

ON THE WOBLING-IN-CONE ANALYSIS OF FLUORESCENCE ANISOTROPY DECAY

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ABSTRACT Interpretation of fluorescence anisotropy decay for the case of restricted rotational diffusion often requires a model. To investigate the extent of model dependence, two models are compared: a strict cone model, in which a fluorescent probe wobbles uniformly within a cone, and a Gaussian model, where the stationary distribution of the probe orientation is of a Gaussian type. For the same experimental anisotropy decay, analysis by the Gaussian model predicts a smaller value for the rate of wobbling motion than the strict cone analysis, but the difference is 35% at most; the cone angle obtained by the strict cone analysis agrees closely with the effective width of the Gaussian distribution. The results suggest that, when only two parameters (the rate and the angular range) are extracted from an experiment, the choice of a model is not crucial as long as the model contains the essential feature, e.g., the more-or-less conical restriction, of the motion under study. Model-independent analyses are also discussed.

INTRODUCTION

Time-resolved measurements of optical anisotropy decay, such as the fluorescence or phosphorescence depolarization and the transient absorption dichroism, allow the analysis of complex rotational motion of probe molecules in supramolecular systems. In contrast to the free rotation in aqueous media, motion in organized structures, such as membrane or muscle, is limited in angular range, because the surrounding architecture usually imposes certain restrictions on the orientations of the probe. Moreover, friction within the structure often reduces the rate of reorientational motion from the value that would be expected in aqueous media. The optical anisotropy decay measurements can thus provide two different types of information, structural (the range) and dynamical (the rate).

In previous papers (1, 2), we proposed a wobbling-in-cone model mainly for the analysis of the motion of a rod-shaped fluorescent probe 1,6-diphenyl-1,3,5-hexatriene in lipid membranes. The model assumes that the major axis of the probe wobbles uniformly within a cone of semiangle θ_c with a wobbling diffusion constant D_w . The values of the parameters, θ_c (the range) and D_w (the rate), can be estimated from an experimental anisotropy decay $r(t)$ obtained after a flash excitation at $t = 0$. The analysis is simple, involving a minimal number of parameters, and is therefore powerful. However, the square-well approximation for the orientational distribution of the probe axis may appear too unrealistic. In view of the wide applica-

tions of the model (3, 4), we thought it necessary to clarify the nature of the approximation. We therefore calculated theoretical $r(t)$ for a Gaussian distribution of the probe orientation and compared the results with the original strict cone model. As shown below, the results suggest that the original analysis yields reasonable information even if the actual distribution is a smooth one.

GAUSSIAN MODEL

In the original strict cone model (1, 2), the stationary distribution $w^s(\theta)$ of the probe orientation is given by

$$w^s(\theta) = \text{nonzero constant for } 0^\circ \leq \theta \leq \theta_c \text{ and} \\ 180^\circ - \theta_c \leq \theta \leq 180^\circ; \\ w^s(\theta) = 0 \text{ for } \theta_c < \theta < 180^\circ - \theta_c, \quad (1)$$

where θ is the angle between the probe axis (direction of the optical transition moment) and the symmetry axis of the wobbling motion (cone axis). The rate of rotation, D_w , is assumed to be independent of the orientation. Under these assumptions, theoretical $r(t)$ can be closely approximated by the expression

$$r(t) = (r_0 - r_\infty) \exp(-t/\phi) + r_\infty, \quad (2)$$

where $r_0 = r(0)$ is the fundamental (or limiting) anisotropy, and r_∞ , the residual anisotropy, is related to the cone angle θ_c by

