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TIME-DEPENDENT ROTATIONAL RATES OF EXCITED FLUOROPHORES

A LINKAGE BETWEEN FLUORESCENCE DEPOLARIZATION AND SOLVENT RELAXATION

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It is generally assumed that the rotational diffusion coefficients of fluorophores are independent of time subsequent to excitation, and that the rotational diffusion coefficients of the ground and the excited states are the same. We now describe a linkage between the extent of solvent relaxation and the rate of fluorescence depolarization. Specifically, if a fluorophore displays time-dependent solvent relaxation it may also show a time-dependent decrease in its rotational rate. A decreased rate of rotation could result from the increased interaction with polar solvent molecules which occurs as a result of solvent relaxation. The decays of anisotropy predicted from our model closely mimic those often observed for fluorophores which are bound to macromolecules. For example, the decays are more complex than a single exponential, and the time-resolved anisotropy can display a limiting value which does not decay to zero. The effect of solvent relaxation upon the rates of rotational diffusion is expected to be most dramatic for solvent-sensitive fluorophores in a viscous environment. These conditions are frequently encountered for fluorophore-macromolecule complexes. Consideration of the linkage between solvent relaxation and rotational diffusion leads to two unusual predictions. First, even spherical fluorophores in an isotropic environment could display multi- or nonexponential decays of fluorescence anisotropy. Secondly, for the special case in which the fluorophore dipole moment decreases upon excitation, the theory predicts that the anisotropy decay rate may increase with time subsequent to pulsed excitation. The predictions of this theory are consistent with published data on the effects of red-edge excitation upon the apparent rotational rates of fluorophores in polar solvents.

Introduction

Measurements of fluorescence anisotropies, particularly the time-resolved decays of the anisotropy, are widely utilized to investigate the physical properties of biological macromolecules. We note the following examples. Fluorescence anisotropies of labeled membranes, measured by the steady-state, time-resolved and differential phase methods, were used to estimate the dynamic properties and order parameters of the acyl side chain region of membranes [1–5]. Time-resolved anisotropies of labeled proteins, such as immunoglobulins and membrane-bound proteins [6–9], have been used to quantify the segmental motions of regions and amino acid residues of these proteins. Finally, the time- and lifetime-dependent anisotropies of tryptophan and tyrosine residues in proteins were used to estimate the motional behavior of these residues relative to the entire protein [10–13]. In native proteins the amino acid residues are closely packed [14]. For this reason segmental motions of the tryptophan residues within the protein matrix are thought to reflect the extent of structural fluctuations within the protein matrix.

From the examples described above, it is evident why anisotropy measurements are useful for quantifying the dynamics of macromolecules. The

decay of anisotropy requires rotational displacement of the fluorophore during the lifetime of the excited state. Since typical excited state lifetimes are near 10 ns, rotational displacements on this time scale can be detected and quantified. For the case of fluorophores bound to macromolecules the measured decays of anisotropy are frequently non- or multiexponential. Additionally, the anisotropies frequently decay not to zero, as is found in homogeneous solution, but rater to nonzero limiting values [2,3,9,15]. For completeness we note that multiexponential decays of anisotropy are expected [16–7] and observed [18,19] for nonsymmetrical and ellipsoidal fluorophores in isotropic solutions. However, relative to the dramatic effects seen for fluorophores bound to macromolecules, the behavior in solution is rather ideal.

In all the theoretical and experimental analyses mentioned above it is assumed that the decay of anisotropy reflects the viscous drag and degree of hindrance imposed on the fluorophore by its surrounding environment. And moreover, it is assumed that the rotational diffusion coefficient of the fluorophore is constant during the lifetime of the excited state. That is, for a spherical molecule, the anisotropy decays as $r(t) = r_0 e^{-6Dt}$ where r_0 is the anisotropy in the absence of rotational diffusion, t the time, and D the rotational diffusion coefficient. The rotational correlation time (ϕ) is related to the diffusion coefficient by $\phi = (6D)^{-1}$.

In this paper we describe an alternative origin for multi- or nonexponential decays of anisotropy. We propose that the rotational correlation time of a fluorophore can be itself dependent upon time. In our opinion such time dependence can reasonably originate with the well known phenomenon of solvent relaxation (ref. 20, and references therein) and with the known effects of increased hydrogen bonding on the rates of rotation diffusion. To be more specific, it appears that the rotational rates of small molecules in solution are decreased several-fold by hydrogen bonding [19,21–23]. Exceptions have been reported [24]. Furthermore, for polar fluorophores in polar viscous solvents, the emission spectra are known to display time-dependent shifts to longer emission wavelengths [5,15 25,26]. These shifts are a result of increased dipole-dipole interactions between the fluorophore and the surrounding solvent molecules, which in turn result from the altered (generally increased) dipole moment of the excited state. We suggest that these time-dependent solvent-fluorophore interactions can result in time-dependent changes in the rotational diffusion coefficient, and hence complex decays of fluorescence anisotropy.

