EFFECT OF LIBRATIONAL MOTION ON FLUORESCENCE DEPOLARIZATION AND NUCLEAR MAGNETIC RESONANCE RELAXATION IN MACROMOLECULES AND MEMBRANES

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ABSTRACT The theory of fluorescent emission anisotropy [$r(t)$] of a cylindrical probe in a membrane suspension is developed. It is shown, independent of any model, that the limiting anisotropy [$r(\infty)$] is proportional to the square of the order parameter of the probe. The order parameter determines the first nontrivial term in the expansion of the equilibrium orientational distribution function of the probe in a series of Legendre polynomials. Following Kinosita, Kawato, and Ikegami, the motion of the probe is described as diffusion ("wobbling") within a cone of semiangle θ_0. Within the framework of this model, an accurate single-exponential approximation for $r(t)$ is considered. An analytic expression relating the effective relaxation time, which appears in the above approximation, to θ_0 and the diffusion coefficient for wobbling is derived. The model is generalized to the situation where the probe is attached to a macromolecule whose motion cannot be neglected on the time scale of the fluorescence experiment. Finally, by exploiting the formal similarity between the theory of fluorescence depolarization and ^{13}C-NMR dipolar relaxation, expressions for T_1, T_2, and the nuclear Overhauser enhancement are derived for a protonated carbon which is nonrigidly attached to a macromolecule and undergoes librational motion described as diffusion on a spherical "cap" of semiangle θ_0.

INTRODUCTION

Time-resolved fluorescence depolarization and ^{13}C nuclear magnetic relaxation (NMR) studies of probes attached to macromolecules or embedded in membranes can provide detailed information about the internal dynamics of these systems. Suitable probes are often available naturally (e.g., amino acid side chains) but can be introduced artificially. The observables (the emission anisotropy or the relaxation times) depend both on the overall motion of the host as well as on the internal motion of the probe. If the host is a large membrane fragment, the

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Note added in proof: After our work had been submitted for publication, a paper by M. P. Heyn entitled "Determination of lipid order parameters and rotational correlation times from fluorescence depolarization experiments" appeared (*FEBS. [Fed. Eur. Biochem. Soc.] Lett.* **108**:359 [1979]). Heyn points out that $r(\infty)/r(0) = S^2$ for probes with parallel μ_a and μ_e which coincide with the long axis ($\hat{\mu}$) of the molecule and stresses the importance of this relation. Our paper shows that this equation is valid even when μ_a and μ_e are not parallel as long as one of these vectors coincides with $\hat{\mu}$, as can be seen by dividing Eq. 8 by Eq. 5. Moreover, we present the relationship between $r(\infty)/r(0)$ and the order parameter [i.e., $r(\infty)/r(0) = P_2(\cos\theta_e) P_2(\cos\theta_a) S^2/P_2(\cos\delta)$; see Eqs. 5 and 9] which is valid in the general case where μ_a, μ_e, and $\hat{\mu}$ all point in different directions. We thank Dr. Heyn for sending us a copy of his paper.

overall motion can be neglected and, for example, the decay of the fluorescence depolarization anisotropy is determined only by the motion of the probe within the lipid bilayer.

The motion of a probe in an orienting environment such as a membrane is clearly different from what it is in solution. In a membrane the probe does not assume all possible orientations with equal probability, and consequently the fluorescence emission anisotropy [$r(t)$] does not decay to zero at long times [i.e., $r(\infty) \neq 0$]. In an important paper, Kinosita et al. (1) proposed a simple model to describe the motion of a cylindrical probe in a membrane. They assumed that the unique symmetry axis of the probe diffuses freely ("wobbles") within a cone of semiangle θ_0. Thus, in this model, the equilibrium orientational distribution function is a constant for $0 \leq \theta \leq \theta_0$ and zero otherwise. For the special case that either the emission or absorption dipole of the probe is coaxial with its unique symmetry axis, they derived a simple expression for the limiting anisotropy [$r(\infty)$] in terms of θ_0, thus allowing the extraction of the cone semiangle from the measurement of $r(\infty)$. Lakowicz et al. (2, 3) objected to this procedure because it is clearly model-dependent. Partly based on the work of Weber (4), they proposed a model-independent interpretation (to be described subsequently) for $r(\infty)$. One of the purposes of this paper is to present a different model-independent interpretation which we feel is to be preferred to theirs. We show that $r(\infty)$ is directly proportional to the square of the order parameter of the probe. The concept of an order parameter has long played a central role in electron spin and nuclear magnetic resonance studies of membranes (5). For cylindrical fluorescent probes in membranes, the order parameter determines the first nontrivial term in the expansion of the equilibrium orientational distribution of the probe in a series of Legendre polynomials. Thus the determination of the order parameter provides model-independent information about this distribution.

The limiting anisotropy depends only on the equilibrium orientational distribution of the probe and thus contains no dynamical information. Kinosita et al. (1), within the framework of the diffusion in a cone model, obtained an exact expression for $r(t)$ as an infinite sum of exponentials whose amplitudes and time constants (relaxation times) depend on θ_0 and the wobbling diffusion constant, D_w. Moreover, they presented a simple but accurate approximation to $r(t)$ containing a single exponential with an effective relaxation time (τ_{eff}) which was inversely proportional to D_w. They displayed the dependence of the proportionality constant on θ_0 graphically. In this paper we shall clarify the nature of their approximation and derive a closed-form analytic expression for the functional dependence of τ_{eff} on θ_0. Our expression readily allows the determination of the wobbling diffusion coefficient (D_w) from the measurement of the effective relaxation time and $r(\infty)$ within the framework of the model.

In addition, we extend the analysis to include the overall diffusive motion of the host. Such an extension is required for the analysis of the experiments of Munro et al. (6) on the fluorescence depolarization of tryptophan residues in proteins. We discuss the assumptions under which their "empirical" expression for $r(t)$ is valid and present the correct relationship between the effective relaxation time for wobbling and D_w, θ_0.

The theory of fluorescence depolarization is quite analogous to the theory of ^{13}C-NMR dipolar relaxation. The correspondence is especially transparent when both are formulated using Wigner rotation matrices (7). The ^{13}C-NMR analogue of the diffusion in a cone model of Kinosita et al. (1) for fluorescence depolarization (when generalized to incorporate the overall motion of the host) is a protonated carbon nonrigidly attached to a macromolecule

moving in such a way that the ^{13}C—H vector diffuses within a cone of semiangle θ_0. This is the simplest possible model which can be used to describe librational motion of an α-carbon in a protein. Howarth (8) first approached this problem by adapting the model of Woessner (9), who considers a carbon rigidly attached to a macromolecule with a single internal rotation (e.g., a methyl group). This amounts to assuming that the ^{13}C—H vector diffuses on the surface of a cone of semiangle θ_0, i.e., the angle θ between the vector and the symmetry axis is constant rather than taking on all values between zero and θ_0. More recently, Howarth (10) developed a less artificial model by considering a finite number (n) of possible values of θ and then (approximately) taking the limit $n \rightarrow \infty$. Here we establish the relation between his approximate spectral density and the one obtained within the diffusion in a cone model. In addition, we make explicit the conditions under which his result is valid.

