ROTATIONAL RESOLUTION OF METHYL-GROUP SUBSTITUTION AND ANISOTROPIC ROTATION OF FLAVINS AS REVEALED BY PICOSECOND-RESOLVED FLUORESCENCE DEPOLARIZATION

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The rotational correlation times of the natural flavin compounds riboflavin and FMN and the flavin models lumiflavin, tetra-O-acetyl-riboflavin and their N(3)-methylated derivatives, dissolved in water, were determined with the time-resolved fluorescence depolarization technique. Owing to the time resolution the effect of methyl group substitution could be determined as a few picoseconds longer correlation time, in agreement with the slightly larger molecular volume. Anisotropic rotation of lumiflavin was demonstrated by observing the change in correlation time when excitation was in the second excited singlet state of the flavin. The angle between the emission transition moment and the long axis of the flavin ring system is approximately 22°.

1. Introduction

The experimental assessment of rotational diffusion is an important tool to investigate structure and dynamics of biomolecules and biopolymers. Extension of this method into the picosecond regime will provide the experimental counterpart of molecular dynamics simulations, which is particularly useful in testing theoretical descriptions of rotational diffusion [1]. Both experimental and theoretical approaches have made considerable progress in recent years.

In this paper we have investigated rotational diffusion of several natural and synthetic flavin derivatives (fig. 1), all dissolved in aqueous solution. Two N(3)-methylated flavin compounds were compared with flavins nonmethylated at that position. It is demonstrated that the effect of methyl substitution on the rotational diffusion is unambiguously present. As established earlier steady-state fluorescence depolarization of several flavins also revealed size-dependent changes [2]. In fig. 1 the optical transitions from ground state to the first two singlet excited states have been indicated, as determined by Johansson et al. [3]. Also presented is the direction of the permanent dipole moment, calculated to be

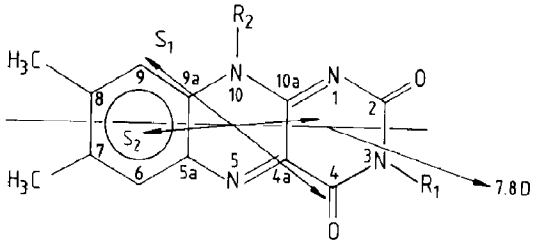

Fig. 1. Structure of flavins investigated. Lumiflavin: $R_1=H$ and $R_2=CH_3$; N(3)-methyllumiflavin: $R_1=R_2=CH_3$; tetra-O-acetylriboflavin: $R_1=H$ and $R_2=CH_2-[CH(OCOCH_3)]_4-H$; N(3)-methyl-tetra-O-acetylriboflavin: $R_1=CH_3$ and $R_2=CH_2-[CH(OCOCH_3)]_4-H$; riboflavin: $R_1=H$ and $R_2=CH_2-[CHOH]_4-H$; FMN: $R_1=H$ and $R_2=CH_2-[CHOH]_3-CHOPO_3$. The directions of dipole moment (7.8 D) and optical transition moments (from ground state to first two excited singlet states) are also indicated.

7.8 D by Platenkamp et al. [4]. The flavin skeleton is intrinsically anisotropic. When the molecule possesses uniaxial symmetry, the fluorescence anisotropy decay profile should change when excitation is either in the first or in the second electronic state, since the anisotropy decay function depends upon the angle of the absorption transition moment with the main symmetry axis. The theoretical background

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and experimental verification using similar molecules has been provided recently [5].

Since the time resolution of pulse fluorimeters has been improved [6], the time-dependent fluorescence behaviour of the flavins was re-investigated. The fluorescence of all compounds decays predominantly in an exponential fashion with lifetimes of about 5 ns, the actual value depending on the particular flavin used.

A preliminary account of part of the work has been presented elsewhere [7].

