

Molecular relaxation spectroscopy of lumichrome

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Abstract

Molecular relaxation spectroscopic experiments on lumichrome compounds dissolved in propylene glycol have resulted in the determination of dipolar relaxation times and the dipole moment difference in the ground and first excited singlet states. As for the closely related flavin compounds, this difference was found to be small (approximately 1 D with a slightly higher dipole moment for the excited singlet). A close examination of the emission spectra obtained on main-band and red-edge excitation revealed that lumichrome can exist in different ground state solvent configurations probably arising from different hydrogen-bonded forms.

1. Introduction

The optical spectroscopy of lumiflavin and lumichrome has received considerable attention during the last two decades [1, 2]. Quite recently, we have exploited the sensitivity of the center of gravity of the fluorescence band of flavins and flavoproteins to obtain dipolar relaxation properties of flavin surroundings [3, 4]. The exact position of the fluorescence band is dependent on the dielectric constant and refractive index of the solvent. Because of the thermal motion of solvent molecules around the solute, there is a distribution of singlet–singlet transition frequencies leading to inhomogeneous spectral broadening [5]. Excitation can take place either in the central portion of the light absorption band or at its red edge. Both main-band and red-edge excited states will have a tendency to relax to a solute–solvent arrangement of minimum energy with a characteristic dipolar relaxation time τ_r (s). This dipolar relaxation time can be determined from an analysis of the emission spectra and fluorescence decay [3, 4]. In addition, information on the change in dipole moment between the ground and excited singlet states can be estimated for a solute dissolved in polar solvents [6]. We have already obtained this quantity for 3-methyllumiflavin [3]. In this paper, we describe similar relaxation experiments on lumichrome and

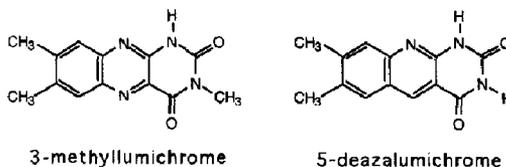


Fig. 1. Chemical structures of 3-methyllumichrome and 5-deazalumichrome.

deazalumichrome (Fig. 1) in the polar solvent propylene glycol. A new aspect of these relaxation studies is that red-edge excitation yields lumichrome species that are solvated differently from lumichrome in bulk solution. The reason for this phenomenon is that both absorption and emission spectra (and not only emission spectra) of edge-excited lumichrome are shifted to the red relative to those of main-band excited lumichrome.

2. Experimental details

2.1. Compounds

3-Methyllumichrome (3ML) and 5-deazalumichrome (5DL) were synthesized as described previously [7]. The solvent was 1,2-propanediol (propylene glycol, distilled under reduced pressure).

2.2. Molecular relaxation spectroscopy

Emission and excitation spectra were recorded on an SLM DMX-1000 spectrofluorometer (SLM

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Aminco, Urbana, IL). Lumichrome fluorescence spectra were scanned with excitation at 385 nm (main band), or at 420 and 445 nm (red edge). Deazalumichrome fluorescence spectra were obtained on excitation at 350 nm (main band) and 400 nm (red edge). The bandwidths of the excitation and emission monochromators were 2 nm. A liquid nitrogen flow unit in combination with a temperature controller was used to regulate the temperature between 203 and 313 K. Details of this temperature control system have been published elsewhere [4]. The spectra were converted into wavenumber scale and the center of gravity or mean wavenumber was numerically evaluated on a personal computer. The results of three experiments at a given temperature were averaged.

Fluorescence decay measurements and the associated data analysis have been described previously [8]. For both compounds the excitation wavelength was 345 nm and emission was detected at 455 nm using a Schott (Mainz, Germany) interference filter.