Briefly, the outline of this paper is as follows.

First, we consider the model-independent interpretation of the limiting fluorescence emission anisotropy [$r(\infty)$] of probes embedded in membrane suspensions. Second, we consider the time-dependence of $r(t)$ within the framework of the diffusion in a cone model of Kinosita et al. (1), focusing on the single exponential approximation they introduced, and we derive a closed-form expression for the effective relaxation time in terms of the cone angle and the wobbling diffusion coefficient. Third, we generalize the expression for $r(t)$ to incorporate the overall diffusive motion of the host. Finally, we exploit the formal analogy between fluorescence depolarization and ^{13}C-NMR dipolar relaxation and obtain expressions for T_1, T_2, and the nuclear Overhauser enhancement of a protonated carbon which can wobble on the surface of a macromolecule. To keep this paper as accessible as possible, we relegated most of the derivations to appendices.

FLUORESCENCE EMISSION ANISOTROPY IN MEMBRANE SUSPENSIONS

We consider an immobile, isotropic suspension of membrane "fragments" doped with a fluorescent probe of cylindrical symmetry. Since the membrane "fragments" are assumed to take on all possible orientations within the macroscopic sample on which the experiment is performed, the theory developed below is not only applicable to planar fragments but also to spherical membrane vesicles. The theory applicable to macroscopically oriented membranes has been recently considered by one of us (11) somewhat along the lines of the present paper.

We let $\hat{\mu}$ be a unit vector directed along the unique (C_∞) axis of the probe (Fig. 1). We assume that the host has uniaxial symmetry so that the equilibrium orientational distribution of the probe depends only on the angle (θ in Fig. 1) between $\hat{\mu}$ and the symmetry axis of the membrane (Z_M in Fig. 1). We let $p_{eq}(\theta)$ be the normalized equilibrium orientation distribution function, i.e.,

$$\int_0^{2\pi} d\phi \int_0^{\pi} \sin\theta d\theta p_{eq}(\theta) = 1. \tag{1}$$

The orientation of the membrane suspension in the laboratory is described by the coordinate system $X_L Y_L Z_L$. We consider a fluorescence emission experiment in which light propagates along the Y_L axis and is polarized along the Z_L axis. The emitted light is monitored along the X_L axis. If $I_{ij}(t)$ is the intensity of emitted light at time t with polarization along the j axis when the absorbed light is polarized along the i axis, the emission anisotropy, $r(t)$, is

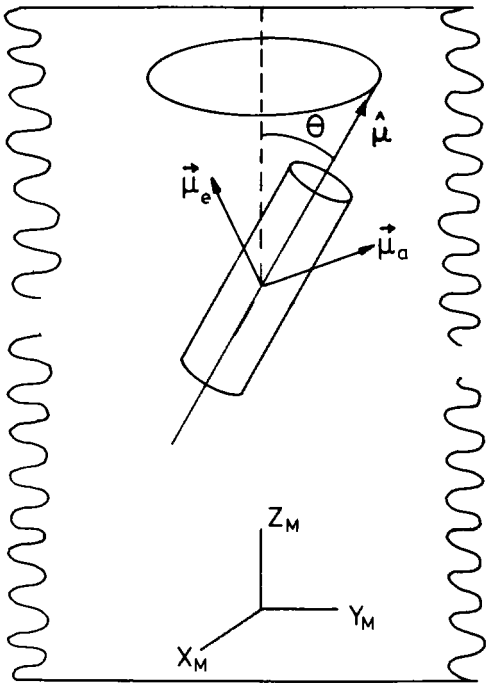

FIGURE 1 Schematic representation of a cylindrical fluorescent probe embedded in a membrane.

$$r(t) = \frac{I_{zz}(t) - I_{zy}(t)}{I_{zz}(t) + 2I_{zy}(t)} \tag{2}$$

The time-dependence of $r(t)$ results solely from the motion of the probe within the membrane, since we have assumed that the membrane fragments or vesicles are immobile. Subsequently we shall extend the analysis to the situation where the probe is attached to a system (e.g., a macromolecule) whose motion cannot be neglected on the time scale of the experiment. In the case that either the emission dipole (μ_e) or the absorption dipole (μ_a) of the probe point along its unique symmetry axis (i.e., $\hat{\mu}$), $r(t)$ can be expressed in terms of a correlation function (1, 12) as[1]

$$r(t) = \frac{2}{5} P_2(\cos\delta)\langle P_2[\hat{\mu}(0) \cdot \hat{\mu}(t)]\rangle, \tag{3}$$

where δ is the angle between μ_a and μ_e and $P_2(x) = (3x^2-1)/2$ is the second Legendre polynomial. In Eq. 3 the unit vector $\hat{\mu}(t)$ specifies the orientation of the probe at time t in a coordinate system attached to the membrane ($X_M Y_M Z_M$ in Fig. 1). The angular brackets denote an equilibrium average, i.e.,

$$\langle(\ldots)\rangle = \int_0^{2\pi} d\phi \int_0^{\pi} \sin\theta d\theta(\ldots)p_{eq}(\theta). \tag{4}$$

[1]It is not absolutely clear from references 1 and 12 that this equation is correct under the stated conditions. However, it can easily be derived by adapting the method outlined in Appendix B.

To obtain the explicit time-dependence of $r(t)$ from Eq. 3 one must evaluate the correlation function using some model for the dynamics of the probe. However, $r(0)$ and $r(\infty)$ can be obtained in a model-independent way. $r(0)$ can be found trivially as follows:

$$r(0) = \frac{2}{5} P_2(\cos \delta) \langle P_2[\hat{\mu}(0) \cdot \hat{\mu}(0)] \rangle = \frac{2}{5} P_2(\cos \delta). \tag{5}$$

To determine $r(\infty)$ we use the addition theorem for spherical harmonics and rewrite Eq. 3 as:

$$r(t) = \frac{2}{5} P_2(\cos \delta) \sum_{m=-2}^{2} \langle C_{2m}^*[\theta(0), \phi(0)] C_{2m}[\theta(t), \phi(t)] \rangle, \tag{6}$$

where $C_{lm}(\theta, \phi)$ are the modified spherical harmonics of Brink and Satchler (13) and θ and ϕ specify the orientation of $\hat{\mu}$ in the $X_M Y_M Z_M$ frame. Using the property of correlation functions

$$\lim_{t \to \infty} \langle A(0) B(t) \rangle = \langle A \rangle \langle B \rangle \tag{7}$$

it follows that

$$r(\infty) = \frac{2}{5} P_2(\cos \delta) \sum_{m=-2}^{2} \langle C_{2m}^*(\theta, \phi) \rangle \langle C_{2m}(\theta, \phi) \rangle$$

$$= \frac{2}{5} P_2(\cos \delta) | \langle C_{20} \rangle |^2 = \frac{2}{5} P_2(\cos \delta) \langle P_2(\cos \theta) \rangle^2, \tag{8}$$

