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The influence of flavo nucleotide (FMN) dimerization on the efficiency of the FMN triplet states generation

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Abstract

The efficiency of triplet state generation in flavin mononucleotide (FMN) water solutions at various pigment concentrations illuminated at two wavelengths (380 nm and 430 nm) was established by the Laser Induced Optoacoustic Spectroscopy (LIOAS) method. It was found that the efficiency of triplet generation by the singlet–triplet intersystem crossing after illumination at 430 nm was usually lower than that after illumination at 380 nm. The yield of triplet generation in FMN at low concentrations in solutions at 380 nm illumination is rather high, about 0.6 but its value is diminished with the increase in the sample temperature and the dye concentration and depends also on the wavelength of excitation. The increase in FMN dimers content gives an increase in fast internal nonradiative conversion of excitation and a decrease in the yield of the fluorescence. LIOAS measurements of the sample and the reference dye in buffered water solutions at 3 °C suggest that only a small part of the excitation energy is used for some structural changes of FMN or possibly for the changes in FMN interactions with its surrounding solvent molecules. The changes in both types should ensue some modification in the volume of the absorbing species giving nonthermal contributions to LIOAS signal. The LIOAS results were corrected for FMN volume changes. The different yields of triplet states generation obtained by two wavelengths of illumination are not only due predominantly to various contributions to absorption from dimeric FMN but also can be partially related to different grade of the superposition of n–π* and π–π* transitions in a region of 380 nm and 430 nm absorption. The absorption at 430 nm band is due predominantly to π–π* transition, whereas in 380 nm region the n–π* transition could have higher contribution which can exhibit different efficiency of triplet generation than π–π* transition. For the samples with low FMN concentrations the yields of fluorescence of FMN excited at these two regions are similar, but at higher dye concentrations they are different.

Keywords: Flavin mononucleotide; Triplet state; Fluorescence; Optoacoustic spectra

1. Introduction

Flavins take part in many biochemical reactions as coenzymes and photoreceptors [1–5]. It was also found that flavins in some organisms can act as anti-oxidant or anti-carcinogenic factors. The illumination by the blue and near ultraviolet light, absorbed by flavins, regulates several processes in plants such as chloroplast migration to places of appropriate light intensity, the opening of the stomatal guard cells and others [6]. The complexes containing flavin photoreceptors occur not only in plants but also in prokaryotes [5]. The photoreceptors involved in all these important processes are the plasma membrane associated phototropins and their homologues [6,7]. Phototropins contain two blue light sensitive domains LOV1 and LOV2 (LOV: light, oxygen and voltage sensitive) [6–9]. Phototropins contain FMN as a chromophoric molecule. The FMN exhibits exceptionally high (about 0.6) yield of triplet states generation [10–12]. The efficient generation of triplet states of dyes or pigments enhances the photoreactions occurring with their participation [13–16]. Upon

Abbreviations: FMN, flavin mononucleotide; LIOAS, Laser Induced Optoacoustic Spectroscopy; FRET, Foerster Resonans Energy Transfer; TDF, fast thermal deactivation; BCP, bromocresol purple.

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illumination LOV domains undergo the photocycle comprising the triplet states [6,7,12]. The mechanisms of these reactions and the interactions between various LOV domains are still investigated [5,7–9,12]. The efficiency of triplet states generation depends on adduct formation by FMN in LOV and by close surroundings of LOV domains [6,7,10]. The FMN properties in LOV and in solutions are different [7,8,11], but the knowledge of triplet states' properties of FMN in solutions can help to understand their properties in LOV. In this work the efficiency of FMN triplet generation was established using the Laser Induced Optoacoustic Spectroscopy (LIOAS) method developed by Braslavsky [17–20]. Previously triplet states of FMN in LOV and in solutions were investigated by LIOAS [5,12], by time-resolved electron paramagnetic resonance [6,10], flash photolysis [5] and transient absorption spectroscopy [7]. The contributions to LIOAS signal from thermal effects and from FMN structural changes generated by light were estimated by the measurement of LIOAS signals of buffered water solutions of FMN and reference dye at two temperatures [17–24]. For FMN free and in LOV structural contributions are similar and rather low [5]. It is much less literature results concerning free FMN [6,10,11] than for FMN in LOV [5–8,12], therefore we decided to investigate FMN in solution.

