

TIME-DEPENDENT ABSORPTION ANISOTROPY AND ROTATIONAL DIFFUSION OF PROTEINS IN MEMBRANES

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ABSTRACT The decay of flash-induced absorption anisotropy, $r(t)$, of a chromophore in a membrane protein is closely correlated with rotational diffusion of the protein in the membrane. We develop a theory of time-dependent absorption anisotropy which is applicable to both linear chromophores and planar chromophores which have two different absorption moments at right angles to one another. The theory treats two types of rotational diffusion of membrane proteins: one is rotation of the whole protein about the normal to the plane of the membrane, and the other is restricted wobbling of the whole or part of the protein molecule. In the former case, $r(t)$ is determined by a rotational diffusion coefficient and an angle between the absorption moment(s) and the normal to the plane of the membrane. Rotation of rigid transmembrane proteins can be described by this treatment. In the latter case, $r(t)$ is characterized by a wobbling diffusion coefficient and the degree of orientational constraint. This treatment may be applicable to independent wobbling of the hydrophilic part of membrane proteins. We further show that, for linear and circularly degenerate chromophores, the effect of the excitation flash intensity on $r(t)$ can be accounted for by a constant scaling factor.

INTRODUCTION

Recently, methods of investigating slow rotational diffusion of membrane proteins have been developed that exploit the long lifetime of the triplet state of probe molecules (1–7). These methods detect the decay of either dichroism or phosphorescence polarization after flash excitation of the triplet probe. A similar flash-induced transient dichroism approach has also been used to investigate rotation of proteins with intrinsic chromophores, which have long-lived photoproducts (8–12).

A variety of theories have been presented to describe quantitatively the fluorescence polarization decay in the case of isotropic rotation of macromolecules in solution (13, 14), independent rotation of probes about an axis in macromolecules (15) and restricted wobbling of probes in membranes (16, 17). These theories are also useful for phosphorescence polarization decay and flash-induced absorption anisotropy of linear chromophores such as retinal and eosin derivatives (3, 6). So far no theories have treated the flash-induced absorption anisotropy decay of chromophores that have two absorption moments lying in the plane of the chromophore (nonlinear chromophore).

Here we present a general treatment of time-dependent absorption anisotropy applicable to both linear and nonlinear chromophores. The latter case is important, because it applies to

hemes and other metalloporphyrins. First we develop a general formalism, then two well-defined models are presented: one is rotation of proteins about the normal to the plane of the membrane, and the other is restricted wobbling of the whole or part of the protein molecule in the membrane. Application of the theory is illustrated by measurements of the rotational diffusion of heme proteins (cytochrome oxidase, cytochrome *P*-450 and cytochrome *b*₅).

In absorption experiments, it is often necessary to excite a significant proportion of chromophores in order to obtain good signal to noise, resulting in a flash intensity-dependent decrease in $r(t)$ (9). Here we show that this effect can be taken into account for linear and circularly degenerate chromophores by multiplying $r(t)$ by a constant scaling factor.

ABSORPTION ANISOTROPY OF A MACROSCOPICALLY ISOTROPIC SYSTEM

Since membrane suspensions containing proteins are isotropic as a whole, the rotation of the whole system in space does not affect the result of an observation. Following the recent theoretical treatment of fluorescence polarization decay in membranes (16), we develop a formal theory of absorption anisotropy decay applicable to any such isotropic system.

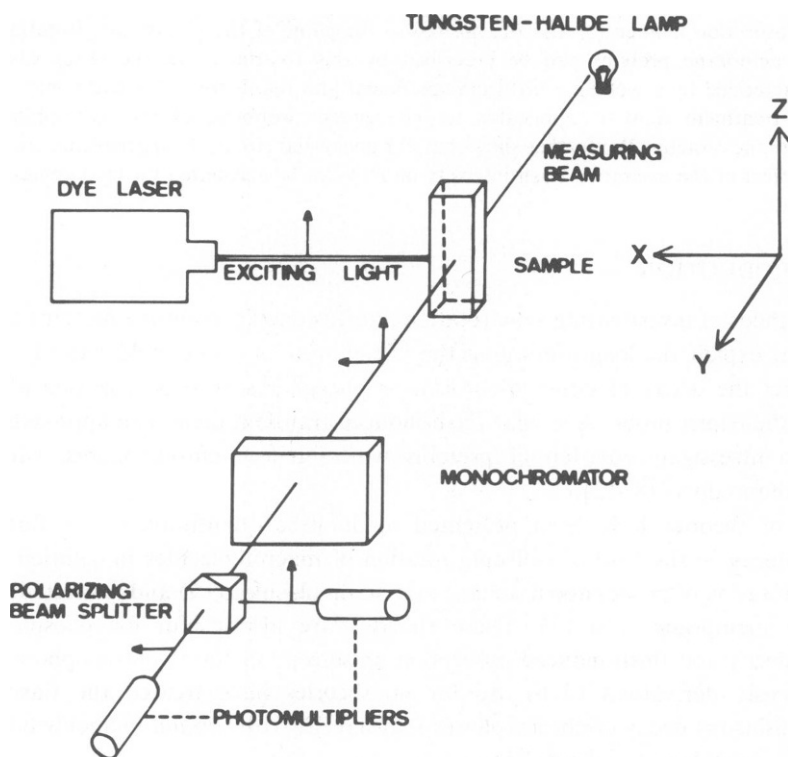


FIGURE 1 Schematic diagram of the detection system of the flash-induced absorption anisotropy.

First we adopt the following convention: a letter printed in bold face type denotes a unit vector; an integration such as $\int d\mathbf{F}$ represents $\int \int d\cos\theta d\phi$ where θ and ϕ are the polar and azimuthal angles of \mathbf{F} with respect to a certain fixed direction.

Taking mutually orthogonal unit vectors \mathbf{X} , \mathbf{Y} , \mathbf{Z} in space (see Fig. 1), we excite (photolyse) a sample with a flash polarized in the direction of \mathbf{Z} (vertically polarized flash). The absorption anisotropy $r(t)$ is defined by

$$r(t) = [A_v(t) - A_h(t)]/[A_v(t) + 2A_h(t)] \quad (1)$$

where $A_v(t)$ and $A_h(t)$ are the respective absorbance changes for vertical and horizontal polarization at time t after the flash.

Absorbance changes result from the formation of any photoproduct with a suitable lifetime. In the case of triplet probes, absorbance changes may be measured as either an increase in absorbance (e.g. triplet-triplet absorption) or as a decrease of absorbance in the singlet-singlet absorption band because of ground-state depletion (2). In the case of photodissociation (e.g. heme-CO complex) an increase or decrease in absorption is observed depending on whether the measurement is made within the absorption band of the original species or of the photoproduct. In this paper we treat the case where the transition dipole moments at the wavelengths of excitation and measurement are parallel. This assumption is valid for

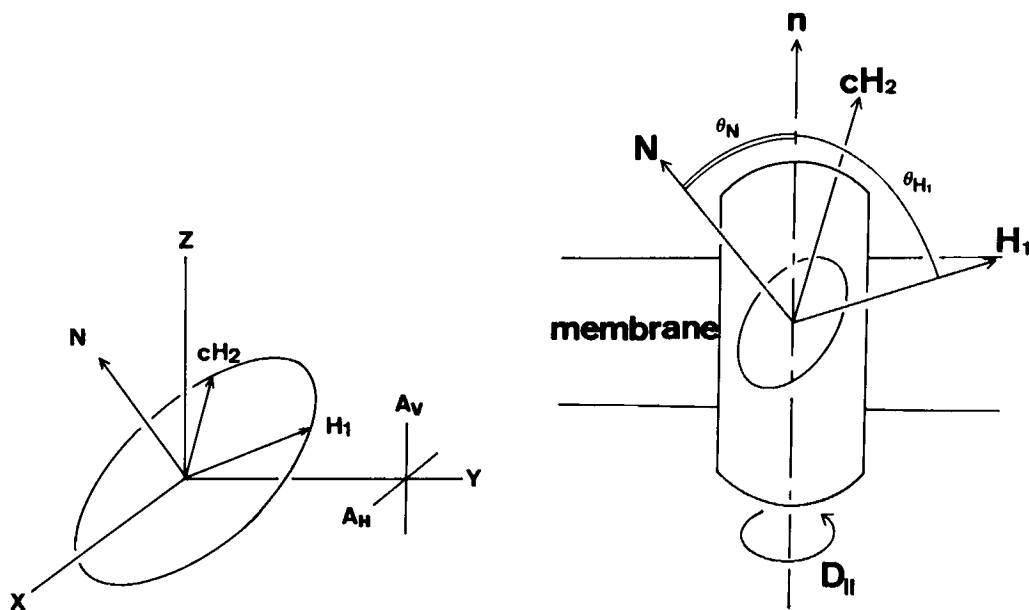


FIGURE 2 Absorption moments \mathbf{H}_1 and \mathbf{cH}_2 lie in the plane of the chromophore at right angles to one another. \mathbf{N} is perpendicular to the plane of the chromophore. A_v and A_h represent absorbance changes in vertical and horizontal directions.