where we have used the fact that the equilibrium orientation distribution function is independent of the azimuthal angle ϕ. This result is consistent with Eq. 25 of the work of Kinosita et al. (1). Eq. 8 can be generalized to the situation where neither μ_a or μ_e point along the unique symmetry axis of the probe. In this case (A. Szabo; to be published) the result is

$$r(\infty) = \frac{2}{5} P_2(\cos \theta_e) P_2(\cos \theta_a) \langle P_2(\cos \theta) \rangle^2, \tag{9}$$

where θ_a and θ_e are the angles between μ_a and μ_e and the unique symmetry axis of the probe. Eq. (5) still holds in this case. The only assumption used in obtaining Eq. 9 is that the equilibrium distribution of the probe depends only on θ. Eq. 9 is valid not only for a "rod-shaped" probe as shown in Fig. 1, but also for a "disk-shaped probe." The most probable orientation of a rod-shaped probe is such that $\hat{\mu}$ is perpendicular to the plane of the membrane, while for a disk-shaped probe it is parallel to this plane.

The importance of Eqs. 8 and 9 is that they relate the limiting behavior of the fluorescence anisotropy to the order parameter (S) of the probe (14) defined as

$$S = \langle P_2(\cos \theta) \rangle. \tag{10}$$

The order parameter plays an important role in electron spin resonance and NMR studies of membranes (5). The significance of the order parameter is that it determines the first nontrivial term in the expansion of the equilibrium of the orientational distribution in a series

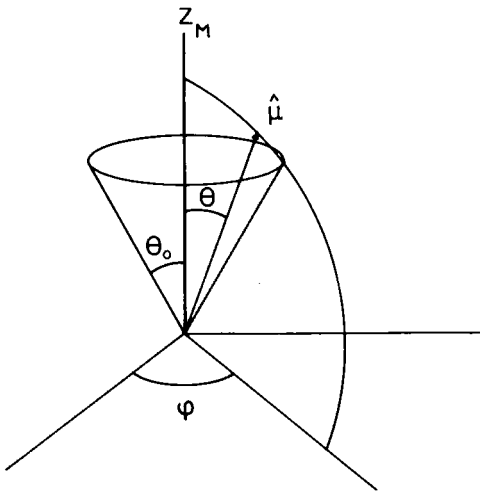

FIGURE 2 Diffusion-in-a-cone model. The unique symmetry axis of the probe ($\hat{\mu}$) wobbles within a cone of semiangle θ_0.

of Legendre polynomials, i.e.,

$$p_{eq}(\theta) = \sum_{l=0}^{\infty} \frac{4l+1}{4\pi} \langle P_{2l}(\cos\theta)\rangle P_{2l}(\cos\theta) = \frac{1}{4\pi} + \frac{5}{4\pi}\langle P_2(\cos\theta)\rangle P_2(\cos\theta) + \cdots \quad (11)$$

Thus the order parameter provides model-independent information about the equilibrium orientational distribution or, equivalently, the potential in which the probe moves.

Alternately, $r(\infty)/r(0)$ can be interpreted within the framework of some model. Kinosita et al. (1, 15) considered a model in which $\hat{\mu}$ can diffuse freely within a cone of semiangle θ_0 (Fig. 2). This corresponds to the normalized equilibrium distribution

$$\begin{aligned} p_{eq}(\theta) &= [2\pi(1-\cos\theta_0)]^{-1} \qquad 0 \leq \theta \leq \theta_0 \\ &= 0 \qquad\qquad\qquad\qquad\quad \theta > \theta_0 \end{aligned} \quad (12)$$

Using this equilibrium distribution it immediately follows that

$$S = \langle P_2(\cos\theta)\rangle = \frac{1}{2}\cos\theta_0(1+\cos\theta_0). \quad (13)$$

Combining this result with Eqs. 5 and 8 (which is valid only if either μ_a or μ_e point along $\hat{\mu}$) we have

$$\frac{r(\infty)}{r(0)} = \langle P_2(\cos\theta)\rangle^2 = \left[\frac{1}{2}\cos\theta_0(1+\cos\theta_0)\right]^2 \equiv A_\infty, \quad (14)$$

where for future reference, we introduced a new notation (A_∞) for $r(\infty)/r(0)$ within the model. Thus the measurement of $r(\infty)/r(0)$ can be used to obtain the cone angle θ_0. Lakowicz et al. (2, 3) noted that this interpretation of $r(\infty)/r(0)$ is clearly model-dependent. The model-independent interpretation they proposed, which is quite different from the one involving the

order parameter, can be described as follows. Using the expression for $r(t)$ in terms of a correlation function (Eq. 3) along with Eq. 5, it is clear that

$$\frac{r(\infty)}{r(0)} = \lim_{t\to\infty} \langle P_2[\cos \alpha(t)] \rangle = \lim_{t\to\infty} \frac{3}{2} \langle \cos^2 \alpha(t) \rangle - \frac{1}{2}, \tag{15a}$$

where $\alpha(t)$ is the angle between $\hat{\mu}(0)$ and $\hat{\mu}(t)$. If one defines (4) the angle $\bar{\alpha}$ by the relation $\cos^2 \bar{\alpha} = \lim_{t\to\infty} \langle \cos^2 \alpha(t) \rangle$, then Eq. 15a becomes

$$\frac{r(\infty)}{r(0)} = P_2(\cos \bar{\alpha}). \tag{15b}$$

Thus this equation is merely a restatement of the relation between the emission anisotropy and the correlation function at long times. Lakowicz et al. (3) refer to $\bar{\alpha}$ (actually θ in their paper) as "the average angular distribution of the probe at times long compared with the fluorescent lifetime," a terminology we find imprecise. We believe that their analysis is not particularly illuminating because it brings one no closer to the goal of learning something about the equilibrium orientational distribution $[p_{eq}(\theta)]$ of the probe. Our analysis goes further since we actually evaluate the limit in Eq. 15a for an arbitrary $p_{eq}(\theta)$ and show that it is proportional to the square of the order parameter S(Eqs. 8 and 9). S in turn determines the first nontrivial expansion of the orientational distribution in a series of Legendre polynomials (Eq. 11). This is the most unique information that can be extracted from the measurement of $r(\infty)/r(0)$ about $p_{eq}(\theta)$.