$$r_\infty/r_0 = [\frac{1}{2} \cos \theta_c (1 + \cos \theta_c)]^2. \quad (3)$$

TABLE I
COMPARISON BETWEEN STRICT CONE AND GAUSSIAN
MODELS

r_{∞}/r_0	Strict cone model		Gaussian model		σ_G/σ_s
	θ_c	σ_s	θ_c	σ_G	
1.000	0.0	0.0	0.0	0.0	
0.989	5.0	0.0022	5.0		
0.955	10.0	0.0088	10.0		
0.901	15.0	0.0196	15.0		
0.831	20.0	0.0342	20.1		
0.746	25.0	0.0522	25.2		
0.653	30.0	0.0731	30.3		
0.555	35.0	0.0962	35.7		
0.458	40.0	0.121	41.6	0.101	0.83
0.364	45.0	0.146	48.1	0.119	0.82
0.279	50.0	0.170	55.1	0.132	0.78
0.204	55.0	0.193	61.9	0.143	0.74
0.141	60.0	0.214	67.7	0.152	0.71
0.0904	65.0	0.231	72.3	0.158	0.68
0.0527	70.0	0.245	75.7	0.162	0.66
0.0265	75.0	0.253	78.1	0.165	0.65
0.0104	80.0	0.257	79.9	0.166	0.65
0.0022	85.0	0.255	81.2	0.167	0.65
0.0	90.0	0.250	82.2	0.167	0.67

The apparent relaxation time, ϕ , is given by

$$\phi = \sigma_s / D_w, \quad (4)$$

where σ_s , the relaxation time in units of $1/D_w$, is a known function of θ_c or, equivalently, of r_{∞}/r_0 . Thus, experimental determination of θ_c and D_w is very simple: first, observed $r(t)$ is fitted with Eq. 2 to obtain ϕ and r_{∞} values.¹ Then, θ_c is calculated from Eq. 3. Finally, σ_s is estimated from r_{∞}/r_0 , using Table I or the closed form expression given by Lipari and Szabo (5), and D_w is obtained from Eq. 4.

In the Gaussian model, we postulate, instead of Eq. 1,

$$w^s(\theta) \propto \exp(q^2 \cos^2 \theta / 2) \quad (5)$$

$$[\approx \exp(q^2/2) \exp(-q^2 \theta^2/4)],$$

where q is a parameter that determines the width of the distribution. As seen in Fig. 1, this distribution is smooth and spreads over the entire angles. In this model, therefore, the probe axis can rotate across the equator ($\theta = 90^\circ$), in contrast to the strict cone model. A theory by

¹In the determination of ϕ (and r_{∞}) by a curve-fitting procedure, we recommend that r_0 be fixed to a predetermined value, e.g., the value obtained in glycerol at a low temperature. This not only reduces the number of variables but is necessary for the subsequent analysis, because the theoretical σ_s or σ_G are calculated on the assumption that r_0 in Eq. 2 represents the value for immobilized probe molecules. If r_0 is let free in the curve fitting, r_0 tends to be underestimated and ϕ overestimated, especially when ϕ is not much larger than the instrumental resolution time. When the sample is turbid, as in the case of membrane suspensions, the depolarization due to light scattering should be corrected for before the analysis (see, e.g., reference 3, where the last line of footnote 2 should read "Values of D ranged between 1.0 and 0.9").

Jähmig (6) has suggested that orientational distribution in lipid bilayer is Gaussian-like.

By again approximating theoretical $r(t)$ with Eq. 2, we calculated ϕ ($= \sigma_G/D_w$) and r_{∞}/r_0 for the Gaussian model for various values of q : first, r_{∞}/r_0 was estimated from the following equation (see reference 2):

$$r_{\infty}/r_0 = \left[\int_0^{180^\circ} \frac{3\cos^2\theta - 1}{2} w^s(\theta) \sin\theta d\theta \right] / \left[\int_0^{180^\circ} w^s(\theta) \sin\theta d\theta \right]^2. \quad (6)$$