3. Results and discussion

3.1. Dipolar relaxation times from spectral shifts

Mazurenko and Bakhshiev [9] have developed a continuous relaxation model which relates the mean wavenumber of the steady state emission spectrum ($\bar{\nu}$, in cm^{-1}) to the dipolar relaxation time (τ_r , in s) and the fluorescence lifetime (τ_f , in s)

$$\frac{\bar{\nu} - \bar{\nu}_\infty}{\bar{\nu}_0 - \bar{\nu}_\infty} = \frac{\tau_r}{\tau_r + \tau_f} \quad (1)$$

At low temperatures there will be no motion of the dipoles of the solute-solvent complex ($\tau_r \gg \tau_f$) and the unrelaxed spectrum with mean wavenumber $\bar{\nu}_0$ is obtained. At the other extreme, at elevated temperatures with $\tau_r \ll \tau_f$, the emission comes from the equilibrium excited state yielding the relaxed spectrum with mean frequency $\bar{\nu}_\infty$.

It has been shown that the completely relaxed spectrum can also be measured by excitation of the fluorophore at the red edge of the absorption band and a modified form of eqn. (1) can be obtained by replacing $\bar{\nu}_\infty$ by $\bar{\nu}^{\text{edge}}$ [10]

$$\frac{\bar{\nu} - \bar{\nu}^{\text{edge}}}{\bar{\nu}_0 - \bar{\nu}_0^{\text{edge}}} = \frac{\tau_r}{\tau_r + \tau_f} \quad (2)$$

where $\bar{\nu}^{\text{edge}}$ is the mean wavenumber of the fluorescence spectrum on red-edge excitation. From

the latter expression, the dipolar relaxation time can be determined from the shift $\bar{\nu} - \bar{\nu}^{\text{edge}}$ between the fluorescence spectra at two excitation frequencies at a given temperature, the maximal shift at low temperature and the known value of τ_f .

If there is a unique solute-solvent arrangement in the ground state, it can be expected that both equations would lead to the same spectral band positions, *i.e.* $\bar{\nu}_\infty \approx \bar{\nu}^{\text{edge}}$. A diagnostic aid in this respect is that, at elevated temperatures ($\tau_r \ll \tau_f$), the emission band position should be unaltered irrespective of excitation wavelength. Indeed, such a result was obtained for flavins on main-band (458 nm) and extreme red-edge (514 nm) excitation [3, 4]. On the other hand, when the molecule can adopt more than one solute-solvent configuration, red-edge excitation can reveal this through the appearance of a red-shifted emission spectrum even at higher temperature with $\tau_r \ll \tau_f$. Because the concentration of these differently solvated molecules can be rather low (much lower than the usual submicromolar concentration used for fluorescence spectroscopy), red-edge spectroscopy is therefore a valuable tool to demonstrate the co-existence of various ground state solute-solvent configurations.

3.2. Excitation and emission spectra

In Fig. 2, the excitation and emission spectra of 3ML are shown. The three fluorescence spectra were obtained on excitation at the main absorption band (385 nm, see arrow in Fig. 2(A)) and at the red edge (420 and 445 nm, see arrows). A progressive long-wave shift of the spectrum can be seen at 203 K on red-edge excitation compared with excitation at the main band (Fig. 2(B)). At an excitation wavelength of 420 nm there is hardly any shift in the 293 K spectrum suggesting the existence of relaxed fluorescence spectral distributions (Fig. 2(C)). It is clear from the far-red-edge excited (445 nm) emission (Figs. 2(B) and 2(C)) that a second emission spectrum with a maximum around 480 nm develops. Since this extra emission was not observed in the closely related fluorophore 3-methylflavin [3], taken under identical conditions of main-band and extreme red-edge excitation, this emission must be assigned to a new species present at low concentration. It has been demonstrated previously that lumichrome can exist in several forms characterized by a different degree of hydrogen bonding [11]. Therefore it is tempting to assign this bathochromic spectrum to another configuration of lumichrome hydrogen bonded to propylene glycol. In Fig. 3, a similar spectral set is presented for 5DL in propylene