2. Experimental methods

Riboflavin and FMN were obtained from Sigma. FMN was purified with anion-exchange chromatography prior to use [8]. Lumiflavin, N(3)-methyl-lumiflavin, tetra-O-acetyl-riboflavin and N(3)-methyl-tetra-O-acetyl-riboflavin were gifts of Dr. F. Müller (Basel). Water was purified on Millipore filters. Ethanol was fluorescent grade from Merck. The time-correlated single-photon-counting equipment with the mode-locked continuous wave argon-ion laser, in combination with the frequency-doubled, synchronously pumped dye laser, as excitation source has been amply described [9–12]. The time resolution of the detection system has been improved by the incorporation of a microchannel plate detector (Hamamatsu R 1645 U-01). The 476.7 nm line of the laser was selected for excitation in the first electronic absorption band since it is close to the 0,0 vibronic transition from ground state to the lowest excited singlet state [13]. Fluorescence was measured at 544.0 nm through an interference filter (Balzer 544) with half bandwidth of 10 nm. Excitation in the second electronic absorption band of the flavin was performed by the output of the frequency-doubled DCM dye laser at 340.0 nm. Data analysis was performed as recently described [14,15]. To derive the instrumental response function we used erythrosin B in water as reference compound (see refs. [7,16] for experimental details). Molecular volumes of the flavin compounds were obtained from the molecular weights and the density, which is taken as 1.60 g/cm^3 for each flavin derivative [17]. Van der Waals volumes of the different flavin compounds were determined using the Chem-X package, developed and distributed by Chemical Design Ltd., Oxford, England. The molecular dimensions were estimated from the van der Waals radii of each constituent atom taking into account the various bond lengths and assuming an extended conformation of the various side chains.

3. Results and discussion

3.1. Fluorescence lifetimes

From a close analysis of the fluorescence decay it turned out that, by comparing fitting criteria using single- and double-exponential decay models, the best fit was obtained for a bi-exponential function with a predominant relative contribution ($=\alpha_i\tau_i/\sum_i\alpha_i\tau_i$) of the long lifetime component of around 5 ns (the exact value depending on the flavin compound). The shorter lifetime component has a value of around 2 ns. Addition of the shorter lifetime component was required to obtain an optimum fit to the data, as illustrated in fig. 2, showing the fluorescence decay analysis of lumiflavin in water. This slight heterogeneity is observed for each flavin studied, independent of the concentrations within the range used, i.e. 0.4–1 μM. The long lifetimes and relative contributions are collected in table 1. This observation also seems to be independent of the solvent, because ethanol instead of water yielded the same slight heterogeneity for flavins soluble in ethanol (cf. table 1). Since the instrumental response functions were inferior in the past (i.e. from 2 ns [18] to 0.5 ns [9–11] until 0.1 ns (this work), this might be the reason for the fact that the effect remained unattended. The apparent heterogeneity remains to be investigated into more detail, but it might be related with the presence of different conformers in the ground state of the flavin, with different excited-state properties. It has been demonstrated recently, by geometry-optimized molecular orbital calculations [19], that the three-ring system is highly flexible even in the oxidized state requiring only 1–2 kcal/mol for deformation.

