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Time-Resolved Fluorescence Relaxation of 3-Methyllumiflavin in Polar Solution

N. V. Shcherbatska,[1] A. van Hoek,[1] P. I. H. Bastiaens,[1] and A. J. W. G. Visser[1,2]

We have studied the fluorescent properties of a well-defined model flavin compound (3-methyllumiflavin) in a relatively polar solvent like propylene glycol or ethanol. Inhomogeneous spectral broadening effects were directly time-resolved by detection at the extreme blue and red edges of the fluorescence band of 3-methyllumiflavin using excitation in the main absorption band. At the high-energy side of the emission band a rapid decay component (tens of picoseconds) was resolved indicative for the disappearance of the initially prepared, nonequilibrium state with a characteristic dipolar relaxation time. At the low-energy side the rise of a solvent relaxed fluorescent species could be time-resolved. The wavelength-dependent effects on the dipolar relaxation were abolished when excitation was at the low-energy side of the absorption band. The experimental decays of the flavin "solvate" at different energies of fluorescence and excitation are presented as they represent an easy diagnosis for energy dependent solvation dynamics. Wavelength dependent rotation of 3-methyllumiflavin, examined by fluorescence anisotropy decay, turned out to be absent for 3-methyllumiflavin in propylene glycol between 263 and 293 K, probably because of the small change in dipole moment upon flavin excitation.

KEY WORDS: Inhomogeneous broadening; dipolar relaxation; flavin; fluorescence anisotropy; solvation dynamics.

INTRODUCTION

The investigation of solvation dynamics of aromatic dye molecules in polar liquids has received longstanding attention during the last decade (see, e.g., Refs. 1–3). In general, due to thermal motions of solvent molecules, the chromophore exhibits a statistical deviation of dipolar interaction energy, resulting in inhomogeneous broadening of the electronic transitions of the dye molecule. We have applied fluorescence relaxation spectroscopy to flavoproteins to obtain information on the dynamical properties of the direct environment of the flavin prosthetic group [4]. The flavin prosthetic group can be considered as a natural fluorescent reporter group that can probe the dynamical structure of the active site of flavoproteins. Depending on the exposure of the flavin to the solvent, the contribution to dipolar relaxation was found to arise from solvent and/or protein dipoles. For separation of both relaxation processes it is very important to acquire precise, quantitative knowledge of the photophysical behavior of a well-defined model flavin compound in a homogeneous polar solvent. As a model system for solvation dynamics 3-methyllumiflavin (MLF) in propylene glycol or ethanol was chosen. Steady-state fluorescence spectra of MLF in propylene glycol as a function of temperature showed a progressive blue shift of the center of gravity of the emission band at decreasing temperature when excitation was in the main absorption band [5]. From these data and by applying Bakhshiev's theory of solvent relaxation [6], dipolar relaxation times of the flavin environment were determined in the temperature range 203–303 K [5]. In

[1] Department of Biochemistry, Agricultural University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands.
[2] To whom correspondence should be addressed.

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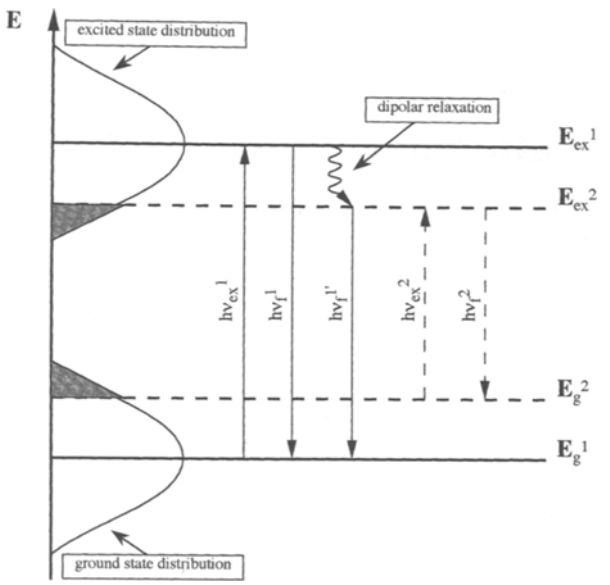

