

Local motions of fluorophores

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Summary. We further describe the general formulation of fluorescence depolarization in which the depolarization results from exchanges between a number of oscillator orientations in thermal equilibrium¹. Temperature and pressure affect the polarization by changing the relative populations of the allowed orientations as well as the rate of exchanges during the fluorescence lifetime. This treatment satisfactorily describes the limited motions that fluorophores undergo when they are either attached to a macromolecule such as the local tryptophan rotations in proteins, or embedded in a biological membrane.

Key words: Fluorescence depolarization – Viscosity – Membranes

It does not seem possible to draw up a completely general description of the depolarization of the fluorescence of an electronic oscillator attached to a macromolecule when it is due to both the free motion of the macromolecule and certain limited local motions of the fluorophore. Similar difficulties arise when the resistance offered by the medium to the rotational diffusion of the fluorophore is highly heterogeneous, as in biological membranes. These circumstances have inspired a number of *ad hoc* treatments of the depolarization that are useful in particular cases, in which the fluorophore forms part of a rigid macromolecule (Gottlieb and Wahl 1963), a flexible polymer (Valeur and Monnerie 1976), a micelle (Shinitzky et al. 1971) or a membrane (Kawato et al. 1977; Zannoni 1981; Szabo 1984).

In the case of globular polymers, the first problem to be solved is the separation of the local motions of the fluorophore from those of the whole particle. In peptides and proteins this separation can be satisfactorily

accomplished by observations of the temperature dependence of the fluorescence polarization of their solutions in 60%–90% glycerol/water mixtures (Weber et al. 1984; Scarlata et al. 1984; Rholam et al. 1984). In these solutions the solvent viscosity is sufficient effectively to abolish the overall motions of particles of a few kilodaltons in the range of about -30°C to $+30^{\circ}\text{C}$. Any depolarization observed under these conditions must be attributed to local fluorophores rotations.

The dependence of the flow viscosity of a homogeneous medium, $\eta(T)$, over the indicated range of temperature is appropriately described by an expansion of the form

$$\eta(T) = \eta(0) \exp[b(T - T_0)] \quad (1)$$

where $\eta(0)$ is the viscosity at a temperature T_0 conveniently chosen to be close to the midpoint of the temperature range and b is the thermal coefficient of the viscosity. Introducing the value of $\eta(T)$ from Eq. (1) into the Perrin equation (Perrin 1926) gives

$$[a_0/a(T)] - 1 = RT\tau/[V\eta(0) \exp[b(T - T_0)]] \quad (2)$$

where $a(T)$ is the stationary anisotropy of emission at temperature T , a_0 the limiting anisotropy, and τ the fluorescence lifetime. In logarithmic form Eq. (2) gives

$$Y = \ln[a_0/a(T)] - 1 - \ln[RT\tau/V\eta(0)] = b(T - T_0). \quad (3)$$

It has been experimentally shown (Weber et al. 1984) that the plot of Y , the left-hand side of (3), against $T - T_0$ for several fluorophores, including tyrosine and tryptophan, yield straight lines with a slope that reproduces quite closely the thermal coefficient of the flow viscosity of Eq. (1). Scarlata et al. (1984) have demonstrated that for a variety of peptides with molecular masses in the range of 1–3 kDa these Y plots consist of two straight-line regions with slopes b_s and b_u that abruptly change into each other at a critical temperature T_c . Rholam et al. (1984) found that several single-chain proteins show very similar behaviour. More recently the Y plots have been used by Royer and Alpert (1987) to describe the local motions of the porphyrin in myoglobin^{DesFe} and hemoglobin^{DesFe}. They also found

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abrupt changes in slope: in myoglobin^{DesFe} two very similar slopes are separated by an interval of constant polarization covering some 10 K. A tentative interpretation of the abrupt changes in slope in the Y plots in terms of equilibria between differently solvated forms of the peptide was given in the initial publication of Scarlata et al. (1984). More recently one of us (Weber 1989) has developed a novel approach to the restricted rotations of a fluorophore attached to a particle that does not undergo appreciable overall rotations during the fluorescence lifetime. It appears able to give a very satisfactory explanation of the existing observations. It also predicts the possible existence of thermal fluorescence repolarization, an effect which has not yet been observed.

