Journal of The Physical Society of Japan
Vol. 59, No. 7, July, 1990, pp. 2584–2595

A Theory of Optical Anisotropy Decay in Membranes

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(Received March 5, 1990)

The theory of optical anisotropy decay $r(t)$ for a protein in a membrane suspension is developed. Our new model describes rotational diffusion of membrane anchored proteins consisting of two different modes occurring simultaneously: one is restricted wobbling of the whole or part of the protein molecule, and the other is rotation of the whole protein about a fixed axis. The anisotropy decay $r(t)$ is characterized by a diffusion coefficient, a cone angle and an angle between the symmetric axis of the wobbling part of the protein and the optical moment of the probe fixed rigidly in the protein. Although the exact $r(t)$ consists of infinite number of exponential functions, an accurate monoexponential plus constant approximation for $r(t)$ is shown to be sufficient for the analysis of experimental data. We derived an analytic expression for the effective relaxation time which appears in this approximation. This treatment may be applicable to complex wobbling plus axial rotation of membrane anchored proteins such as cytochrome b_5.

§1. Introduction

Studies on the dynamics of molecules in biological and artificial membranes are of importance in understanding the dynamic structures and physiological mechanics of functional proteins in biological membranes. Especially, dynamic protein-protein interactions in biological membranes is essential in complex enzyme systems, for example, electron transfer reactions in oxydative phosphorylation, drug metabolism and steroid hormone synthesis.

There are increasing amount of experiments on slow rotational diffusion of membrane proteins using triplet probes with long lifetime of the triplet state or intrinsic chromophores having long-lived photoproducts. These measurements detect the decay of either dichroism or phosphorescence polarization after flash excitation.[1-7]

A variety of theories have been presented to describe quantitatively the optical polarization decay $r(t)$ in the case of anisotropic rotation of macromolecules in the membrane such as an axial rotation of integral membrane protein about membrane normal and a restricted wobbling of proteins in membranes.[8-12] Lipari, Szabo and other people gave a theory to analyze NMR data on anisotropic rotation of aminoacid residues in proteins which is applicable to the above membrane systems.[13-20]

In our previous paper,[21] we have also treated the theory of absorption and emission anisotropy decay for axial rotation and restricted wobbling of membrane proteins. It is useful when these two motions occur in a completely different time range. However it is not applicable when wobbling and axial rotation occur in the same time scale (wobbling plus axial rotation). Practically, membrane proteins such as cytochrome b_5 and glycophorin A may undergo both rotation modes simultaneously, that is, rotating about the membrane normal axis with wobbling in the same time scale.

In this paper we propose a new wobbling model which includes the rotational effect. Using this model, we have derived simple expressions for the limiting anisotropy $r(\infty)$ and convenient approximate expressions for $r(t)$ which can be used to analyze the experimental results. We calculated the effective relaxation time analytically by the method given by Lipari *et al.*[13] Although we develop the theory for rotation of membrane proteins, our theory is also applicable to a variety of protein motions in supramolecular systems.

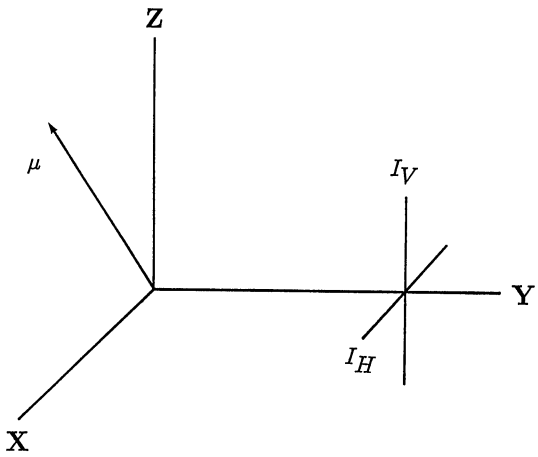

Fig. 1. Geometry of the system. The vector μ represents the direction of emission transition moment of an optical probe in the sample which is located at the origin of the coordinate. $I_V(t)$ and $I_H(t)$ are intensity components of emitted light for vertical and horizontal polarization.

First we follow a general formalism. In §3, we give our new model followed by the detailed calculation. Application of our theory is illustrated by measurements of the rotational diffusion of heme proteins (cytochrome b_5) in §4.

§2. Optical Anisotropy in Membrane Suspensions

In this paper, we consider the case where a chromophore is bound to proteins which are anchored in planner membrane fragments. Membranes are equivalent to an ensemble of planner membrane fragments which are immobile in the present time scale. A distinct feature of such a system is that it is isotropic as a whole.