FIGURE 3 Rotation-about-membrane normal model. Angles θ_N and θ_{H_1} are constant. $D_{||}$ is the rotational diffusion coefficient.

ground-state depletion of eosin triplet probes and metalloporphyrins and retinal in bacteriorhodopsin at appropriate wavelengths.

In Fig. 2 let \mathbf{H}_1 and $c\mathbf{H}_2$ be the absorption moments that lie in the plane of the chromophore at right angles to one another. Let \mathbf{N} be the normal to the plane of the chromophore. The set $\{\mathbf{H}_1, \mathbf{H}_2\}$ completely defines the orientation of the chromophore in space. Here, the set $\{\mathbf{H}_1, c\mathbf{H}_2\}$ represents nonlinear chromophores in the case of $c \neq 0$ (e.g. hemes, metalloporphyrins), or linear chromophores in the case of $c = 0$ (e.g. eosin derivatives, erythrosin derivatives, retinal). The set $\{\mathbf{H}_1, \mathbf{H}_2, \mathbf{N}\}$ satisfies the relationship

$$(\mathbf{H}_1 \cdot \mathbf{Z})^2 + (\mathbf{H}_2 \cdot \mathbf{Z})^2 + (\mathbf{N} \cdot \mathbf{Z})^2 = 1. \quad (2)$$

The absorbance change $A_v(t)$ is proportional to $(\mathbf{H}_1 \cdot \mathbf{Z})^2 + (c\mathbf{H}_2 \cdot \mathbf{Z})^2$, and we obtain

$$A_v(t) = S(t) \langle (\mathbf{H}_1 \cdot \mathbf{Z})^2 + c^2(\mathbf{H}_2 \cdot \mathbf{Z})^2 \rangle_t, \quad (3)$$

where $S(t) \propto e^{-t/\tau}$ represents the decay of the number of molecules in the excited state, τ is the lifetime, and $\langle \rangle_t$ denotes the average over all the excited state chromophores. Similarly in isotropic samples

$$\begin{aligned} A_H(t) &= (1/2)S(t) \langle 1 - (\mathbf{H}_1 \cdot \mathbf{Z})^2 + c^2 - c^2(\mathbf{H}_2 \cdot \mathbf{Z})^2 \rangle_t \\ &= [(c^2 + 1)/2]S(t) - (1/2)A_v(t). \end{aligned} \quad (4)$$

Substituting Eqs. 3 and 4 into Eq. 1, we obtain

$$r(t) = [1/(c^2 + 1)] \langle P_2(\mathbf{H}_1 \cdot \mathbf{Z}) + c^2 P_2(\mathbf{H}_2 \cdot \mathbf{Z}) \rangle_t, \quad (5)$$

where $P_2(x) = (1/2)(3x^2 - 1)$ is the Legendre polynomial of order 2.

In order to calculate the average in Eq. 5, we introduce a distribution function $W(\mathbf{H}_1, \mathbf{H}_2, t)$, the probability that we would find a chromophore with orientation $\{\mathbf{H}_1, \mathbf{H}_2\}$ in the sample at time t regardless of (whether or not) it is in the excited state. The function W is normalized such that

$$\int \int W(\mathbf{H}_1, \mathbf{H}_2, t) d\mathbf{H}_1 d\mathbf{H}_2 = 1. \quad (6)$$

For the evolution of W with time, we define $G(\mathbf{H}_1^0, \mathbf{H}_2^0, 0 | \mathbf{H}_1, \mathbf{H}_2, t)$ as the probability that a chromophore with orientation $\{\mathbf{H}_1^0, \mathbf{H}_2^0\}$ at time 0 will rotate into a new orientation $\{\mathbf{H}_1, \mathbf{H}_2\}$ by time t . Thus,

$$W(\mathbf{H}_1, \mathbf{H}_2, t) = \int \int W(\mathbf{H}_1^0, \mathbf{H}_2^0, 0) G(\mathbf{H}_1^0, \mathbf{H}_2^0, 0 | \mathbf{H}_1, \mathbf{H}_2, t) d\mathbf{H}_1^0 d\mathbf{H}_2^0. \quad (7)$$

Here we understand that W contains a factor $\delta(\mathbf{H}_1 \cdot \mathbf{H}_2)$, since \mathbf{H}_1 and \mathbf{H}_2 are mutually perpendicular. For isotropic sample, the stationary distribution W^s is given by

$$W^s(\mathbf{H}_1, \mathbf{H}_2) = (1/8\pi^2) \delta(\mathbf{H}_1 \cdot \mathbf{H}_2). \quad (8)$$

The excitation probability of a chromophore by a vertically polarized flash is proportional to

$$1 - \exp \{ -\kappa [(\mathbf{H}_1^0 \cdot \mathbf{Z})^2 + c^2(\mathbf{H}_2 \cdot \mathbf{Z})^2] \} \quad (9)$$

where $\kappa \equiv 3QI\sigma/(1 + c^2)$, Q is the quantum yield, I the integrated flash intensity in photons/cm² and σ the absorption cross section in cm² (18).¹ A unit excitation requires the normalization factor

$$1/\Delta_c \equiv 1/\int (1 - \exp \{-\kappa[(\mathbf{H}_1^0 \cdot \mathbf{Z})^2 + c^2(\mathbf{H}_2^0 \cdot \mathbf{Z})^2]\} W^s d\omega) \quad (10)$$

where W^s , G and $\int d\omega$ represent $W^s(\mathbf{H}_1^0, \mathbf{H}_2^0)$, $G(\mathbf{H}_1^0, \mathbf{H}_2^0, 0 | \mathbf{H}_1, \mathbf{H}_2, t)$ and $\int \int d\mathbf{H}_1^0 d\mathbf{H}_2^0$, respectively. Eq. 5 can be rewritten as

$$r(t) = \frac{1}{(c^2 + 1)\Delta_c} \int [P_2(\mathbf{H}_1 \cdot \mathbf{Z}) + c^2 P_2(\mathbf{H}_2 \cdot \mathbf{Z})] \times (1 - \exp \{-\kappa[(\mathbf{H}_1^0 \cdot \mathbf{Z})^2 + c^2(\mathbf{H}_2^0 \cdot \mathbf{Z})^2]\}) W^s G d\Omega \quad (11)$$

where $\int d\Omega$ represents $\int \int \int d\mathbf{H}_1^0 d\mathbf{H}_2^0 d\mathbf{H}_1 d\mathbf{H}_2$.

In the following, we discuss $r(t)$ in three special cases: *I*, circularly degenerate chromophore (fourfold symmetry), i.e., $c = 1$; *II*, linear chromophore, i.e., $c = 0$; *III*, $\kappa \ll 1$, i.e., the excitation flash is weak, so that the number of excited chromophores is negligible (c can be any value).

Since $r(t)$, Δ_c do not depend on the direction of \mathbf{Z} in space in a macroscopically isotropic sample, we can average these over \mathbf{Z} without changing their values. We perform the integration with respect to \mathbf{Z} in Appendix I. Here we show the resultant expressions.

Case I: Circularly Degenerate Chromophore

$$\Delta_{c=1} = 1 - e^{-\kappa} \sum_{n=0}^{\infty} \frac{\kappa^n}{n!(2n+1)} \quad (12)$$

$$r(t)/r(0) = \int P_2(\mathbf{N}^0 \cdot \mathbf{N}) W^s G d\Omega \quad (13)$$

$$r(0) = \left[\frac{3}{2\kappa} - \left(1 + \frac{3}{2\kappa} \right) e^{-\kappa} \sum_{n=0}^{\infty} \frac{\kappa^n}{n!(2n+1)} \right] / 4 \left[1 - e^{-\kappa} \sum_{n=0}^{\infty} \frac{\kappa^n}{n!(2n+1)} \right] \quad (14)$$

where $r(0) \rightarrow 0.1$ ($\kappa \rightarrow 0$) (19) and $r(0) \rightarrow 0$ ($\kappa \rightarrow \infty$). Thus, $r(t)/r(0)$ is independent of the intensity of the photoselecting flash, and only the initial anisotropy $r(0)$ depends on the flash intensity.

