 2023
Accepted May 29, 2024

This paper presents the results of experimental studies of excited state dynamics of FAD in free form and FAD bound with bacterial diaphorase. The studies were carried out by recording the time-resolved polarized fluorescence using time-correlation single photon counting technique after excitation of FAD by femtosecond laser pulses. It was found that excited state dynamics of FAD-diaphorase complexes differs significantly from that of free FAD. Free FAD exhibited four fluorescence decay times ranging from a few picoseconds to a few nanoseconds, while two fluorescence decay times were observed in FAD-diaphorase complex. The analysis of fluorescence polarization decay of FAD-diaphorase complex revealed a subnanosecond decay time of $\tau_{bv} = 130$ ps. It was shown that this fluorescence depolarization time was due to anisotropic vibrational relaxation in FAD excited state which leads to rotation of transition dipole moment due to rearrangement of the molecular nuclei configuration after excitation.

Keywords: FAD, Diaphorase, polarized fluorescence, TCSPC, anisotropy, fluorescence lifetime.

DOI: 10.61011/EOS.2024.05.59125.6160-24

Introduction

Flavin adenine dinucleotide (FAD) is an important natural coenzyme that plays an important role in redox reactions in living cells metabolism. FAD exist in living cells in several forms with fully reduced ($FADH_2$) and oxidized (FAD^+) forms being the most stable among them. Spectral properties of these forms differ considerably. The oxidized form, FAD^+, has a wide absorption spectrum in blue and ultraviolet (UV) region with maxima at 450 nm and 370 nm and fluoresces in the green spectral range with a peak at 532 nm. The reduced form, $FADH_2$, in turn does not fluoresce [1]. This feature makes it possible to use FAD^+ as a fluorescent intracellular marker for non-invasive monitoring of redox reactions in living cells. Fluorescence lifetime imaging microscopy (FLIM) [2] is currently the most informative method for monitoring of FAD fluorescence in living cells and tissues. FLIM is based on the determination of spatial distributions of fluorescence lifetimes of the given fluorophore in cells and tissues. In particular, the analysis of FAD fluorescence lifetimes can be used to differentiate between free and protein-bound FAD, folded and unfolded FAD conformations, and to distinguish FAD fluorescence signals from those of other intracellular coenzymes, such as nicotinamide adenine dinucleotide (NAD(P)H) [3,4].

According to the results of early studies [1,5–7], free FAD in solutions exhibit several fluorescence lifetimes. These times depend considerably on the experimental conditions: pH, oxygen concentration, polarity and temperature.

The fluorescence lifetimes of bound FAD are known to vary in a range from hundreds picoseconds to several nanoseconds depending on the type of enzyme [8,9]. Two fluorescence lifetimes of 220 ps and 2.7 ns were determined in the the study of FAD bound to cholesterol oxidase [6] in solution. Nanosecond lifetime within 2.3−2.7 ns was also observed for other enzymes, in particular in FAD bound with D-amino acid oxidase [8] and lipoamide dehydrogenase (LipDH) [10–12]. Bound FAD also exhibited sub-nanosecond fluorescence lifetimes with the values depending considerably on the enzyme and varied in a wide range from 40 ps to 300 ps [8,10]. Different authors have given different interpretations of the nature of these sub-nanosecond fluorescence lifetimes. In particular, one of the widely used hypothesis is a reversible electron transfer reaction in the FAD excited state between isoalloxazine and one of the amino acids in the enzyme binding site [8,10,11].

The heterogeneous fluorescence dynamic in free and bound FAD makes the interpretation of the observed fluorescence lifetime in living cells [13] quite challenging. Thus, two fluorescence lifetimes of about 200 ps and 2.5 ns were observed during the analysis of FLIM signals of FAD in living cells [14]. However, the main challenge was how to establish a correlation between these fluorescence lifetimes and bound and free form of FAD and to explain particular excited-states relaxation processes in FAD. Therefore, the investigation of fluorescence anisotropy decay can be used to obtain the additional information about the relaxation processes in the excited states of molecules [15]. The fluorescence anisotropy and rotational diffusion time were reported to be significantly different in free and bound FAD [10,16,17]. An increase in the rotational diffusion time by two orders of magnitude in bound FAD was reported

in studies [9,13,18] and may be used to separate free and bound states of FAD in living cells cells.