TIME-DEPENDENCE OF THE EMISSION ANISOTROPY IN MEMBRANE SUSPENSIONS: DIFFUSION IN A CONE

To obtain the time-dependence of $r(t)/r(0)$ for the situation where either the emission or absorption dipoles of the probe point along its C_∞ axis one must evaluate the correlation function $\langle P_2[\hat{\mu}(0) \cdot \hat{\mu}(t)] \rangle$ (see Eqs. 3 and 5) using some model of the dynamics of the probe. For example, one can assume that the probe diffuses in a potential $V(\theta)$ and thus the time-dependent probability distribution satisfies the Smoluchowski equation. The simplest choice for $V(\theta)$ is $V(\theta) = 0$ for $0 \le \theta \le \theta_0$ and $V(\theta) = \infty$ otherwise; i.e., the C_∞ axis of the probe can diffuse freely in a cone of semiangle θ_0 (Fig. 2). The equilibrium orientational distribution consistent with this model is given by Eq. 12. This model has been considered by Kinosita et al.(1, 15) who showed that

$$\frac{r(t)}{r(0)} = \langle P_2[\hat{\mu}(0) \cdot \hat{\mu}(t)] \rangle = \sum_i A_i e^{-D_w t/\sigma_i}, \tag{16}$$

where D_w is the "wobbling" diffusion constant. The coefficients A_i and σ_i cannot be expressed as closed analytical functions of θ_0. Kinosita et al. (1) displayed the functional dependence of these coefficients graphically. In addition, Kinosita et al. (1) presented a convenient approximate expression for $r(t)/r(0)$,

$$\left[\frac{r(t)}{r(0)}\right]_{\text{approx}} = A_\infty + (1 - A_\infty) \exp(-D_w t/\langle \sigma \rangle), \tag{17}$$

where $A_\infty = r(\infty)/r(0)$ is given by Eq. 14 and

$$\langle \sigma \rangle = \sum_{i \neq \infty} A_i \sigma_i / (1 - A_\infty). \tag{18}$$

They showed that Eq. 17 was virtually indistinguishable from the exact result (Eq. 16) for cone angles which are $<50°$.

We now wish to clarify the nature of the approximation which leads to Eq. 17 and to derive a relatively simple closed-form expression for $\langle \sigma \rangle$ in terms of θ_0. For the sake of generality, we consider a correlation function $C(t)$. Suppose we know its exact value at $t = 0$ [i.e., $C(0)$] and at $t = \infty$ [i.e., $C(\infty)$]. Then the simplest expression for the time-dependence of $C(t)$, which is exact at $t = 0$ and at $t = \infty$ must have the form:

$$C_{\text{approx}}(t) = C(\infty) + [C(0) - C(\infty)]\exp(-t/\tau_{\text{eff}}), \tag{19}$$

where τ_{eff} is an effective relaxation time. To determine τ_{eff} we can insist that $C_{\text{approx}}(t)$ shares with the exact $C(t)$ some additional property. A reasonable, but not unique, choice is to require that the time integral of $C_{\text{approx}}(t) - C(\infty)$ be exact, i.e.,

$$\int_0^\infty [C_{\text{approx}}(t) - C(\infty)]dt \equiv \tau_{\text{eff}}[C(0) - C(\infty)] = \int_0^\infty [C(t) - C(\infty)]dt. \tag{20}$$

Letting $C(t) \equiv r(t)/r(0) = \langle P_2[\hat{\mu}(0) \cdot \hat{\mu}(t)] \rangle$, since $r(\infty)/r(0) = \langle P_2(\cos\theta) \rangle^2$ condition (20) becomes

$$\tau_{\text{eff}}[1 - \langle P_2(\cos\theta) \rangle^2] = \int_0^\infty [\langle P_2[\hat{\mu}(0) \cdot \hat{\mu}(t)] \rangle - \langle P_2(\cos\theta) \rangle^2]dt. \tag{21}$$

In the special case that the motion of the probe can be described as diffusion in a cone, using Eqs. 16 and 14 in Eq. 21 and evaluating the integral we find:

$$\tau_{\text{eff}}(1 - A_\infty) = \sum_{i \neq \infty} A_i \sigma_i / D_w. \tag{22}$$

Comparing this with Eq. 19 we see that

$$\tau_{\text{eff}} = \langle \sigma \rangle / D_w. \tag{23}$$

Thus, the approximation (Eq. 17) of Kinosita et al. (1) is exact at $t = 0$ and at $t = \infty$ and has the property that the area (in the sense of Eq. 20) is exact within the model.

As discussed by one of us elsewhere (11), another way of determining τ_{eff} is to require that the correlation function be exact at short times (approximation A of reference 11) (i.e., have the exact slope at $t = 0$). Since (1) $\langle P_2[\hat{\mu}(0) \cdot \hat{\mu}(t)] \rangle = 1 - 6D_w t + \cdots$, it is readily verified that in this case the appropriate choice for τ_{eff} is $(1 - A_\infty)/6D_w$, which is different from the result in Eq. 22 which was based on the requirement that the area under the correlation function be exact (approximation B of reference 11). Approximation A is clearly better at very short times but approximation B (on which we shall focus in the rest of this paper) gives a much better description of the entire time-course of the fluorescence anisotropy.

The method outlined above requires the knowledge of the complete solution (i.e., Eq. 16) to determine $\langle \sigma \rangle$ or τ_{eff}. Since the A_i's and σ_i's cannot be given as closed-form functions of θ_0 it might appear hopeless to sum Eq. 18 to give a closed-form result for $\langle \sigma \rangle$ in terms of θ_0.

However, in Appendix A we show that it is possible to obtain a closed-form expression for $\langle \sigma \rangle$ by bypassing the solution of the complete time-dependent problem. The method we employ has recently been used by Szabo (11), within the framework of the Smoluchowski description of the dynamics, in a similar context. This method is a generalization of a technique used in the theory of first passage times (see reference 16). In addition, it is of interest to note that it is also closely related to an approach used in atomic physics (17) to calculate second-order properties such as the polarizability. In Appendix A we show that

$$D_w\tau_{\text{eff}}(1 - A_\infty) = -x_0^2(1 + x_0)^2\{\log[(1 + x_0)/2] + (1 - x_0)/2\}/[2(1 - x_0)] + (1 - x_0)(6 + 8x_0 - x_0^2 - 12x_0^3 - 7x_0^4)/24, \quad (24)$$

where $x_0 = \cos\theta_0$. We note that when $\theta_0 = \pi$ (i.e., the motion is unrestricted) $\tau_{\text{eff}} = 1/6D_w$ as to be expected. Thus if θ_0 is determined from A_∞, the wobbling diffusion constant, D_w, can be determined from the effective relaxation time of $r(t)/r(0)$. It should be pointed out that even if the decay of $r(t)/r(0)$ is not adequately represented by a single exponential, Eq. 24 allows the extraction of D_w from the decay of the emission anisotropy. As Eq. 21 shows $\tau_{\text{eff}}(1 - A_\infty)$ is exactly the area under $[r(t) - r(\infty)]/r(0)$. Thus, if this area is measured and θ_0 is known from $r(\infty)$, then D_w can be found from Eq. 24.

The above expressions for the time-dependence of the fluorescence anisotropy are only valid when either μ_a or μ_e lies along the C_∞ axis of the probe. If this is not the case, the situation is more complicated and will be discussed in detail elsewhere. However, an approximate result for $r(t)/r(0)$ valid in the general case is quoted below (Eq. 30 with $\tau_M \to \infty$) for the sake of completeness.