Fig. 1. Simplified scheme of excitation and emission energy-dependent fluorescence decay kinetics. For clarity the scheme is restricted to dipolar relaxation and not to vibronic relaxation. Inhomogeneously broadened ground- and excited-state distributions are shown together with high- and low-energy levels in both electronic states. High-energy excitation (hv_{ex}^1) may lead to observable dipolar relaxation as a rapid component in the fluorescence decay monitored at the blue side of the emission band (hv_f^1) and as a rise time at the red edge of the emission spectrum ($hv_f^{1'}$). Red edge excitation (hv_{ex}^2) may result into a relaxed excited state in which no further relaxation is observed, and no energy-dependent fluorescence decay kinetics (hv_f^2).

addition, information on the difference in dipole moment between the singlet ground and the excited states of the flavin was obtained [5].

In this paper another approach is described to follow these dipolar relaxation processes by time-resolved fluorescence experiments using main-band excitation and detection at the blue and red edges of the fluorescence spectrum. When excitation is at the red edge of the absorption band, wavelength-dependent kinetic effects may disappear and these experiments therefore provide an easy diagnosis for the occurrence or absence of ultrarapid solvation dynamics. A schematic representation of these effects is given in Fig. 1.

EXPERIMENTAL

3-Methyllumiflavin (MLF) was synthesized as described earlier [7]. Propylene glycol (Merck) was distilled under reduced pressure. Spectroscopic-grade ethanol was obtained from Merck. MLF solutions were always 5 μM or lower.

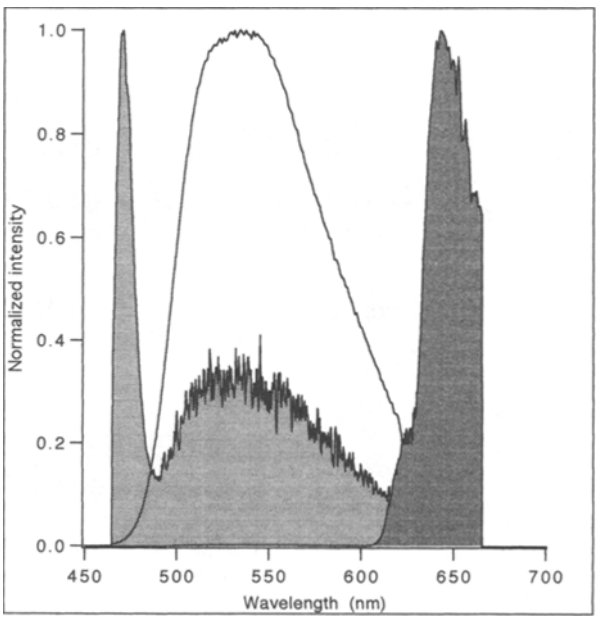

Fig. 2. Corrected steady-state fluorescence spectrum of MLF in propylene glycol at 293 K (λ_{exc} = 458 nm). Also shown are emission spectra when blue and red edge selective filters were put between the sample and the emission monochromator.

Two laser lines from a mode-locked argon ion laser source have excellent characteristics for selective flavin excitation. The 457.9-nm line at high energy is right within the main absorption band, while the 514.5-nm line (low energy) is at the extreme red edge. Polarized fluorescence decay curves were measured by the time-correlated single-photon counting technique. Full details concerning experimental and analytical (maximum entropy method and global analysis) approaches are given in Ref. 8. In Fig. 2 the corrected fluorescence spectrum of MLF in propylene glycol is shown together with the fluorescence transmitted through the filters for extreme blue (Schott GG475 cutoff and Schott 480.5-nm interference filters) and red edge (Schott KV550 cutoff and Balzers K65 bandpass filters) selection.