It was suggested in literature that the 380 nm absorption band for FMN and also for other flavins is the superposition of $\pi-\pi^*$ transitions with some admixture of $n-\pi^*$ transitions, whereas the 430 nm band has pure $\pi-\pi^*$ character [2,25]. In a case of different efficiencies of triplet states generation for these two types of transitions the slow thermal deactivations measured for these two regions of excitation wavelengths should be different.

It is known [26–31] that FMN molecules occur in monomeric and dimeric forms. The dimers are excellent traps for energy of excitation by the light and the energy transferred to them from monomers by nonradiative energy transport [32,33]. In the rigid solutions (solid PVA) the dimers of FMN are weakly fluorescent [27,30,34] and excitation energy migrates between both forms (monomers and dimers) by Foerster Resonans Energy Transfer (FRET) mechanism [27,28,30]. The relative contributions from these forms to various radiative and nonradiative paths of deexcitation depend on the sample concentration, temperature and the solvent in which the pigment is investigated [27,28,32,34]. Thermal deactivation efficiency is usually dependent on the dimers content, because they can exchange part of excitation energy into heat in fast thermal deactivation (TDF) process. The increase in temperature causes decrease in the fluorescence quantum yield [27,28,32].

Spectral properties of the aggregates depend on their structure [35,36]. The phenomena of flavins' photoreception processes in biological systems, mechanisms responsible for excitation energy transfer between various forms of flavins, as well as the various paths of excited flavin deexcitation need further investigation. It is not excluded that the flavins occurring in biological systems could be forced by the protein surroundings to aggregate. The dimer formation influences the process of excitation energy migration. The occurrence of flavin dimers in living organisms was evidenced [37,38]. Guo et al. [7] even suggest that the existence of FMN dimers in LOV influences the photocycles occurring in such complexes. But the dimers role in such biological processes is not clear yet. The participation of flavin triplet states in photocycle of LOV containing dimers suggests investigation of the efficiency of triplet state generation for samples containing dimers. In this work the spectral properties of FMN, predominantly the yields of triplet states generation, are investigated in a simple model system of FMN in buffered water solutions. The change in the sample concentration and temperature shifts the monomer–dimer equilibrium. The evaluation of the part of the excitation energy used for triplet excitation at different excitation wavelengths, dye concentrations and temperatures, provides information about a possible photochemical activity of FMN located in biological systems. Up to now important biological mechanisms of reactions of FMN triplet with aromatic amino acids have been investigated predominantly by the time-resolved nuclear magnetic resonance [15]. It is planned that participation of FMN triplet states in this type of reactions, established by LIOAS, will be a subsequent stage of our study.

2. Materials and methods

FMN (riboflavin-5′-monophosohate sodium salt), from Fluka was used without further purification. Aqueous solutions of FMN in 66 mM potassium phosphate buffer of pH = 7.03 at several dye concentrations were prepared.

The absorption, fluorescence emission and fluorescence excitation spectra were measured using a Cary 4000 UV–VIS spectrophotometer and a fluorescence spectrophotometer Hitachi F4500, respectively.

The measurements of time-resolved photothermal signals were carried out by the LIOAS method developed by Braslavsky [17–24] using the experimental setup constructed in our laboratory [39,40]. The wavelengths of the used nitrogen–dye laser flash were 380 nm and 430 nm.