FLUORESCENCE OF PROBES ATTACHED TO MACROMOLECULES

Here we consider the generalization of the above analysis to the situation where the fluorescent probe is attached to a spherical macromolecule whose diffusional motion cannot be neglected on the time scale of the experiment. We again assume that the probe has a C_∞ axis with either μ_a or μ_e pointing along it. This unique symmetry axis "wobbles" in a cone of semiangle θ_0. If it is assumed that the overall rotational motion of the macromolecule is independent of the wobbling motion, then the previous expressions for $r(t)/r(0)$ can be rigorously generalized by simply multiplying them by $\exp(-6D_Mt)$ where D_M is the rotational diffusion coefficient of the macromolecule. In particular, the approximation in Eq. 17 becomes

$$\left[\frac{r(t)}{r(0)}\right]_{\text{approx}} = A_\infty \exp(-t/\tau_M) + (1 - A_\infty)\exp[-t(\tau_M^{-1} + \tau_{\text{eff}}^{-1})] \quad (25)$$

where

$$\tau_M = 1/6D_M, \quad (26)$$

with A_∞ given by Eq. 14 and τ_{eff} is related to the wobbling diffusion coefficient via Eq. 24. It is of interest to compare Eq. 25 (which has a sound theoretical basis) with the "empirical" expression used by Munro et al. (6) to analyze their data on the fluorescence emission anisotropy of tryptophans in a variety of proteins. Their expression (in our notation) can be

written as

$$\left[\frac{r(t)}{r(0)}\right]'_{\text{approx}} = A_\infty \exp(-t/\tau_M) + (1 - A_\infty)\exp(-t/\tau'), \tag{27}$$

where their effective correlation time (τ') is related to the wobbling diffusion coefficient by

$$\tau' = 2\theta_0^2/3D_w\pi^2. \tag{28}$$

The form of Eq. 27 follows from Eq. 25 if it is assumed that the wobbling motion is much faster than the overall rotational motion of the macromolecule (i.e., $\tau_M \gg \tau_{\text{eff}}$). This is an excellent approximation in many cases. However, the relationship between τ' and D_w (i.e., Eq. 28) does not appear to have a sound theoretical basis (i.e., $\tau' \neq \tau_{\text{eff}}$) even in the limit of small θ_0. In this limit, Eq. 24 reduces to

$$\tau_{\text{eff}} = 7\theta_0^2/24D_w, \tag{29}$$

where θ_0 is in radians. Incidentally, Eq. 29 is a reasonably accurate approximation to Eq. 24, even for fairly large cone-angles. For example, if $\theta_0 = 30°$, Eq. 29 errs by <10%.

It should be stressed that Eq. 25 is valid only in the case that either μ_a or μ_e is parallel to the axis which wobbles (i.e., $\hat{\mu}$). It is not clear that this is the case for tryptophans in proteins. When neither μ_a nor μ_e are coaxial with $\hat{\mu}$ (e.g., as in Fig. 1) the theory is more complicated. It is clear that, in addition to the wobbling of the C_∞ axis of the probe, rotational diffusive motion about this axis also contributes to the decay of the fluorescence anisotropy. For the sake of completeness we quote (A. Szabo; to be published) an approximate expression for $r(t)/r(0)$ which can be regarded as the generalization of Eq. 25, i.e.,

$$\left[\frac{r(t)}{r(0)}\right]_{\text{approx}} = \frac{e^{-t/\tau_M}}{P_2(\cos\delta)} \sum_{m=-2}^{2} e^{-m^2t/6\tau'_e}\left[S^2 + (1 - S^2)\, e^{-t(1-m^2/6)/\tau''_e}\right] \times d_{m0}(\theta_e)d_{m0}(\theta_a)\cos m\phi_{ae}, \tag{30}$$

where θ_e and θ_a are the angles between μ_a and μ_e and $\hat{\mu}$, respectively, δ is the angle between μ_a and μ_e, ϕ_{ae} is the difference between their azimuthal angles, τ'_e is an effective correlation time for diffusion about $\hat{\mu}$, τ''_e is an effective correlation time for the wobbling of the $\hat{\mu}$ axis, and $d^2_{m0}(\beta)$ are reduced Wigner rotation matrices (13), i.e.,

$$d^{(2)}_{00}(\beta) = (3\cos^2\beta - 1)/2 \tag{31a}$$

$$d_{\pm 10}(\beta) = \mp(3/2)^{1/2}\sin\beta\cos\beta \tag{31b}$$

$$d_{\pm 20}(\beta) = (3/8)^{1/2}\sin^2\beta \tag{31c}$$

As a result of the symmetry of the Wigner rotation matrices, the sum over m in Eq. 30 contains only three unique terms. Eq. 30 has some interesting properties: (*a*) when either θ_a or θ_e equals zero it reduces to Eq. 25 with $\tau''_e = \tau_{\text{eff}}$; (*b*) when $\theta_0 = \pi$ (i.e., the motion is unrestricted) and $\tau_M = \infty$, it reduces to the result for a freely diffusing cylinder ("symmetric top") with $\tau'_e = 1/6D_Z$ and $\tau''_e = 1/6D_X$ (see, for example, reference 18). The ^{13}C-NMR

analogue of Eq. 30 (see below) is discussed in more detail by Brainard and Szabo (manuscript submitted for publication) who are concerned with the interpretation of ^{13}C-NMR relaxation studies of cholesterol in membranes.

EFFECT OF LIBRATIONAL MOTION ON ^{13}C-NMR RELAXATION

The NMR relaxation of protonated ^{13}C nuclei is determined by the dipolar interaction between the carbon and hydrogen nuclei which fluctuates as a result of spatial motion. In particular, the relaxation times are determined by the reorientation of the ^{13}C—H vector(s) with respect to the magnetic field. The theory of fluorescence depolarization and dipolar relaxation are quite analogous. This is especially transparent when both theories are formulated in terms of correlation functions involving Wigner rotation matrices (7).

Basically, the ^{13}C—H vector plays the same role as the emission and absorption dipoles when these point in the same direction. The ^{13}C—NMR analogue of the fluorescence depolarization problem considered in the previous section is shown in Fig. 3. The ^{13}C nucleus is nonrigidly attached to a macromolecule with overall diffusion constant D_M in such a way that the ^{13}C—H vector wobbles in a cone of semiangle θ_0. Alternately, one can imagine the ^{13}C nucleus diffusing on spherical "cap" of semiangle θ_0. This constitutes the simplest possible model for the liberational or wobbling motion of the α-carbons of proteins.