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

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

$$r(t)=\frac{I_V(t)-I_H(t)}{I_V(t)+2I_H(t)}. \tag{1}$$

In the above, $I_V(t)$ and $I_H(t)$ are intensity components of either absorption change or emission for vertical and horizontal polarization at time t after the flash.

Hereafter, we use the notations of emission depolarization. Let μ be the direction of the emission moment. The following description is straightforwardly applicable to absorption anisotropy by reading μ as the absorption moment for linear chromophores or the direction perpendicular to the plane of the chromophore for circularly symmetric chromophores.[21] For simplicity, we assume that the absorption dipole at the wavelength of excitation and the emission dipole μ are parallel. We introduce a normalized distribution function $w(\mu, t)$, the probability that we would find the emission dipole with orientation μ, i.e.,

$$\int w(\mu, t)\,\mathrm{d}\mu=1. \tag{2}$$

For the evolution of w with time, we define $g(\mu^0, 0|\mu, t)$ as the probability that the emission dipole with orientation μ^0 at time 0 will rotate into a new orientation μ by time t; thus,

$$w(\mu, t)=\int w(\mu^0, 0)g(\mu^0, 0|\mu, t)\,\mathrm{d}\mu^0. \tag{3}$$

In particular, g reduces to $\delta(\mu^0-\mu)$ for $t=0$. The stationary distribution w^s is given by letting $t\to\infty$,

$$w^s(\mu)=g(\mu^0, 0|\mu, \infty). \tag{4}$$

Following the derivation in refs. 11, 12, $r(t)$ can be expressed in terms of these functions as

$$\frac{r(t)}{r(0)}=\iint P_2(\mu^0\cdot\mu)w^s(\mu^0)g(\mu^0, 0|\mu, t)\,\mathrm{d}\mu^0\,\mathrm{d}\mu, \tag{5}$$

where $P_2(x)=(3x^2-1)/2$ is the second Legendre polynomial and $r(0)=0.4$. For $t\to\infty$, we obtain

$$\frac{r(\infty)}{r(0)}=\left[\int P_2(\boldsymbol{n}\cdot\mu)w^s(\mu)\,\mathrm{d}\mu\right]^2, \tag{6}$$

where $\boldsymbol{n}$ is the normal to the membrane. Here we have used eq. (4) and the addition theorem for $P_2(\mu^0\cdot\mu)$. The quantity $r(\infty)/r(0)$ cor-

responds to the degree of orientational constraint. When the absorption and the emission moment make a fixed angle λ, $r(0)$ in eqs. (5) and (6) should be replaced by $r(0)=0.4P_2(\cos\lambda)$ instead of 0.4.

§3. Wobbling under Rotational Effect

Wobbling motion is of frequent observation in supramolecular systems, such as, internal motion of a subunit in a macromolecule or flexible motion of a fibrous structure. A typical type of wobbling of proteins is expressed as "wobbling-in-cone".[11,13,22] Let $\boldsymbol{\nu}$ be the unit vector directed along the symmetry axis of the wobbling part of the protein. This model allows $\boldsymbol{\nu}$ to diffuse freely within a cone around membrane normal $\boldsymbol{n}$. The cone, with half angle θ_c, may be formed by steric hindrance of surrounding structures. The restricted motion of the probe molecule results in the non-zero value of $r(\infty)$. Kinosita *et al.*[11] solved the above problem in the cases of rod-shaped molecule with emission dipole $\boldsymbol{\mu}$ parallel and perpendicular to the long axis.

In this paper, we generalize our discussion to the case where the emission dipole $\boldsymbol{\mu}$ has an arbitrary but fixed angle $\alpha(0°\leq\alpha\leq90°)$ with respect to $\boldsymbol{\nu}$ (see Fig. 2). At the first part of this section, we consider the wobbling motion without axial rotation; $\boldsymbol{\mu}$ does not rotate around $\boldsymbol{\nu}$. Then, in the succeeding subsection, we examine the effect of axial rotation of $\boldsymbol{\mu}$ around $\boldsymbol{\nu}$ which combines with the wobbling. A concise review of the results for "wobbling-in-cone" model and "axial rotation" model are given in Appendix A and C, respectively.