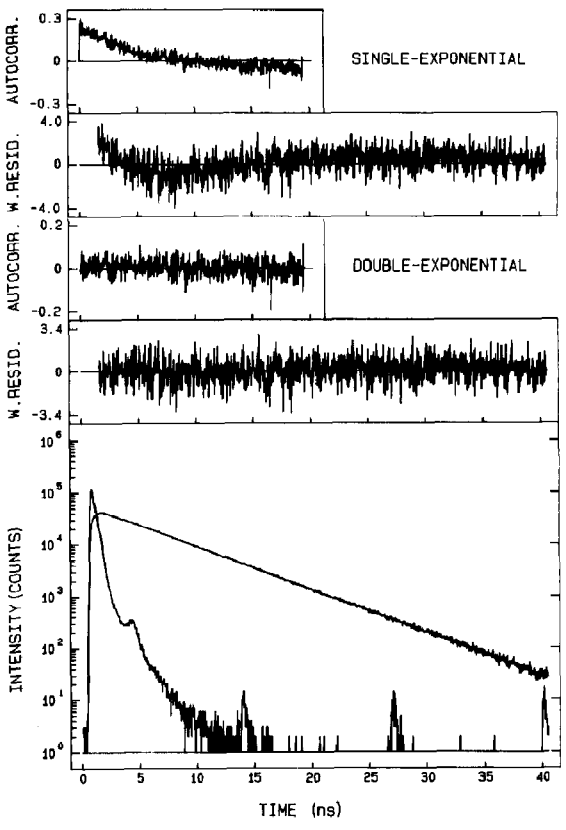

Fig. 2. Analysis of fluorescence decay of 1 μM lumiflavin in water at 20°C. The excitation and emission wavelengths were 476.7 and 544.0 nm, respectively. Fluorescence was measured through a polarizer set at the magic angle (54.7°). The time equivalence per channel was 39.6 ps/channel and the fit range was between channels 40 to 1024. The fluorescence response of erythrosin B in water and the experimental and calculated fluorescence response of lumiflavin according to a bi-exponential decay model are shown. The upper panels show the weighted residuals between calculated and observed fluorescence and the correlation function of the residuals. The recovered parameters are: $\alpha_1 = 0.956 \pm 0.003$, $\tau_1 = 5.21 \pm 0.01$ ns, $\alpha_2 = 0.044 \pm 0.003$, $\tau_2 = 2.1 \pm 0.2$ ns. Statistical parameters for this fit are $\chi^2 = 1.05$, Durbin–Watson parameter DW = 2.04 and the number of zero passages in the autocorrelation function ZP = 252. For comparison the weighted residuals and autocorrelation function for a single-exponential decay analysis are also shown. The parameters for this fit are $\alpha = 1.000 \pm 0.001$, $\tau = 5.162 \pm 0.002$ ns with statistical parameters $\chi^2 = 1.37$, DW = 1.54 and ZP = 89.

3.2. Rotational correlation times

From experiments with polarized excitation and emission one can relate, in principle, the measured

Table 1
Fluorescence lifetimes of flavin compounds in water or ethanol at 20°C (λ_{exc} is 476.7 nm and λ_{em} is 544.0 nm)

Compound	Solvent	τ (ns) (±0.03 ns)	$\alpha\tau$ [a] (%) (±1%)
lumiflavin	water	5.20	98
	ethanol	6.55	99
N(3)-methyllumiflavin	water	4.67	96
	ethanol	5.88	98
tetra-O-acetylriboflavin	water	5.10	97
	ethanol	6.30	97
N(3)-methyl-tetra -O-acetyl-riboflavin	water	4.53	96
	ethanol	5.55	96
riboflavin	water	4.85	95
FMN	water	4.78	98

[a] Percentage of fluorescence contribution, arising from the long lifetime component.

emission anisotropy,

$$r(t) = [I_{\parallel}(t) - I_{\perp}(t)]/[I_{\parallel}(t) + 2I_{\perp}(t)],$$

to the correlation function describing the reorientation of the fluorescent molecule:

$$r(t) = \tfrac{2}{5}\langle P_2[\hat{\mu}_{abs}(0)\,\hat{\mu}_{em}(t)]\rangle$$

with $\langle P_2[\hat{\mu}_{abs}(0)\,\hat{\mu}_{em}(t)]\rangle$ the orientational correlation function expressed in terms of the second-order Legendre polynomial of the vector product of the absorption transition moment $\hat{\mu}_{abs}$ at time $t = 0$ and emission transition moment $\hat{\mu}_{em}$ at time t. For spherical molecules the anisotropy takes a relatively simple form:

$$r(t) = \tfrac{2}{5}P_2(\cos\theta)\exp(-t/\phi), \qquad (2)$$

where $P_2(\cos\theta)$ is the second-order Legendre polynomial of the cosine of the angle θ between absorption and emission transition moment: ϕ is the rotational correlation time, $\phi = 1/6D$, with D the rotational diffusion coefficient. In fig. 3A an example of a fluorescence anisotropy decay analysis is presented for lumiflavin in water. The apparent anisotropy is smaller than the true one, which turned out to be very close to the maximum value of 0.40. The lower apparent anisotropy is caused by the finite pulse width as shown by computer simulations [20]. In fig. 4A we have also plotted two statistical fit parameters versus fixed correlation times with preset initial anisotropy $r(0) = 0.38$. The relatively sharp