The mathematical formulation of this model is considered in Appendix B. Using the results derived there in conjunction with Table I of reference 19, the longitudinal (T_1) and transverse (T_2) relaxation times and the nuclear Overhauser enhancement (NOE) for the model are

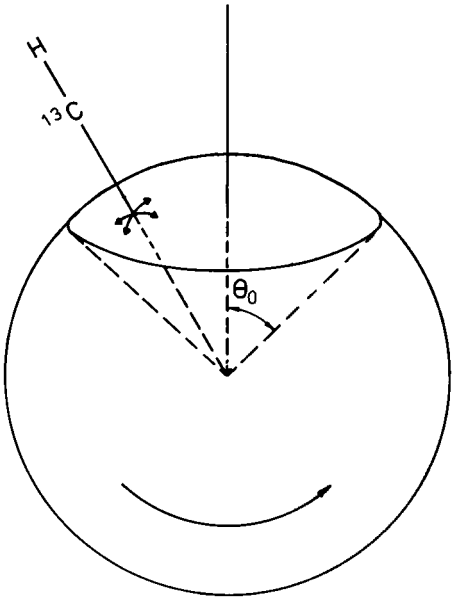

FIGURE 3 A ^{13}C nucleus nonrigidly attached to a macromolecule undergoing librational motion. The ^{13}C—H vector wobbles within a cone of semiangle θ_0.

shown in Fig. 3 are presented.

given by

$$T_1^{-1} = c[J(\omega_C - \omega_H) + 3J(\omega_C) + 6J(\omega_C + \omega_H)] \tag{32a}$$

$$T_2^{-1} = c[4J(0) + J(\omega_C - \omega_H) + 3J(\omega_C) + 6J(\omega_H) + 6J(\omega_C + \omega_H)] \tag{32b}$$

$$\text{NOE} = 1 + \frac{\gamma_H[6J(\omega_C + \omega_H) - J(\omega_C - \omega_H)]}{\gamma_C[J(\omega_C - \omega_H) + 3J(\omega_C) + 6J(\omega_C + \omega_H)]}, \tag{32c}$$

where $c = \hbar^2\gamma_C^2\gamma_H^2 r_{CH}/4$ with $\omega_i = \gamma_i H_0$ where H_0 is the external magnetic field. The spectral density $J(\omega)$ is

$$J(\omega) = 2\int_0^\infty \cos\omega t C(t)\, dt = \frac{2}{5}\int_0^\infty \cos\omega t e^{-t/\tau_M}\langle P_2[\hat{\mu}(0)\cdot\hat{\mu}(t)]\rangle\, dt, \tag{33}$$

where the unit vector $\hat{\mu}$ specifies the orientation of the ^{13}C—H vector in a coordinate frame rigidly attached to the macromolecule. The exact (i.e., within the model) $J(\omega)$ can be obtained by using Eq. 16 for $\langle P_2[\hat{\mu}(0)\cdot\hat{\mu}(t)]\rangle$ in Eq. 33. As we will show shortly, the single exponential approximation for this correlation function (i.e., Eq. 17) is adequate for most cases. Using this approximation in Eq. 33 and evaluating the integral we find

$$J_{\text{approx}}(\omega) = \frac{2}{5}A_\infty\frac{\tau_M^{-1}}{\tau_M^{-2} + \omega^2} + \frac{2}{5}(1 - A_\infty)\frac{(\tau_M^{-1} + \tau_{\text{eff}}^{-1})}{(\tau_M^{-1} + \tau_{\text{eff}}^{-1})^2 + \omega^2}, \tag{34}$$

where τ_M is given by Eq. 26, τ_{eff} by Eq. 24, and A_∞ by Eq. 14.

Eq. 34 has the identical form as an expression recently obtained by Howarth (10) using a completely different approach. Our derivation has the advantage that it clearly shows the nature of the approximations implicit in this result. Moreover, we establish the correct relation between τ_{eff} and the wobbling diffusion constant D_w (Eq. 24). Finally, it is clear from our formulation that Eq. 34 is only valid when the ^{13}C—H vector is coaxial with the wobbling axis as in Fig. 3, a point not mentioned by Howarth.

To investigate the accuracy of Eq. 34, in Figs. 4 *a* and *b* we present exact and approximate calculations of the variation of T_1 with τ_w ($\equiv 1/6D_w$) at two magnetic fields for $\theta_0 = 36.9°$ (Fig. 4 *a*) and $\theta_0 = 60°$ (Fig. 4 *b*). The exact results were calculated using Eq. 16 with the coefficients kindly supplied to us by Dr. Kinosita (Institute of Physical and Chemical Research, Japan). For $\theta_0 = 36.9°$ the two calculations give virtually indistinguishable results. The approximation is better for smaller angles and deteriorates as the cone angle increases. However, as Fig. 4 *b* shows, even for a cone angle as large as 60°, the approximation gives reasonable results. In Fig. 5 we present exact and approximate calculations of the NOE as a function of τ_w at high field for the same two values of the cone angle. The situation is the same as in the case of T_1.

In analogy with the discussion at the end of the last section, Eq. 34 is only applicable when the ^{13}C—H vector is coaxial with the "wobbling" axis. If the ^{13}C—H vector make a nonzero angle (say β) with this axis, then rotational diffusion about this axis influences the relaxation. Even in the limit that the rotational correlation time about the "wobbling" axis is infinite, Eq. 34 holds only if $\beta = 0$. The extension to nonzero values of β is considered by Brainard and Szabo (manuscript submitted for publication) who use the resulting expression to analyze ^{13}C-NMR relaxation experiments on cholesterol in membranes.

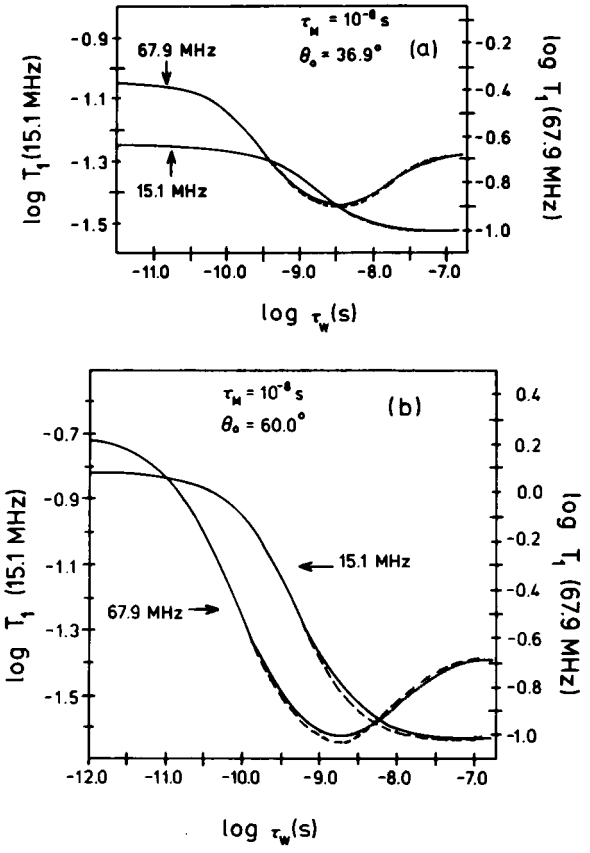

FIGURE 4 Spin-lattice relaxation times as a function of $\tau_w (\equiv 1/6D_w)$ at two magnetic fields for (*a*) $\theta_0 = 36.9°$ and (*b*) $\theta_0 = 60°$. The exact (solid lines) and approximate (dashed lines) results within the model

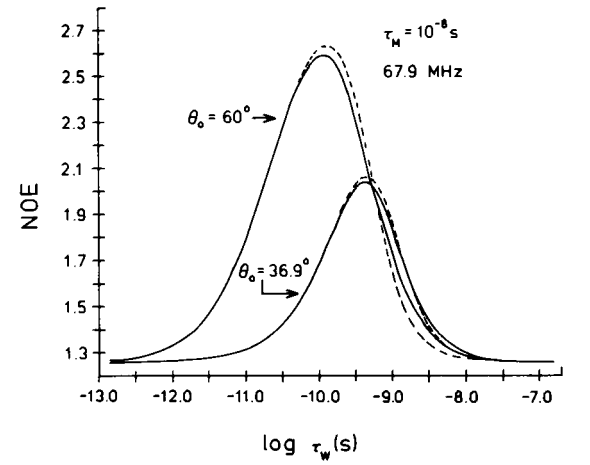

FIGURE 5 The NOE at high field as a function of τ_w for $\theta_0 = 36.9°$ and 60°. Exact results, solid lines; approximate results, dashed lines.