The LIOAS signals could be analyzed by two methods that were proposed by Marti et al. [41] and Rudzki-Small et al. [42]. The first method [41] is based on the following formula:

$$\Phi_T E_T = (1-\alpha)E_{LAS} - \Phi_F E_F \quad (1)$$

where, α is the part of the absorbed energy of the laser light (E_{LAS}) converted into heat in a time shorter than 0.8 μs, Φ_F and E_F are the fluorescence yield and the energy of singlet state, Φ_T and E_T are the yield of the triplet state generation and its energy. Times longer than 8 μs could not be measured by our experimental setup. The above formula has been derived assuming that the part of the absorbed energy not emitted as fluorescence and not quickly converted into heat is used for triplet states generation. It is of course a crude approximation but even such an approximation gives an opportunity to compare the yields of triplet generation for a set of chemically similar samples [39,40].

The value of α was obtained as a ratio of the first maximum of the LIOAS signal of the sample assigned to the TDF to the first maximum of the LIOAS signal measured for the reference sample. For reference, practically the whole absorbed excitation energy was converted into heat in a very fast process of internal conversion. The reference was the water solution of bromocresol purple (BCP) [43]. It is one of the dyes recommended for LIOAS measurements, but it creates some problems, because it was reported [43] that in nonbuffered water solution its absorption is unstable [43]. It can be used only below pH = 3 or at pH above 9. In our LIOAS experiments the BCP solvent buffer at pH = 7.03 was used as references. The solvent of the sample has to be identical or at least very similar to that of the reference, because their thermoelastic properties have to be the same. For this reason the LIOAS measurements for FMN samples were made in buffer solutions at pH = 7.03. The set of normalized absorption spectra of BCP solutions for the various concentrations, temperatures and pH values used in the present investigations shows that BCP was in the whole set of measurements in the same form (not shown). At pH = 7.03 BCP was stable. On the basis of Rudzki-Small [42] method the LIOAS signal, obtained by means of our arrangement, can be analyzed only for slow (triplet) decays shorter than 8 μs, for slower decays only information about their occurrence can be obtained.

3. Results

In the FMN samples studied, the content of monomeric and dimeric forms depends on the sample concentration and temperature [26,32,44]. Fig. 1 shows the FMN absorption spectra, normalized at 430 nm, measured for several dye concentrations at room temperature (20 °C). The shapes of these spectra are practically similar till 1.4×10^{-4} M concentration but changes are stronger at higher concentration, for example, at

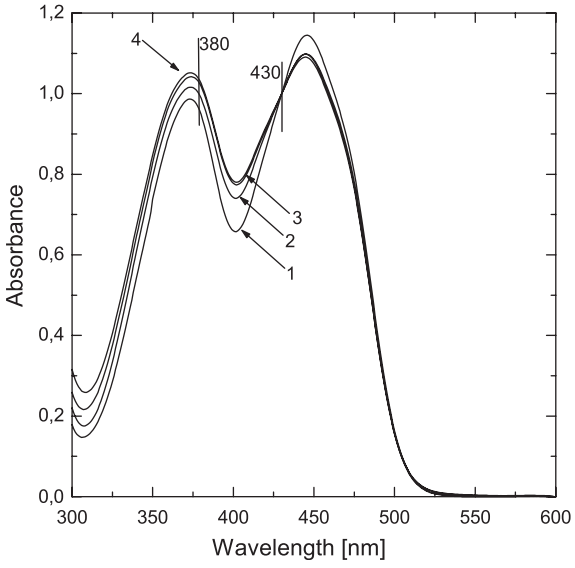

Fig. 1. Normalized at 430 nm absorption spectra of FMN water solution measured at 20 °C. Concentrations: curve 1 – 3.0×10^{-4} M; curve 2 – 2.2×10^{-4} M; curve 3 – 1.4×10^{-4} M; curve 4 – 6.6×10^{-5} M.