RESULTS AND DISCUSSION

Fluorescence Decay Curves of MLF in Propylene Glycol as a Function of Excitation and Emission Energies

In Fig. 3 experimental fluorescence decay curves of MLF in propylene glycol at 253 K are presented under different conditions of excitation and emission wave-

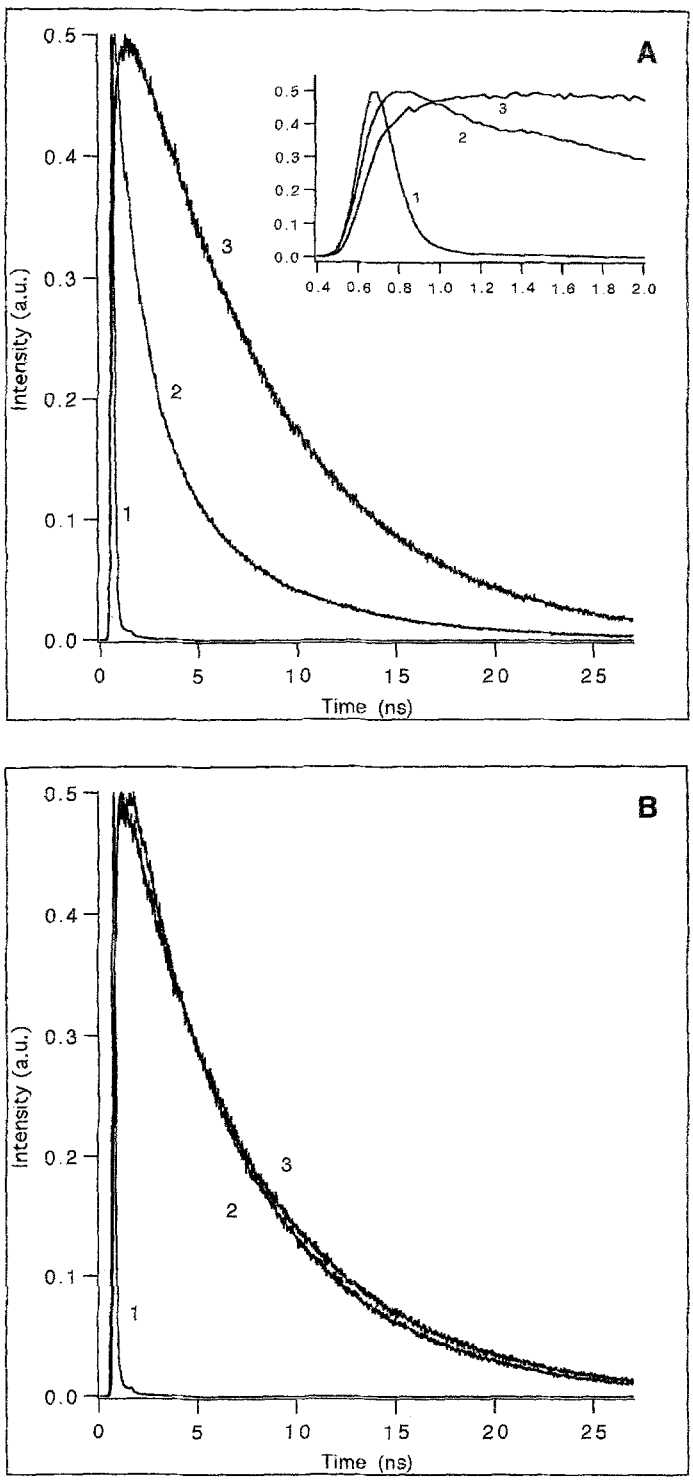

Fig. 3. Experimental fluorescence decay detected at the blue edge (curve 2) and red edge (curve 3) of the emission band of MLF in propylene glycol at 253 K. The instrumental response (curve 1) is also shown. (A) λ_{exc} = 457.9 nm; the inset shows an enlarged portion. (B) λ_{exc} = 514.5 nm.