3.1 Wobbling without axial rotation

If the rod-shaped molecule does not rotate about its long axis $\boldsymbol{\nu}$, the orientation of the dipole $\boldsymbol{\mu}$ is still confined within a cone around

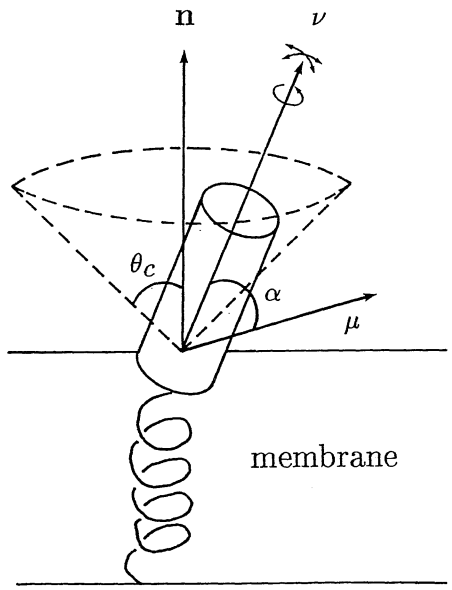

Fig. 2. Schematic representation of an optical probe fixed rigidly on the hydrophilic head of the membrane anchored protein. There is an axial rotation about $\boldsymbol{\nu}$ which in turn wobbles in a cone (dashed line) of half angle θ_c. $\boldsymbol{\mu}$ has a fixed angle α with respect to $\boldsymbol{\nu}$.

$\boldsymbol{n}'$ tilted by α from $\boldsymbol{n}$. The half angle of this cone is also θ_c (see Fig. 3). Here, we insist that the same discussions given for "wobbling-in-cone" (see Appendix A) are applicable to such "wobbling-in-*tilted*-cone" case. This can be understood as follows.

First, eq. (5) can be used as it is, since it is given irrespective of $\boldsymbol{n}$. Equation (6), however, should suffer a slight modification, since the motion under consideration is no longer symmetric around $\boldsymbol{n}$. We let $t\to\infty$ in eq. (5). With the aid of eq. (4), we obtain

$$\frac{r(\infty)}{r(0)}=\iint P_2(\boldsymbol{\mu}^0\cdot\boldsymbol{\mu})\,w^s(\boldsymbol{\mu}^0)\,w^s(\boldsymbol{\mu})\,\mathrm{d}\boldsymbol{\mu}^0\,\mathrm{d}\boldsymbol{\mu}. \quad (7)$$

We substitute next addition theorem to eq. (7),

$$P_2(\boldsymbol{\mu}^0\cdot\boldsymbol{\mu})=P_2(\boldsymbol{n}'\cdot\boldsymbol{\mu}^0)P_2(\boldsymbol{n}'\cdot\boldsymbol{\mu})+\frac{1}{3}P_2^1(\boldsymbol{n}'\cdot\boldsymbol{\mu}^0)P_2^1(\boldsymbol{n}'\cdot\boldsymbol{\mu})\cos(\varphi^0-\varphi)$$
$$+\frac{1}{12}P_2^2(\boldsymbol{n}'\cdot\boldsymbol{\mu}^0)P_2^2(\boldsymbol{n}'\cdot\boldsymbol{\mu})\cos 2(\varphi^0-\varphi), \quad (8)$$

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

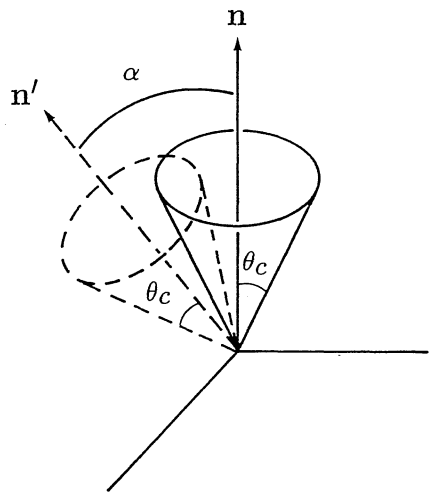

Fig. 3. "Wobbling-in-cone" model and "wobbling-in-*tilted*-cone" model. The half angle of each cone is θ_c. n' is tilted by α from n.

$$\frac{r(\infty)}{r(0)}=\left[\int P_2(n'\cdot\mu)w^s(\mu)\,d\mu\right]^2. \tag{9}$$

When we take θ and φ to be the polar and azimuthal angles of μ with respect to n' (not n), the same diffusion equation for "wobbling-in-cone" model (see later eq. (10)) holds for $w(\mu, t)$. Therefore, the remaining discussions completely follow the ones given for "wobbling-in-cone" model (see Appendix A).

Another interpretation is possible by noticing that the membrane suspension is isotropic as a whole. Therefore the relative orientation of the cone with respect to the membrane fragment has no influence on the experimentally obtained anisotropy $r(t)$.