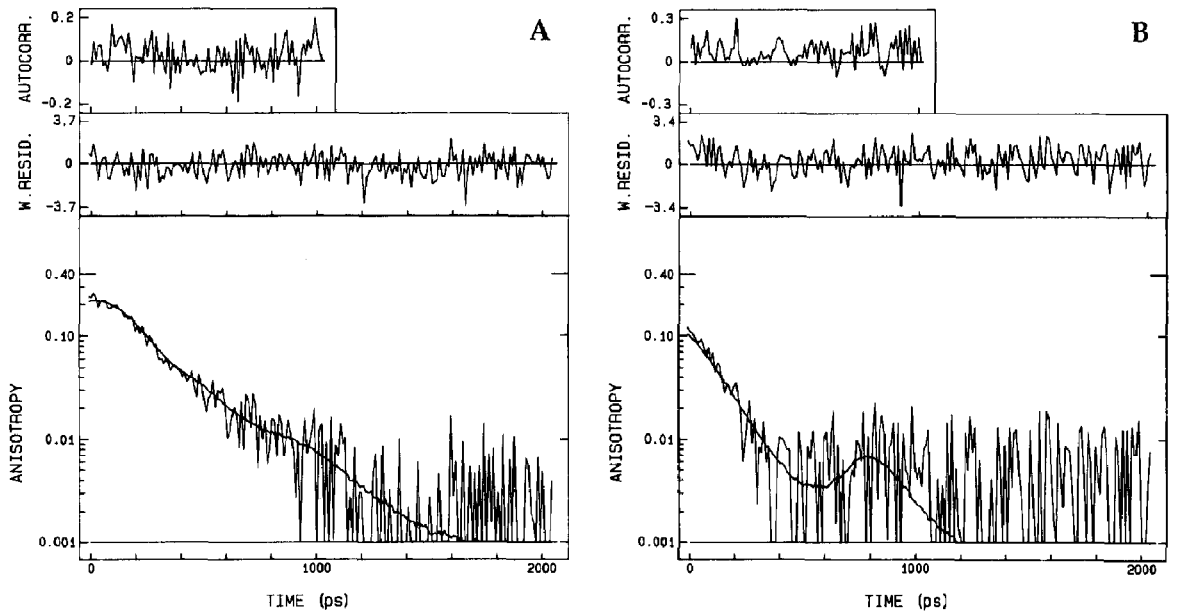

Fig. 3. Direct analyses of fluorescence anisotropy decay of 1 μM lumiflavin in water at 20°C after excitation in the first (A) and second (B) absorption band, respectively. (A) Excitation and emission wavelengths were at 476.7 and 544.0 nm, respectively. The time equivalence was 10.5 ps/channel and the range for fitting was between channels 30 and 300. The experimental and calculated anisotropies are presented between channels 30 and 230. The initial anisotropy was fixed to 0.38 and the recovered correlation time is 82 ± 1 ps. Statistical parameters (see legend to fig. 2): $\chi^2 = 1.34$, DW = 1.66, $ZP_\parallel = 44$ and, $ZP_\perp = 51$. (B) Excitation and emission wavelengths were at 340.0 and 544.0 nm, respectively. The time equivalence was 10.2 ps/channel and the range for fitting was between channels 57 and 257. The experimental and calculated anisotropies are presented between channels 57 and 257. The initial anisotropy was fixed to 0.25 and the recovered correlation time is 97 ± 2 ps. Statistical parameters: $\chi^2 = 1.86$, DW = 1.19, $ZP_\parallel = 40$ and, $ZP_\perp = 29$.

maximum and minimum, coinciding on the ϕ-axis, is indicative for a reliable value for the correlation time. In order to obtain consistent results for all flavin compounds, the initial anisotropy was preset to 0.38 in all subsequent analyses while the correlation time was the adjustable parameter.

In a previous publication [7] the initial anisotropy was preset to 0.4 in the analysis. From a careful analysis of the fluorescence anisotropy of flavins in solid matrices it was found recently that the initial anisotropy is 0.38 ± 0.01 [21]. Prefixing the initial anisotropy to 0.38 in the analysis of the fluorescence anisotropy decay gave the same fit quality, but the correlation times were a few picoseconds longer than the previously reported ones. From eq. (2) with $r(0) = 0.38$ it follows then immediately that the angle between the transition moments of absorption and emission amounts to 10.5° (excitation within the first electronic absorption band).

The correlation times of all flavin compounds have been collected in table 2. The effective molecular volume of the flavins is correctly reflected by the correlation time. For instance, addition of a methyl group at the N(3) position yields a few picoseconds longer correlation time. From these results it can be concluded that picosecond resolution can be obtained with time-correlated single-photon counting and a relatively broad instrumental response function. Such a resolution was also obtained using phase fluorometry with GHz modulation frequencies [22].