Case II: Linear Chromophore

$$\Delta_{c=0} = 1 - (1/\sqrt{\kappa}) \text{Erf} \sqrt{\kappa} \quad (15)$$

where $\text{Erf} x$ is the Gauss error function:

$$\text{Erf} x = \int_0^x e^{-t^2} dt = \sum_{n=0}^{\infty} \frac{(-1)^n x^{2n+1}}{n!(2n+1)} \quad (16)$$

$$r(t)/r(0) = \int P_2(\mathbf{H}_1^0 \cdot \mathbf{H}_1) W^s G d\Omega \quad (17)$$

¹ σ is related to the extinction coefficient ϵ by $\sigma = 3.82 \times 10^{-21} \epsilon$.

$$r(0) = \left[\frac{3}{2\kappa} e^{-\kappa} + \left(1 - \frac{3}{2\kappa} \right) (1/\sqrt{\kappa}) \operatorname{Erf} \sqrt{\kappa} \right] / 2 [1 - (1/\sqrt{\kappa}) \operatorname{Erf} \sqrt{\kappa}] \quad (18)$$

where $r(0) \rightarrow 0.4$ ($\kappa \rightarrow 0$) and $r(0) \rightarrow 0$ ($\kappa \rightarrow \infty$). Thus, $r(t)/r(0)$ is again independent of the flash intensity, while $r(0)$ is a function of the excitation flash.

Case III: Weak Excitation

$$\Delta_c = (\kappa/3)(c^2 + 1) \quad (\kappa \ll 1). \quad (19)$$

$$r(t)/r(0) = \frac{1}{c^2(c^2 - 1) + 1} \int \{ c^4 P_2(\mathbf{N}^0 \cdot \mathbf{N}) + (c^2 - 1) P_2(\mathbf{H}_1^0 \cdot \mathbf{H}_1) \\ + c^2(c^2 - 1) [P_2(\mathbf{H}_1^0 \cdot \mathbf{N}) + P_2(\mathbf{N}^0 \cdot \mathbf{H}_1)] \} W^s G d\Omega. \quad (20)$$

$$r(0) = 0.4 [c^2(c^2 - 1) + 1] / (c^2 + 1)^2. \quad (21)$$

ANISOTROPIC MOTION OF PROTEINS IN MEMBRANES

When proteins are embedded in membranes, the probability of the reorientation $\{\mathbf{N}^0, \mathbf{H}_1^0\} \rightarrow \{\mathbf{N}, \mathbf{H}_1\}$ depends on the orientation of the membrane in which the molecule is placed. We have to correlate $r(t)$ with a distribution function defined within a particular segment of membrane, where the motion of the protein can be described by a differential equation.

We assume that a suspension of membranes is equivalent to an ensemble of planar membrane segments. Within a segment designated by its normal \mathbf{n} , we define the evolution function $g_{\mathbf{n}}(\mathbf{N}^0, \mathbf{H}_1^0, 0 | \mathbf{N}, \mathbf{H}_1, t)$ and the stationary distribution $w_{\mathbf{n}}^s(\mathbf{N}^0, \mathbf{H}_1^0)$. Now the product $W^s G$ in Eqs. 13, 17, 20 can be replaced with the average of $w_{\mathbf{n}}^s g_{\mathbf{n}}$ over \mathbf{n} , for example for circularly symmetric chromophores

$$r(t)/r(0) = (1/M) \sum_{\mathbf{n}} \int P_2(\mathbf{N}^0 \cdot \mathbf{N}) w_{\mathbf{n}}^s g_{\mathbf{n}} d\Omega \quad (22)$$

where $w_{\mathbf{n}}^s g_{\mathbf{n}}$ represents $w_{\mathbf{n}}^s(\mathbf{N}^0, \mathbf{H}_1^0) g_{\mathbf{n}}(\mathbf{N}^0, \mathbf{H}_1^0, 0 | \mathbf{N}, \mathbf{H}_1, t)$.

We have assumed that all M segments contain an equal number of proteins. We have neglected the rotation of the whole segments. Since all segments are essentially identical to each other, $w_{\mathbf{n}}^s$ and $g_{\mathbf{n}}$ for different \mathbf{n} s are related simply by rotation in space. Thus we can choose any particular membrane segment for the calculation of $r(t)$:

$$(\text{case I}) \quad r(t)/r(0) = \int P_2(\mathbf{N}^0 \cdot \mathbf{N}) w^s g d\Omega. \quad (23)$$

$$(\text{case II}) \quad r(t)/r(0) = \int P_2(\mathbf{H}_1^0 \cdot \mathbf{H}_1) w^s g d\Omega. \quad (24)$$

$$(\text{case III}) \quad r(t)/r(0) = \frac{1}{c^2(c^2 - 1) + 1} \int \{ c^4 P_2(\mathbf{N}^0 \cdot \mathbf{N}) \\ + (c^2 - 1)^2 P_2(\mathbf{H}_1^0 \cdot \mathbf{H}_1) + c^2(c^2 - 1) [P_2(\mathbf{H}_1^0 \cdot \mathbf{N}) + P_2(\mathbf{N}^0 \cdot \mathbf{H}_1)] \} w^s g d\Omega. \quad (25)$$

ROTATION OF MEMBRANE PROTEINS ABOUT THE NORMAL TO THE PLANE OF THE MEMBRANE

In this section, we treat the rotational diffusion of a chromophore fixed rigidly in a protein that rotates about the normal to the plane of the membrane. In this case, both \mathbf{N} and \mathbf{H}_1 have

fixed angles with respect to the membrane normal \mathbf{n} , and the difference in the azimuthal angles between \mathbf{N} and \mathbf{H}_1 around \mathbf{n} is constant (see Fig. 3).

Thus the diffusion equation of the chromophore is

$$\partial W(\psi_N, t)/\partial t = D_1 \partial^2 W(\psi_N, t)/\partial \psi_N^2 \quad (26)$$

where ψ_N is the azimuthal angle of \mathbf{N} around \mathbf{n} at time t , $w(\psi_N, t)$ is the distribution function that locates the plane of the chromophore, and D_1 is the rotational diffusion coefficient of the protein about the membrane normal. For cylindrical proteins whose axis of symmetry is normal to the plane of the membrane, D_1 is expressed as

$$D_1 = kT/4\pi a^2 h \eta \quad (27)$$

where a is the radius of the cylinder, h the length immersed in the membrane, η the membrane viscosity, k the Boltzmann constant, and T the absolute temperature (20).

The Green function g (i.e. the evolution function) and the stationary solution w^s of Eq. 26 with the boundary condition of $w^s(\psi_N + 2\pi, t) = w^s(\psi_N, t)$ are readily obtained by the method of separation of variables:

$$g(\psi_N, 0 | \psi_N, t) = (1/2\pi) \sum_{l=-\infty}^{\infty} e^{-l^2 D_1 t} e^{il(\psi_N - \psi_N^0)}. \quad (28)$$

$$w^s(\psi_N) = 1/2\pi. \quad (29)$$

Substituting Eqs. 28 and 29 into Eqs. 23–25, we calculate $r(t)/r(0)$. In Appendix II, we show that $r(t)/r(0)$ is expressed as

$$r(t)/r(0) = A_1 e^{-D_1 t} + A_2 e^{-4D_1 t} + A_3. \quad (30)$$

Cases I and II: Linear and Circularly Symmetric Chromophores

$$r(t)/r(0) = 3 \sin^2 \theta_A \cos^2 \theta_A e^{-D_1 t} + (3/4) \sin^4 \theta_A e^{-4D_1 t} + (1/4)(3 \cos^2 \theta_A - 1)^2 \quad (31)$$

where $A = H_1$ for linear chromophores and $A = N$ for circularly symmetric chromophores.