Besides rather slow rotational diffusion processes, other anisotropic processes with much shorter fluorescence polarization decay times were observed in the FAD-enzyme complexes [9,10,11,16,19,20]. In study [19], besides a long rotational diffusion time from 50 ns to 60 ns, a shorter fluorescence depolarization time of about 0.5 ns was observed, in other works this time was at about 1.8–7 ns in [9,16]. However, the nature of these short depolarization times has not been understood yet and is a matter of discussions. Resonant energy transfer between two FAD molecules in two different dimer binding sites is one of the possible reasons for the presence of short fluorescence depolarization time in FAD bound with LipDH [16]. The presence of a local rotational motion of FAD in the LipDH binding site due to the interaction with amino acids was proposed as another explanation [10]. Our recent study [21] found a short (about 1 ns) anisotropic relaxation time in the excited state of NADH bound with alcohol dehydrogenase (ADH) that was explained as a rotation of the fluorescence dipole moment due to the rearrangement of nuclei configuration of NADH molecules after excitation by a short femtosecond pulses. Meanwhile, the investigations of the fluorescence polarization decay in enzyme-coenzyme complexes have not got widespread use yet, and the potential of analysis of the corresponding anisotropic relaxation processes has been poorly implemented

This study first investigated the polarized fluorescence decay in FAD bound to a bacterial diaphorase under single-photon excitation at 450 nm. Diaphorase is a lipoamide dehydrogenase that has high diaphorase activity. This enzyme acts as a catalyst for the oxidation of NADH to NAD^+ in the presence of oxygen and plays an important role in metabolism and production of energy in mitochondria [22,23]. In addition, about 50% of FAD fluorescence in mitochondria is associated with FAD bound to lipoamide dehydrogenase [13,24]. One of the important results of this study is that a relatively short fluorescence depolarization time τ_{bv} of about 130 ps was observed in the FAD-diaphorase complex. The analysis of the depolarization time of bound FAD was based on a model that had been recently developed in our previous publication [21]. According to this publication, a short depolarization time of the FAD-diaphorase complex characterizes the anisotropic vibrational relaxation in the excited state of FAD that leads to the rotation of the transition dipole moment of FAD as a result of the rearrangement nuclear reconfiguration after excitation.

Materials

A commercial reagent of diaphorase from *Clostridium kluyveri* (Sigma-Aldrich, lot D5540, 5.6 unit/mg, EC 1.8.1.4, CAS 9001-18-7) was used as a FAD-dependent enzyme. The diaphorase was dissolved in the PBS (10 mM, pH 7.3) buffer in the amount of 1–1.5 mg/ml. Test experiments were conducted using free FAD (98% pure, DIA-M) that was dissolved in the PBS buffer in a concentration of 60 μM. The solutions were freshly prepared before the experiment at room temperature.

Experiment procedure

The recording of the polarized fluorescence decay signals was based on the method described in detail in our previous publications [21,25–27]. Briefly, FAD excitation was conducted by linearly-polarized laser radiation at 450 nm that corresponded to the transition to the first excited state. The second harmonic of the femtosecond Ti:Sapphire oscillator (Mai Tai HP DS, Spectra Physics) with a pulse duration of 100 fs and repetition rate of 80 MHz was used as the excitation source. The laser beam was focused into the center of a quartz cell containing the investigated molecular solution. The average power of laser radiation focused on the cell was about 500 μW. The fluorescence radiation was recorded perpendicularly to the laser excitation beam in a spectral range of 532 ± 10 nm that corresponded to the fluorescence maximum of FAD. The orthogonal polarization components of fluorescence $I_{\parallel}$ and $I_{\perp}$ were separated by a Glan prism and then recorded simultaneously and independently by two fast avalanche photodetectors (ADP050-CTC, MPD) operated in the photon counting mode. The instrumental response function width was about 50 ps. The fluorescent decay signals were acquired using a time-correlated single photon counting system (TCSPC, PicoHarp 300, PicoQuant). The collection time was 300 s with a time bin of 4 ps. To avoid the photobleaching effect, the solution was pumped through the cell using a peristaltic pump.

Analysis of experimental signals

Experimental signals $I_{\parallel}(t)$ and $I_{\perp}(t)$ of the polarized fluorescence were analyzed using the following expressions [25,28]:

$$I_{\parallel}(t) = G \int_0^t IRF(t') I_{tot}(t-t')[1 + 2r(t-t')]dt', \quad (1)$$

$$I_{\perp}(t) = \int_0^t IRF(t') I_{tot}(t-t')[1 - r(t-t')]dt', \quad (2)$$

where $I_{tot}(t-t')$ is the polarization-independent component of fluorescence intensity that describes its isotropic decay, $r(t-t')$ is the polarization-dependent component of fluorescence intensity (fluorescence anisotropy) that describes its anisotropic decay, $IRF(t)$ is the instrumental response function, G is the coefficient that takes into account the difference in the sensitivity of two detection system channels. The instrumental response function $IRF(t)$ was

measured experimentally by observing the scattered second-harmonic radiation of the femtosecond laser at 532 nm. The details of the instrumental response function measurements are discussed in our previous publication [21].