It is possible to calculate the steady-state fluorescence anisotropy $\langle r \rangle$ from the initial anisotropy, the fluorescence lifetime τ, and correlation time ϕ using the following relationship:

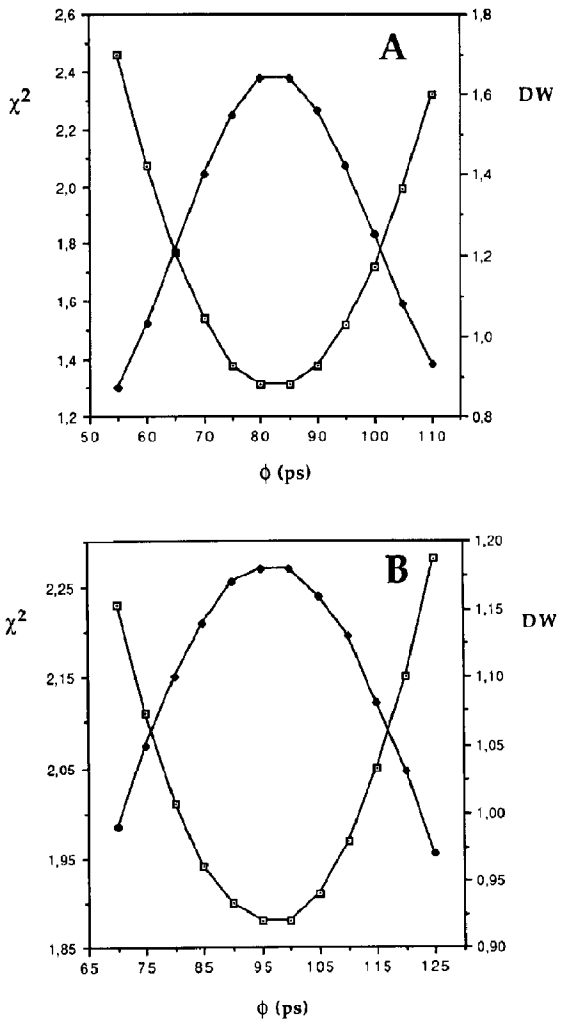

Fig. 4. Fixed correlation times analyses of fluorescence anisotropy decay of 1 μM lumiflavin in water at 20°C after excitation in the first (A) and second (B) absorption band, respectively. (A) Curves of χ^2 and DW versus rotational correlation time ϕ for the experiment detailed in fig. 3A, with $r(0)=0.38$ and correlation time fixed as indicated; (●●●) χ^2 and (□□□) DW. (B) Same as (A) but now for the experiment detailed in fig. 3B (fixed value for $r(0)=0.25$).

$$\langle r\rangle = \frac{\int_0^\infty r(0)\exp(-t/\phi)\exp(-t/\tau)\,\mathrm{d}t}{\int_0^\infty \exp(-t/\tau)\,\mathrm{d}t}$$

$$= r(0)\frac{\phi}{\tau+\phi}, \qquad (3)$$

in which exponential functions describing anisotropy and total fluorescence decays were substituted into eq. (3). These data are also collected in table 2. Experimental values, already reported more than twenty years ago [2], are included as well to demonstrate the excellent agreement.

A comparison with theoretically predicted values can be made by referring to the Stokes–Einstein relationship:

$$\phi = \frac{\eta V}{kT} = \frac{1}{6D}, \qquad (4)$$

where η is the viscosity of water, k is the Boltzmann constant, T is the temperature, and V is the molecular volume. The volumes were obtained as outlined in section 2. From the data collected in table 2, it can be clearly observed that the calculated correlation times based on the van der Waals volumes are significantly shorter than the ones based on the molecular weight and density. Both calculated correlation times are shorter than the measured ones. An explanation can be found in the high polar character of the flavin molecule. The calculated ground-state dipole moment of the isoalloxazine ring is 7.8 D [4] and it is directed between the two carbonyl residues at an angle of 16° with respect to the horizontal axis (see fig. 1). It has been concluded from the effect of pressure on the fluorescence spectral distribution of flavins that ground- and excited-state solvations are similar [23]. Therefore, the results can be explained by assuming (weak) dipole–dipole interactions between the excited flavin molecules and water molecules. Stick-boundary conditions will certainly prevail in this system thereby increasing the effective volume of the flavin by solvent attachment. Another explanation could be the fact that the flavin molecule is not a spherical rotor. This aspect will be discussed in section 3.3.