Case III: Weak Excitation

$$A_1 = \frac{1}{c^2(c^2 - 1) + 1} [3c^4 \cos^2 \theta_N \sin^2 \theta_N + 3(c^2 - 1)^2 \cos^2 \theta_{H_1} \sin^2 \theta_{H_1} - 6c^2(c^2 - 1) \cos^2 \theta_N \cos^2 \theta_{H_1}]. \quad (32)$$

$$A_2 = \frac{1}{c^2(c^2 - 1) + 1} \{ (3/4)c^4 \sin^4 \theta_N + (3/4)(c^2 - 1)^2 \sin^4 \theta_{H_1} + 3c^2(c^2 - 1)[\cos^2 \theta_N \cos^2 \theta_{H_1} - (1/2) \sin^2 \theta_N \sin^2 \theta_{H_1}] \}. \quad (33)$$

$$A_3 = \frac{1}{c^2(c^2 - 1) + 1} \{ (c^4/4) (3 \cos^2 \theta_N - 1)^2 + [(c^2 - 1)^2/4] (3 \cos^2 \theta_{H_1} - 1)^2 + [c^2(c^2 - 1)/2] (3 \cos^2 \theta_N - 1)(3 \cos^2 \theta_{H_1} - 1) \}. \quad (34)$$

In the case of linear and circularly degenerate chromophores, we can determine the angle θ_N or θ_H , from the time-independent residual anisotropy ratio $r(\infty)/r(0) \equiv A_3$:

$$\theta_A = \cos^{-1} \{ [1 \pm 2(r(\infty)/r(0))^{1/2}] / 3 \}^{1/2} \quad (35)$$

where $A = N$ for circularly symmetric chromophores, and $A = H_1$ for linear chromophores (see Fig. 3). For circularly symmetric chromophores, θ_N coincides with the tilt angle of the chromophore plane from the membrane plane. Fig. 4 shows the angular dependence of $r(\infty)/r(0)$. In the case of $\kappa \ll 1$ (weak excitation flash), Heyn et al. (21) have obtained the same result as Eq. 35 for linear chromophores. Generally, values of c and θ_{H_1} for nonlinear chromophores depend on the absorption wavelength, so that we may be able to choose the wavelength where the chromophore is circularly symmetric ($c = 1$) (22, 23) (see Appendix III).

In this section, we have assumed a single rotating species of proteins. Actually, however, different rotating species of proteins in membranes have been observed for band 3 in erythrocytes and cytochrome oxidase in mitochondria due to protein-protein interactions (24, 25). In this case, the measured $r(t)$ is the weighted sum of the individual $r(t)$ s with different D_i . It should be noted that the time-independent residual anisotropy ratio $r(\infty)/r(0)$ is not changed, provided that the rotation of all species is sufficiently rapid to completely randomize the absorption moments around the membrane normal during the time of observation.

RESTRICTED WOBBLING OF PROTEINS IN MEMBRANES

Wobbling motion is a general phenomenon that is observed in supramolecular systems, for example, internal motion of a subunit in a macromolecule and flexing motion in a fibrous structure. In this section, we treat the wobbling diffusion of a protein with either a circularly degenerate chromophore ($c = 1$) or a linear chromophore ($c = 0$) fixed rigidly in the protein (see Fig. 5). The model is equally applicable to wobbling of the whole protein molecule or to independent wobbling of part of the protein molecule.

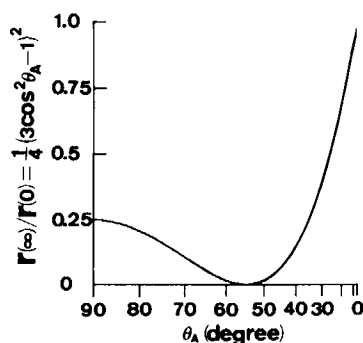


FIGURE 4 Angular dependence of the time-independent residual anisotropy ratio in rotation-about-membrane normal model. $A = N$ for circularly symmetric chromophores and $A = H_1$ for linear chromophores.

Wobbling-in-Cone Model

A typical type of wobbling of proteins is expressed as "wobbling in cone" where the orientation of the normal **N** to the plane of the circularly symmetric chromophore is confined within a cone around the normal **n** to the plane of the membrane, or the absorption moment of the linear chromophore wobbles within a cone around **n** (Fig. 5). The cone may be formed by steric hindrance of surrounding structures.

Assuming that the wobbling diffusion coefficient D_w of **N** (circularly symmetric chromophore) or **H₁** (linear chromophore) has a uniform value throughout the cone of half angle θ_c , we have shown (16)

$$r(t) = \sum_{i=1}^{\infty} r_i \exp(-D_w t / \sigma_i) \quad (36)$$

$$r(\infty)/r(0) \equiv r_{\infty}/r(0) = [(1/2) \cos \theta_c (1 + \cos \theta_c)]^2 \quad (37)$$

where r_i and σ_i are constants that depend only on θ_c , and the stationary term r_{∞} corresponds to $\sigma_{\infty} = \infty$. Furthermore, $r(t)$ is closely approximated by the simple expression

$$r(t) = [r(0) - r_{\infty}] \exp(-D_w t / \langle \sigma \rangle) + r_{\infty} \quad (38)$$

where $\langle \sigma \rangle / D_w = \phi$ is the mean rotational relaxation time and

$$\langle \sigma \rangle = \sum_{i=1}^{\infty} r_i \sigma_i / [r(0) - r_{\infty}]. \quad (39)$$

The angular dependence of r_i , σ_i and $\langle \sigma \rangle$ are shown in Fig. 3 in reference 16.

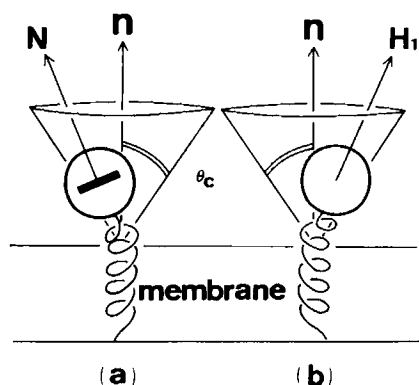


FIGURE 5

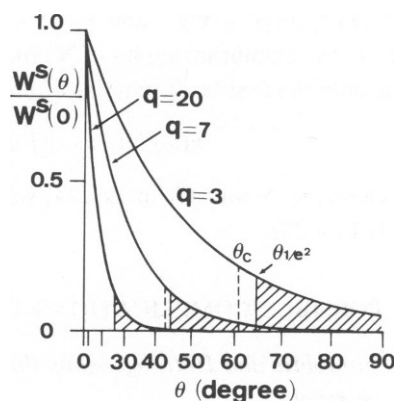


FIGURE 6

FIGURE 5 Wobbling-in-cone model. **N** (circularly symmetric chromophore) or **H₁** (linear chromophore) wobbles uniformly in the cone of half-angle θ_c .

FIGURE 6 Comparison of the cone angle θ_c with $\theta_{1/2}$ in cosine type potential with different distribution width (q) for restricted wobbling of proteins in membranes. θ_c is indicated by the vertical dashed line and $\theta_{1/2}$ is indicated by the vertical solid line. Accurate values are $(\theta_c, \theta_{1/2}, q) = (25.6^\circ, 25.8^\circ, 20), (43.0^\circ, 44.3^\circ, 7), (61.0^\circ, 64.8^\circ, 3)$. $w^s(\theta)$ is the stationary distribution of chromophores around the membrane normal. The population of chromophores between $\theta \sim \theta + \Delta\theta$ is proportional to $w^s(\theta) \sin\theta \Delta\theta$.

In the general case when the barrier against wobbling motion cannot be assumed to be a rigid wall, the wobbling-in-cone model with the square-well potential may appear unrealistic. However, even when the potential is a Gaussian type, the above wobbling-in-cone model can be a good approximation (Kinosita, to be published). In Appendix IV, we discuss the meaning of θ_c in a simple non-square-well potential.

Model Free Wobbling Diffusion Coefficient and Order Parameter

Another possibility to describe the wobbling motion in an unknown potential is to use a model-free diffusion coefficient $\langle D_w \rangle$ and an order parameter S . The $\langle D_w \rangle$ is obtained by the initial decay of $r(t)$ (16):

$$r(t)/r(0) \approx 1 - 6 \langle D_w \rangle t \quad (40)$$

The orientational constraint of wobbling motion, $r(\infty)/r(0)$, can be correlated to the conventional order parameter S (26–28). For a circularly degenerate chromophore, setting $t \rightarrow \infty$ in Eq. 23 and considering the relationship $g(\mathbf{N}^0, \mathbf{H}_1^0, 0 | \mathbf{N}, \mathbf{H}_1, \infty) = w^s(\mathbf{N}, \mathbf{H}_1)$

$$r(\infty)/r(0) = \int \int P_2(\mathbf{N}^0 \cdot \mathbf{N}) \hat{w}(\mathbf{N}^0) \hat{w}(\mathbf{N}) d\mathbf{N}^0 d\mathbf{N} \quad (41)$$

where

$$\hat{w}(\mathbf{N}) = \int w(\mathbf{N}, \mathbf{H}_1) d\mathbf{H}_1 \quad (42)$$

and a similar relationship defines $w(\mathbf{N}^0)$. We use the addition theorem

$$P_2(\mathbf{N}^0 \cdot \mathbf{N}) = P_2(\mathbf{n} \cdot \mathbf{N}^0) P_2(\mathbf{n} \cdot \mathbf{N}) + (1/3) P_2^1(\mathbf{n} \cdot \mathbf{N}^0) P_2^1(\mathbf{n} \cdot \mathbf{N}) \cos(\phi^0 - \phi) \\ + (1/12) P_2^2(\mathbf{n} \cdot \mathbf{N}^0) P_2^2(\mathbf{n} \cdot \mathbf{N}) \cos 2(\phi^0 - \phi) \quad (43)$$

where $P_2^1(x) = 3x(1 - x^2)^{1/2}$ and $P_2^2(x) = 3(1 - x^2)$ are the associate Legendre polynomials, ϕ^0 and ϕ are the azimuthal angles of \mathbf{N}^0 and \mathbf{N} around \mathbf{n} . Since $w(\mathbf{N})$ and $w(\mathbf{N}^0)$ are symmetric around \mathbf{n} , only the first term remains after integration over \mathbf{N}^0 or \mathbf{N} :

$$r(\infty)/r(0) = [\int P_2(\mathbf{n} \cdot \mathbf{N}) \hat{w}(\mathbf{N}) d\mathbf{N}]^2 = S^2. \quad (44)$$

Simply changing \mathbf{N} into \mathbf{H}_1 in Eq. 44, we get the formula for linear chromophores derived previously (26–28).