3.0×10^{-4} M FMN concentration. As it follows from Fig. 1 the ratio of absorptions measured at 380 nm to that measured at 430 nm is changing with dye concentration. Such changes are also seen at various temperatures (not shown). The contributions to absorption from monomeric and dimeric forms at two wavelengths of light used for LIOAS measurements are different for various samples. Table 1 shows the concentrations of FMN monomers C_M and dimers C_D at various sample concentrations and temperatures calculated on the grounds of analysis of the absorption spectra and data presented in literature [26,32,33,44] from the formula $C_M = xC$ and $C_D = ((1-x)/2)C$, where $x = \left(\sqrt{1-8K_c}-1\right)/4K_c$ is the contribution from monomers and K is the dimerization constant. Calculation were done for $K = 314\ M^{-1}$ (at $t = 3$ °C), $K = 194.4\ M^{-1}$ (at $t = 15$ °C), and $K = 100.48\ M^{-1}$ (at $t = 30$ °C). At room temperature and at low dye concentrations the dimers concentration is usually by two orders of magnitude lower than that of monomers. At 3 °C the dimerization is more effective than in room temperature. Of course, dimerization influences the yield of FMN fluorescence [32,33]. Monomeric FMN at room temperature in water solution at pH = 7 exhibits the yield of fluorescence independent of wavelength of excitation $\Phi_F = 0.26$ [2].

The yield of FMN fluorescence depends on temperature [28,32]. The increase in temperature causes the decrease in the fluorescence quantum yield. It is due to the increase in the probability of nonradiative transition $S_1 \rightarrow S_0$. The increase in FMN concentration causes the decrease in relative fluorescence quantum yield showing greater contribution of dimers in fluorescence quenching [32,33].

In formula (1), proposed by Marti et al. [41] the values of the yield of fluorescence applied for the calculation of triplet state efficiency were taken from literature [27,28]. The yield of fluorescence was practically independent of wavelengths of excitation.

For all LIOAS measurements the concentration of the reference dye was adjusted so that the absorptions of the sample and the reference at the wavelength of the laser flash were the same. The LIOAS measurements were performed at the following temperatures: 3 °C, 15 °C and 30 °C.

Table 1
Molar concentrations of FMN monomers (C_M) and dimers (C_D) in the samples of various dye concentrations (C_{FMN}) and temperatures (Temp.) (calculated on the basis of literature data [25,26])

FMN concentration $\times 10^{-4}$ (M)	Temp. (°C)	$C_M \times 10^{-4}$ (M)	$C_D \times 10^{-6}$ (M)
0.66	3	0.63	1.26
1.40	3	1.29	5.26
2.20	3	1.96	12.10
3.00	3	2.58	20.90
0.66	15	0.64	0.80
1.40	15	1.33	3.44
2.20	15	2.04	8.08
3.00	15	2.71	14.30
0.66	30	0.65	0.42
1.40	30	1.36	1.87
2.20	30	2.11	4.48
3.00	30	2.84	8.09

The FMN concentrations, the dependences of triplet states generation and fast thermal deactivation on the FMN concentrations are shown in Table 2. A comparison of Table 2 with Table 1 showing the contents of monomeric and dimeric forms in FMN water solutions at a given temperature and dye concentration, calculated at the basis of previously [26,32,44] and presently measured and analyzed absorption spectra shows that the amounts of dimers in the samples are much lower than those of monomeric form, but that dimer presence has measurable influence on the yield of fluorescence of the sample.

Fig. 2 represents the examples of LIOAS signals. In pure water at 3 °C $\beta = (\delta V/\delta T)/V$ the volume expansion coefficient is close to zero [17–23], therefore, the observed LIOAS signal is predominantly due to volume changes in absorbing species which can be a result of some structural changes in the absorbing macromolecule. In buffer, the value of β is usually slightly different than in water [23], but as follows from Fig. 2A and C, the first LIOAS maxima measured at about 3 °C (at the temperature at which the maxima are the lowest) for both reference dye (BCP) (curves 2) and FMN (curves 1), in the same buffer and at low concentrations of the dye, are both very low, but the maximum for FMN is higher than for BCP reference. It is reasonable because usually references are dyes not exhibiting structural changes as a result of illumination. It is known that BCP does not exhibit structural changes causing the volume expansion effect. Small structural volume changes of FMN were reported in literature [12]. Fig. 2 suggests also that FMN at low concentrations does not undergo strong structural changes as a result of illumination. The results from Fig. 2A and C are used for the correction of amplitude of H_{max} due to fast thermal deactivation. The LIOAS signals for the same sample as in Fig. 2A and C but measured at 30 °C are shown in Fig. 2B and D. LIOAS signals at this temperature are much higher, because they contain contributions from slow TD related predominantly with triplet state. From the comparison of Fig. 2B and D follows that the LIOAS signals for both wavelengths of excitations are for low FMN concentration similar. For calculation of α (from formula (1)) the LIOAS signals were corrected by subtraction of the structural contributions. Yields of triplet generation in several FMN samples obtained from LIOAS signals on the basis of formula (1) are given in Table 2. As it was reported in literature [5–7,9–12] the efficiency of triplet states generation, at low dimers content is high, even higher than usually reported 0.6 value. The values shown in Table 2 can be overestimated, but the efficiencies for various FMN concentrations and temperatures can be compared. From this comparison it clearly follows that at higher FMN dimers content the efficiency of triplet states generation is lower.