Isotropic signals of the fluorescence decay $I_{tot}(t)$ were determined according to expressions (1) and (2) as follows [21,29]:

$$I_{tot}(t) = \frac{I_{\parallel}(t) + 2I_{\perp}(t)}{3}. \quad (3)$$

The isotropic component of the fluorescence decay $I_{tot}(t)$ in expressions (1), (2) and (3) is described in the general case by a sum of several exponents:

$$I_{tot}(t) = I_0 \sum_{i=1}^{n} a_i \exp\Big(-\frac{t}{\tau_i}\Big), \quad (4)$$

where I_0 is the initial fluorescence intensity, τ_i are the fluorescence lifetimes, a_i are the corresponding weighting coefficients normalized to unity: $\sum_i a_i = 1$.

The fluorescence anisotropy $r(t)$ in expressions (1) and (2) was determined from the orthogonal polarization components of fluorescence $I_{\parallel}(t)$ and $I_{\perp}(t)$ [21,29]:

$$r(t) = \frac{\frac{1}{G}I_{\parallel}(t) - I_{\perp}(t)}{\frac{1}{G}I_{\parallel} + 2I_{\perp}(t)}. \quad (5)$$

In the general case, the signal $r(t)$ cannot be represented by a sum of several exponents. Generally, when a single group of fluorescent molecules is observed in a solution, the fluorescence anisotropy decay $r(t)$ (5) may be adequately described by a single-exponential model that is characterized by the rotational diffusion time τ_r [29]:

$$r(t) = r_0 \exp\Big(-\frac{t}{\tau_r}\Big), \quad (6)$$

where r_0 is the anisotropy coefficient. However, when it comes to the investigation of molecular complexes, the fluorescence anisotropy decay often has a more complex behavior [9,10,11,16,19,20].

For analysis of the observed polarized fluorescence decay signals in FAD-diaphorase complex, this study used a model developed in our recent publications [21,30] where the anisotropic relaxation processes in the free NADH and NADH bound with ADH enzyme have been investigated and described. According to the model, the fluorescence anisotropy decay of the coenzyme-enzyme complex may be described as

$$r(t) = \Big(r_{b1} + r_{b2}e^{\frac{-t}{\tau_{bv}}}\Big)e^{\frac{-t}{\tau_{br}}}, \quad (7)$$

where τ_{br} is the rotational diffusion time of the whole complex and τ_{bv} is the anisotropic excited state relaxation time of the coenzyme only.

r_{b1} and r_{b2} in expression (7) characterize the angle θ between the transition dipole moments of excitation and fluorescence at the excitation time $t = 0$ and after the vibrational relaxation $t = t_{rel}$ when the excited molecule appears to be in the equilibrium state:

$$r_{b1} = \langle P_2(\cos\theta)\rangle|_{t=t_{rel}},$$

$$r_{b2} = \langle P_2(\cos\theta)\rangle|_{t=0} - \langle P_2(\cos\theta)\rangle|_{t=t_{rel}}. \quad (8)$$

Analysis of the decay signals of the orthogonal fluorescence polarization components was performed according to expressions (1) and (2) and models (6) or (7) by the global fitting procedure using a program written in Python3.

Table 1. Fluorescence lifetimes and weighting coefficients for free FAD molecules and FAD-diaphorase complexes

Item	τ_1, ns (a_1)	τ_2, ns (a_2)	τ_3, ns (a_3)	τ_4, ns (a_4)
Free FAD	0.02 (0.47)	0.22 (0.11)	2.26 (0.25)	3.82 (0.17)
FAD-diaphorase	0.23 (0.18)	4.36 (0.82)	–	–

Table 2. Fluorescence anisotropy and depolarization time of free FAD and FAD-diaphorase complex

Item	r_0, r_{b2}	τ_r, τ_{bv}
Free FAD	0.34	0.160 ns
FAD-diaphorase	0.36	0.130 ns

Results

The fluorescence lifetimes and weighting coefficients in free FAD in the solution and in FAD-diaphorase complexes determined using the global fit procedure are presented in Table 1.