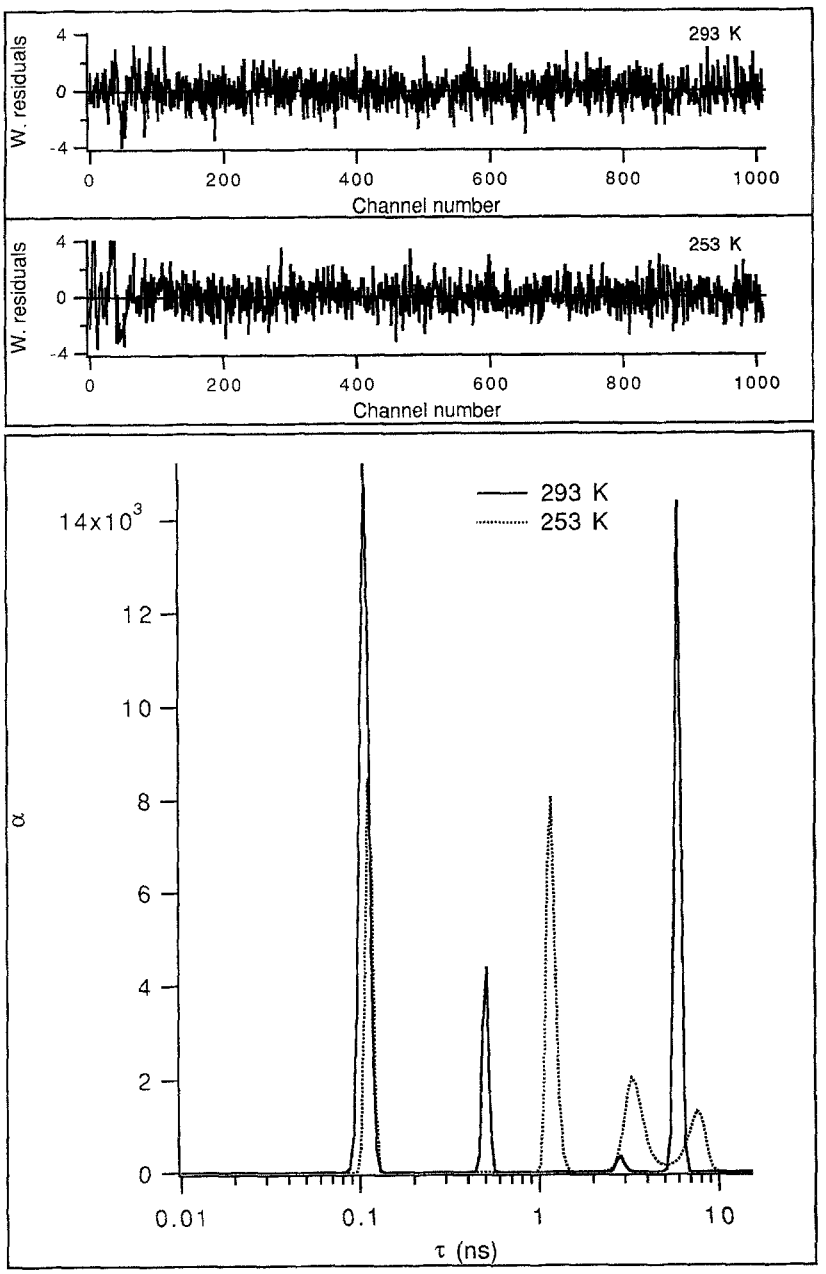

Fig. 4. Examples of MEM analysis of fluorescence decay of MLF in propylene glycol at 293 K (solid) and 253 K (dashed) into a lifetime distribution. The weighted residuals between calculated and experimental curves are shown at the top. λ_{exc} = 457.9 nm, λ_{em} = 480.5 nm.

lengths. In Fig. 3A decay curves obtained at a high excitation energy appear to be completely different. The blue fluorescent photons decay much faster than the red ones. In the inset in Fig. 3A it is emphasized that the "red" emission shows a rise prior to the decay. In Fig. 3B decay curves obtained at a lower excitation energy appear to be similar. The decay patterns at temperatures between 253 and 293 K have a similar appearance, exhibiting only a variation in decay rate and in relative intensities (data not shown). The characteristics of the experimental decay curves can be fully explained by inhomogeneous broadening of the electronic transitions by fluctuating chromophore–solvent interactions. For a better understanding of these results one should refer to the diagram in Fig. 1. Excitation in the main band of the absorption spectrum results in the population of non-

