TIME RESOLVED FLUORESCENCE OF FLAVIN ADENINE DINUCLEOTIDE

Ph. WAHL, J. C. AUCHET
Centre de Biophysique Moléculaire, 45045 Orléans Cedex, France

and

A. J. W. G. VISSER, F. MÜLLER
Department of Biochemistry, Agricultural University, Wageningen, The Netherlands

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1. Introduction

From a comparison of the quatum efficiencies and lifetimes of the isoalloxazine fluorescence in flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) and from the slightly different extinction coefficients of both compounds Spencer and Weber have calculated and approximated the rate constants which describes the strong intramolecular quenching in FAD [1].

One of the assumptions in their derivation is that the observed fluorescence is due to the open or unstacked form of FAD, while the closed or stacked structures have no appreciable fluorescence. These assumptions are among others based on the experimental fact that the shape of the fluorescence spectra of FAD and FMN is almost identical. However, one can also approach this point from another direction. Suppose that the more complete kinetic scheme can be applied for FAD (fig. 1).

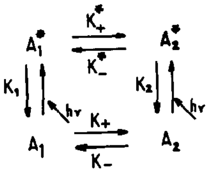

Fig. 1. Kinetic scheme describing the folding and unfolding of FAD in the ground-state and in the excited state.

If both folded and unfolded forms are fluorescent and the emission spectra of both species are overlapping, one should expect that the time course of the fluorescence intensity ($I(t)$) at a given wavelength of emission, after a pulse excitation can be built up by a sum of two exponentials with different amplitude factors:

$$I(t, \lambda) = A'_1(\lambda) \exp(-t/\tau'_1) + A'_2(\lambda) \exp[-t/\tau'_2]$$

The derivation of this equation and the expressions of the parameters $A'_1(\lambda)$, $A'_2(\lambda)$, τ'_1, τ'_2 are given in a previous work [2]. These parameters are functions of k_+^*, k_-^*, k_1, k_2 and K, where k_+^* and k_-^* are the rate constants in $\sec^{-1}$ for folding and unfolding in the excited state, $k_1 = I/\tau_1$ and $k_2 = I/\tau_2$ are the rate constants in $\sec^{-1}$ for fluorescence and radiationless transitions for the isolated forms and K is the association constant in the ground state, index 1 and 2 refer to the open and closed conformations respectively (see fig. 1).

Furthermore, $A'_1(\lambda)$ and $A'_2(\lambda)$ are functions of the emission wavelength λ.

On the other hand, when there is only one fluorescent form, it is expected that the exponential decay is characterized by one time constant and is independent of the emission wavelength. If the unfolded form is fluorescent, the fluorescence lifetime is then given by $\tau'_1 = I/(k^* + k_1)$.

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We investigated the time resolved emission of FAD to detect whether or not there was a heterogeneous character of the fluorescence which might be a direct experimental evidence for the existence of more than one fluorescent species. For this study, the single photon counting method was used, which is superior to the phase and modulation fluorometer method [3] because it is more sensitive with respect to the detection of a heterogeneous character of the fluorescence decay. Especially when measurements are made on samples with low quantum yields of the emission, as is the case with FAD (in the order of 0.03 at neutral pH and at room temperature [1], the single photon counting method provides a reasonable resolution of the fluorescence in time and in wavelength.

2. Materials and methods

FMN and FAD (grade III) were obtained from the Sigma Company (St. Louis, USA). FAD was purified with chromatography on DEAE-cellulose (Whatman DE 52) [4]. 0.05 M sodium phosphate buffer at pH 6.9 made from doubly distilled water was used as solvent. The chromophore concentration was always 20 μM.

Fluorescence decays have been obtained with a single photoelectron counting apparatus which has been described earlier [5]. We used a free running flash lamp operating in nitrogen at high pressure. The exciting wavelength and the emission wavelength are selected by grating monochromators. The wavelength band were 10 nm and 6 nm for excitation and emission respectively. The response function g(t) of the apparatus, which is used for the decay analysis, was obtained with a reference compound by a method described elsewhere [6]. The reference used here was 1,1,4,4 tetraphenyl 1–3 butadiene, (TPB) obtained from Koch Light. Its time constant is 1.78 nsec [7]. Analysis of the data has been performed with a computer program based on the method of modulatrix functions [8]. The program was designated to determine the three parameters relative to decays which are a sum of two exponential terms according to the following expression:

$$I(t) = [C_1\, e^{-t/\tau_1} + (1-C_1)\, e^{-t/\tau_1}]\, A \qquad (1)$$

where A is a factor of normalization. The fit of these parameters was checked by calculating the convolution of the decay (1) with g(t). This computed curve was then compared visually with the experimental curve and the weighted residual was calculated [9].

The average time constant $<\tau>$ was also calculated. It is defined by the following expression:

$$<\tau> = \frac{M_1\ [\mathrm{i}(t)]}{M_0\ [\mathrm{i}(t)]} - \frac{M_1\ [\mathrm{g}(t)]}{M_0\ [\mathrm{g}(t)]}$$

M_1 [i(t)] and M_0 [i(t)] designate the moments of order one and of order zero of an experimental decay i(t), M_1 [g(t)] and M_0 [g(t)] the moments of the response function g(t).