The existing descriptions of the fluorescence depolarization by local rotations follow the original approach of Perrin (1926), in that a fixed geometry is postulated and the depolarization is calculated from the expected motion of the oscillators between excitation and emission. In the new approach the depolarization is assumed to result from reorientational jumps between a number N of permitted oscillator positions populated as determined by $N-1$ ground-state thermodynamic equilibria between pairs of directions. No explicit assumptions are made regarding the relative orientations of the oscillators. One of the simplest situations is shown in Fig. 1, a three-orientations model, sufficient however to describe the presently known observations. The relative orientations 1, 2, 3 determine the exchange anisotropies a_{12} and a_{23} . At any given temperature the allowed orientations exist as fractions $f_1(T)$, $f_2(T)$ and $f_3(T)$ of the total so that $f_1 + f_2 + f_3 = 1$. The effects of temperature demand the specification of the equilibria $K_1 = f_1/f_2$ and $K_2 = f_2/f_3$ at a given temperature as well as the standard enthalpies ΔH_1 and ΔH_2 of the respective equilibria. Likewise we require to specify the rates of the orientational transitions $k_{12} = k_{21}f_2/f_1$ and $k_{23} = k_{32}f_3/f_2$ and their dependence upon the temperature. Because of the interdependence of equilibrium constants of pairs of orientations and their rates of mutual interconversion, the dependence of $\langle a \rangle$, the observable anisotropy, has the relatively simple form:

$$a_0 - \langle a \rangle = (a_0 - a_{12}) 2f_1 k_{12} \tau / [1 + k_{12} \tau (1 + f_1/f_2)] + (a_0 - a_{23}) 2f_2 k_{23} \tau / [1 + k_{23} \tau (1 + f_2/f_3)]. \quad (4)$$

When the exchanges of orientation are very fast, that is $k_{12} \tau \gg 1$ and $k_{23} \tau \gg 1$, Eq. (4) adopts the even simpler form

$$a_0 - \langle a \rangle = (a_0 - a_{12}) f_{12}^H + (a_0 - a_{23}) f_{23}^H \quad (5)$$

with

$$f_{12}^H = 2/(1/f_1 + 1/f_2) \quad f_{23}^H = 2/(1/f_2 + 1/f_3) \quad (6)$$

where f_{12}^H and f_{23}^H are the harmonic means of the fractional populations involved in the two separate equilibria. It is noticeable that Eq. (5) does not contain the kinetic parameters k_{12} and k_{23} . The stationary anisotropies then reflect directly the thermodynamic equilibria between the orientations. Orientation exchanges that

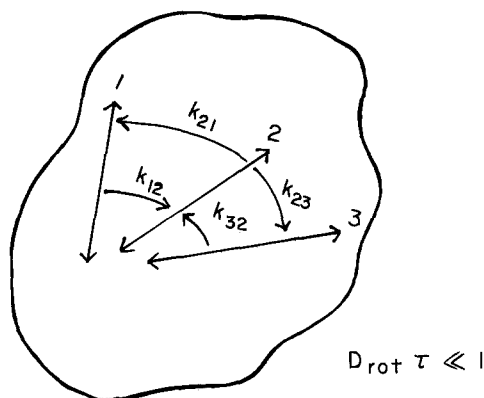


Fig. 1. Schematic representation of the exchanging oscillator directions in a particle that is effectively motionless during the fluorescence lifetime