According to the results shown in the first line, Table 1, free FAD in the buffer solution was characterized by four fluorescence lifetimes: $\tau_1 = 20$ ps, $\tau_2 = 220$ ps, $\tau_3 = 2.26$ ns and $\tau_4 = 3.82$ ns. The highest contribution to the signal ($a_1 = 0.46$) was made by the shortest fluorescence lifetime $\tau_1 = 20$ ps. As shown in the second line of Table 1, when FAD bound with diaphorase, two-exponential decay of the fluorescent signal with fluorescence lifetimes of $\tau_1 = 230$ ps and $\tau_2 = 4.36$ ns was observed. In this case, the highest contribution to the signal ($a_2 = 0.82$) was made by the nanosecond fluorescence lifetime $\tau_2 = 4.36$ ns that is comparable with, but a little longer than the longest lifetime in free FAD $\tau_4 = 3.82$ ns. Note that the FAD-diaphorase complex had no the shortest picosecond lifetime of 20 ps that made the largest contribution to the fluorescence decay in free FAD.

The fluorescence depolarization analysis results for free FAD and FAD-diaphorase complex are listed in Table 2. The results for free FAD in the buffer solution listed in the first line of Table 2 contain the initial anisotropy r_0 and rotational diffusion time τ_r that describe the anisotropy

decay signals according to expression (6). Values of r_0 and τ_r in free FAD in Table 2 are in good agreement with the results of the previous studies [31].

The results obtained for the FAD-diaphorase complex using expression (7) are shown in the second line of Table 2. As can be seen, these results also contain only two parameters: initial anisotropy r_{b2} and anisotropy decay time τ_{bv}, while the rotational diffusion time of the whole complex τ_{br} (7), that achieved several dozens of nanoseconds in the early studies in FAD-LipDH [9,13,18], was not recorded in our experiments.

The depolarization time τ_{bv} determined for this case and shown in the second line of Table 2 was a little lower than the rotational diffusion time in free FAD and was equal to $\tau_{bv} = 0.130$ ns. For comparison, the figure shows the isotropic $I_{tot}(t)$ and anisotropic $r(t)$ fluorescence decays of free FAD and FAD-diaphorase complex obtained on the basis of the experimental signals using equations (3) and (5), respectively.

Discussion

The obtained experimental results have demonstrated that the fluorescence decay in free FAD in the solution and FAD-diaphorase differed considerably. As can be seen from Table 1, free FAD is characterized by four fluorescence lifetimes falling in the range from several picoseconds to several nanoseconds, while in the FAD-diaphorase complexes only two fluorescence lifetimes are observed.

The results of our investigations of the fluorescence decay in free FAD in solutions summarized in the first line of Table 1 agree well with the results obtained by other authors. Thus, in studies [32,33], four fluorescence lifetimes were observed $\tau_1 = 7$ ps ($a_1 = 0.66$), $\tau_2 = 220$ ps ($a_2 = 0.03$), $\tau_3 = 2.09$ ns ($a_3 = 0.17$) and $\tau_4 = 3.97$ ns ($a_4 = 0.14$) with weighting coefficients specified in parentheses. Here, it should be noted that the high value of short fluorescence lifetime $\tau_1 = 20$ ps recorded in this study, compared with 7 ps observed in [7,32,33], was probably attributed to the characteristic response time of the detector that was equal to 50 ps. The nature of these lifetimes is the subject of discussion until recently. Shorter lifetimes 7 ps and 220 ps were associated by the several authors [32,33] with the interaction processes (stacking) between adenine and isoalloxazine in the folded configuration of FAD, and the lifetimes 2 ns and 4 ns were associated with relaxation processes in the unfolded conformations of FAD.