Then, σ_G was calculated according to the method of Szabo

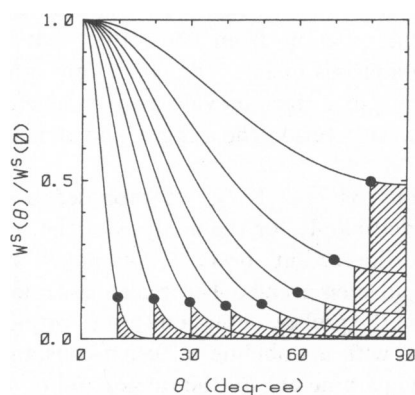


FIGURE 1 Stationary distribution of probe orientations in the Gaussian model. The distribution is normalized to 1 at $\theta = 0^\circ$. The shaded area under each distribution curve denotes the region for which the fractional population is $1/e^2$ of the total; the vertical line at the left edge of the area points to the effective width, θ_c , of the distribution. The closed circles represent the cone angle θ_c that would be obtained by applying the strict cone analysis to the Gaussian distribution.

(5, 7). For the integration of differential equations involved in the method, we adopted a series expansion in powers of q (see Appendix). The series failed to converge at large q , and σ_G could not be calculated for $r_\infty/r_0 > 0.5$. From a general principle, however, we expect that σ_G approaches σ_s for narrow distributions, as is already evident in the last column of Table I.

In Table I, the strict cone distribution and the Gaussian distribution that give a same r_∞/r_0 value are compared in each row. As may be seen, both models predict similar dimensionless relaxation times, σ_s or σ_G . In other words, D_w estimated from experimental ϕ and r_∞/r_0 , through the relation $D_w = \sigma/\phi$, is not much different between the two models. The systematic deviation of at most 35% is considered satisfactory in view of the experimental uncertainties. Also note that D_w is a quantity that varies by more than an order of magnitude, depending on the sample and conditions (3).

For a given r_∞/r_0 , we can calculate the effective width of the Gaussian distribution. The width, θ_e , is defined as the angle beyond which the fractional population is $1/e^2$ ($=0.1353$):

$$\int_{\theta_e}^{180^\circ - \theta_e} w^2(\theta) \sin \theta \, d\theta \bigg/ \int_0^{180^\circ} w^2(\theta) \sin \theta \, d\theta = 1/e^2 \quad (7)$$

The same r_∞/r_0 may be translated into θ_e by Eq. 3. As seen in Fig. 1 and Table I, θ_e and θ_c agree closely. That is, θ_c obtained by applying the strict cone analysis is a measure of the angular range in which the probe resides for most of the time.²

In summary, analysis of an experimental $r(t)$ with either model gives practically the same "rate" and "range" values. Description of restricted rotation with only two parameters is already a simplification; precise shape of $w^2(\theta)$ is immaterial under the simple approach.

As seen in Table I, the apparent relaxation time

$\phi (= \sigma/D_w)$ is not a simple inverse of the rate of rotation, D_w , because σ depends strongly on the angular range. This should be borne in mind in the estimation of the viscosity. One may postulate the Einstein's relation between D_w and the "viscosity" η against the reorientational motion [$\eta = kT/6D_wV_e f$, where V_e and f are the effective volume and the shape factor of the probe, k Boltzmann's constant, and T the absolute temperature (1)], but the relation between η and ϕ is not simple. The viscosity is a dynamic property, whereas ϕ is a complex quantity that depends on both the rate and the range.

MODEL-INDEPENDENT ANALYSES

As has already been shown (2), D_w can be estimated from the initial slope of $r(t)$:

$$r(t)/r_0 \approx 1 - 6D_w t, \quad (8)$$

for $t \approx 0$. This relation is quite general and does not depend on the mode of orientational restriction. Even heterogeneity in a sample, including differences in the lifetime of the excited (photoselected) state, can be taken into account: D_w obtained from the initial slope is the average over all probes. Often, however, limited time resolution of the instrument precludes precise determination of the initial slope. One then has to resort to the relaxation time analysis. Normally, a model is required for the interpretation of ϕ . In the absence of an appropriate model, however, one may postulate that Eq. 2 adequately approximates the actual $r(t)$, including the initial slope. D_w may then be estimated from the approximate relation

$$D_w \approx (1 - r_\infty/r_0)/6\phi. \quad (9)$$

This method generally leads to an underestimate of D_w , and the magnitude of the error depends on the actual orientational distribution. If the actual distribution is of the Gaussian type, the error is <20%.