Table 2
Yields of all fast thermal deactivation (α) and the generation of triplet state (Φ_T) obtained from LIOAS results on the basis of formula (1) for the samples of various FMN concentrations in water solutions

FMN concentration $\times 10^{-4}$ (M)	Temperature (°C)	λ_{exc} (nm)	α	Φ_T
0.66	15	380	0.44	0.78
		430	0.50	0.54
	30	380	0.41	0.83
		430	0.35	0.75
1.4	15	380	0.48	0.76
		430	0.52	0.57
	30	380	0.49	0.74
		430	0.46	0.66
2.2	15	380	0.51	0.72
		430	0.49	0.65
	30	380	0.57	0.64
		430	0.53	0.58
3.0	15	380	0.53	0.71
		430	0.60	0.51
	30	380	0.56	0.66
		430	0.63	0.46

Measured at various temperatures and wavelengths of exciting light (λ_{exc}).

The second method [42] by deconvolution of LIOAS signals of the sample and reference provides the decay times of triplet states and their contributions to thermal deactivation, but reasonable results with our apparatus can be obtained only for slow decay times between 0.8 μs and 8 μs. Time evolution of pressure was assumed to be a sum of single exponential functions, first one is related to fast thermal deactivation (in time shorter than 0.8 μs), others with slow deactivations. The reference dye BCP converts practically the whole energy absorbed into heat in a time shorter than 0.8 μs [39,40,43]. Results of analysis suggest that the slow components are not observed in time range between 0.8 μs and 8 μs. It is in agreement with literature data [2] that FMN triplets decay time is very long from 8 μs to 100 μs. In first component are included all fast thermal deactivations with decay times shorter than 0.8 μs. This amplitude has to be of course similar to value α from Table 2. From formula (1) is evaluated the yield of triplet generation (Table 2). From this table follows that some values of slow and fast TD have to be overestimated, because the sum of slow and fast TD and fluorescence yield is in some cases higher than unity. Any way, even in such cases, the sequence of the increases or decreases of TD delivers proper information about undergoing processes.

4. Discussion

Excitation energy migrates between various FMN excited states [2,33,34,45]. Usually four absorption maxima are observed for flavins [2]. Two of them at about 370 nm and at 450 nm are located in the spectral region investigated by us. As follows from literature [2] and our experiments the fluorescence yields at these both bands are similar. But as it follows from Table 2 the efficiency of triplet state generation is for several samples dependent on wavelengths of illumination. This dependence is rather irregular but in all cases the efficiency of the triplet state generation is higher at 380 nm than at 430 nm illumination. The sample concentration and temperature also have influence on Φ_T value (Table 2). Results suggest that the effect is due not only to the change in dimers content. It is possible that the second effect is related with the superposition of n–π* and π–π* transitions in a region of 380 nm absorption.