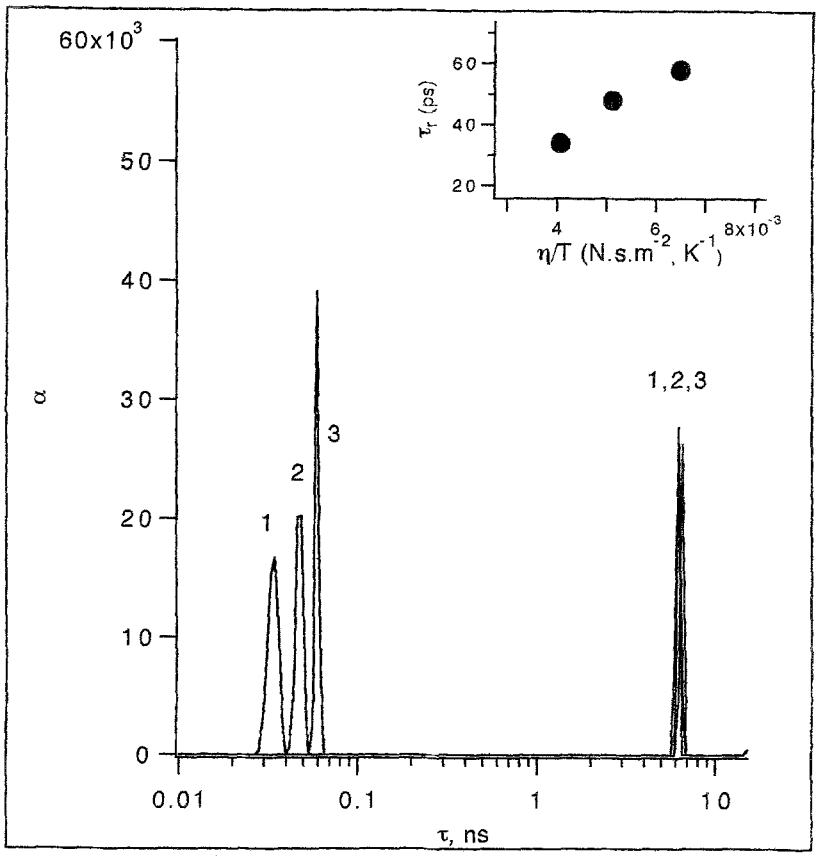

Fig. 5. MEM-recovered fluorescence lifetime distribution patterns of MLF in ethanol at 273 K (curve 1), 263 K (curve 2), and 253 K (curve 3). The inset shows the short lifetime (τ_r) versus η/T. λ_{exc} = 457.9 nm, λ_{em} = 480.5 nm.

equilibrium excited states. The excited-state solvate (chromophore surrounded by solvent dipoles) will have a tendency to relax to the equilibrium excited state with a certain dipolar relaxation time τ_r. This will be reflected by a rapid decay component at the blue edge and a rise time at the red edge of the fluorescence spectrum exactly as observed. Excitation at the red edge of the absorption band photoselects solvates with the smallest electronic transition frequencies [9]. These excited chromophores are already in the equilibrium excited state, and no further dipolar relaxation should be observed irrespective of emission wavelength, which again is in agreement with the results presented in Fig. 3B. It should be noted that when the τ_r is much shorter than the average fluorescence lifetime τ_f, no rise time will be observed as emission always originates from the relaxed fluorescent state.

Maximum Entropy Analysis of Main-Band Excited High-Energy Fluorescence Decay of MLF in Polar Solution

Rather than invoking a continuum model for description of solvent relaxation [1], we decided to use a "non a priori" approach to analyze the fluorescence decay data. In Fig. 4 an example of a maximum entropy data analysis of blue edge detected fluorescence of MLF in propylene glycol at 293 K is given. Excitation was in the main absorption band (457.9 nm). It is clear that the change in representation of the results from the time domain into the lifetime domain leads to a complex fluorescence lifetime distribution pattern with at least three main distributed lifetime components between 10 ps and 10 ns. The distributions were also complex at other temperatures (only results obtained at 253 K are shown in Fig. 4). The main component, located at about 0.1 ns, is connected with relaxation to the fluorescent state, which is much longer lived and manifested by a lifetime component of 6 ns (note that the relaxed fluorescence is partly transmitted through the filter chosen for blue edge selection; see Fig. 2). The remaining distributions may arise from different types of solvates. When fluorescence decay curves of MLF dissolved in ethanol were analyzed with the maximum entropy method, the resulting distribution patterns were much simpler: two peaks, of which the short one (tens of picoseconds) shows a clear tem-