3.2 *Wobbling under rotational effect*

Next we consider the case where the principal mode of motion of μ will be the "spinning" around ν, on which the effect of wobbling motion of ν around n is superposed. We especially focus on the situation when two motions occur in the same time range. To take this effect into account, we assume that the coupled motion can be described by only one wobbling diffusion constant D. We further simplify this complicated motion by assuming that the stationary distribution of μ is uniform over certain restricted region. Within our model, the above restricted region will depend on the relative value of θ_c with respect to α. In the following, we examine $r(\infty)/r(0)$ and $r(t)/r(0)$ in three cases: *I*, $\theta_c<\alpha$; *II*, $\theta_c=\alpha$; *III*, $\theta_c>\alpha$. For our later purpose, we introduce the notations; $\theta_1=|\theta_c-\alpha|$, $\theta_2=\theta_c+\alpha$, $x_1=\cos\theta_1$ and $x_2=\cos\theta_2$.

Case I: $\theta_c<\alpha$

We redefine θ and φ to be the polar and azimuthal angles of μ around n. Under the above assumptions, μ diffuses freely in a potential which has the shape like $V(\theta)=0$ for $\theta_1\le\theta\le\theta_2$, $V(\theta)=\infty$ otherwise (see Fig. 4(a)). The distribution function $w(\mu, t)=w(\theta, \varphi, t)$ obeys the simple diffusion equation

$$\frac{\partial}{\partial t}w(\mu, t)=D\nabla_\mu^2 w(\mu, t), \tag{10}$$

where ∇_μ^2 is the angular part of the Laplacian operating on μ:

$$\nabla_\mu^2=\frac{1}{\sin\theta}\frac{\partial}{\partial\theta}\left(\sin\theta\frac{\partial}{\partial\theta}\right)+\frac{1}{\sin^2\theta}\frac{\partial^2}{\partial\varphi^2}. \tag{11}$$

Diffusion equation (10) is subjected to the boundary conditions,

$$\frac{\partial}{\partial\theta}w(\mu, t)=0 \text{ at } \theta=\theta_1 \text{ and } \theta=\theta_2. \tag{12}$$

The homogeneous solution of eq. (10) with the initial condition

$$w(\mu, 0)=\delta(\mu^0-\mu), \tag{13}$$

gives g in eq. (3). The stationary distribution $w^s(\mu)$ is uniform over the band $\theta_1\le\theta\le\theta_2$, i.e.,

$$\begin{aligned} w^s(\mu)&=\frac{1}{2\pi(x_1-x_2)} && \theta_1\le\theta\le\theta_2,\\ &= 0 && \text{otherwise.} \end{aligned} \tag{14}$$

Using this stationary distribution, we obtain $r(\infty)/r(0)$ from eq. (6)

$$\frac{r(\infty)}{r(0)}=\left[\frac{1}{2}(1-x_1^2-x_1x_2-x_2^2)\right]^2\equiv B_\infty, \tag{15}$$

which is equivalently given in terms of θ_c and α by

$$\frac{r(\infty)}{r(0)}=\left[\frac{1}{4}(\cos 2\theta_c+2\cos 2\theta_c\cos 2\alpha+\cos 2\alpha)\right]^2. \tag{16}$$