The range information can be expressed in several ways. The quantity r_∞/r_0 , termed the "degree of orientational constraint" (2), can be directly estimated from an experimental anisotropy decay,³ and serves as an index of the angular range: generally, narrower angular range results in a higher value of r_∞/r_0 . If the orientational distribution is assumed to be axially symmetric, r_∞/r_0 can be translated into the order parameter S . For the diphenylhexatriene with optical transition moments lying parallel to the probe axis, Eq. 6 holds and thus $r_\infty/r_0 = S^2$ (2, 8, 9); more general cases have been treated in reference 5. The interpretation in terms of the order parameter may be convenient when comparing the results with theoretical work or with the results obtained by magnetic resonance

²Note that Eq. 1 represents two cones opposed to each other. We chose this form for the symmetric diphenylhexatriene molecule. Within the framework of the strict cone model, the two cones are independent in the sense that a probe molecule with a direction in one cone never rotates into the other cone. In actual membranes, however, the probe will eventually cross the equator, and will occupy the two cones with equal probabilities in the long run. The two cones may be considered to be connected with a "leakage path." It is in this sense that we compare θ_c and θ_e . Note that the presence of the leakage path does not affect the value of r_∞ , which is reached when the probe fills up one cone. Even the entire $r(t)$ remains practically the same as long as the rate of the leakage is much smaller than D_w .

Whether a probe crosses the equator in the fluorescence time scale or not is an interesting question. For a wide angular distribution (r_∞ small), the Gaussian model implicates a rapid crossover, whereas the strict cone model does not allow the passage. The fact that the two models predict similar relaxation times (Table I), however, means that the experimental discrimination even between the two extreme cases will be very difficult. Fluorescence anisotropy, being invariant under space inversion, is not very sensitive to rotations beyond the equator; estimation of the "flip-flop" rate would require other experimental techniques.

³Estimation with a curve-fitting procedure is preferable, because the finite decay time and/or the afterglow (even a very weak one) of the excitation flash may increase the apparent r_∞ , especially when true r_∞ is small.

spectroscopy, though direct comparisons are not necessarily warranted.

The description by r_∞/r_0 or S does not require any knowledge (or assumption) about the shape of the orientational distribution other than the (approximate) axial symmetry in the derivation of S . However, when the type of motion can be inferred (e.g., whether it is a wobbling-in-cone type, outside-of-cone type, or surface-of-cone type; precise shape of the distribution [e.g., square-well or Gaussian] is not very important as shown above), a pictorial view based on a model may offer a clearer insight. Also, estimation of the rate usually requires a model. Since r_∞/r_0 , S , and θ_c (or θ_e) are mathematically equivalent, all being simple functions of r_∞/r_0 , there is no absolute preference among the three. For the diphenylhexatriene in membrane, the assumption of conical distribution appears to be reasonable. The wobbling-in-cone analysis then provides both structural (the range) and dynamical (the rate) information about the membrane interior.

APPENDIX

We present here a brief summary of the method of calculating σ , the relaxation time of $r(t)$, for the case of wobbling motion of a fluorescent probe in a local, axially symmetric potential. We assume that the probe motion obeys the Smoluchowski's equation of the form (cf. Eq. 22 in reference 2)

$$\frac{1}{D_w} \frac{\partial w}{\partial t} = \Delta w + w \Delta V + (1 - x^2) \frac{dV}{dx} \frac{\partial w}{\partial x}, \quad (A1)$$

where $w = w(x \equiv \cos \theta, \psi, t)$ is the probability that the probe's transition moment (either emission or absorption) points to (θ, ψ) in spherical coordinates at time t , $V = V(x)$ is the potential, and Δ is the angular part of the Laplacian operator. The dimensionless relaxation time σ is defined by the Equation 5

$$\sigma \left(1 - \frac{r_\infty}{r_0}\right) = \frac{D_w}{r_0} \int_0^\infty [r(t) - r_\infty] dt. \quad (A2)$$