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APPENDIX A

We consider the diffusion of a unit vector $\hat{\mu}$ in a potential of the form $V(\theta) = 0$ for $0 \leq \theta \leq \theta_0$, $V(\theta) = \infty$ otherwise. Physically this describes the wobbling of a rod-shaped probe within a cone of semiangle θ_0. The polar coordinates (θ, ϕ in Fig. 2) of $\hat{\mu}$ are denoted by Ω. The conditional probability $[p(\Omega t|\Omega' 0)]$ that the orientation of $\hat{\mu}$ is Ω at time t if it was Ω' at time $t = 0$ within this model obeys the equation

$$D_w \nabla_\Omega^2 p(\Omega t|\Omega' 0) = \frac{\partial}{\partial t} p(\Omega t|\Omega' 0), \tag{A1}$$

where ∇_Ω^2 is the angular part of the Laplacian operating on Ω, subject to the boundary condition

$$\frac{\partial}{\partial \theta} p(\Omega t|\Omega' 0)\Big|_{\theta=\theta_0} = 0, \tag{A2}$$

and the initial condition

$$p(\Omega 0|\Omega' 0) = \delta(\Omega - \Omega'). \tag{A3}$$

We will set $D_w = 1$ in the subsequent development. For free diffusion the conditional probability is symmetric in Ω and Ω' [i.e., $p(\Omega t|\Omega' 0) = p(\Omega' t|\Omega 0)$]. Consequently its time derivative satisfies Eq. A1 when ∇_Ω^2 is replaced by $\nabla_{\Omega'}^2$. This simple observation plays a crucial role in the following analysis.

We are going to consider the correlation function for this diffusion process,

$$\begin{aligned} C(t) &= \langle P_2[\hat{\mu}(0) \cdot \hat{\mu}(t)] \rangle \\ &= \sum_{m=-2}^{2} \langle C_{2m}^*[\Omega(0)] C_{2m}[\Omega(t)] \rangle, \end{aligned} \tag{A4}$$

with the coefficients given by

$$\begin{aligned} C_{2m} &= (-)^m \frac{(2-m)!}{(2+m)!} P_2^m(\cos\theta) e^{im\phi}, \; m > 0 \\ C_{2-m} &= (-)^m C_{2m}^*, \end{aligned} \tag{A5}$$

where we have used the addition theorem for spherical harmonics in the form given by Brink and Satchler (13); $\Omega(t)$ and $\Omega(0)$ are the angular polar coordinates of vectors $\hat{\mu}(t)$ and $\hat{\mu}(0)$, respectively. The main goal of this appendix is the calculation of

$$\tau_{\text{eff}}(1 - A_\infty) = \int_0^\infty [C(t) - A_\infty] \, dt, \tag{A6}$$

where

$$A_\infty = \lim_{t\to\infty} C(t) = \langle P_2(\cos\theta) \rangle^2.$$

Our strategy will be to bypass the explicit calculation of the correlation function which appears in Eq. A6. We are going to reduce the problem of the evaluation of the integral in Eq. A6 to a quadrature in terms of a function which satisfies a simple differential equation.

We define

$$F(t) = \sum_{m=-2}^{2} \langle F_{2m}^{*}[\Omega(0)]\, F_{2m}[\Omega(t)] \rangle \tag{A7}$$

with

$$F_{2m}(\Omega) = C_{2m} - \delta_{m0} \langle P_2(\cos\theta) \rangle. \tag{A8}$$

The average of $F_{2m}(\Omega)$ vanishes [i.e., $\langle F_{2m}(\Omega) \rangle = 0$]. For $m = \pm 1, \pm 2$, this fact follows directly from the azimuthal symmetry of the problem. For $m = 0$ the $\langle P_2(\cos\theta) \rangle$ term exactly cancels the contribution of C_{20}. Moreover, it is simple to show that

$$F(t) = C(t) - A_{\infty}. \tag{A9}$$

Therefore, substituting Eq. A9 in Eq. A6

$$\begin{aligned} \tau_{\text{eff}}(1 - A_{\infty}) &= \int_0^{\infty} F(t)\, dt \\ &= \sum_{m=-2}^{2} \tau_m, \end{aligned} \tag{A10}$$

where

$$\tau_m = \int_0^{\infty} \langle F_{2m}^{*}[\Omega(0)] F_{2m}[\Omega(t)] \rangle\, dt, \tag{A11}$$

which is equivalent, using the definition of a correlation function, to

$$\tau_m = \int_0^{\infty} dt \iint d\Omega d\Omega' F_{2m}^{*}(\Omega') F_{2m}(\Omega) p_{\text{eq}}(\theta') p(\Omega t \,|\, \Omega' 0). \tag{A12}$$

If we let

$$T_{2m}(\Omega') = \int_0^{\infty} dt \int d\Omega F_{2m}(\Omega) p(\Omega t \,|\, \Omega' 0), \tag{A13}$$

Eq. A11 can be reexpressed as

$$\tau_m = \int d\Omega' F_{2m}^{*}(\Omega') T_{2m}(\Omega') p_{\text{eq}}(\theta'). \tag{A14}$$

We now show that T_{2m} satisfies a simple differential equation. Operating with the angular part of the Laplacian operator $\nabla_{\Omega'}^2$, on both sides of Eq. A13 we find

$$\begin{aligned} \nabla_{\Omega'}^2 T_{2m}(\Omega') &= \int_0^{\infty} dt \int d\Omega \frac{\partial}{\partial t} p(\Omega t \,|\, \Omega' 0) F_{2m}(\Omega) \\ &= \int d\Omega F_{2m}(\Omega)[p_{\text{eq}}(\theta) - \delta(\Omega - \Omega')], \end{aligned} \tag{A15}$$

where we have used Eq. A1 and the property

$$\lim_{t \to \infty} p(\Omega t \,|\, \Omega' 0) = p_{\text{eq}}(\theta).$$

The integral in Eq. A15 can be evaluated immediately using the property that the average of F_{2m} is zero.