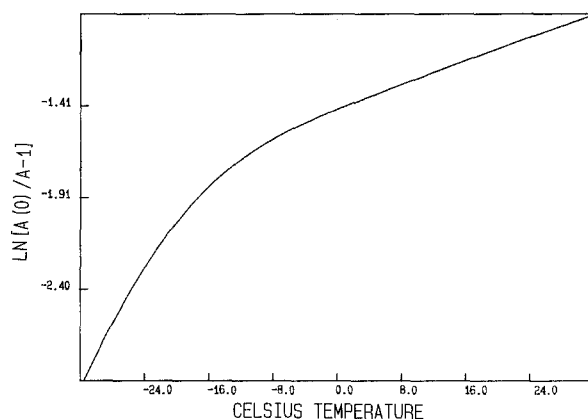


Fig. 2. Y plot according to Eq. (5) with $a_0 = 0.35$, $a_{12} = 0.30$, $a_{23} = 0.05$, $\Delta H_{12} = -22 RT_0$, $\Delta H_{23} = -8 RT_0$, $f_1/f_2(0^\circ \text{C}) = 0.25$, $f_2/f_3(0^\circ \text{C}) = 7$. To be compared with the plots of Figs 1 and 2 of Scarlata et al. (1984) and Figs 1 and 2 of Rholam et al. (1984). In Figs 2 and 3 computations have been made by Eq. (5) rather than Eq. (4) to reduce the number of parameters and to reach the condition of thermodynamic rather than kinetic significance

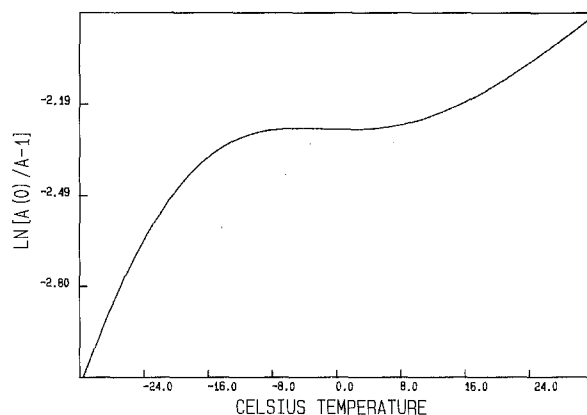


Fig. 3. Y plot with the same parameters of Fig. 2 except that $\Delta H_{12} = -20 RT_0$, $\Delta H_{23} = -12 RT_0$, $f_1/f_2(0^\circ \text{C}) = 0.3$, $f_2/f_3(0^\circ \text{C}) = 28$. To be compared with Fig. 4 (first panel) of Royer and Alpert (1987)

are fast in comparison with the rate of emission occur in tryptophan residues in proteins (Gratton et al. 1986), and in fact Eq. (5) finds ready application to these cases (Figs 2 and 3). We note that in this treatment the exchange anisotropies enter as adjustable parameters so that the fluorophore rotations are not given a precise geometrical context. The loss of information that this necessarily implies is balanced by the simplicity of the resulting equations, the reduction in the number of arbitrary parameters involved in the calculations and the establishment of a direct link between the thermodynamic parameters of the ground-state orientational equilibria and the observed depolarization.

The time-dependent depolarization

The time-dependent anisotropy $a(t)$ can be calculated by the inverse Laplace transform of Eq. (4). We thus require functions $g_1(t)$ and $g_2(t)$ with Laplace transforms that generate the coefficients of τ in the terms in $a_0 - a_{12}$ and $a_0 - a_{23}$ in Eq. (4). We obtain

$$\begin{aligned} g_1(t) &= f_{12}^H \{1 - \exp[-(1 + f_1/f_2)k_{12}t]\} \\ g_2(t) &= f_{23}^H \{1 - \exp[-(1 + f_2/f_3)k_{23}t]\} \end{aligned} \quad (7)$$

and

$$a_0 - a(t) = (a_0 - a_{12})g_1(t) + (a_0 - a_{23})g_2(t). \quad (8)$$

For times much longer than the reorientation times, that is when $k_{12}t \gg 1$ and $k_{23}t \gg 1$, the anisotropy takes on a virtually constant values. Fig. 4 shows a plot of $a(t)$ against the logarithm of the time for a case likely to be found in proteins and peptides (Gratton et al. 1986). We assumed $k_{12} = k_{23} = \tau/10$ and thermal coefficients of the reorientations 0.07 K^{-1} and 0.03 K^{-1} , respectively.