3. Results and discussion

In table 1 the results for the FMN and FAD fluorescence kinetics at variable emission wavelengths, constant excitation wavelength (370 nm) and constant temperature (20°C) are collected. A computer program was used in which the experimental data were analyzed into two time constants with their relative amplitudes (see Materials and methods). In general the weighted residual showed no deviation from its statistical value so that there was good agreement between experimental and convoluted curves. In table 1 only the lifetimes (τ_1) with the largest weight factor c_1 and the average lifetime $< \tau >$ are included. The data obtained with FMN are in good agreement with the results of Spencer et al. [10]. Only one lifetime is observed and the lifetime is constant across the fluorescence band. The same tendency can be obser-

Table 1
Two component analysis of the FAD and FMN fluorescence at different emission wavelengths

Fluorescence wavelength (nm)	FAD			FMN		
	τ_1 (nsec)	C_1	$<\tau>$ (nsec)	τ_1 (nsec)	C_1	$<\tau>$ (nsec)
500	2.72	0.98	2.93	4.55	1.00	4.55
520	2.82	0.95	2.79	4.70	1.00	4.67
560	2.68	0.99	2.84	4.69	1.00	4.58

Excitation wavelength 370 nm, temperature 20°C.

ved with FAD, where the data (table 1) point to one single lifetime, which is within 0.2 nsec constant across the fluorescence band. It was not possible to analyse accurately the fluorescence at wavelength longer than 560 nm because the reference compound (TPB) did not emit at these wavelengths. The lifetimes of the second component, which is present in very low percentage (<5%), were always longer than about 5 nsec and the variation between them is very large. Although impurities cannot be excluded, when weak emissions are detected, it might be possible that the appearance of the small percentage of the second component is a result of the calculation method employed especially in cases where the main component has a short lifetime. As a test for the significance of our results some experimental data were analysed into one single lifetime and in these cases the lifetimes are within 0.1 nsec identical with τ_1. Also the temperature dependence of the fluorescence lifetime at constant emission wavelength (520 nm) was studied. From the data in table 2 it is obvious that especially at the lower temperatures the fluorescence is characterized by one single exponential decay. For FAD the lifetime decreases with increasing temperature from 3.5 nsec at 2°C to 2.1 nsec at 34°C. The large variation of the fluorescence lifetime with temperature illustrates the strong dynamic character of the fluorescence quenching. For FMN the variation is within a much smaller range namely 4.9 nsec at 2°C to 4.5 nsec at 34°C. The same tendency is observed by Spencer and Weber [1]. However our lifetimes of the FAD fluorescence seem to be about 0.2–0.3 nsec longer. The single exponential decay of the FAD fluorescence justifies the assumptions that $1/\tau_1' >> 1/\tau_1'$ and that $1/\tau_1' \approx k_+^* + k_1$ (fig. 2). In the complex, extra radiationless processes are present as compared to the open form. Radiationless deactivation is so rapid that $K_-^* << k_2$ and A_i^* is probably created only by direct excitation. As pointed out earlier, because the stacked form is nonfluorescent the only rate parameter that can be evaluated is k_+^* by taking the observed lifetime of the FAD fluorescence as τ_1' and the observed lifetime of the FMN fluorescence as $1/k_1$ [1]. The k_i^* values at different temperatures are shown in the form of an Arrhenius plot (fig. 2). From the slope of the plot the activation energy for complex formation in the excited state is calculated as 5.45 Kcal/mole. This value is in the same order of magnitude as found by Spencer and Weber [1]. We can conclude from these results, that the fluorescence of FAD in aqueous solution arises from one single species, which corresponds to an aqueous environment of the isoalloxazine moiety.

Table 2
Two component analysis of the FAD and FMN fluorescence at different temperatures

Temperature (°C)	FAD			FMN		
	τ_1 (nsec)	C_1	$<\tau>$ (nsec)	τ_1 (nsec)	C_1	$<\tau>$ (nsec)
2	3.51	0.99	3.69	4.87	1.00	4.79
12	3.11	1.00	3.20	–	–	–
20	2.82	0.95	2.79	4.70	1.00	4.67
27	2.47	0.91	2.76	4.59	1.00	4.55
34	2.07	0.94	2.36	4.52	1.00	4.50

Excitation wavelength 370 nm, emission wavelength 520 nm.

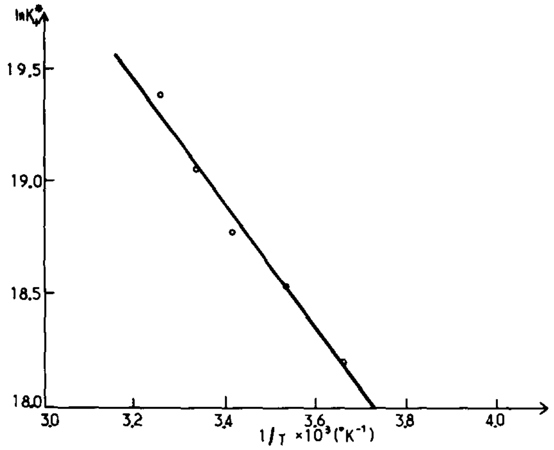

Fig. 2. Plot of $\ln k_+^*$ vs $1/T$. k_+^* can be assumed as the rate constant (in sec^{-1}) of formation of the complex between the excited isoalloxazine and adenine moieties. T = temperature in °K.

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