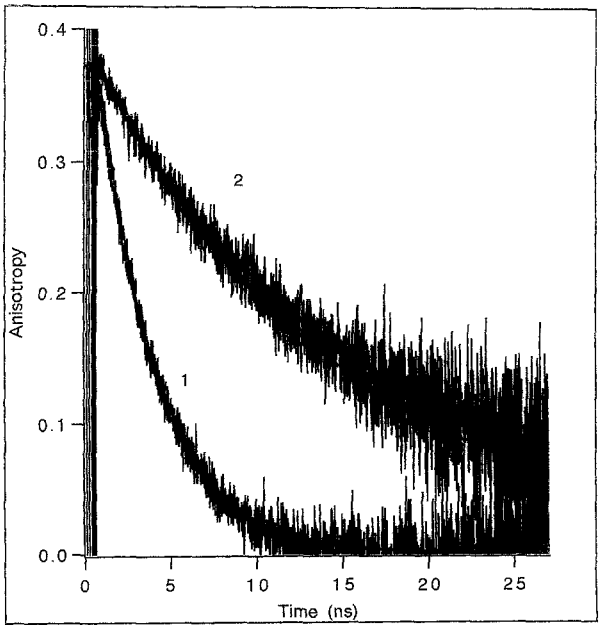

Fig. 6. Experimental and calculated (single-component analysis) fluorescence anisotropy decay curves at 293 K (set of curves 1) and at 273 K (set of curves 2). Each temperature corresponds to two experiments recorded at the blue and red edges of the emission band. λ_{exc} = 457.9 nm.

Table I. Rotational Correlation Times of MLF in Propylene Glycol as a Function of Temperature (λ_{exc} = 457.9 nm)[a]

T (K)	β (-)	Φ (ns)	η/T ($N \cdot s \cdot m^{-2}$, $^{-1}$)
263	0.374 (0.370–0.378)	29.0 (28.0–30.5)	0.0186
273	0.370 (0.369–0.371)	14.0 (13.9–14.1)	0.0074
293	0.365 (0.360–0.373)	3.3 (3.2–3.4)	0.0017

[a]Results from blue and red edge detected emissions were globally linked. Values between parentheses were from a detailed error analysis with a 67% confidence interval.

perature dependence and the long one (6 ns) is temperature invariant between 273 and 293 K (Fig. 5). The rapid component is proportional to η/T (η is the viscosity of ethanol [10] and T the temperature) as shown in the inset in Fig. 5. These results show that the fast decay time is connected with the Debye dipolar relaxation time τ_d:

$$\tau_d = 4\pi\eta a^3/(kT)$$

where a is the radius of the polar, spherical molecule rotating in a continuous medium of viscosity η and k the Boltzmann constant.

Fluorescence Anisotropy Decay of MLF in Propylene Glycol

The question of wavelength-dependent rotation of dye molecules in polar solution has been raised in several publications [1,11,12]. It has been found that solvent relaxation must be linked with rotational diffusion. To investigate this we have measured fluorescence anisotropy decay curves of MLF in propylene glycol using main band excitation and detection at the blue and red edges of the fluorescence band. Analysis of the time-resolved anisotropies between 263 and 293 K revealed that there is hardly any difference between anisotropies detected at high and low emission energies and, in addition, the decay of anisotropy is monoexponential (Fig. 6). Rotational correlation times have been collected in Table I. The fact that there is no clear effect of detection wavelength on the anisotropy decay led us to conclude that there is no change in dipole moment orientation during relaxation of the solvent around MLF. This result is also in keeping with the very small change in dipole moment upon excitation of MLF in the excited singlet [5].

CONCLUSIONS

Solvent relaxation can be visualized by picosecond-resolved fluorescence upon main-band excitation of MLF in propylene glycol or ethanol. A rapid disappearance is observed at the blue edge and a rapid appearance at the red edge of the emission. Upon red edge excitation there is no difference in decay at blue and red edge detected emission. No wavelength-dependent rotation of MLF in propylene glycol could be observed. The results presented are valuable in interpreting flavoprotein fluorescence and in elucidating the dynamical structure of the active site.

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