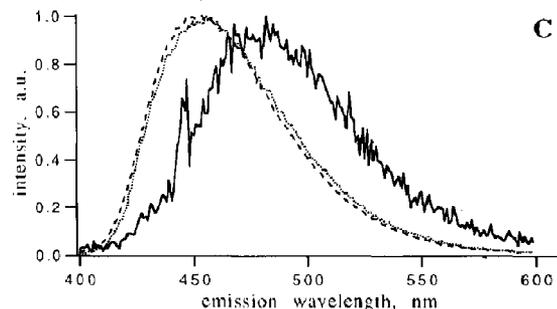
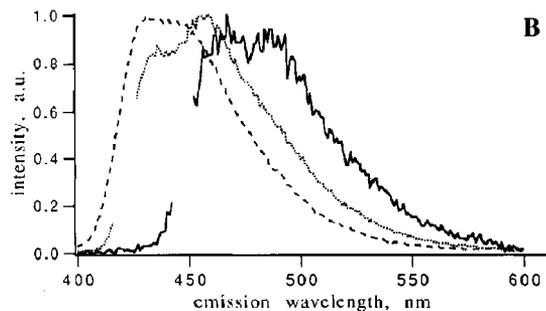
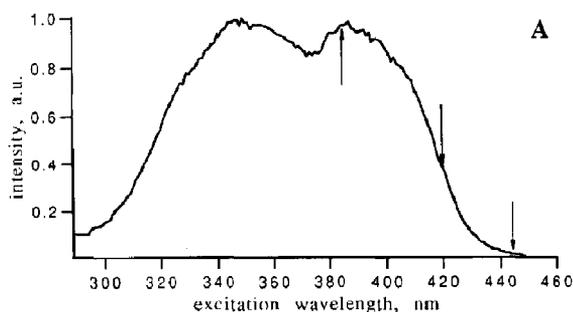


Fig. 2. Excitation and emission spectra of 3ML in propylene glycol. (A) Excitation spectrum at 293 K with $\lambda_{em} = 460$ nm. The arrows indicate $\lambda_{exc} = 380$ nm (main band) $\lambda_{exc} = 420$ nm (red edge) and $\lambda_{exc} = 445$ nm (extreme red edge). (B), (C) Fluorescence spectra with $\lambda_{exc} = 380$ nm (broken curve), $\lambda_{exc} = 420$ nm (dotted curve) and $\lambda_{exc} = 445$ nm (full curve) at 203 K (B) and 293 K (C). In (B) the points in the spectra located around $\lambda_{em} = 420$ nm (dotted curve) and $\lambda_{em} = 445$ nm (full curve) were omitted. In (C) a small contribution from Rayleigh scattering is visible at $\lambda_{em} = 445$ nm in the noisy spectrum (full curve). The spectra shown in (B) and (C) are the average of three individual spectra.

glycol. We selected a far-red-edge excitation wavelength (Fig. 3(A)) which resulted in somewhat noisier spectra. However, the clear shift between the main-band and red-edge excited spectra at lower temperatures (Fig. 3(B)) and the partial restoration at room temperature (Fig. 3(C)) can be seen.

The mean wavenumber of the emission spectrum (center of gravity) is shown as a function of two excitation wavelengths and of temperature in Fig.