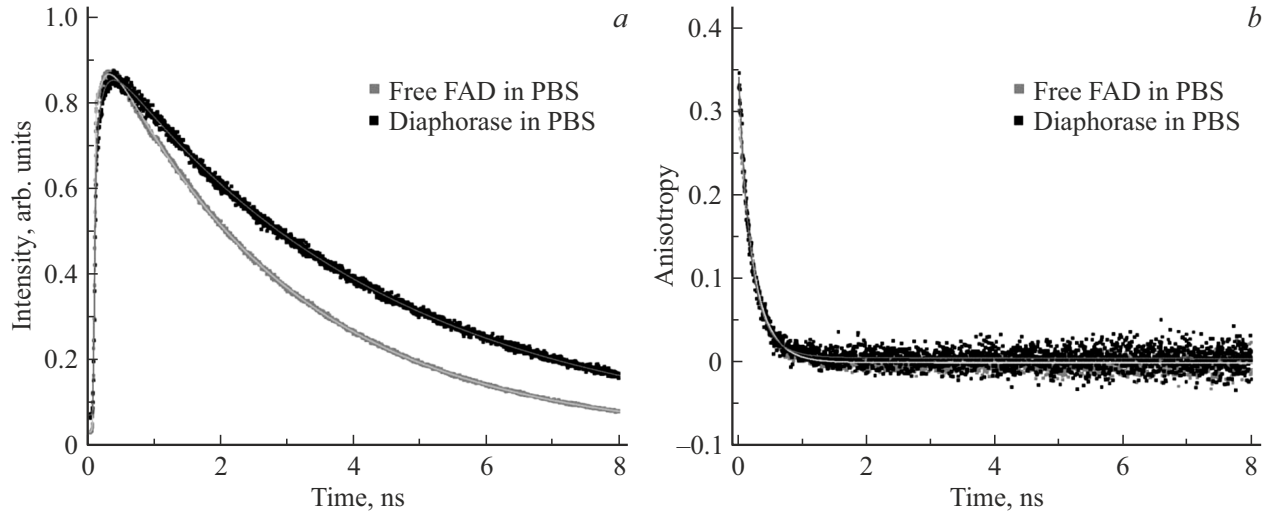
In this study, the investigations of fluorescence decay in the FAD-diaphorase complexes were performed for the first time. The obtained results shown in the second line of Table 1 demonstrate the existence of two fluorescence lifetimes: $\tau_1 = 230$ ps and $\tau_2 = 4.36$ ns. In earlier studies of the FAD-LipDH complexes three distinct fluorescence lifetimes have been recognized: $\tau_1 \sim 100$ ps, $\tau_2 = 300 - 700$ ps and $\tau_3 = 2.5 - 3.14$ ns [8,16,19]. The nanosecond fluorescence lifetime in the FAD-diaphorase complex $\tau_2 = 4.36$ ns obtained in this study was larger than the fluorescence lifetime of the FAD-LipDH obtained earlier in refs. [8,10,16,20]. This may be attributed to the differences in the origin of the enzymes and their activity. This study used the bacterial diaphorase, while studies [8,10,16,19] used animal LipDH that has no diaphorase activity. This study also detected a sub-nanosecond fluorescence lifetime of FAD-diaphorase $\tau_1 = 230$ ps that was in agreement by the order of magnitude with the results of earlier investigations of FAD bound to LipDH [6,8,16].

Based on the obtained experimental results and comparison with the results of other studies, it can be assumed that the observed nanosecond fluorescence lifetime of the FAD-diaphorase complex $\tau_2 = 4.36$ ns is attributed to the relaxation processes in the unfolded conformation of FAD. This assumption is supported by the results of the X-ray analysis of the bacterial diaphorase [11] and animal LipDH [34] that FAD is bound to these enzymes in the unfolded conformation. This assumption also agrees with the results of investigations of the time-resolved fluorescence in free FAD in alcohol solutions where FAD exists only in the unfolded conformation [33,35] and of the investigations of flavin mononucleotide (FMN) [7,36] in which there is no interaction between the adenine and isoalloxazine. In these studies, homogeneous fluorescence decay with fluorescence lifetime of 4.0−4.7 ns was observed [7,33,35,36].

Moreover, as can be seen from Table 1, in the bound state of FAD there is no short fluorescence lifetime of about 20 ps that is observed on free FAD. In studies [32,33], the presence of this short lifetime was suggested to be due to the electron transfer processes between adenine and isoalloxazine in the excited state during π-stacking. We suggested that the absence of this fluorescence lifetime in the FAD-diaphorase complex is attributed to the fact that FAD exists in the enzyme binding site in the unfolded conformation without π-stacking.

Comparing the fluorescence lifetimes of free FAD $\tau_2 = 220$ ps and the FAD-diaphorase complex $\tau_1 = 230$ ps as shown in Table 1, it can be seen that they coincide within the experiment measurement. However, despite such coincidence, they may result from different processes. According to the interpretation proposed by the authors of [32,33], the fluorescence lifetime of free FAD of about 200 ps is attributed to the adenine and isoalloxazine interaction in the excited state of the folded conformation of FAD. For bound FAD, the fluorescence lifetime of about 100−200 ps is attributed to the interaction between isoalloxazine and electron-donor amino acids in the binding site [8,10,11].