Here, it might be interesting that $r(\infty)/r(0)$

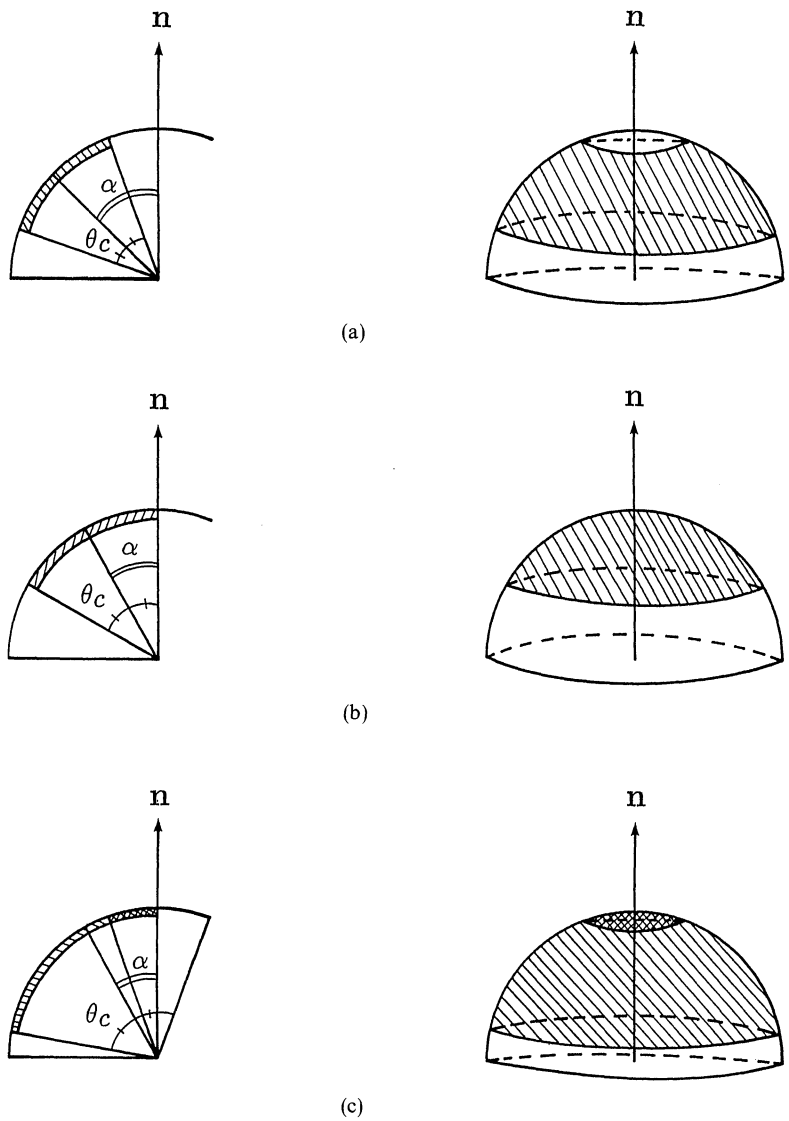

Fig. 4. "Wobbling under rotational effect" model. The shape of the square well potential is classified into three cases. (a) *Case I*: $\theta_c < \alpha$. The cross section of the hemisphere on the right hand side is depicted on the left hand side. The stationary distribution of μ is uniform over the band region hatched with parallel lines and zero otherwise. (b) *Case II*: $\theta_c = \alpha$. This case corresponds to the "wobbling-in-cone" model with the half angle $2\theta_c$. (c) *Case III*: $\theta_c > \alpha$. The stationary distribution of μ in the inverted bowl region hatched with crossed lines is twice as much as that in the band region hatched with parallel lines.

$=1/64$ is determined by only either θ_c or α when one of these is equal to 60°.

The expression for $r(t)/r(0)$ is given as

$$\frac{r(t)}{r(0)} = \sum_{i=1}^{\infty} B_i \exp(-Dt/\xi_i), \tag{17}$$

where B_i and ξ_i are constants that depend on θ_1 and θ_2. These are consistently determined by the boundary conditions (12) for $g(\mu^0, 0|\mu, t)$. Especially, B_∞ is defined by eq. (15) and $\xi_\infty = \infty$. Equation (17) can be approximated by a monoexponential expression

$$\left(\frac{r(t)}{r(0)}\right)_{\text{approx}}=B_\infty+(1-B_\infty)\exp(-t/\phi_I). \quad (18)$$

In order to determine the effective relaxation time ϕ_I, eq. (18) should satisfy the next relation (see (A·5))

$$\int_0^\infty\left[\left(\frac{r(t)}{r(0)}\right)_{\text{approx}}-B_\infty\right]dt=\int_0^\infty\left[\frac{r(t)}{r(0)}-B_\infty\right]dt. \quad (19)$$

By following the same procedure given by Lipari *et al.*,[13] we have calculated the exact analytic expression for ϕ_I (see Appendix B for the details):

$$\begin{aligned}D\phi_I(1-B_\infty)=&\frac{c_1}{2(x_1-x_2)}(1-x_1)(1-x_2)(1+x_1+x_2)\log\frac{1-x_2}{1-x_1}\\&+\frac{c_2}{2(x_1-x_2)}(1+x_1)(1+x_2)(1-x_1-x_2)\log\frac{1+x_1}{1+x_2}\\&+\frac{1}{24}[3(1-4c_1-4c_2+c_5+c_6)+3(-2c_1+2c_2+2c_3+2c_4-c_5+c_6)(x_1+x_2)\\&+(1+8c_1+8c_2-4c_3+4c_4+c_5+c_6-2\langle P_2\rangle)(x_1^2+x_1x_2+x_2^2)]. \quad (20)\end{aligned}$$

In the above

$$c_1=\frac{1}{4}(1-x_1)(1-x_2)(1+x_1+x_2), \quad (21)$$

$$c_2=\frac{1}{4}(1+x_1)(1+x_2)(1-x_1-x_2), \quad (22)$$

$$c_3=\frac{1}{2}(1+x_1)(1+x_2)[1+2x_1x_2+2(1-x_1-x_2)(x_1+x_2)], \quad (23)$$

$$c_4=-\frac{1}{2}(1-x_1)(1-x_2)[1+2x_1x_2-2(1+x_1+x_2)(x_1+x_2)], \quad (24)$$

$$c_5=\frac{1}{4(1-x_1x_2)}(1+x_1)^2(1+x_2)^2[(1-x_1-x_2)^2-x_1x_2], \quad (25)$$

$$c_6=\frac{1}{4(1-x_1x_2)}(1-x_1)^2(1-x_2)^2[(1+x_1+x_2)^2-x_1x_2], \quad (26)$$

$$\langle P_2\rangle=\int P_2(\boldsymbol{n}\cdot\boldsymbol{\mu})w^s(\boldsymbol{\mu})\,d\boldsymbol{\mu}=-\frac{1}{2}(1-x_1^2-x_1x_2-x_2^2). \quad (27)$$