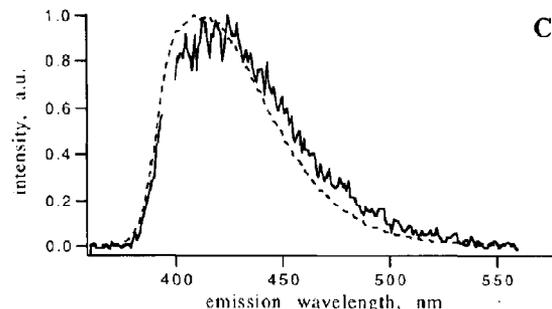
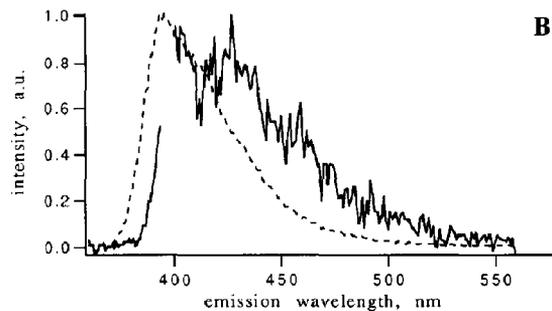
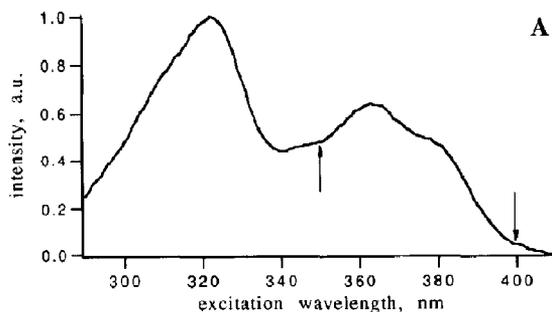


Fig. 3. Excitation and emission spectra of 5DL in propylene glycol. (A) Excitation spectrum at 293 K with $\lambda_{em} = 430$ nm. The arrows indicate $\lambda_{exc} = 350$ nm (main band) and $\lambda_{exc} = 400$ nm (red edge). (B), (C) Fluorescence spectra with $\lambda_{exc} = 350$ nm (broken curve) and $\lambda_{exc} = 400$ nm (full curve) at 203 K (B) and 293 K (C). In (B) and (C) the points in the noisy spectra (full curves) located around $\lambda_{em} = 400$ nm were omitted. The spectra shown in (B) and (C) are the average of three individual spectra.

4 for both compounds. It can be concluded from Fig. 4 that the lumichrome system is not so well behaved as the closely related lumiflavin system, when main-band and red-edge excited emissions are compared (see fig. 2 of ref. 3 or fig. 2(C) of ref. 4). For 3ML, the center of gravity of 420 nm excited fluorescence spectrum is not constant at different temperatures (Fig. 4(A)). This may arise from a mixture of different hydrogen-bonded species which cannot be distinguished by red-edge spectroscopy. This is confirmed by the center of gravity of the emission band on 445 nm excitation which is at much lower energy values than the other two centers at all temperatures (approx-

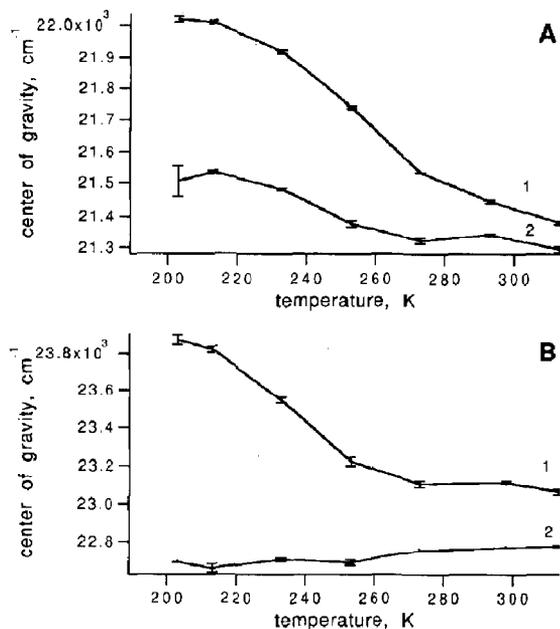


Fig. 4. Center of gravity of fluorescence spectra as a function of temperature. (A) 3ML: curve 1, $\lambda_{\text{exc}} = 380$ nm; curve 2, $\lambda_{\text{exc}} = 420$ nm. (B) 5DL: curve 1, $\lambda_{\text{exc}} = 350$ nm; curve 2, $\lambda_{\text{exc}} = 400$ nm. The error bars are based on three separate experiments.

mately $20.2 \times 10^3 \text{ cm}^{-1}$ between 203 and 293 K). The results obtained with 5DL show the same tendency as for 3ML: the center of gravity of the main-band excited fluorescence spectrum levels off to a constant value at $T > 260$ K, while the red-edge excited fluorescence spectrum remains red shifted (Fig. 4(B)). Therefore the presence of different hydrogen-bonded species is also likely for 5DL. The reason why the center of gravity of main-band excited 5DL levels off at a lower temperature than that of 3ML is the much longer fluorescence lifetime of 5DL (see next section), indicating that dipolar relaxation is already complete at temperatures higher than 260 K.