To illustrate the potential effects of solvent relaxation upon rotational diffusion we consider a two-state model. Solvent relaxation, and its effects on the rotational rates of fluorophores, is certainly more complex than this model. However, this extreme model illustrates the essential features of the concept described above, and in addition, is simple enough to be described analytically and to be understood intuitively. In addition, a continuous model is briefly considered. the quantitative and qualitative predictions of both models are similar.

2. Theory

2.1. Two-state model

To illustrate the effects of solvent relaxation on rotational diffusion we chose a two-state model, as shown in fig. 1. It is assumed that upon excitation, and following rapid internal conversion, the fluorophore arrives in the initially excited or unrelaxed state (F). Many fluorophores with polar substituents have altered dipole moments in the excited state. If the solvent is also composed of polar molecules these dipoles reorient so as to decrease the energy of the excited state. This process, called solvent relaxation, results in a red-shifted emission spectrum. The temperature, viscosity and chemical properties of the solvent govern the rate of solvent relaxation (k). If the solvent is fluid, the relaxation rate is more rapid than the decay rate (Γ) of the fluorophore. That is, when $k \gg \Gamma$ the emission is mainly from the R state. Conversely, if the solvent is extremely viscous then $k \ll \Gamma$ and the blue-shifted emission of the F state is observed. The time-resolved intensity decays of the F and R states ($I_F(t)$ and $I_R(t)$) were described previously [27]. These decays are

$$I_F(t) = \Gamma e^{-(\Gamma+k)t} \tag{1}$$

$$I_R(t) = \Gamma[e^{-\Gamma t} - e^{-(\Gamma+k)t}] \tag{2}$$

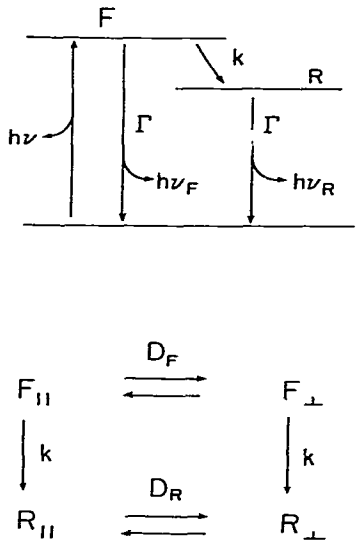

Fig. 1. Two-state model with different rotational rates for the initially excited (F) and solvent relaxed (R) states.

where $\Gamma = \tau_0^{-1}$ is the decay rate of the fluorophore unperturbed by solvent relaxation and $\Gamma + k = \tau_F^{-1}$ the decay rate of the F state. This lifetime of the F state (τ_F) is decreased by relaxation away from the observation wavelength. Other aspects of this model, and more complex cases, were described previously [28]. We have assumed that relaxation is a simple two-state process, that solvent relaxation can be described by a singe rate constant, and that the decay rate of the fluorophore is not altered by the event of relaxation.

We are concerned with the effects of solvent relaxation upon the steady-state and time-resolved anisotropies of the F and the R states. To some extent the F and R states may be separately observable by wavelength selection for the blue and the red sides of the emission, respectively. The physical origin of the difference in the diffusion coefficients is assumed to be the different solvent-fluorophore interactions in the F and R states. It is also assumed that solvent relaxation and rotational diffusion are independent events. Hence, the parallel (||) and the perpendicular ($\perp$) components of the F state ($F_{||}$ and $F_{\perp}$) interchange with the rate constant D_F, and the components of the R state ($R_{||}$ and $R_{\perp}$) interchange with D_R. In this model there is no coupling of solvent relaxation with rotational diffusion. For instance, $F_{||}$ cannot be transformed directly into $R_{\perp}$. More complex models which contain such cross-terms would probably show to a lesser extent the effect of different rotational rates of the F and R states.

The dependence of the anisotropies upon the kinetic constants of the system (Γ, k, D_R and D_F) may be derived from the differential equations describing the populations of the individual states upon steady-state illumination or following pulsed excitation. This derivation is described in detail in the appendix. In the following paragraphs we describe the results of this derivation, and the experimental implications of these results.

2.2. Anisotropy of the F state

The steady-state (r_F) and time-resolved ($r_F(t)$) anisotropies of the F state are rather simple, and characteristic of that expected for a spherical molecular in homogeneous solution. The time-resolved decay of the F state is given by

$$r_F(t) = r_0 e^{-6D_F t}, \tag{3}$$

and the steady-state anisotropy of the F state is

$$r_F = \frac{r_0}{(1+6D_F\tau_F)}. \tag{4}$$

In these equations r_0 is the anisotropy in the absence of rotational motion and $\tau_F = (\Gamma + k)^{-1}$ the lifetime of the F state. This lifetime is less than the lifetime in the absence of solvent relaxation ($\tau_0 = \Gamma^{-1}$) because relaxation is an additional rate process depopulating the excited state, and thereby decreasing the lifetime. The simplicity of eqs. 3 and 4 is a result of assuming solvent relaxation to be irreversible and a result of our assumption that the rotation rates of the fluorophore about its molecular axes are identical. Eq. 3 is the usual exponential decay of anisotropy expected for a spherical fluorophore in a homogeneous environment, and eq. 4 is the Perrin equation [29] as expressed in terms of the anisotropy rather than polarization [30].