3.3. Anisotropic motion

In order to detect anisotropic motion of the isoalloxazine moiety it is appropriate to consider only the lumiflavin case and not the compounds with the bulky side chains. For an irregularly shaped molecule the theoretical anisotropy decay function is composed of a combination of five exponential terms [24]. If the shape of lumiflavin contains symmetry and can be approximated as that of a prolate ellipsoid of revolution, this is reduced to three correlation

Table 2
Rotational correlation times and steady-state fluorescence anisotropies of flavins in water at 20°C (λ_{exc} is 476.7 nm and λ_{em} is 544.0 nm)

Compound	ϕ_{obs} (ps) (±1 ps)	ϕ_{calc} [a] (ps)	ϕ_{calc} [b] (ps)	$\langle r\rangle_{obs}$ [c]	$\langle r\rangle_{calc}$ [d]
lumiflavin	82	66	44	0.005	0.006
N(3)-methyllumiflavin	85	69	45	0.007	0.007
tetra-O-acetylriboflavin	158	139	91	0.011	0.011
N(3)-methyl-tetra-O-acetyl-riboflavin	164	142	92	0.014	0.013
riboflavin	131	96	64	0.009	0.010
FMN	160	117	71	0.010	0.012

[a] From eq. (4) and volume from molecular weight and density.
[b] From eq. (4) and van der Waals volume. [c] From ref. [2]. [d] From eq. (3).

times (ϕ_i, $i=1$, 2, and 3) connected with the anisotropy decay function [25]:

$$r(t)=\beta_1\exp(-t/\phi_1)+\beta_2\exp(-t/\phi_2)+\beta_3\exp(-t/\phi_3)\,. \quad (5)$$

The pre-exponential factors β_i ($i=1$, 2, and 3) are functions of the angles that the absorption and emission transition moments subtend with the main symmetry axis, and of the angle θ between the absorption and emission transition moments (see ref. [5] for details). The location of the absorption transition moments are known [3]. The first absorption transition moment (476.7 nm excitation) makes an angle of 32° with respect to the long axis (the symmetry axis) of the three-membered ring system (see fig. 1). The total length of the axis is 9 Å. The short axis runs through the lines connecting N(5) with N(10) and the carbon atom of the methyl group adjacent to N(10). The length of the short axis amounts to 4.3 Å. The correlation times ϕ_i are functions of the two possible diffusion constants, namely for rotation about the long symmetry axis ($D_\parallel$) and for rotation about the short axis ($D_\perp$) [25]. $D_\parallel$ and $D_\perp$ can be expressed in the axial ratio and in the rotational diffusion coefficient (D) of an equivalent sphere [25]. Therefore the correlation times ϕ_i can be easily expressed in the effective correlation time, ϕ_{eff}, describing the correlation time of an equivalent sphere. The parameters of the anisotropy decay model according to eq. (5) have been collected in table 3. From inspection of table 3 it can be immediately concluded that the anisotropy decay is close to an exponential function since the relative contributions

Table 3
Parameters describing fluorescence anisotropy decay of lumiflavin as prolate ellipsoid [a]

axial ratio $\rho=2.1$	
diffusion constant $D_\parallel=1.252$ D	
diffusion constant $D_\perp=0.649$ D	
angle transition moment [b] $\delta=32°$	
$\phi_1=1.572\phi_{eff}$	
$\phi_2=1.353\phi_{eff}$	
$\phi_3=0.955\phi_{eff}$	
$\lambda_{exc}=476.7$ nm:	$\beta_1=0.185$
	$\beta_2=0.184$
	$\beta_3=0.011$
$\lambda_{exc}=340.0$ nm:	$\beta_1=0.302$
	$\beta_2=-0.052$
	$\beta_3=0.001$

[a] Detailed mathematical expressions are given in ref. [5].
[b] From ref. [3].