As shown in Table 2, the fluorescence polarization decay in the FAD-diaphorase characterized by the one exponential with relatively short lifetime of about 130 ps that was a somehow smaller than the rotational diffusion time of free FAD in the solution $\tau_r = 160$ ps. This short lifetime cannot be assigned to the rotational diffusion of the FAD-diaphorase complex because in accordance with the Einstein-Stokes



(*a*) Isotropic and (*b*) anisotropic fluorescence decay in free FAD and FAD-diaphorase complex. Dots show the experimental data, and solid lines show the fit. Both the experimental data and fitting result were calculated according to expressions (3) and (5).

equation [27], the rotational diffusion time of the FAD-diaphorase complex is more than 30 ns due to its large weight and volume. However, this nanosecond lifetime of bound FAD was not observed in our experimental conditions.

The existence of the short depolarization time in the FAD-LipDH complexes was reported earlier in publications [9,10,16,20] where it's varied from 0.5 ns [18,20] to 9 ns [10]. The fluorescence depolarization time in the FAD-diaphorase complex determined in this study is lower than the short fluorescence depolarization time of the FAD-LipDH complex obtained in the studies [9,10,16,20].

The short fluorescence depolarization time in bound FAD is interpreted differently by different authors. The first model [16] is the resonant energy transfer between two FAD in the LipDH dimer binding sites. Another model [10] is the presence of the local rotational motion of FAD in the LipDH binding site due to the movement of amino acids.

This study proposes the quantitative interpretation of the observed experimental results according to expression (7) that is based on the quantum-mechanical theory [21,30] and describes the time dependence of the fluorescence anisotropy of the molecular complex. According to this model, the short fluorescence depolarization time in FAD-diaphorase complex $\tau_{bv} = 0.13$ ns characterized the anisotropic vibrational relaxation in the FAD excited state, during which rotation of the fluorescence transition dipole moment occurs due to rearrangement of the FAD nuclei configuration after excitation. We suggested that the local rotation of the whole FAD molecule in the diaphorase binding site doesn't occur because FAD has a rather tight structure of non-covalent bonds with amino acids [37,38].

According to this model, the absence of the long rotational diffusion time of the FAD-diaphorase complex is attributed to the fact that the corresponding anisotropy coefficient r_{b1} in (7), (8) was close to zero, which proves that the angle between the transition dipole moments of the excitation and fluorescence was approximately 58° for the bound FAD.

Note that during the investigations of the fluorescence depolarization in the FAD-LipDH complexes under excitation at 450 nm in studies [9,10,16,20] the long rotational diffusion time of about 40–70 ns was observed, and the anisotropy coefficient was about 0.35. The difference of the result obtained in this study from the results in [9,10,16,20] may be explained by the fact that this study used the bacterial diaphorase and studies [9,10,16,20] used animal enzymes that had no diaphorase activity because it is known that the lipoamide dehydrogenase and diaphorase structures may be very different [39].

Conclusion

The time-resolved polarized fluorescence of the FAD-diaphorase complex and free FAD in the PBS buffer were studied. It was found that FAD bound with diaphorase has two fluorescence lifetimes $\tau_1 = 0.27$ ns and $\tau_2 = 4.34$ ns, while free FAD in the solution is characterized by four fluorescence lifetimes: $\tau_1 = 20$ ps, $\tau_2 = 220$ ps, $\tau_3 = 2.3$ ns and $\tau_4 = 3.7$ ns. It was shown that the presence of only two lifetimes in the FAD-diaphorase complex was attributed to the fact that FAD in the binding site was only in one (unfolded) conformation. It was found that the fluorescence polarization decay of the FAD-diaphorase complex is characterized by the sub-nanosecond lifetime $\tau_{rb} = 130$ ps, while the substantially longer rotational diffusion time was not observed at all. During the interpretation of the obtained results, it was suggested that the observed fluorescence depolarization time was attributed to the anisotropic vibrational relaxation in the excited state of coenzymes that was followed by the rotation of the transition dipole moment

due to the nuclei reconfiguration in the FAD molecule. The obtained result may be used for the analysis of interactions between FAD and various enzymes in solutions and living cells.

Funding

I.A. Gorbunova, M.E. Sasin, D.V. Yashkov and D.A. Volkov are grateful to the Russian Science Foundation for the support in the form of grant № 23-22-00230. The authors are grateful to Ioffe Institute, Russian Academy of Sciences, for the equipment provided for the experiment.