3.3. Fluorescence lifetimes and dipolar relaxation times

The fluorescence decays at 293 K for 3ML and 5DL are given in Fig. 5(A). The emission intensity of 3ML decays much more rapidly than that of 5DL. The fluorescence decays were analyzed in a distribution of lifetimes using the maximum entropy method (see ref. 8 for details). This distribution analysis yielded a main peak at 1.4 ns and a minor peak at 0.35 ns for 3 ML (Fig. 5(B)). For 5DL, a single distribution at 6.9 ns was recovered (Fig. 5(B)). In order to evaluate the dipolar relaxation times in each temperature, we need the

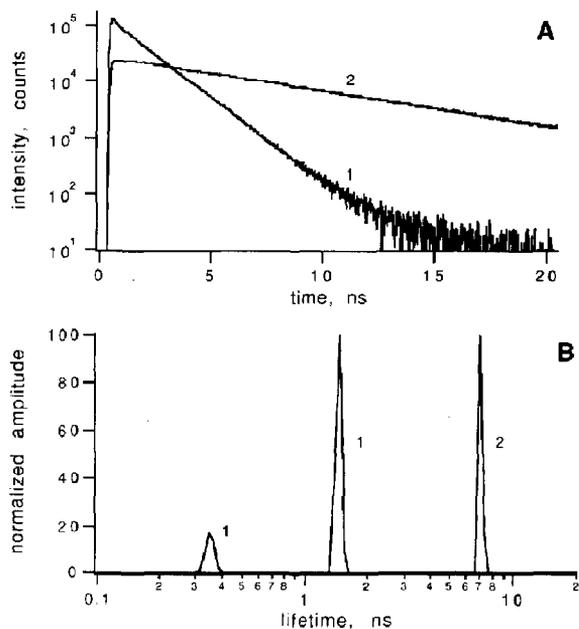


Fig. 5. Fluorescence decay analysis. (A) Fluorescence decay curves of 3ML (curve 1) and 5DL (curve 2). (B) Decay analysis by lifetime distribution for 3 ML (peak 1) and 5DL (peak 2).

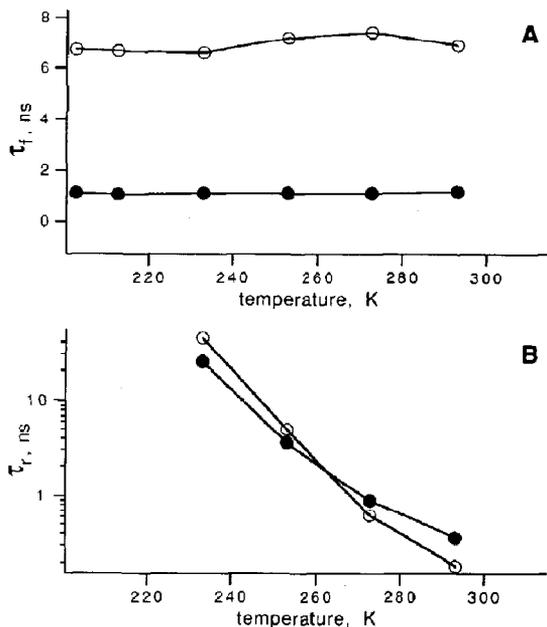


Fig. 6. Fluorescence lifetimes (A) and dipolar relaxation times (B) for 3ML (●) and 5DL (○) between 200 and 300 K.