POSSIBLE COMPLICATIONS IN APPLICATION TO EXPERIMENTS

Immobile and Heterogeneous Populations in Rotation about the Membrane Normal

In actual experiments, we use a limited time window to detect $r(t)$ curves. If the rotational relaxation time, $\phi_1 = 1/D_{\parallel}$, of a fraction of proteins is more than 10 times larger than the experimental time window, this very slowly rotating fraction may be detected as immobile population and the measured $r(\infty)/r(0)$ is no longer expressed by Eqs. 31 and 34. In this case, the experimental $[r(\infty)/r(0)]_{\text{ex}}$ can be written by:

$$[r(\infty)/r(0)]_{\text{ex}} = f_{\text{im}} \cdot 1 + (1 - f_{\text{im}}) [r(\infty)/r(0)]_{\text{th}} \quad (45)$$

where f_{im} is the immobile fraction in a given time window, and $[r(\infty)/r(0)]_{th}$ the theoretical time-independent term in Eqs. 31 and 34. If we can determine the value of $[r(\infty)/r(0)]_{th}$ independently in reconstituted proteoliposomes or by other techniques, then we can calculate f_{im} using Eq. 45. For example, $[r(\infty)/r(0)]_{th} = 0.19$ for retinal of bacteriorhodopsin has been determined by flash-induced absorption anisotropy in synthetic lecithin vesicles (21). The θ_N of heme chromophores of cytochrome oxidase ($\theta_N \approx 90^\circ$) and cytochrome *P*-450 ($\theta_N \approx 0-10^\circ$) has been investigated by optical and ESR techniques in oriented membranes (29, 30). $[r(\infty)/r(0)]_{th}$ can be calculated from these angles using Eq. 31 for linear and circularly degenerate chromophores.

If there is a variety of rotating species, the experimental $(A_1/A_2)_{ex}$ is no longer expressed by $(A_1/A_2)_{th} = 4\cot^2\theta_A$ in Eq. 31 for linear chromophores ($A = H_1$) and circularly symmetric chromophores ($A = N$). Since θ_N or θ_{H_1} can be determined by ESR, linear dichroism or anisotropy decay curves (from A_3 in Eq. 31) studies, we can check the existence of heterogeneous rotating populations by the comparison of $(A_1/A_2)_{ex}$ with $4\cot^2\theta_A$.

The above approach is useful for examining protein-protein interactions in biological membranes where even a single protein species may be rotating as monomer, small oligomer, or immobile. We show examples for circularly degenerate chromophores in Application.

Rapid Restricted Wobbling of a Probe Independent of Slow Protein Motion

In the previous sections, we have assumed that a chromophore is fixed rigidly in a protein and does not show any rapid motion independent of slow protein rotation. This assumption may be likely for intrinsic chromophores (31). However, when we use an extrinsic probe bound to the protein for detecting the slow motion of the protein, the probe often exhibits rapid restricted wobbling in the order of several hundred picoseconds to a few nanoseconds (32-34). The rotation of the whole protein molecule about the membrane normal has been observed to be slower than 1 μ s in all cases examined (35). A third type of motion that conceivably occurs is independent segmental flexibility of part of the protein molecule as observed in immunoglobulins (36) and in F-actin (37). Evidence for such a motion has been obtained for $(Ca^{2+}-Mg^{2+})ATPase$ in sarcoplasmic reticulum (38). In the case that the above three motions are independent to one another and the segmental motion is much faster than rotation of the whole protein molecule, $r(t)$ may be written as:

$$r(t) = r_p(t)r_s(t)r_r(t) \quad (46)$$

where $r_p(t)$ is the anisotropy decay due to probe wobbling, $r_s(t)$ is due to the segmental motion of the protein, and $r_r(t)$ is due to protein rotation about the membrane normal.

Almost all investigations of slow rotation with eosin derivatives show a relatively low experimental initial anisotropy $r_{ex}(0) \leq 0.2$ compared with the theoretical value $r_{th}(0) = 0.4$ (3, 24). This may be due to the rapid decay of $r_p(t)$ and/or $r_s(t)$ which might be much faster than the instrumental time resolution. In fact, a large loss of anisotropy due to $r_p(t)$ has been observed; for example, ~35% loss for *N*-(1-anilino-4-naphthyl)-4-maleimide bound to cytochrome oxidase (32), and ~50% loss for eosin-maleimide bound to $(Ca^{2+} - Mg^{2+})ATPase$ (Kataoka, to be published), or to band 3 in erythrocytes (Cherry, to be published).

APPLICATION

Here we show experimental applications of the present theory to circularly degenerate metalloporphyrins.

Cytochrome Oxidase

Recently, Kawato et al. (25) have observed the rotational diffusion of cytochrome oxidase in the inner membrane of mitochondria. Cytochrome oxidase is a completely transmembrane protein, implying that rotation occurs about the normal to the plane of the membrane. They analyzed the data with the present "rotation-about-membrane normal" model. The absorption anisotropy of the heme-CO complex in mitochondria was measured after photolysis by a vertically polarized laser flash. The anisotropy decayed within 2 ms to a constant value. The experimental data were analyzed by the following equation:

$$r_r(t) = r_1 e^{-t/\phi_1} + r_2 e^{-4t/\phi_1} + r_3 \quad (47)$$

where ϕ_1 is the rotational relaxation time. In this way, they obtained $\phi_1 = 350 \pm 20 \mu\text{s}$, $r_1/r_2 = 2.1 \pm 1.4$, $r_3/r_r(0) = 0.54 \pm 0.05$ in 50% sucrose at 37°C. ESR and optical studies show that θ_N of heme a_3 chromophore is $\approx 90^\circ$ (29). It follows that if all cytochrome oxidase are mobile in the time range of 2 ms, the residual anisotropy ratio $[r_r(\infty)/r_r(0)]_{\text{th}}$ should be ≈ 0.25 according to Eq. 31 (see Fig. 4), since fourfold symmetry is a good approximation in their experimental conditions [photodissociation at 590 nm, measurement at 446 nm, $r_r(0) \approx 0.1$]. The failure of the $r(t)$ curves to fall to the predicted value therefore indicates the existence of an immobile cytochrome oxidase ($\phi_1 > 30$ ms). The fraction of immobile population, f_{im} , can be calculated as $\sim 40\%$ in mitochondria by Eq. 45. Moreover, a single rotating species should show $r_1/r_2 = 4\cot^2\theta_N \approx 0$ by Eq. 31. Therefore, the measured value of $r_1/r_2 \approx 2.1$ suggests a variety of rotating species such as monomer, dimer, etc. Immobile cytochrome oxidase in mitochondrial membranes might be oligomeric cytochrome oxidase or cytochrome oxidase complexed with other membrane proteins.

The rotation of cytochrome oxidase has also been investigated in phosphatidylcholine-phosphatidylethanolamine-cardiolipin vesicles. The $r_r(t)$ is expressed by Eq. 47 with $\phi_1 = 517 \pm 46 \mu\text{s}$ and $r_3/r_r(0) = 0.28 \pm 0.02$ in 60% sucrose at room temperature (23). Since $r_3/r_r(0)$ is close to the value expected from independent measurements of θ_N , this result strongly supports the validity of our theory and indicates that nearly all cytochrome oxidase ($>95\%$) are rotating in these lipid vesicles. However, the measured value of $r_1/r_2 \approx 1$ suggests multiple mobile populations, which is consistent with the observation by freeze-fracture electron micrographs (23).