Conflict of interest

The authors declare that they have no conflict of interest.

References

[1] S.D.M. Islam, T. Susdorf, A. Penzkofer, P. Hegemann. Chem. Phys., **295**, 137–149 (2003). DOI: 10.1016/j.chemphys.2003.08.013
[2] M.V. Shirmanova, I.N. Druzhkova, M.M. Lukina, V.V. Dudenkova, N.I. Ignatova, L.B. Snopova, V.I. Shcheslavskiy, V.V. Belousov, E.V. Zagaynova. Sci. Rep., **7**, 8911 (2018). DOI: 0.1038/s41598-017-09426-4
[3] C. Stringari, L. Abdeladim, G. Malkinson, P. Mahou, X. Solinas, I.I. Lamarre, S. Brizion, J.-B. Galey, W. Supatto, R. Legouis, A.-M. Pena, E. Beaurepaire. Sci. Rep., **7**, 3792 (2017). DOI: 10.1038/s41598-017-03359-8
[4] X. Wang, Y. Xie, M. Huang, L. Yao, Y. Wang, Y. Fei, J. Ma, L. Mi. IEEE J. Selected Topics in Quant. Electron., **25**, 12019 (2019). DOI: 10.1109/JSTQE.2018.2889429
[5] A.A. Heikal. Biomark. Med., **4** (2), 241–263 (2010). DOI: 10.2217/bmm.10.1
[6] R. Schmitz, K. Tweed, C. Walsh, A. J. Walsh, M. C. Skala. J. Biomed. Optics, **26**, (2021). DOI: 10.1117/1.jbo.26.5.056502
[7] A. Sengupta, R.V. Khade, P. Hazra. J. Photochem. Photobiol. A, **221** (1), 105–112 (2011). DOI: 10.1016/j.jphotochem.2011.04.033
[8] N. Nakashima, K. Yoshihara, F. Tanaka, K. Yagi. J. Biol. Chem., **255**, 5261–5263 (1980). DOI: 10.1016/s0021-9258(19)70779-0
[9] P.A.W. van den Berg, A. van Hoek, A.J.W.G. Visser. J. Biophys., **87**, 2577–2586 (2004). DOI: 10.1529/biophysj.104.040030
[10] E. Nishimoto1, Y. Aso, T. Koga, S. Yamashita. J. Biochem., **140**, 349–357 (2006). DOI: 10.1093/jb/ mvj156
[11] T. Fukamichi, E. Nishimoto. J. Fluoresc., **25** (3), 577–583 (2015). DOI: 10.1007/ s10895-015-1537-x
[12] P.I.H. Bastiaens, A.V. Hoek, W.J.H.V. Berkel, A.D. Kok, A.J.W.G. Visser. J. Biochem., **31**, 7061–7068 (1992). DOI: 10.1021/bi00146a006
[13] S. Huang, A.A. Heikal, W.W. Webb. Photodiagnosis Photodyn. Ther., **82** (5), 2811–2825 (2002). DOI: 10.1016/S0006-3495(02)75621-X
[14] R. Cao, H.K. Wallrabe, A. Periasamy. J. Biom. Opt., **25**, 1 (2020). DOI: 10.1117/1.jbo.25.1.014510
[15] Q. Yu, A.A. Heikal. J. Photochem. Photobiol. B, **95** (1), 46–57 (2009). DOI: 10.1016/j.jphotobiol.2008.12.010
[16] P.I. Bastiaens, A. van Hoek, J.A. Benen, J.-C. Brochon, A.J. Visser. J. Biophys., **63**, 839–853 (1992). DOI: 10.1016/S0006-3495(92)81659-4
[17] J.R. Lakowicz, H. Szmacinski, K. Nowaczyk, M.L. Johnson. Proc. Nat. Acad. Sci. USA, **89**, 1271–1275 (1992). DOI: 10.1073/pnas.89.4.1271
[18] P.A.W. van den Berg, K.A. Feenstra, A.E. Mark, H.J.C. Berendsen, A.J.W.G. J. Phys. Chem. B, **106**, 8858–8869 (2002). DOI: 10.1021/jp020356s
[19] A.J. Visser, A. van Hoek. Photochem. Photobiol., **33**, 35–40 (1981). DOI: 10.1111/j.1751-1097.1981.tb04293.x
[20] A.J.W.G. Visser, H.-J. Grande, C. Veeger. Bioph. Chem., **12**, 35–49 (1980). DOI: 10.1016/0301-4622(80)80037-8