$\langle P_2\rangle$ is the equilibrium average of $P_2(\cos\theta)$ over the distribution function eq. (14). The meanings of c_1 to c_6 is also given in Appendix B. When θ_c and α (hence x_1 and x_2) are determined, the diffusion constant D can be determined by the use of eq. (20) with experimentally observed ϕ_I.

In the limit $x_1\to1(\theta_1\to0)$, eq. (15) reduces to

$$\frac{r(\infty)}{r(0)}=\left[\frac{1}{2}x_2(1+x_2)\right]^2, \quad (28)$$

and eq. (20) to

$$D\phi_I(1-B_\infty)=-\frac{x_2^2(1+x_2)^2}{2(1-x_2)}\left(\log\frac{1+x_2}{2}+\frac{1-x_2}{2}\right)+\frac{1}{24}(1-x_2)(6+8x_2-x_2^2-12x_2^3-7x_2^4)\equiv f(x_2). \quad (29)$$

Equations (28) and (29) correspond to (A·1) and (A·6), respectively, with x_c being replaced by x_2.

When $\theta_c=0$ with constant α, eq. (16) is simplified as

$$\frac{r(\infty)}{r(0)}=\left[\frac{1}{2}(1-3\cos^2\alpha)\right]^2, \tag{30}$$

which coincides with (C·2). This can be easily understood by noticing that no wobbling motion occurs when $\theta_c=0$. For $r(t)/r(0)$, however, eq. (18) does not directly reduce to the correponding expression for axial rotation only. This stems from the difference between physical dimension of D_r and D. Taking this difference into account, the discrepancy is eliminated. Details of the calculations are relegated to Appendix C.

Case II: $\theta_c=\alpha$

This case corresponds to the "wobbling-in-cone" model with the half angle $2\theta_c$ (see Fig. 4(b)). Therefore parallel expressions in Appendix A hold for this case with θ_c being replaced by $2\theta_c$. They are

$$\frac{r(\infty)}{r(0)}=\left[\frac{1}{2}x_2(1+x_2)\right]^2$$
$$=\left[\frac{1}{2}\cos 2\theta_c(1+\cos 2\theta_c)\right]^2\equiv C_\infty, \tag{31}$$

$$\frac{r(t)}{r(0)}=\sum_{i=1}^{\infty}C_i\exp(-Dt/\zeta_i), \tag{32}$$

$$\left(\frac{r(t)}{r(0)}\right)_{\text{approx}}=C_\infty+(1-C_\infty)\exp(-t/\phi_{II}), \tag{33}$$

and

$$D\phi_{II}(1-C_\infty)=f(x_2). \tag{34}$$

The functional form of $f(x)$ is defined by eq. (29). In eq. (32), C_i and ζ_i depend only on θ_c or α as before.

Case III: $\theta_c>\alpha$

This case needs a particular consideration since the stationary distribution of $\mu(w^s(\mu))$ has two different values. The width of the band which has been considered in the *Case I*($\theta_1\le\theta\le\theta_2$) becomes wider as θ_c is increased. When θ_c exceeds α (*Case III*), the edge of the band crosses the membrane normal direction $\boldsymbol{n}$ and there appears a certain overlapping region like an inverted bowl $0\le\theta\le\theta_1$ (see Fig. 4(c)). The probability density that we would find μ within the area $0\le\theta\le\theta_1$ is twice as much as that within the band area $\theta_1\le\theta\le\theta_2$. Therefore the total area that can be occupied by μ is regarded to be made up of two parts, namely, area A and B: area A ($0\le\theta\le\theta_1$) is put on the larger inverted bowl area B ($0\le\theta\le\theta_2$). The total area of A and B is given by the sum of each area,

$$2\pi(1-x_1)+2\pi(1-x_2)=2\pi(2-x_1-x_2). \tag{35}$$

Accordingly, the stationary distribution $w^s(\mu)$ is

$$w^s(\mu)=\frac{1}{\pi(2-x_1-x_2)} \quad 0\le\theta\le\theta_1,$$
$$=\frac{1}{2\pi(2-x_1-x_2)} \quad \theta_1\le\theta\le\theta_2,$$
$$=0 \quad \text{otherwise.} \tag{36}$$