temperature-dependent average fluorescence lifetimes (τ_f), which are presented for both compounds in Fig. 6(A). For reasons outlined in the previous section we could not use eqn. (2) for the evaluation

of the dipolar relaxation times. Therefore we restrict ourselves to eqn. (1). For $\bar{\nu}_0$ the values at 203 K were selected. The other extreme wave-number $\bar{\nu}_\infty$ was estimated as follows. For 5DL, the values at the three higher temperatures (Fig. 4(B)) were averaged. For 3ML, the center of gravity of the emission spectrum at the highest temperature was taken to be representative of the completely relaxed spectrum. The dipolar relaxation times thus obtained were plotted as a function of temperature (Fig. 6(B)). The values are of comparable magnitude as found previously [3].

3.4. Difference between ground and excited state dipole moments

The maximal shift $\bar{\nu}_0 - \bar{\nu}_\infty$ is related to the difference in dipole moments between the S_1 and S_0 states $\mu_e - \mu_g$ ($\Delta\mu$), the static dielectric constant ϵ_0 and the refractive index n of the solvent by [9]

$$\bar{\nu}_0 - \bar{\nu}_\infty = \frac{2(\mu_e - \mu_g)^2}{ch\epsilon_0 a^3} \left(\frac{\epsilon_0 - 1}{\epsilon_0 + 2} - \frac{n^2 - 1}{n^2 + 2} \right) \quad (3)$$

where c is the velocity of light, h is Planck's constant, ϵ_0 is the dielectric constant in a vacuum and a is the Onsager radius of the chromophore. The maximal shift $\bar{\nu}_0 - \bar{\nu}_\infty$ was obtained from the center of gravities at the highest and lowest temperatures as described in the previous section. Substituting published values for ϵ_0 (55.7 at 203 K) [12] and n (1.4329) of propylene glycol [13] and assuming a certain value for the Onsager radius, the difference in dipole moments can be calculated using eqn. (3). The Onsager radius was taken to be 0.5 nm, which is 0.05 nm longer than the longest diameter of 3ML. The value of the refractive index of propylene glycol is given at room temperature (293 K), but its value at 203 K can be estimated using Maxwell's equation ($n \propto (\epsilon_0)^{1/2}$) and noting that $\epsilon_0 = 32$ at 293 K [13]. There is a slight temperature dependence of the term in eqn. (3) containing the dielectric constant and the refractive index (this term is equal to 0.786 at 293 K and to 0.705 at 203 K), but this makes the absolute value of $\Delta\mu$ only a factor of 1.05 larger at 203 K compared with that at 293 K. Although the absolute value of μ_e cannot be established from eqn. (3), the fact that the emission spectra exhibit a significant red shift makes it likely that $\mu_e > \mu_g$. This is in agreement with values obtained from semi-empirical molecular orbital calculations on alloxazines in the gas phase [14, 15]. Experimental (203 K) and theoretical results are collected in Table 1. It is clear from these data that $\Delta\mu$ is approximately 1 D from experiment and about 2 D from

TABLE 1. Difference between the dipole moments of ground and excited state lumichrome

Compound	Maximal shift (cm^{-1})	$\Delta\mu(\text{obs.})$ (D)	$\Delta\mu(\text{calc.})$ (D)	Reference
3-Methyllumichrome	725	1.09		
5-Deazalumichrome	780	1.12		
Alloxazine			1.93	14
Alloxazine			2.57	15
Lumichrome			1.97	15

quantum chemical determination. The experimental dipole moment difference is of a similar magnitude as found for 3-methyllumiflavin [3].

4. Conclusions

Red-edge fluorescence spectroscopy of lumichrome in a viscous polar solvent has revealed the presence of differently solvated lumichrome molecules in the ground state. This observation is characteristic for lumichrome and not for the chemically related flavin chromophoric group. Comparable solvent dipolar relaxation times were obtained for two different lumichrome compounds. From the data a slight difference in the dipole moments of the ground and excited states was obtained.

Acknowledgment

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