2.3. Anisotropy of the R state

Somewhat more complex expressions are needed to describe the anisotropy of the relaxed state. These expressions are

$$r_R = \frac{r_F}{(1+6D_R\tau_0)} \tag{5}$$

$$r_R(t) = \frac{kr_0}{(6D_F + k - 6D_R)} \times \left[\frac{e^{-(\Gamma+6D_R)t} - e^{-(\Gamma+k+6D_F)t}}{e^{-\Gamma t} - e^{-(\Gamma+k)t}}\right] \tag{6}$$

First, consider eq. 5 for the steady-state anisotropy. In words, this equation indicates that the steady-state anisotropy of the R state is determined by the steady-state anisotropy of the F state (r_F), the intrinsic lifetime of the R state if this state could be directly excited (τ_0), and the rotational rate of the fluorophore in this state (D_R). Notice that the form of this equation is analogous to eq. 4 except that r_0 is replaced by r_F. Since the F state populates the R state, the anisotropy of the F state partially determines that of the R state.

Consideration of eqs. 4 and 5 suggests experimental means by which one could measure the rotational rates of the individual states. Assume that these states could be selectively observed by choice of appropriate emission wavelengths. Then, the diffusion coefficient of each state can be calculated. Specifically, D_F could be obtained from τ_F and r_F (eq. 4) and D_R could be obtained from r_F and τ_0 (eq. 5). We note that τ_F can be measured directly, and that τ_0 may be obtained from the phase difference between the red and the blue sides of the emission [28], or from the time-resolved intensity decay of the R state (eq. 2). Spectral overlap of the emission of the F and the R states could prevent the successful determination of the individual rotational rates.

A first examination the time-resolved anisotropy of the R state is seemingly complex (eq. 6). We note the following limiting cases which are evidence that this expression is correct. If $D_F = D_R = D$ then as expected, $r_R(t) = r_0 e^{-6Dt}$. Also, if $D_F = D_R = 0$ then $r_R(t) = r_0$. If $k = 0$ then there is no relaxed emission, which is also the result at $t = 0$. Finally, integration of eq. 6 using the decay law for the R state yields the expected steady-state anisotropy (eq. 5);

$$r_R = \frac{\int_0^\infty I_R(t) r_R(t) dt}{\int_0^\infty I_R(t) dt} \tag{7}$$

2.4. Anisotropy of the total emission

Anisotropy measurements are commonly performed using emission filters which transmit most of the emission. As a result, both the F and the R state emissions are observed simultaneously. It is known that for multiple emitting species, the measured anisotropy is the weighted sum of the anisotropies of the individual anisotropies [29]. Hence, for our two-state model, the total anisotropy is given by

$$r(t) = f_F(t) r_F(t) + f_R(t) r_R(t) \tag{8}$$

where $f_F(t)$ and $f_R(t)$ are the fractional intensities of the F and the R states at various times after excitation. These values may be obtained from eqs. 1 and 2,

$$f_F(t) = \frac{I_F(t)}{I_F(t) + I_R(t)} = e^{-kt} \tag{9}$$

$$f_R(t) = \frac{I_R(t)}{I_F(t) + I_R(t)} = 1 - e^{-kt} \tag{10}$$

Combination of eq. 8 with eqs. 3 and 6 yields the time-resolved anisotropy for the total emission,

$$r(t) = \frac{r_0}{(6D_F + k - 6D_R)} \times \left[ke^{-6D_R t} + (6D_F - 6D_R)e^{-(k+6D_F)t}\right] \tag{11}$$

The steady-state anisotropy is given by

$$r = f_F r_F + f_R r_R \tag{12}$$

where the steady-state fractional intensities are $f_F = \Gamma/(\Gamma + k)$ and $f_R = k/(\Gamma + k)$. Eq. 11 is unusual because if $D_R > D_F$ it can contain a term with a negative preexponential factor, which appears similar to that found for the emission from the product of an excited-state process (eq. 2). Hence, unusual behavior of $r(t)$ may be expected when $D_R > D_F$. For most fluorophores we expect

solvent relaxation to decrease the rate of rotational diffusion, i.e., $D_R < D_F$. Then, eq. 12 predicts a doubly-exponential decay of anisotropy.

It is informative to consider the case where solvent relaxation is faster than either rate of rotation; i.e., $k \gg D_R$ and $k \gg D_F$. Then the decay of the total anisotropy (eq. 11) becomes a single exponential with

$$r(t) = r_0 e^{-6D_R t}. \tag{13}$$

Under these circumstances the fluorophore quickly relaxes to the R state and the rotational rate of this state determines the decay of the anisotropy. Such behavior would prevent the observation of a time-dependent rate of rotation. It seems probably that this is the usual occurrence in polar solvents because the solvent relaxation generally precedes rotational diffusion [27]. This disparity of rates is probably a consequence of the larger molecular motions necessary for rotational diffusion as compared with those required for solvent relaxation.