($=\beta_i/\Sigma_i\beta_i$) of the first two correlation times (ϕ_1 and ϕ_2) are similar, while the exponential term with the shorter correlation time (ϕ_3) only contributes for 3% to the decay. We have used the experimental fluorescence anisotropy decay of lumiflavin for analysis according to the ellipsoidal model with the parameters listed in table 3. The effective correlation time, ϕ_{eff}, was systematically varied until the best match between experimental and calculated decay was obtained. The results, presented in fig. 5 as a plot of statistical parameters against ϕ_{eff}, are in favour of anisotropic rotation since the effective correlation time is shorter than the one obtained from direct analysis (i.e. 55 ps instead of 82 ps).

Anisotropic motion of lumiflavin was unambiguously demonstrated by also conducting experiments

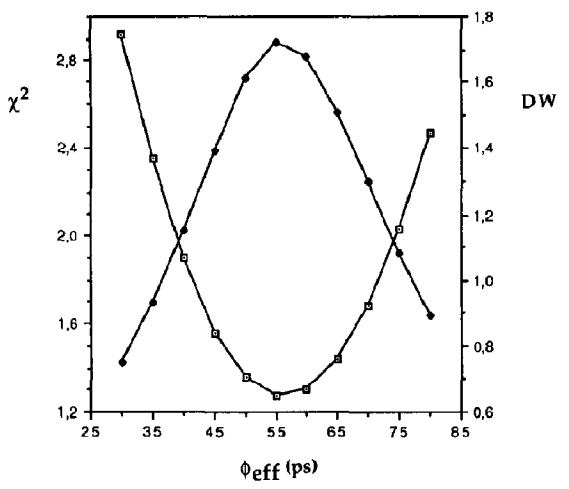

Fig. 5. Analysis of fluorescence anisotropy decay of lumiflavin according to a prolate ellipsoidal rotor. The experiment detailed in fig. 3A was analyzed using the parameters summarized in table 3. The effective correlation time, ϕ_{eff}, was systematically varied until an optimum fit was obtained (ϕ_{eff}=55 ps). See text for details (●●●) χ^2 and (□□□) DW.

at 340.0 nm excitation, which encompasses the second electronic absorption band. The transition moment of this band makes an angle of 39° with the first optical transition moment and is nearly parallel to the symmetry axis [3]. The near collinearity of the second transition moment and the long axis would predict a slower, largely exponential anisotropy decay. At 340.0 nm excitation experiments with two flavin compounds were performed: FMN and lumiflavin. FMN has electronic properties identical to those of lumiflavin. Because of its much longer correlation time the establishment of the initial anisotropy using 340.0 nm excitation and 544.0 nm emission turned out to be very precise, $r(0) = 0.25 \pm 0.01$ (data not shown). The value of $r(0) = 0.25$ was fixed in the analysis of the anisotropy decay of lumiflavin. From the analysis a distinctly longer correlation time of 97 ps was obtained (fig. 4B). Presented in a semilogarithmic way, the anisotropy taken with 340.0 nm excitation (fig. 3B) apparently shows a faster decay than the anisotropy with 476.7 nm excitation (fig. 3A). However, the frequency-doubled dye laser pulse width is 4 ps as compared to the 100 ps argon ion laser pulse, which leads to a distinctly smaller instrumental response function (about 100 ps as compared to 200 ps when excitation was at 476.7 nm). It is essential to apply proper deconvolution methods to retrieve the correct correlation times at high accuracy. A fixed correlation time analysis, similarly as performed in fig. 4A, revealed that the optimum correlation time is shifted to higher values, but the maximum Durbin–Watson and minimum χ^2 values ar much less pronounced than in the former case. The latter effect is due to the lower initial anisotropy, which decreases the dynamic range of measurable anisotropy.

From the initial anisotropies using two excitation wavelengths in combination with the directions of the absorption transition moments in the molecular framework of the isoalloxazine ring system as determined by Johansson et al. [3], the angle between the emission transition moment and the symmetry axis could be determined, θ_e is $22 \pm 5°$.

4. Conclusions

The shortest rotational correlation times obtained in this study are approximately two or three times shorter than the width at half maximum of the instrumental response function. Both the precision and reproducibility (within a few picoseconds) of the method are excellent. The precision is demonstrated by the sensitivity of the correlation time for a slightly increasing molecular volume by a methyl group. Anisotropic rotation of the isoalloxazine moiety is clearly demonstrated by the fact that the correlation times turn out to be different when excitation was either within the first or second electronic absorption band.

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