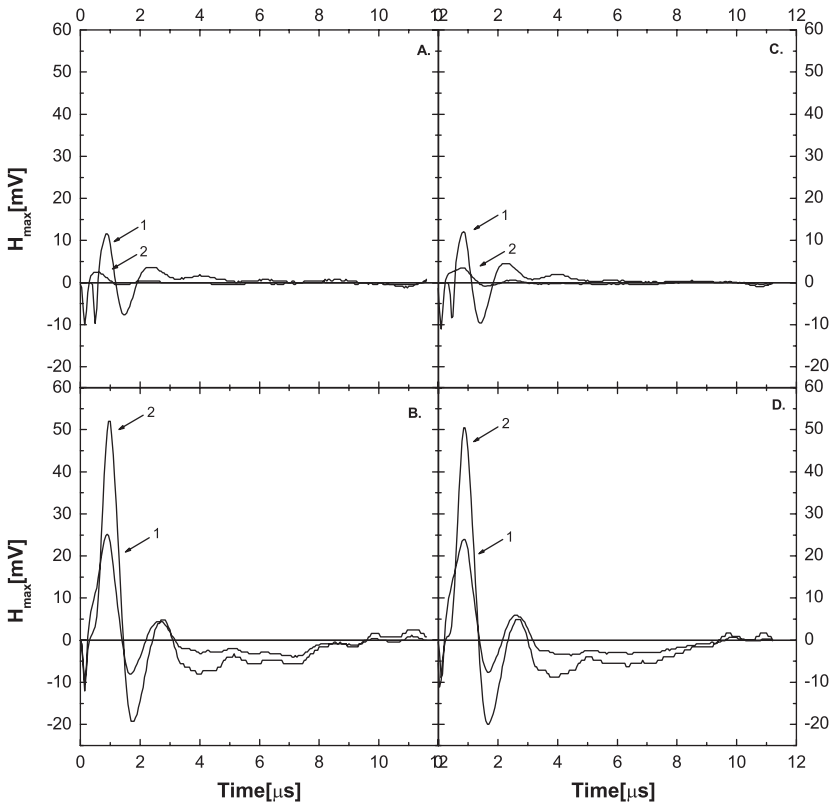

Fig. 2. Examples of FMN (curve 1) and reference dye (BCP, curve 2) LIOAS signals. A and B – at 380 nm light illumination, C and D – at 430 nm light illumination. Temperatures: 3 °C for A and C, 30 °C for B and D; concentration of FMN 6.6×10^{-5} M.

Hitherto, it has been assumed [2] that the energy transfer between these two bands is very efficient. If it was true, the both efficiencies of triplet state generation as well as the fluorescence yield should be at both excitation wavelengths similar. According to the energy levels and the transitions scheme proposed previously [2], practically whole energy absorbed by the dye in 380 nm band is transferred to the energy level related with 430 nm band. The fluorescence emission and intersystem crossing (ISC) transitions are proposed to be generated only from most long wavelength 430 nm band.

Our results (Table 2) show that in some cases a part of energy absorbed in 370 nm region is by ISC transferred to some additional triplet state instead of being transferred to singlet 430 nm level and only after there by ISC by partially transferred to its triplet and partially emitted as fluorescence light. As a result, the whole ISC transfer from 380 nm region has to be more efficient than ISC transfer from 430 nm, therefore, the whole slow TD of the sample is larger, and as a result also the yield of triplet generation calculated from formula (1) is higher at 380 nm than at 430 nm illumination. But the change in the contents of monomeric and dimeric forms with the change in FMN concentration and sample temperature gives rather complicated dependences of the yield of triplet generation from spectral region used, probably because of various contributions to absorption from $n-\pi^*$ and $\pi-\pi^*$ for different FMN forms in various spectral range.

The deconvolution of LIOAS signals gives only very fast compound of TD. In literature [2] is reported for flavins decays of triplet states, which are too slow to be measured by our LIOAS arrangement. It seems that effective migration of excitation energy between FMN forms can also have influence on observed data.

The exact quantitative interpretation of efficiency of triplet generation data needs further investigations, but actually it is possible to conclude that several molecular mechanisms are engaged in a process of FMN triplet generation.

The results suggest that photochemical properties of FMN in living cells could also strongly depend on the close surrounding in which FMN is located and on the wavelength of the incident light.

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