2.5. Continuous model

As an alternative to the two-state model described above one may assume that the rotational rate of the fluorophores changes continuous from the initial value D_F to the final value D_R. Hence, we will assume that the time-dependent diffusion coefficient ($D(t)$) is given by

$$D(t) = D_R + (D_F - D_R)e^{-kt} \tag{14}$$

where k is the rate of solvent relaxation. During each time interval Δt, occurring at time t, the depolarization is given by $e^{-6D(t)\Delta t}$. The total depolarization at time t is given by the product of these individual depolarization factors. Hence,

$$r(t) = \prod_{t_i=0}^{t} r_0 e^{-6D(t)\Delta t} \tag{15}$$

which can be rearranged to

$$r(t) = r_0 \exp\left(-6 \sum_{t_i=0}^{t} D(t)\Delta t\right). \tag{16}$$

As Δt approaches zero the sum in eq. 16 becomes

$$f(D,t) = \int_0^t D(t')\mathrm{d}t' = D_R t + \frac{(D_F - D_R)}{k}(1 - e^{-kt}) \tag{17}$$

Hence, for the case of an exponential decay of the diffusion coefficient from D_F to D_R the anisotropy at time t is given by

$$r(t) = r_0 e^{-f(D,t)}. \tag{18}$$

3. Results

3.1. Modeling of the time-resolved anisotropies

It is informative to examine model calculations which illustrate the effect of solvent relaxation and an altered rate of rotational diffusion on the time-resolved anisotropies. We will consider the total emission, since this is the most commonly used experimental circumstance. Initially, we consider the effect of a decreased rotational rate of the R state relative to the F state. Decreased rotational rates are expected for solvent-sensitive fluorophores because hydrogen bonding interactions are known to be correlated with slower rotational rates [19,21–23], and of course, solvent relaxation is a consequence of increased fluorophore-solvent interactions. The parameters chosen for modeling are comparable in magnitude with those found for

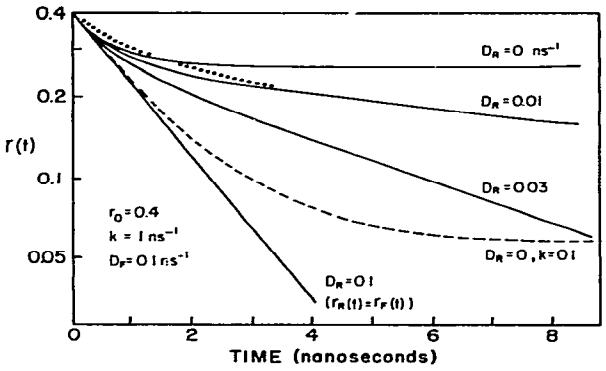

Fig. 2. Effect of slower rotational diffusion of the R state on the time-resolved decays of anisotropy. The chosen parameters are given on the figure. The dashed line (- - - - - -) illustrates the effect of a slower relaxation rate on the apparent limiting anisotropy of the total anisotropy $r(t)$; $k = 0.1$ ns^{-1}, $D_F = 0.1$ ns^{-1}, and $D_R = 0$. The dotted line (· · · · · ·) shows the expected anisotrophy decay of the R state, $r_R(t)$, for $k = 1$ ns^{-1}, $D_F = 0.1$ ns^{-1}, and $D_R = 0.01$ ns^{-1}.

many fluorophores. In particular, r_0 was assumed to be 0.4, the relaxation rate (k) was assumed to be 1 ns^{-1}, and the rotational rate (D_F) of the F state was assumed to be 0.1 ns^{-1}. The somewhat more rapid rate of solvent relaxation relative to the rotational rate is consistent with the results found for fluorophores in viscous solvents [20,27]. The effect of progressively decreasing values of D_R on the time-resolved anisotropies are shown in fig. 2. When $D_R = D_F = 0.1$ ns^{-1} the decay of the total anisotropy is a single exponential, as is evident from the linear dependence of log $r(t)$ upon time. This line ($D_R = 0.1$ ns^{-1}) also represents the decays of $r_F(t)$ and $r_R(t)$ for this special case. When D_R is decreased to one-third of the magnitude of D_F ($D_R = 0.03$ ns^{-1}) the decay of anisotropy clearly appears to be multi- or nonexponential. Still greater nonlinearity is seen when D_R is decreased 10-fold relative to D_F. When D_R is decreased still further (to $D_R = 0$) one notices that the decay of anisotropy becomes comparable to that found for a fluorophore in an environment which hinders its motions beyond a maximum angle [31]. This apparent limiting anisotropy (r_∞) is given by

$$r_\infty = \frac{r_0 k}{6D_F + k} \tag{19}$$

and is hence dependent upon the rotational rate of the F state and the rate of solvent relaxation. If the relaxation rate is decreased, then there is more time for rotational diffusion of the fluorophore prior to the fluorophore arriving in the assumed immobile R state, and ence a lower value for the apparent limiting anisotropy r_∞ (- - -).