Cytochrome P-450

Richter et al. (11) have demonstrated the rotational diffusion of cytochrome *P-450* in phenobarbital-induced rat liver microsomes at 23°C. The observed decay of $r(t)$ implies that, in disagreement with the ESR results (30), the heme plane does not lie in the plane of the membrane. Since cytochrome *P-450* is also a transmembrane protein, they analyzed their data by rotation-about-membrane normal model. Similar rotational diffusion of cytochrome *P-450* has been observed in phenobarbital-induced rabbit liver *P-450* at 21°C (39). The measured $r_r(\infty)/r_r(0)$ is ~ 0.6 – 0.8 (rat liver) and ~ 0.8 (rabbit liver). Further study of

rotational diffusion was performed with purified rat liver cytochrome *P*-450 in phosphatidylcholine-phosphatidylethanolamine-phosphatidylserine vesicles.² The $r_r(\infty)/r(0)$ in this vesicle system is ~ 0.12 . If we assume that all cytochrome *P*-450 are rotating in this vesicle system in analogy with cytochrome oxidase vesicles, the angle θ_N can be calculated as $\sim 40^\circ$ or 70° from Fig. 4. Assuming $[r_r(\infty)/r_r(0)]_{\text{th}} = 0.12$ it follows that between two-thirds and one-half of cytochrome *P*-450 is immobile in rat liver microsomes and around two-thirds of cytochrome *P*-450 is immobile in rabbit liver microsomes at room temperature. The rotational relaxation time was $\sim 100 \mu\text{s}$ for rat liver cytochrome *P*-450 in both microsomes and lipid vesicles in 60% sucrose at room temperature.

Cytochrome *b*₅

The rotational diffusion of cytochrome *b*₅ in dimyristoyl-lecithin vesicles has been demonstrated by Vaz et al. (4). The anisotropy of cytochrome *b*₅ was measured after flash photolysis of rhodium (III)-protoporphyrin IX which was incorporated into apocytochrome *b*₅. They interpreted their data in terms of wobbling of the whole protein around the membrane normal. The averaged orientation of the normal to the metalloporphyrin plane, *N*, has not yet been determined. If the average direction of *N* is parallel to the plane of the membrane, $r(\infty)/r(0)$ should be below 0.25 according to the "spinning-in-equatorial-band" model by Kinoshita et al. (16). Vaz et al. (4) observed the same $r(\infty)/r(0) = 0.6$ both below and above the lipid phase transition, implying that *N* is not parallel to the plane of the membrane. Data were, therefore, analyzed by the wobbling-in-cone model assuming that the average direction of *N* is perpendicular to the membrane plane. The rotational relaxation time is then $\phi = 9 \mu\text{s}$ in the gel phase and $\phi = 0.4 \mu\text{s}$ in the liquid-crystalline phase. The cone angle is $\theta_c = 33^\circ$ in both phases.

Local Lateral Diffusion

An interesting application of the rotational diffusion measurements may be to investigate the local lateral diffusion of integral membrane proteins. In the case of rotation-about-membrane normal, the local (free) lateral diffusion coefficient D_L^{loc} can be written as:

$$D_L^{\text{loc}} \approx \left(\ln \frac{\eta}{\eta'} - \gamma \right) a^2 D_{\parallel} \quad (48)$$

where *a* is the radius of the cylindrical protein, η' the viscosity of aqueous phase, η the membrane viscosity (1–10 P), γ the Euler's constant (0.5772) (20). In this way we may obtain $D_L^{\text{loc}} = 5 \times 10^{-9}$ – $5 \times 10^{-10} \text{ cm}^2/\text{s}$ for both cytochrome oxidase and cytochrome *P*-450 in both natural membranes and reconstituted lipid vesicles (23). The local lateral diffusion is of importance for understanding the electron transfer process in mitochondria and liver microsomes, since electrons may be transferred by local collisions between component enzymes (23, 40). Although there are several methods for measuring the long range lateral diffusion such as fluorescence photobleaching recovery method etc., no method exists for direct measurement of the local lateral diffusion. Generally for integral membrane proteins,

²Gut, J., C. Richter, R. J. Cherry, K. H. Winterhalter, and S. Kawato. Submitted for publication.

the long-range lateral diffusion coefficient D_L ($<10^{-10}$ cm²/s) is at least an order of magnitude smaller than D_L^{loc} (35). This difference is probably because the long range lateral diffusion is reduced by protein-protein collisions or by barriers against diffusion (e.g. cytoskeletal structures), while D_L^{loc} corresponds to free diffusion.

CONCLUSION

The essence of the present paper can be summarized as follows:

Rotation of Proteins about the Normal to the Plane of the Membrane

The time dependence of $r(t)/r(0)$ is described by Eqs. 30–34. For linear and circularly symmetric chromophores, the angle between the absorption moment(s) and the membrane normal can be calculated by Eq. 35 from the time-independent residual anisotropy ratio $r(\infty)/r(0)$. For general nonlinear chromophores, the angle between absorption moments and the membrane normal can be obtained by the combination of Eqs. 21, and 32–34, when the intensity of photoselection flash is weak.

In an accompanying paper (31), the validity of the theory is tested for a simple model system consisting of bacteriorhodopsin incorporated into lipid bilayers. It is found that Eq. 31 provides an excellent fit to the experimental data under all conditions where only monomeric bacteriorhodopsin is present.

Restricted Wobbling of either Part of the Protein Molecule or the Whole Protein Molecule

The time dependence of $r(t)/r(0)$ is described by Eqs. 36–39. The orientational constraint of the wobbling (cone angle, order parameter) can be obtained by Eqs. 37 and 44 from $r(\infty)/r(0)$.

Effect of Excitation Intensity

Intense flash excitation is often necessary in order to get good signal to noise. For linear and circularly symmetric chromophores, $r(t)/r(0)$ is independent of the flash intensity and only $r(0)$ is dependent on the intensity of photoselecting flash.

Protein-Protein Interactions

The theory is useful for investigating the current topic of protein-protein interactions in biological and reconstituted lipid membranes. Since protein rotation is very sensitive to the size of the protein, the deviation of experimental decay parameters from theoretically predicted values for a single rotating species indicates the existence of protein-protein interactions. Examples of this approach are illustrated for cytochrome oxidase and cytochrome P-450 in Application.

The above approach was previously used to demonstrate different populations of band 3 in the erythrocyte membrane (41). In the accompanying paper (31), it is shown that in bacteriorhodopsin-lipid vesicles a very small proportion of protein aggregates causes significant deviation from the theoretical curve.

APPENDIX I

Here we show the derivation of Eqs. 12–21 in the text. Since $r(t)$ and Δ_c do not depend on the direction of \mathbf{Z} in space in a macroscopically isotropic sample, we can average these over \mathbf{Z} without changing their values.

Case I: Circularly Degenerate Chromophore

$$\begin{aligned}\Delta_{c-1} &= (1/4\pi) \int [1 - e^{-\kappa} e^{\kappa(\mathbf{N}^0 \cdot \mathbf{Z})^2}] W^s d\omega d\mathbf{Z} \\ &= 1 - e^{-\kappa} \sum_{n=0}^{\infty} \frac{\kappa^n}{n!(2n+1)}\end{aligned}\quad (\text{I } 1)$$

$$\begin{aligned}r(t) &= \frac{-1}{8\pi\Delta_{c-1}} \int P_2(\mathbf{N} \cdot \mathbf{Z}) [1 - e^{-\kappa} e^{\kappa(\mathbf{N}^0 \cdot \mathbf{Z})^2}] \\ &\quad \times W^s G d\Omega d\mathbf{Z}.\end{aligned}\quad (\text{I } 2)$$

In order to perform the integration with respect to \mathbf{Z} , we use the addition theorem

$$\begin{aligned}P_2(\mathbf{N} \cdot \mathbf{Z}) &= P_2(\mathbf{N} \cdot \mathbf{N}^0) P_2(\mathbf{N}^0 \cdot \mathbf{Z}) + (1/3) P_2^1(\mathbf{N} \cdot \mathbf{N}^0) P_2^1(\mathbf{N}^0 \cdot \mathbf{Z}) \cos(\phi - \phi') \\ &\quad + (1/12) P_2^2(\mathbf{N} \cdot \mathbf{N}^0) P_2^2(\mathbf{N}^0 \cdot \mathbf{Z}) \cos 2(\phi - \phi')\end{aligned}\quad (\text{I } 3)$$

where $P_2^1(x)$, $P_2^2(x)$ are the associate Legendre polynomials (see Eq. 43 in the text), ϕ and ϕ' are the azimuthal angles of \mathbf{Z} and \mathbf{N} around \mathbf{N}^0 . Taking into account the property that the evolution function G is not dependent on the direction \mathbf{Z} but only on the relative arrangement between $\{\mathbf{N}^0, \mathbf{H}_1^0\}$ and $\{\mathbf{N}, \mathbf{H}_1\}$, the second and third terms in Eq. I3 drop out upon integration over ϕ . Thus

$$\begin{aligned}r(t) &= \frac{-1}{8\pi\Delta_{c-1}} \int P_2(\mathbf{N}^0 \cdot \mathbf{Z}) [1 - e^{-\kappa} e^{\kappa(\mathbf{N}^0 \cdot \mathbf{Z})^2}] d\mathbf{Z} \\ &\quad \times \int P_2(\mathbf{N} \cdot \mathbf{N}^0) W^s G d\Omega\end{aligned}\quad (\text{I } 4)$$