Using this stationary distribution, we obtain $r(\infty)/r(0)$ as

$$\frac{r(\infty)}{r(0)}=\left[\frac{x_1+x_2}{2(2-x_1-x_2)}(1-x_1^2+x_1x_2-x_2^2)\right]^2, \tag{37}$$

or equivalently

$$\frac{r(\infty)}{r(0)}=\left[\frac{\cos\theta_c\cos\alpha}{4(1-\cos\theta_c\cos\alpha)}\right.$$
$$\left.\times(\cos 2\theta_c-2\cos 2\theta_c\cos 2\alpha+\cos 2\alpha)\right]^2. \tag{38}$$

If we put $\alpha=0$ keeping θ_c constant, eq. (38) reduces to

$$\frac{r(\infty)}{r(0)}=\left[\frac{1}{2}\cos\theta_c(1+\cos\theta_c)\right]^2, \tag{39}$$

which is derived for wobbling motion alone (with the half angle θ_c). This limit corresponds to the case when there is no axial rotation.

It is worthwhile to note that both eqs. (15) and (37) reduce to eq. (31) in the limit of $\theta_c=\alpha$. Hence $r(\infty)/r(0)$ is continuous over the entire angle region of θ_c and α.

To calculate $r(t)/r(0)$ we treat area A and B separately so that wobbling motion occurs independently within each area. We assume that the measured $r(t)/r(0)$ is the weighted sum of the individual $r_A(t)/r(0)$ and $r_B(t)/r(0)$ which are obtained by replacing θ_c in Appendix A with θ_1 and θ_2, respectively. With this assumption $r(t)/r(0)$ is given by

$$\frac{r(t)}{r(0)}=\frac{1-x_1}{2-x_1-x_2}\sum_{i=1}^{\infty}E_i\exp(-Dt/\kappa_i)+\frac{1-x_2}{2-x_1-x_2}\sum_{i=1}^{\infty}F_i\exp(-Dt/\lambda_i), \tag{40}$$

where E_i, κ_i depend only on θ_1 while F_i, λ_i on θ_2. E_∞ and F_∞ are given by

$$E_\infty=\left[\frac{1}{2}x_1(1+x_1)\right]^2,\quad F_\infty=\left[\frac{1}{2}x_2(1+x_2)\right]^2. \tag{41}$$

The approximated expression for $r(t)/r(0)$ is also averaged as

$$\left(\frac{r(t)}{r(0)}\right)_{\text{approx}}=\frac{1-x_1}{2-x_1-x_2}[E_\infty+(1-E_\infty)\exp(-t/\phi_A)]+\frac{1-x_2}{2-x_1-x_2}[F_\infty+(1-F_\infty)\exp(-t/\phi_B)], \tag{42}$$

where

$$D\phi_A(1-E_\infty)=f(x_1),\quad D\phi_B(1-F_\infty)=f(x_2), \tag{43}$$

(see eq. (29)). For the purpose of reducing eq. (42) into a monoexponential expression, we require that this would fulfill the relation (19). Then we have

$$\left(\frac{r(t)}{r(0)}\right)_{\text{approx}}=G_\infty+(1-G_\infty)\exp(-t/\phi_{III}). \tag{44}$$

In the above

$$G_\infty=\frac{1-x_1}{2-x_1-x_2}E_\infty+\frac{1-x_2}{2-x_1-x_2}F_\infty, \tag{45}$$

and

$$D\phi_{III}(1-G_\infty)=\frac{1-x_1}{2-x_1-x_2}f(x_1)+\frac{1-x_2}{2-x_1-x_2}f(x_2). \tag{46}$$

The above approximation is reasonable, but might not be unique.

Formally speaking, $r(t)/r(0)$ should be given in the following way. We shall denote by the indices 1 and 2 the distribution function $w(\mu, t)$ referring, respectively, to the region $0\le\theta\le\theta_1$ and $\theta_1\le\theta\le\theta_2$. Both $w_1(\mu, t)$ and $w_2(\mu, t)$ behave in accordance with the diffusion equation (10) in each region, and subject to the boundary conditions,

$$w_1(\mu, t)=2w_2(\mu, t),$$
$$\frac{\partial}{\partial\theta}w_1(\mu, t)=\frac{\partial}{\partial\theta}w_2(\mu, t)\quad\text{at }\theta=\theta_1, \tag{47}$$
$$\frac{\partial}{\partial\theta}w_2(\mu, t)=0\quad\text{at }\theta=\theta_2. \tag{48}$$

Under such conditions, however, the method[13] to obtain D in terms of x_1, x_2 and the effective relaxation time is of no use. This is because the conditional probability g in this case no longer possesses such symmetry as

$$g(\mu^0, 0|\mu, t)=g(\mu, 0|\mu^0, t), \tag{49}$$

which plays a crucial role in their procedure.