Instead of observing the entire emission through a broad-band filter one may choose to use selected wavelengths to observe, to the extent possible, the emission of the F or the R states. Of course, the anisotropy of the F state decays exponentially. The time-resolved anisotropy of the R state (· · · ·) is shown in fig. 2 for the case of $D_R = 0.01$ ns^{-1}. One notices that the multiexponential nature of this decay is less apparent than that of the total decay. When the total decay is monitored, the population of fluorophores which is observed changes with time according to eqs. 9 and 10. When the R state alone is observed the changing contribution of the F and R states to the total emission does not alter the observed anisotropy. It is important to note that the form of the time-resolved decay calculated using our simple model is clearly comparable to that found for membrane bound fluorophores [2,15], protein-bound fluorophores [6,7,9] and for the intrinsic tryptophan residues of proteins [11]. We are not claiming that our model correctly describes these diverse observations of nonexponential decays of anisotropy. This is clearly not the case, since some molecules which display substantial limiting anisotropies in membranes, such as as diphenylhexatriene and perylene [2,32], are not likely to form substantial hydrogen bonds even in the excited state. Rather, we wish to indicate that in a polar medium the anisotropy of a polar fluorophore may decay first rapidly, and then more slowly, as a result of a time-dependent change in its interactions with the surrounding environment.

Our model allows a unique prediction which, to the best of our knowledge, has not been detected experimentally. In the discussion of fig. 2 we considered the case in which the relaxed state of the fluorophore interacted more strongly with the solvent. This is commonly the case because the dipole moments of excited states are generally larger than those of ground states. Now we consider a molecule whose dipole moment decreases in the excited state, i.e., its distribution of electronic charges becomes more uniform. Such molecules are known to exist [33,34], but their fluorescence spectral properties have not been reported in detail. Our model predicts that such molecules may display unique time-resolved decays of anisotropy. As a result of the larger ground-state dipole moment, the solvent-fluorophore interactions are expected to be stronger in the ground state than in the excited state. Consequently, the solvent-fluorophore interactions become weaker subsequent to excitation, and the rate of fluorophore rotation may increase. The time-resolved anisotropy of such a fluorophore is modeled in fig. 3. We assumed that $D_R > D_F$ by factors of 3- and 10-fold. Remarkably, the time-resolved anisotropy of such a molecular would display an initial slow decay, followed by a faster decay as the molecule becomes detached from the surrounding solvent.

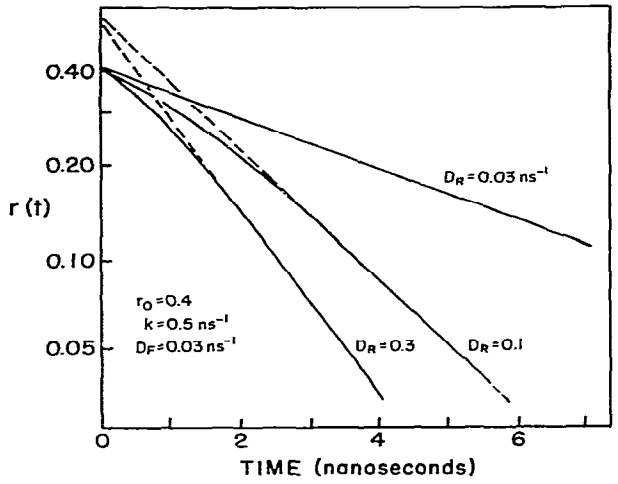

Fig. 3. Effect of faster rotational diffusion of the R state on the time-resolved decays of anisotropy.

Notice also that the anisotropy, extrapolated to $t = 0$ (— — —), would exceed the r_0 value of the fluorophore. The increase could be substantial. For example, for the case described in fig. 3 the apparent value of r at $t = 0$ would be near 0.55.

One important characteristic of these predicted results is that they are distinct from those which may be observed for a fluorophore in an environment which hinders its motion [31] or those possible for an asymmetric molecule [16–19]. For these molecules the average rate of rotational motion can only appear to decrease with time. In contrast, for a fluorophore which can uncouple from the solvent in the excited state, the apparent anisotropy decay rate can show a time-dependent increase. To the best of our knowledge such unusual time-dependent decays of anisotropy have not been observed for any sample containing a fluorophore in a single environment. The possibility that fluorophores can display a time-dependent decrease in their dipole-dipole interactions with polar solvents has been demonstrated by Nemkovich et al. [35]. Upon excitation on the extreme red edge of the absorption band these workers observed a time-dependent increase in the anti-Stokes emission.

We also performed model calculations using the continuous model (eqs. 17 and 18). These are not shown because the results are similar to those presented in Figs. 2 and 3. Using the same assumed parameters, the calculated time-resolved decays were quantitatively comparable to those obtained using the two-state model. Hence, whether on regards solvent relaxation as a stepwise or a continuous process, the effects on the decays of anisotropy are similar.