[21] I. Gorbunova, M. Sasin, D. Golyshev, A. Semenov, A. Smolin, Y.M. Beltukov, O.S. Vasyutinskii. J. Phys. Chem. B, **125** (34), 9692–9707 (2021). DOI: 10.1021/acs.jpcb.1c04226
[22] A. Ambrus, B. Torocsik, L. Tretter, O. Ozohanics, V. Adam-Vizi. Human Molecular Genetics, **20**, 2984–2995 (2011). DOI: 10.1093/hmg/ddr202
[23] N.E. Babady, Y.P. Pang, O. Elpeleg, G. Isaya. Proc. Nat. Acad. of Sci., **104** (15), 6158–6163 (2007). DOI: 10.1073/pnas.0610618104
[24] W.S. Kunz, W. Kunz. Biochim. et Biophys. Acta — General Subjects, **841** (3), 237–246 (1985). DOI: 10.1016/0304-4165(85)90064-9
[25] S. Denicke, K.-H. Gericke, A.G. Smolin, P.S. Shternin, O.S. Vasyutinskii. J. Phys. Chem. A, **114**, 9681–9692 (2010). DOI: 10.1021/jp101403x
[26] M.E. Sasin, A.G. Smolin, K.-H. Gericke, E. Tokunaga, O.S. Vasyutinskii. Phys. Chem. Chem. Phys., **20**, 19922–19931 (2018). DOI: 10.1039/c8cp02708k.
[27] I.A. Gorbunova, M.E. Sasin, J. Rubayo-Soneira, A.G. Smolin, O.S. Vasyutinskii. J. Phys. Chem. B, **124**, 10682–10697 (2020). DOI: 10.1021/acs.jpcb.0c07620
[28] M.E. Sasin, I.A. Gorbunova, N.O. Bezverkhnii, Y.M. Beltukov, O.S. Vasyutinskii, J. Rubayo-Soneira. Tech. Phys. Lett., **45**, 672–674 (2019). DOI: 10.1134/s1063785019070101
[29] J.R. Lakowicz. *Principles of Fluorescence Spectroscopy*, 3rd ed. (Springer, NY, 2006). DOI: 10.1007/978-0-387-46312-4
[30] I.A. Gorbunova, M.E. Sasin, Y.M. Beltukov, A.A. Semenov, O.S. Vasyutinskii. Phys. Chem. Chem. Phys., **22**, 18155–18168 (2020). DOI: 10.1039/D0CP02496A
[31] M.K. Krasnopevtseva, V.P. Belik, I.V. Semenova, A.G. Smolin, A.A. Bogdanov, O.S. Vasyutinskii. Tech. Phys. Lett., **46** (6), 614–616 (2020). DOI: 10.1134/S1063785020060218
[32] M.S. Islam, M. Honma, T. Nakabayashi, M. Kinjo, N. Ohta. Int. J. Mol. Sci., **14** (1), 1952–1963 (2013). DOI: 10.3390/ijms14011952
[33] T. Nakabayashi, M.S. Islam, N. Ohta. J. Phys. Chem. B, **114** (46), 15254–15260 (2010). DOI: 10.1021/jp1063066
[34] T. Toyoda, K. Suzuki, T. Sekiguchi, L.J. Reed, A. Takenaka. J. Biochem, **123**, 668–674 (1998). DOI: 10.1093/oxfordjournals.jbchem.a021989
[35] L. Radoszkowicz, D. Huppert, E. Nachliel, M. Gutman. J. Phys. Chem. A, **114** (2), 1017–1022 (2010). DOI: 10.1021/jp908766e
[36] H. Grajek, I. Gryczynski, P. Bojarski, Z. Gryczynski, S. Bharill, L. Kulak. Chem. Phys. Lett., **439**, 151–156 (2007). DOI: 10.1016/j.cplett.2007.03.042

[37] S. Chakraborty, M. Sakka, T. Kimura, K. Sakka. Biosci. Biotechnol. Biochem., **72** (4), 982–988 (2008). DOI: 10.1271/bbb.70724
[38] E. Szabó, A. Ambrus. Biologia Futura, **74** (1), 109–118 (2023). DOI: 10.1007/s42977-023-00155-6
[39] V. Massey, O. Gibson, C. Veeger. J. Biochem., **77**, 341–351 (1960). DOI: 10.1042/bj0770341

Translated by E.Ilinskaya