Using the following formula (n : integer):

$$\int x^n e^{ax^2} dx = \frac{x^{n-1} e^{ax^2}}{2a} - \frac{n-1}{2a} \int x^{n-2} e^{ax^2} dx \quad (\text{I } 5)$$

we obtain

$$\begin{aligned}r(0) &= \frac{-1}{8\pi\Delta_{c-1}} \int P_2(\mathbf{N}^0 \cdot \mathbf{Z}) [1 - e^{-\kappa} e^{\kappa(\mathbf{N}^0 \cdot \mathbf{Z})^2}] d\mathbf{Z} \\ &= \left[\frac{3}{2\kappa} - \left(1 + \frac{3}{2\kappa} \right) e^{-\kappa} \sum_{n=0}^{\infty} \frac{\kappa^n}{n!(2n+1)} \right] / 4 \left[1 - e^{-\kappa} \sum_{n=0}^{\infty} \frac{\kappa^n}{n!(2n+1)} \right].\end{aligned}\quad (\text{I } 6)$$

Case II: Linear Chromophore

$$\begin{aligned}\Delta_{c-0} &= \frac{1}{4\pi} \int [1 - e^{-\kappa(\mathbf{H}_1^0 \cdot \mathbf{Z})^2}] W^s d\omega d\mathbf{Z} \\ &= 1 - \sum_{n=0}^{\infty} \frac{(-1)^n \kappa^n}{n!(2n+1)}.\end{aligned}\quad (\text{I } 7)$$

$$r(t) = \frac{1}{4\pi\Delta_{c=0}} \int P_2(\mathbf{H}_1^0 \cdot \mathbf{Z}) [1 - e^{-\kappa(\mathbf{H}_1^0 \cdot \mathbf{Z})^2}] d\mathbf{Z} \\ \times \int P_2(\mathbf{H}_1 \cdot \mathbf{H}_1^0) W^s G d\Omega \quad (\text{I } 8)$$

$$r(0) = \frac{1}{4\pi\Delta_{c=0}} \int P_2(\mathbf{H}_1^0 \cdot \mathbf{Z}) [1 - e^{-\kappa(\mathbf{H}_1^0 \cdot \mathbf{Z})^2}] d\mathbf{Z} \\ = \left[\frac{3}{2\kappa} e^{-\kappa} + \left(1 - \frac{3}{2\kappa}\right) \sum_{n=0}^{\infty} \frac{(-1)^n \kappa^n}{n!(2n+1)} \right] \left/ 2 \left[1 - \sum_{n=0}^{\infty} \frac{(-1)^n \kappa^n}{n!(2n+1)} \right] \right. \quad (\text{I } 9)$$

where again we use the addition theorem for $P_2(\mathbf{H}_1 \cdot \mathbf{Z})$ and Eq. I5. We should note that G is independent of the direction \mathbf{Z} .

Case III: Weak Excitation

When $\kappa \ll 1$, Eq. 9 simplifies to

$$1 - \exp \{-\kappa[(\mathbf{H}_1^0 \cdot \mathbf{Z})^2 + c^2(\mathbf{H}_2^0 \cdot \mathbf{Z})^2]\} \simeq \kappa[(\mathbf{H}_1^0 \cdot \mathbf{Z})^2 + c^2(\mathbf{H}_2^0 \cdot \mathbf{Z})^2] \quad (\text{I } 10)$$

$$\Delta_c \simeq (\kappa/4\pi) \int [(\mathbf{H}_1^0 \cdot \mathbf{Z})^2 + c^2(\mathbf{H}_2^0 \cdot \mathbf{Z})^2] W^s d\omega d\mathbf{Z} = (\kappa/3)(c^2 + 1). \quad (\text{I } 11)$$

Since $r(t) = (1/4\pi) \int r(t) d\mathbf{Z}$, $r(t)$ can be written by

$$r(t) = [1/(c^2 + 1)^2] \int [P_2(\mathbf{H}_1 \cdot \mathbf{Z}) \\ + c^2 P_2(\mathbf{H}_2 \cdot \mathbf{Z})] (3/4\pi) [(\mathbf{H}_1^0 \cdot \mathbf{Z})^2 + c^2(\mathbf{H}_2^0 \cdot \mathbf{Z})^2] W^s G d\Omega d\mathbf{Z}. \quad (\text{I } 12)$$

We use the following relationship for integration with respect to \mathbf{Z} :

$$\int P_2(\mathbf{H}_i \cdot \mathbf{Z}) (3/4\pi) (\mathbf{H}_j^0 \cdot \mathbf{Z})^2 W^s G d\Omega d\mathbf{Z} = 0.4 \int P_2(\mathbf{H}_i \cdot \mathbf{H}_j^0) W^s G d\Omega \quad (\text{I } 13)$$

where we use the addition theorem $P_2(\mathbf{H}_i \cdot \mathbf{Z}) = P_2(\mathbf{H}_i \cdot \mathbf{H}_j^0) P_2(\mathbf{H}_j^0 \cdot \mathbf{Z}) + \dots$. Thus, we obtain

$$r(t) = [0.4/(c^2 + 1)^2] \int \{P_2(\mathbf{H}_1 \cdot \mathbf{H}_1^0) + c^2[P_2(\mathbf{H}_1 \cdot \mathbf{H}_2^0) + P_2(\mathbf{H}_2 \cdot \mathbf{H}_1^0)] \\ + c^4 P_2(\mathbf{H}_2 \cdot \mathbf{H}_2^0)\} W^s G d\Omega. \quad (\text{I } 14)$$

The set $\{\mathbf{H}_1, \mathbf{H}_2, \mathbf{N}\}$ or $\{\mathbf{H}_1^0, \mathbf{H}_2^0, \mathbf{N}^0\}$ satisfies the relationship

$$P_2(\mathbf{U} \cdot \mathbf{H}_1) + P_2(\mathbf{U} \cdot \mathbf{H}_2) + P_2(\mathbf{U} \cdot \mathbf{N}) = 0 \quad (\text{I } 15)$$

where \mathbf{U} is any unit vector. It follows that

$$r(t) = [0.4/(c^2 + 1)^2] \int \{c^4 P_2(\mathbf{N} \cdot \mathbf{N}^0) + (c^2 - 1)^2 P_2(\mathbf{H}_1 \cdot \mathbf{H}_1^0) \\ + c^2(c^2 - 1)[P_2(\mathbf{H}_1^0 \cdot \mathbf{N}) + P_2(\mathbf{H}_1 \cdot \mathbf{N}^0)]\} W^s G d\Omega. \quad (\text{I } 16)$$

Setting $t = 0$, since $\mathbf{H}_1^0 \cdot \mathbf{N}^0 = 0$, we obtain

$$r(0) = \frac{0.4}{(c^2 + 1)^2} (c^4 - c^2 + 1). \quad (\text{I } 17)$$

APPENDIX II

Here we show the derivation of Eqs. 30–34 in the text. Let \mathbf{n} be the unit vector which is normal to the membrane plane. An addition theorem for unit vectors \mathbf{I}, \mathbf{J} is written as:

$$P_2(\mathbf{I} \cdot \mathbf{J}) = P_2(\mathbf{n} \cdot \mathbf{I})P_2(\mathbf{n} \cdot \mathbf{J}) + (1/3)P_2^1(\mathbf{n} \cdot \mathbf{I})P_2^1(\mathbf{n} \cdot \mathbf{J}) \cos(\phi_I - \phi_J) \\ + (1/12)P_2^2(\mathbf{n} \cdot \mathbf{I})P_2^2(\mathbf{n} \cdot \mathbf{J}) \cos 2(\phi_I - \phi_J) \quad (\text{II } 1)$$

where ϕ_I and ϕ_J are the azimuthal angles of \mathbf{I} and \mathbf{J} around \mathbf{n} , respectively. Applying Eq. II1 to Eqs. 23–25, it follows that