4. Discussion

It is of interest to describe the experimental circumstances where a linkage of solvent relaxation and rotational diffusion may be observed. Since solvent relaxation requires interaction between polar solvent molecules and a polar fluorophore, solvent relaxation is not observed if either the solvent or the fluorophore is nonpolar. Consequently, a time-dependent change in the rotational diffusion coefficient is not expected in nonpolar solvents, irrespective of the viscosity. Similarity, fluorophores lacking polar substituents do not display significant solvent-dependent shifts and the rotational diffusion coefficients are not expected to shown any significant time dependence. For a polar fluorophore in polar solvents the observations of a time-dependent diffusion coefficient requires that solvent relaxation and rotational diffusion occur at comparable rates. Such conditions are found in solvents of moderate viscosity, and are often found for fluorophores bound to macromolecules [5,20,25,36]. It seems possible that the covalent linkage of fluorophores to macromolecules could enhance the effects of excited-state interactions on rotational diffusion. For instance, if the fluorophore is also covalently bound to the macromolecule, the formation of a single hydrogen bond between the excited state of a fluorophore and the macromolecule may substantially decrease its diffusive motion. Supporting this speculation is experimental evidence which seems to indicate that the diffusive rates of fluorophores bound to proteins are decreased by hydrogen bonding [9].

It is important to emphasize that time-dependent diffusion coefficients are not expected for all polar fluorophores in polar solvents. Such effects

are only expected if the excited fluorophore displays a time-dependent shift of the emission spectrum to lower energies, indicating an increased extent of dipole-dipole interaction with the solvent. Many polar fluorophores do not show such solvent-dependent shifts, even though they are hydrogen bonded to the solvent. Examples of such molecules include fluorescein and rhodamine 6G.

Finally, it seem appropriate to examine the experimental evidence which may indicate that solvent relaxation can affect the rates of rotational diffusion. In general such a correlation seems reasonable, since the apparent molecular volumes of solutes are known to increase with the number of fluorophore-solvent hydrogen bonds [19,21–24,37]. Moreover, multiexponential decays of anisotropy are frequency observed for polar fluorophores in membranes [9,38] and bound to proteins [9,11]. A more definitive observation supporting this concept may have been accomplished Valeur and Weber [39,40]. These workers found that excitation on the red edge of the absorption spectrum of many fluorophores resulted in an increase in the fluorescence anisotropy. These results were interpreted as indicating a larger apparent molecular volume, which in turn was explained as the result of an out-of-plane electronic transition. Valeur and Weber suggested that these out-of-plane transitions are selectively excited upon red-edge excitation.

We suggest an alternative explanation of these data, which is a decreased rotational rate of the fluorophore in the relaxed state. Upon excitation on the red edge of an absorption band it is known that the emission spectra are shifted to longer wavelengths relative to that observed with centrally located excitation wavelengths (refs. 41–43; and J.R. Lakowicz and S. Keating-Nakamoto, unpublished results). In general, the spectral characteristics of the excited state formed upon red-edge excitation are similar to those of the solvent relaxed states. That is, on red-edge excitation, one selectively excites a subclass of the fluorophores which are interacting more strongly with the polar solvent molecules. Hence, one may expect these 'relaxed' fluorophores to rotate more slowly. This is precisely the result found by Valeur and Weber [39]. The polar solvent propylene glycol was used in all their measurements, and the temperatures were adjusted to yield solutions of moderate viscosity. Hence, the rates of solvent relaxation and rotational diffusion may have been comparable. As predicted from our model, only those molecules which are known to display significant solvent relaxation also showed the decreased rotational rate on red-edge excitation. To support our interpretation further we note that decreased rotation rates on red-edge excitation were not found for fluorophores which were not capable of hydrogen bonding to the solvent. For clarity and completeness we note the following details concerning the fluorophores examined by Valeur and Weber. Decreased rotational rates upon red-edge excitation were observed for indole and five other aromatic amines. Such an effect was not observed for perylene or 9-aminoacridine. Of course, perylene is nonpolar so no effect is expected. 9-Aminoacridine is polar, but like fluorescein, it does not display substantial solvent-dependent spectral shifts. In seeming contradiction to our interpretation, anthracene did show a decreased rotational rate on red-edge excitation. However, examination of its polarization spectrum in the absence of rotational diffusion indicates that the long-wavelength absorption band is composed of two or more electronic transitions. Hence, the decrease in the apparent rotational rate of anthracene was probably due to selective excitation of one of these states. It is well known that the apparent rotational rates of nonspherical fluorophores are dependent upon the angle between absorption and emission dipoles [16–18]. Hence, the red-edge excitation experiments of Valeur and Weber [39] are essentially in complete agreement with our model linking solvent relaxation and rotational diffusion. In spite of this seemingly good agreement we caution that the fluorescence lifetimes were not measured for the red-edge excitation conditions for most of the compounds studied. The inclusion of such additional data in calculation of the rotational rates may alter the outcome of their analysis. We note that other workers have suggested that fluorophores may have different rotational rates in the ground and the excited states [44,45]. This suggested difference was used to explain the possibly different rota-

tional rates measured by either transient polarized absorption or time-resolved emission. However, to the best of our knowledge, time-dependent changes in the diffusive rates have not be suggested previously.

In summary, we suggest that the time-dependent interactions between a polar fluorophore and its environment can result in complex decays of fluorescence anisotropy. For such fluorophores, the rotational correlation times may themselves be time dependent. Multiexponential decays of anisotropy are expected even for spherical molecules in homogeneous solutions. Such effects should also be considered in the interpretation of the anisotropies of fluorophores that are bound to biological macromolecules.