By a procedure similar to the one in reference 5 (see also reference 7), we can show that σ is expressed as a sum of five terms:

$$\sigma = \sum_{m=-2}^2 \sigma_m, \quad (A3)$$

where

$$\sigma_m = -\frac{(2-m)!}{(2+m)!} \int_{-1}^1 [P_2^m(x) - \delta_{m0} \langle P_2 \rangle] w^s(x) S_{2m}(x) dx, \quad (A4)$$

for $m \geq 0$ and $\sigma_{-m} = \sigma_m$. $P_2^m(x)$ is the associated Legendre polynomial, δ_{m0} the Kronecker delta, $\langle P_2 \rangle = \langle P_2^0 \rangle = \int_{-1}^1 P_2^0(x) w^s(x) dx$, and $w^s(x)$ is the stationary solution of Eq. A1 normalized so that $\int_{-1}^1 w^s(x) dx = 1$. $S_{20}(x)$ is given by the integral

$$S_{20}(x) = \int_1^x \left[\frac{e^{V(z)}}{1-y^2} \int_1^y [P_2(z) - \langle P_2 \rangle] e^{-V(z)} dz \right], \quad (A5)$$

and $S_{2m}(x)$ for $m = 1$ or 2 satisfies the following differential equation:

$$(1-x^2) \frac{d^2 S_{2m}}{dx^2} - 2x \frac{dS_{2m}}{dx} - \frac{m^2}{1-x^2} S_{2m} - (1-x^2) \frac{dV}{dx} \frac{dS_{2m}}{dx} = P_2^m(x). \quad (A6)$$

To solve Eq. A6, we expand $S_{2m}(x)$ as

$$S_{2m}(x) = \sum_{i=0}^{\infty} \frac{S_{2m}^{(i)}(x)}{i!}, \quad (A7)$$

with $S_{2m}^{(0)}(x) = -\frac{1}{6} P_2^m(x)$ as the "unperturbed" solution corresponding to $V(x) = 0$. High-order terms ($i \geq 1$) satisfy

$$(1-x^2) \frac{d^2 S_{2m}^{(i)}}{dx^2} - 2x \frac{dS_{2m}^{(i)}}{dx} - \frac{m^2}{1-x^2} S_{2m}^{(i)} - i(1-x^2) \frac{dV}{dx} \frac{dS_{2m}^{(i-1)}}{dx} = 0. \quad (A8)$$

When $V(x)$ can be expanded in a power series of x , we can sequentially determine $S_{2m}^{(i)} \rightarrow S_{2m}^{(i+1)}$'s as sums of associated Legendre polynomials. In the case of the Gaussian model, in particular, $V(x) = -\frac{1}{2} q^2 x^2$. Then, $S_{2m}^{(i)}(x)$ is obtained as

$$S_{2m}^{(i)}(x) = q^{2i} \sum_{j=1}^{i+1} a_{2m}^{(i)}(j) P_{2j}^m(x). \quad (A9)$$

Numerical values of $a_{2m}^{(i)}(j)$ can be calculated from a recursive formula obtained from Eq. A8 [$a_{2m}^{(0)}(1) = -\frac{1}{6}$, and $a_{2m}^{(i)}(j) = 0$ for $j = 0$ and $j > i+1$]. For the calculation of σ_G in Table I, $a_{2m}^{(i)}(j)$ up to $i = 100$ were stored in a FACOM 230-75 computer, and the integrations in Eqs. A4 and A5 were performed numerically after suitable conversion of variables.

We thank Dr. A. Szabo for helpful comments on the manuscript.

This work was supported by a research grant for Solar Energy Conversion-Photosynthesis given by the Japanese Science and Technology Agency.

Received for publication 16 June 1981 and in revised form 6 September 1981.

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