$$\int P_2(\mathbf{N}^0 \cdot \mathbf{N}) w^s g d\mathbf{N}^0 d\mathbf{N} = P_2(\cos \theta_N)^2 + (1/3)P_2^1(\cos \theta_N)^2 e^{-D_1 t} \\ + (1/12)P_2^2(\cos \theta_N)^2 e^{-4D_1 t} \quad (\text{II } 2)$$

$$\int P_2(\mathbf{H}_1^0 \cdot \mathbf{H}_1) w^s g d\mathbf{N}^0 d\mathbf{N} = P_2(\cos \theta_{H_1})^2 + (1/3)P_2^1(\cos \theta_{H_1})^2 e^{-D_1 t} \\ + (1/12)P_2^2(\cos \theta_{H_1})^2 e^{-4D_1 t} \quad (\text{II } 3)$$

$$\int P_2(\mathbf{N}^0 \cdot \mathbf{H}_1) w^s g d\mathbf{N}^0 d\mathbf{N} = \int P_2(\mathbf{H}_1^0 \cdot \mathbf{N}) w^s g d\mathbf{N}^0 d\mathbf{N} \\ = P_2(\cos \theta_N)P_2(\cos \theta_{H_1}) + (1/3)P_2^1(\cos \theta_N)P_2^1(\cos \theta_{H_1}) \cos(\phi_N - \phi_{H_1}) e^{-D_1 t} \\ + (1/12)P_2^2(\cos \theta_N)P_2^2(\cos \theta_{H_1}) \cos 2(\phi_N - \phi_{H_1}) e^{-4D_1 t} \quad (\text{II } 4)$$

where we use $\phi_N - \phi_{H_1} = \phi_{N^0} - \phi_{H_1^0} = \text{constant}$ and w^s, g are given by Eqs. 28 and 29. Therefore, Eqs. 23–25 can be summarized as Eq. 30 (double exponential plus constant). Eqs. 32–34 are readily obtained by substituting Eqs. II2–II4 into Eq. 25 and using the following relationship:

$$\mathbf{N} \cdot \mathbf{H}_1 = 0 = \cos \theta_N \cos \theta_{H_1} + \sin \theta_N \sin \theta_{H_1} \cos(\phi_N - \phi_{H_1}). \quad (\text{II } 5)$$

APPENDIX III

Here we treat the anisotropy decay for nonlinear chromophores when the magnitude of cH_2 differs between excitation and measurement wavelengths. Let $c_e H_2$ and $c_m H_2$ be, respectively, cH_2 at excitation and measurement wavelengths. Eqs. 5, 10 and 11 can be rewritten as follows:

$$r(t) = \frac{1}{c_m^2 + 1} \langle P_2(\mathbf{H}_1 \cdot \mathbf{Z}) + c_m^2 P_2(\mathbf{H}_2 \cdot \mathbf{Z}) \rangle_t \quad (\text{III } 1)$$

$$1/\Delta_c \equiv 1/\int (1 - \exp\{-\kappa[(\mathbf{H}_1^0 \cdot \mathbf{Z})^2 + c_e^2(\mathbf{H}_2^0 \cdot \mathbf{Z})^2]\}) W^s G d\Omega. \quad (\text{III } 2)$$

$$r(t) = \frac{1}{(c_m^2 + 1)\Delta_c} \int [P_2(\mathbf{H}_1 \cdot \mathbf{Z}) + c_m^2 P_2(\mathbf{H}_2 \cdot \mathbf{Z})] \\ \times (1 - \exp\{-\kappa[(\mathbf{H}_1^0 \cdot \mathbf{Z})^2 + c_e^2(\mathbf{H}_2^0 \cdot \mathbf{Z})^2]\}) W^s G d\Omega \quad (\text{III } 3)$$

where $\kappa = 3QI\sigma/(1 + c_e^2)$. In case III: weak excitation, Eqs. 19–21, 25 become

$$\Delta_c = (\kappa/3)(c_e^2 + 1) \quad (\kappa \ll 1) \quad (\text{III } 4)$$

$$r(t)/r(0) = (1/F) \int [P_2(\mathbf{H}_1^0 \cdot \mathbf{H}_1) + c_e^2 P_2(\mathbf{H}_2^0 \cdot \mathbf{H}_1) \\ + c_m^2 P_2(\mathbf{H}_1^0 \cdot \mathbf{H}_2) + c_e^2 c_m^2 P_2(\mathbf{H}_2^0 \cdot \mathbf{H}_2)] W^s G d\Omega \quad (\text{III } 5)$$

$$r(0) = 0.4 \frac{F}{(c_e^2 + 1)(c_m^2 + 1)} \quad (\text{III } 6)$$

$$r(t)/r(0) = (1/F) \int [P_2(\mathbf{H}_1^0 \cdot \mathbf{H}_1) + c_e^2 P_2(\mathbf{H}_1^0 \cdot \mathbf{H}_1) + c_m^2 P_2(\mathbf{H}_1^0 \cdot \mathbf{H}_2) + c_e^2 c_m^2 P_2(\mathbf{H}_2^0 \cdot \mathbf{H}_2)] w^4 g \, d\Omega \quad (\text{III } 7)$$

where $F = c_e^2 c_m^2 - (c_e^2 + c_m^2)/2 + 1$. Eqs. 30 and 32–34 for rotation about membrane normal in the case of weak excitation are then replaced by

$$r(t)/r(0) = A_1 e^{-D_1 t} + A_2 e^{-4D_1 t} + A_3, \quad (\text{III } 8)$$

$$A_1 = (3/F) [\sin^2 \theta_{H_1} \cos^2 \theta_{H_1} - (c_e^2 + c_m^2) \cos^2 \theta_{H_1} \cos^2 \theta_{H_2} + c_e^2 c_m^2 \sin^2 \theta_{H_2} \cos^2 \theta_{H_2}] \quad (\text{III } 9)$$

$$A_2 = (3/4F) [\sin^4 \theta_{H_1} - (c_e^2 + c_m^2) (2 \cos^2 \theta_{H_1} \cos^2 \theta_{H_2} - \sin^2 \theta_{H_1} \sin^2 \theta_{H_2}) + c_e^2 c_m^2 \sin^4 \theta_{H_2}] \quad (\text{III } 10)$$

$$A_3 = (1/4F) [(3 \cos^2 \theta_{H_1} - 1)^2 + (c_e^2 + c_m^2) (3 \cos^2 \theta_{H_1} - 1) (3 \cos^2 \theta_{H_2} - 1) + c_e^2 c_m^2 (3 \cos^2 \theta_{H_2} - 1)^2] \quad (\text{III } 11)$$

where $F = c_e^2 c_m^2 - (c_e^2 + c_m^2)/2 + 1$.

The heme a_3 · CO complex of cytochrome oxidase is a nonlinear chromophore whose $r(0)$ has been shown to depend on both excitation and measurement wavelengths (22). However, it has also been shown for this chromophore that c_e is close to 1 at 590 nm and c_m is 1 at 447 nm (23). Therefore, when the heme a_3 · CO complex is excited at 590 nm by a laser flash of Rhodamine 6G in methanol and measured at 447 nm, the chromophore can be treated as circularly symmetric (23).

APPENDIX IV

Here we discuss the meaning of the cone angle θ_c in the wobbling-in-cone model when the actual potential is not the square-well type. We consider a simple cosine type potential V given by

$$V = \alpha |\cos \theta| \quad (\text{IV } 1)$$

where θ is the angle between the membrane normal and \mathbf{N} (circularly symmetric chromophore) or \mathbf{H}_1 (linear chromophore) (see Fig. 5). The stationary distribution is given by

$$w^s(\theta) = \frac{q}{e^q - 1} e^{q |\cos \theta|} \quad (\text{IV } 2)$$

where $q = -\alpha/kT$ determines the width of the distribution. We define θ_c by the order parameter S as expressed by Eqs. 37 and 44:

$$S = 1 - (3/q) + (3/q^2) + \left[3 \left(1 - \frac{2}{q} \right) / 2(e^q - 1) \right] = \frac{1}{2} \cos \theta_c (1 + \cos \theta_c) \quad (\text{IV } 3)$$

We define θ_{1/e^2} such that the fractional population between θ_{1/e^2} and 90° is equal to $1/e^2$ of the total chromophores:

$$\int_{\cos \theta_{1/e^2}}^1 w^s \, d \cos \theta = 1 - e^{-2} \quad (\text{IV } 4)$$

Fig. 6 shows that there is a close correspondence between θ_c and θ_{1/e^2} . Thus, θ_c is a useful parameter for determining the angular width of the chromophore distribution independent of the actual potential.

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