Therefore, Eq. A15 becomes

$$\nabla^2_{\Omega} T_{2m}(\Omega') = -F_{2m}(\Omega'). \tag{A16}$$

It is apparent that T_{2m} has to satisfy the boundary condition of Eq. A2.

We are going to solve the differential equation A16, then we will evaluate the integral in Eq. A14. It can be shown that

$$\tau_m = \tau_{-m}. \tag{A17}$$

Therefore we have to solve Eq. A16 for $m = 0,1,2$. For $m = 0$ Eq. A16 becomes, after letting $x = \cos\theta$,

$$\frac{\partial}{\partial x}(1 - x^2)\frac{\partial}{\partial x} T_{20}(x) = -P_2(x) + \langle P_2 \rangle. \tag{A18}$$

Eq. A18 can be integrated twice to give

$$T_{20}(x) = -\langle P_2 \rangle \log [(1 + x)/2] - (1 - x^2)/4 + c. \tag{A19}$$

$T_{20}(x)$ given by Eq. A19 satisfies the boundary condition at $\theta = \theta_o$ and is well-behaved at $x = 1$ for an arbitrary value of the constant c. However, from the definition of F_{20}, the net contribution of c to the integral in Eq. A14 for $m = 0$ vanishes. Therefore, the arbitrariness of c is irrelevant for the calculation of $\tau_{\text{eff}}(1 - A_\infty)$, and we can set $c = 0$.

For $m = 1$, Eq. A16 becomes

$$\frac{\partial}{\partial x}(1 - x^2)\frac{\partial}{\partial x} K_{21}(x) - K_{21}(x)/(1 - x^2) = 6P^1_2(x), \tag{A20}$$

where we have set

$$T_{21}(x, \phi) = e^{i\phi} K_{21}(x)/6\sqrt{6}. \tag{A21}$$

The solution of Eq. A20 can be written as a linear combination of a particular solution of Eq. A20 and the general solution of the associated homogeneous equation.

It is apparent that

$$K_{21}(x) = P^1_2(x) \tag{A22}$$

is a particular solution of Eq. A20 which is just Legendre's associated equation for $l = 2$, $m = 1$.

The general solution of the associated homogeneous equation of Eq. A20 can be found using the expression given by Margenau and Murphy (20), (Eq. 14–55), for the Green function of the associated Legendre equation which is well-behaved at $x = \pm 1$

$$G(x, x') = \frac{1}{2m}\left[\frac{(1 + x)(1 - x')}{(1 - x)(1 + x')}\right]^{m/2} \qquad m \neq 0. \tag{A23}$$

Then, setting $m = 1$, the solution of the homogeneous equation which is well-behaved at $x = 1$ is

$$K_{21}(x) = \frac{1}{2}\left(\frac{1 - x}{1 + x}\right)^{1/2}. \tag{A24}$$

It is easy to verify that

$$T_{21}(x, \phi) = -e^{i\phi}\left[x(1 - x^2)^{1/2} + (1 - 2x_0^2)(1 + x_0)\left(\frac{1 - x}{1 + x}\right)^{1/2}\right]\Big/ 2\sqrt{6} \tag{A25}$$

where $x_0 = \cos\theta_0$, is the solution of Eq. A16 well-behaved at $x = 1$, and satisfying the boundary condition at $\theta = \theta_0$.

Using a similar argument, it is found that, for $m = 2$, the solution with the required properties is

$$T_{22}(x, \phi) = e^{2i\phi}\left[(1 - x)^2 - x_0(1 + x_0)^2\left(\frac{1 - x}{1 + x}\right)\right]\Big/ 4\sqrt{6}. \tag{A26}$$

Evaluating the integrals in Eq. A14, using Eqs. A17 and A10, and reintroducing the wobbling diffusion constant, we recover Eq. 24 of the text.

APPENDIX B

In this appendix we outline the derivation of the correlation function $C(t)$ which determines the spectral density via Eq. 33. We use the notation of reference 19 as closely as possible. If Ω_{LF} denotes the Euler angles which specify the orientation of the ^{13}C—H vector in the laboratory coordinate system, then the required correlation function is (7, 20)

$$C(t) = \langle D^{(2)*}_{q0}[\Omega_{LF}(0)] D^{(2)}_{q0}[\Omega_{LF}(t)] \rangle, \tag{B1}$$

where $D^{(2)}_{ab}(\Omega)$ are Wigner rotation matrices (12). We let $X_M Y_M Z_M$ be the coordinate system rigidly attached to the spherical macromolecule with the Z_M axis pointing along the direction about which the wobbling occurs, and $X_F Y_F Z_F$ be the coordinate system attached to the ^{13}C nucleus with Z_F pointing along the ^{13}C—H vector. Using the transformation properties of Wigner rotation matrices (13) we have

$$D^{(2)}_{q0}(\Omega_{LF}) = \sum_{a=-2}^{2} D^{(2)}_{q0}(\Omega_{LM}) D^{(2)}_{a0}(\Omega_{MF}), \tag{B2}$$

where Ω_{IJ} are the Euler angles which specify the orientation of the Jth coordinate frame in the Ith frame. Using Eq. B2 in Eq. B1 and assuming that the overall and wobbling motions are independent, we have

$$C(t) = \sum_{aa'} \langle D^{(2)*}_{qa}[\Omega_{LM}(0)] D^{(2)}_{qa'}[\Omega_{LM}(t)] \rangle \times \langle D^{(2)*}_{a0}[\Omega_{MF}(0)] D^{(2)}_{a'0}[\Omega_{MF}(t)] \rangle. \tag{B3}$$

Since (7).

$$\begin{aligned} \langle D^{(2)*}_{qa}[\Omega_{LM}(0)] D^{(2)}_{qa'}[\Omega_{LM}(t)] \rangle &= \delta_{aa'} e^{-6D_M t}/5 \\ &= \delta_{aa'} e^{-t/\tau_M}/5 \end{aligned} \tag{B4}$$

Eq. B3 becomes

$$C(t) = \frac{e^{-t/\tau_M}}{5} \sum_{a=-2}^{2} \langle D^{(2)*}_{a0}[\Omega_{MF}(0)] D^{(2)}_{a0}[\Omega_{MF}(t)] \rangle. \tag{B5}$$

Using the relationship (13) between Wigner rotation matrices and modified spherical harmonics we have

$$C(t) = \frac{e^{-t/\tau_M}}{5} \sum_{a=-2}^{2} \langle C_{2a}[\theta(0), \phi(0)] C^*_{2a}[\theta(t), \phi(t)] \rangle, \tag{B6}$$

where θ and φ are the polar angles which specify the orientation of the Z_F axis (and hence the ^{13}C—H vector) in the M frame. If we define $\hat{\mu}$ as a unit vector pointing along Z_F and use the addition theorem for spherical harmonics (13), Eq. B6 becomes

$$C(t) = \frac{e^{-t/\tau_M}}{5} \langle P_2[\hat{\mu}(0) \cdot \hat{\mu}(t)] \rangle, \tag{B7}$$

which is the required result.

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