Appendix

A1. Derivation of the steady-state anisotropies

Consider a fluorophore randomly oriented in homogeneous solution, and excited with light which is linearly polarized along the Z axis (fig. 4). The equations describing the steady-state and time-resolved anisotropies can be derived using the mathematical procedures described earlier [46]. The kinetic equations describing the excited-state population of the F state fluorophores are

$$\frac{dF_{\parallel}}{dt} = \gamma_{\parallel} f(t) - (\Gamma + k + 4D_F) F_{\parallel} + 4D_F F_{\perp} \tag{A1}$$

$$\frac{dF_{\perp}}{dt} = \gamma_{\perp} f(t) + 2D_F F_{\parallel} - (\Gamma + k + 2D_F) F_{\perp} \tag{A2}$$

$F_{\parallel}$ and $F_{\perp}$ are the time-dependent populations of the individual polarized components of the F state,

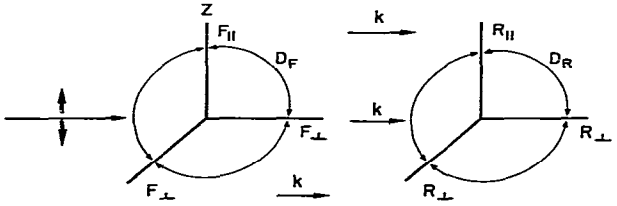

Fig. 4. F state and R state fluorophores in a coordinate system.

Γ the decay rate of the fluorophore, k the rate of relaxation to the R state, and $f(t)$ the time-dependent excitation. $\gamma_{\parallel}$ and $\gamma_{\perp}$ are the extent to which the light is absorbed by fluorophores aligned along each axis. For ventrically polarized and constant excitation $f(t)$ can be set equal to unity. The selection factors are

$$\gamma_{\parallel} = \tfrac{1}{3}(1 + 2r_0) \tag{A3}$$

$$\gamma_{\perp} = \tfrac{1}{3}(1 - r_0) \tag{A4}$$

where r_0 is the anisotropy in the absence of rotational diffusion. Using $\Gamma' = \Gamma + k$ and $D = d/dt$ the kinetic equations become

$$(D + \Gamma' + 4D_F) F_{\parallel} - 4D_F F_{\perp} = \delta_{\parallel} f(t) \tag{A5}$$

$$-2D_F F_{\parallel} + (D + \Gamma' + 2D_F) F_{\perp} = \delta_{\perp} f(t) \tag{A6}$$

For steady-state illumination the derivatives are equal to zero. Eqs. A1 and A2 become

$$\gamma_{\parallel} = (\Gamma + k + 4D_F) F_{\parallel} - 4D_F F_{\perp} \tag{A7}$$

$$\gamma_{\perp} = -2D_F F_{\parallel} + (\Gamma + k + 2D_F) F_{\perp} \tag{A8}$$

Application of Cramer's rule to eqs. A7 and A8 yields expression for $F_{\parallel}$ and $F_{\perp}$,

$$\Delta F_{\parallel} = \gamma_{\parallel}(\Gamma + k + 2D_F) + \gamma_{\perp} 4D_F \tag{A9}$$

$$\Delta F_{\perp} = \gamma_{\perp}(\Gamma + k + 4D_F) + \gamma_{\parallel} 2D_F \tag{A10}$$

where Δ is the determinant. For $D = 0$

$$\Delta = \begin{vmatrix} (\Gamma + k + 4D_F) & -4D_F \\ -2D_F & (\Gamma + k + 2D_F) \end{vmatrix} = (\Gamma + k)(\Gamma + k6D_F) \tag{A11}$$

For $d/dt \neq 0$ the determinant is $\Delta = (D + \Gamma + k + 6D_F)(D + \Gamma + k)$. Using eqs. A9 and A10 and the definition of the anisotropy

$$r_F = \frac{F_{\parallel} - F_{\perp}}{F_{\parallel} + 2F_{\perp}} \tag{A12}$$

one obtains the steady-state anisotropy of the F state;

$$r_F = \frac{r_0}{1 + 6D_F \tau_0} \tag{A13}$$

where $\tau_F = (\Gamma + k)^{-1}$ and $r_0 = \gamma_{\parallel} - \gamma_{\perp}$. Since the anisotropy is a ratio is not necessary to consider the emissive rate of the fluorophore.