Rotational motions in membranes

It is noticeable in Fig. 4 that the long-time-constant anisotropy decreases as the temperature is raised, an ob-

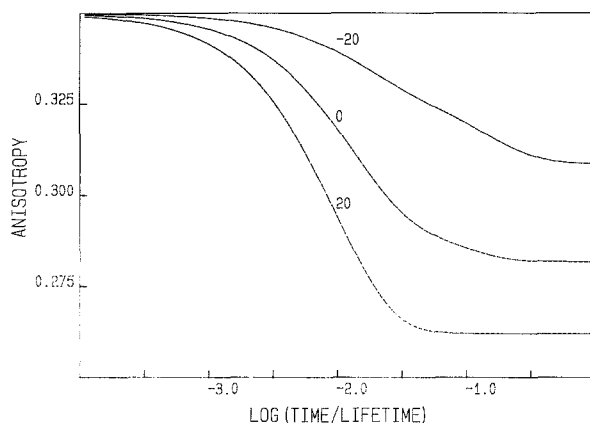


Fig. 4. Plots of the anisotropy against $\log_{10} t$, in units of the fluorescence lifetime τ , computed from Eqs. (7) and (8). Same parameters as those in Fig. 2, with the addition of $k_{12}\tau = k_{23}\tau = 10$ and thermal coefficients of the $1 \rightarrow 2$ and $2 \rightarrow 3$ transitions of 0.07 K^{-1} and 0.03 K^{-1} . The three curves are for $-20, 0$ and 20°C

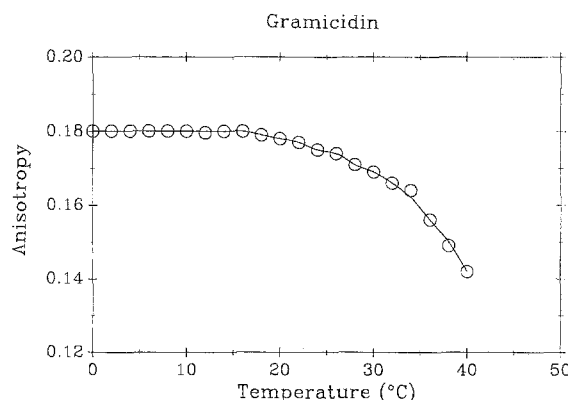


Fig. 5. Experimental data for the temperature dependence of the anisotropy of the fluorescence from gramicidin dissolved in dimyristoylglycerophosphocholine bilayers (from Scarlata 1988), and the fitted curve calculated by Eq. (4), with parameters given in text

servation that has repeatedly been made in studies of the real time decay of the fluorescence of fluorophores incorporated in biological membranes. To describe such effects Kawato et al. (1977) introduced the additional parameter a_{inf} which is physically determined by a potential barrier that limits the rotations within a prescribed angular amplitude. In our proposed treatment the long-time-constant anisotropy results from the equilibration of the several allowed orientations within the fluorescence lifetime and not from the existence of a physical rotational barrier. Its dependence on the temperature is not arbitrary but is determined by the enthalpies of the orientational equilibria involved. A discussion of the application of the parametric approach to membranes is given by Scarlata (1989). Its application to the depolarization of the fluorescence from gramicidin incorporated into bilayers is shown in Fig. 5. In applications to peptides and proteins, the enthalpy changes associated with the reorientations $1 \leftrightarrow 2$ and $2 \leftrightarrow 3$ are difficult to specify precisely as they depend greatly upon the fractional values f_1, f_2, f_3 assumed for a given temperature. The unusual anisotropy dependence upon the temperature observed for gramicidin (Fig. 5) permits one to determine the change in enthalpy associated with the change in slope in the Y plot (Scarlata et al. 1984), while virtually only two orientations appear to contribute appreciably to the polarization. The curve in the figure has been calculated assuming $\Delta H_{12} = -40 \text{ RT}$, $\Delta H_{23} = -10 \text{ RT}$, $a_0 = 0.273$, $a_{12} = 0.186$, $a_{23} = 0.0$, $f_1/f_2 = 150$, $f_2/f_3 = 100$, $k_{12}\tau = 1$ and $k_{23}\tau = 0.3$. We chose to present the simple plot of anisotropy against temperature instead of the Y plot since it is more appropriate to demonstrate the coincidence of experimental and fitted values. The figure shows the predominance of a unique orientation, or set of rapidly exchanging orientations, over a considerable range of temperatures, a consequence of the strong interactions of the tryptophan and other parts of the molecule, that predicate an unusually large enthalpy of reorientation.

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