Similar procedures may be used to obtain the

steady-state anisotropy of the R state. However, instead of the photoselection factors $\gamma_{\parallel}$ and $\gamma_{\perp}$, the pumping functions of $R_{\parallel}$ and $R_{\perp}$ are given by $kF_{\parallel}$ and $kF_{\perp}$, respectively. From fig. 4 it is evident that the differential equations for the R state are

$$\frac{dR_{\parallel}}{dt} = kF_{\parallel} - (\Gamma + 4D_R)R_{\parallel} + 4D_R R_{\perp} \quad \text{(A14)}$$

$$\frac{dR_{\perp}}{dt} = kF_{\perp} + 2D_R R_{\parallel} - (\Gamma + 2D_R)R_{\perp} \quad \text{(A15)}$$

For steady-state illumination the derivatives are zero, and the F_i values become equal to the steady-state values. Using Cramer's rule one obtains

$$\Delta R_{\parallel} = kF_{\parallel}(\Gamma + 2D_R) + kF_{\perp}4D_R \quad \text{(A16)}$$

$$\Delta R_{\perp} = kF_{\parallel}2D_R + kF_{\perp}(\Gamma + 4D_R) \quad \text{(A17)}$$

where Δ is the determinant for this system of eqs. A14 and A15. Adding and subtracting eqs. A16 and A17 according to the definition of the anisotropy yields

$$r_R = r_F \frac{\Gamma}{\Gamma + 6D_R} = \frac{r_F}{1 + 6D_R\tau_0} \quad \text{(A18)}$$

A2. Derivation of the time-resolved anisotropies

The time-resolved anisotropies can be obtained by solving eqs. A1 and A2, A14 and A15 subject to the appropriate boundary conditions. After the pulsed excitation $f(t) = 0$, and eqs. A1 and A2 yield

$$(D + \Gamma')(D + \Gamma' + 6D_F)F_{\parallel} = 0 \quad \text{(A19)}$$

$$(D + \Gamma')(D + \Gamma' + 6D_F)F_{\perp} = 0 \quad \text{(A20)}$$

where $\Gamma' = \Gamma + k$ and $D = d/dt$. The solutions to these homogeneous equations are of the form

$$F_i(t) = A_i e^{-\Gamma' t} + B_i e^{-(\Gamma' + 6D_F)t} \quad \text{(A21)}$$

The constants A_i and B_i can be determined by inserting the boundary condition at $t = 0$. These are $A_{\parallel} + B_{\parallel} = \gamma_{\parallel}$ and $A_{\perp} + B_{\perp} = \gamma_{\perp}$. This exercise yields the usual expressions for the time-resolved decays of the individual polarized components [47]. These are

$$F_{\parallel}(t) = \tfrac{1}{5}e^{-(\Gamma + k)t} + \tfrac{2}{5}r_0 e^{-(\Gamma + k + 6D_F)t} \quad \text{(A22)}$$

$$F_{\perp}(t) = \tfrac{1}{5}e^{-(\Gamma + k)t} - \tfrac{1}{5}r_0 e^{-(\Gamma + k + 6D_F)t} \quad \text{(A23)}$$

Substitution into the anisotropy definition (eq. A12) yields the usual single-exponential decay of fluorescence anisotropy (eq. 3).

The time-resolved anisotropy of the R state can be obtained in a similar manner. Using eqs. A14 and A15 one obtains

$$(D + \Gamma + 4D_R)R_{\parallel} - 4D_R R_{\perp} = kF_{\parallel} \quad \text{(A24)}$$

$$-2D_R R_{\parallel} + (D + \Gamma + 2D_R)R_{\perp} = kF_{\perp} \quad \text{(A25)}$$

The time-dependent values of $R_{\parallel}$ and $R_{\perp}$ are one again obtained using Cramer's rule.

$$\Delta R_{\parallel} = (D + \Gamma + 2D_R)kF_{\parallel} + 4D_R kF_{\perp} \quad \text{(A26)}$$

$$\Delta R_{\perp} = 2D_R kF_{\parallel} + (D + \Gamma + 4D_R)kF_{\perp} \quad \text{(A27)}$$

where $\Delta = (D + \Gamma)(D + \Gamma + 6D_R)$. The solution of these equations can be assumed to be a sum of that expected for the homogeneous portion ($F_i = 0$) and the nonhomogeneous portion ($F_i \neq 0$). Hence, the solution can be assumed to be of the form

$$R_i(t) = a_i e^{-\Gamma t} + b_i e^{-(\Gamma + 6D_R)t} + c_i e^{-(\Gamma + k)t} + d_i e^{-(\Gamma + k + 6D_F)t} \quad \text{(A28)}$$

In this expression the last two terms (nonhomogeneous) originate with the similar decays found for $F_{\parallel}$ and $F_{\perp}$ (eqs. A22 and A23). The terms a_i–d_i are obtained from the boundary conditions. At $t = 0$, $R_{\parallel}(t) = R_{\perp}(t) = 0$. Substitution of eq. A28 into eqs. A14 and A15, followed by equating of similar exponential terms, yields expression for the polarized components of the R state. These expressions are

$$R_{\parallel}(t) = R(t) + \tfrac{2}{5}G(t) \quad \text{(A29)}$$

$$R_{\perp}(t) = R(t) - \tfrac{1}{5}G(t) \quad \text{(A30)}$$

where

$$R(t) = \tfrac{1}{5}e^{-\Gamma t} - \tfrac{1}{5}e^{-(\Gamma + k)t} \quad \text{(A31)}$$

$$G(t) = \frac{kr_0}{(6D_R - k - 6D_F)}\left[e^{-(\Gamma + k + 6D_F)t} - e^{-(\Gamma + 6D_R)t}\right] \quad \text{(A32)}$$

Using the definition of the anisotropy these equations (eqs. A29–A32) yield eq. 6.

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