§4. Discussion

For the newly proposed "wobbling under rotational effect" model, $r(t)/r(0)$ can be written as an infinite sum of exponentials (see eqs. (17), (32) and (40)). To analyze the experimental data, it is necessary to derive a simple but good approximate expression for $r(t)/r(0)$, whose parameters can be analytically related to the potential and the diffusion coefficient. A possible approximation to $r(t)/r(0)$ that has exact values at $t=0$ and $t=\infty$ is given as eq. (18) (or (33), (44)) which is expressed in terms of monoexponential damping. As was pointed out by Szabo,[19] there are several ways to determine the effective relaxation time satisfying the above conditions. Here we consider two of them.

The first one[11,13,17] is to require eq. (19) which implies that the area under $r(t)/r(0)$ is the same between the exact solution and the approximated one. For our square-well potential, the analytical expression relating the effective relaxation time to the diffusion coefficient is obtained following the method of Lipari and Szabo[13] (see also Appendix B). This ap-

proximation is reasonably accurate and fits well to the exact $r(t)/r(0)$ over a wide range of time. For arbitrary potential, however, the effective relaxation time defined in this way still has to be determined numerically.

Another way[17] of determining effective relaxation time is to require that $r(t)/r(0)$ is exact up to linear order in time (initial decay). On the basis of this approximation, Lipari and Szabo[13] gave an approximate expression for $r(t)/r(0)$ when the wobbling of the unique axis of the probe and rotational diffusive motion about this axis occur simultaneously for an arbitrary shape of potential, i.e.,

$$\left(\frac{r(t)}{r(0)}\right)_{\text{approx}} = \sum_{n=-2}^{2} \exp(-n^2 D_r t)[\langle P_2\rangle^2 + (1-\langle P_2\rangle^2)\exp\{-(6-n^2)D_w t/(1-\langle P_2\rangle^2)\}][d^{(2)}_{n0}(\alpha)]^2. \tag{50}$$

Here we have omitted the effect of the overall rotational motion of the macromolecule which is independent of the wobbling motion and $d^{(2)}_{n0}(\alpha)$ are reduced Wigner rotation matrices, i.e.,

$$d^{(2)}_{00}(\alpha) = (3\cos^2\alpha - 1)/2, \tag{51}$$

$$d^{(2)}_{\pm 10}(\alpha) = \mp(3/2)^{1/2}\sin\alpha\cos\alpha, \tag{52}$$

$$d^{(2)}_{\pm 20}(\alpha) = (3/8)^{1/2}\sin^2\alpha. \tag{53}$$

Szabo[19] gave a generalization of eq. (50) for special potentials that are symmetric about $\theta=\pi/2$. His final expression contains several exponentials and four parameters.

As Brainard *et al.* pointed out, there is significant difference between the above two approximations; the diffusion constant obtained from the initial decay may not be reliable.[18] Equation (50) for wobbling plus axial rotation is determined from the initial decay and is still too complicated to analyze the experimental data. By contrast, we have given in this paper a simpler approximate expression for $r(t)/r(0)$ which is characterized by 2 decay parameters and gives a better description of the entire time-course. Thus our theory can be applied straightforwardly to computer curve-fitting of the experimental data. As an example, we show the application of our theory to the experiment on cytochrome b_5.

The rotational diffusion of cytochrome b_5 in dimyristoyl-lecithin vesicles has been demonstrated by Vaz *et al.*[4] The anisotropy decay of cytochrome b_5 was measured after flash photolysis of rhodium (III)-protoporphyrin IX which was incorporated into apocytochrome b_5. The observed $r(\infty)/r(0)$ are 0.63 and 0.58 for below and above the phase transition of dimyristoyl-lecithin, respectively. It might be reasonable to assume that below the phase transition the rotational motion is inhibited and only the wobbling motion occurs, and that above the phase transition both wobbling and rotation occur simultaneously. The half angle of the cone is $\theta_c=31.2°$ which is obtained by eq. (A·1). It might be also reasonable to postulate that θ_c does not change above the transition point since it is determined by the steric hindrance of the lipid bilayer. Then the average direction of heme normal has a fixed angle $\alpha=6°$ with respect to the membrane normal. We have estimated this value from the graphical plot of $r(\infty)/r(0)$ against α for $\theta_c=31.2°$ (see Fig. 5).

So far, we have assumed that an optical probe 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.[23] However, it is sometimes observed that an extrinsic probe often exhibits rapid restricted wobbling in the order of several hundred picoseconds to a few nanoseconds.[24,25] The rotation of the whole protein molecule about the membrane normal has been observed to be slower than 1 μsec in all cases examined.[26] There are also proteins whose catalytic sites undergo independent segmental motion which has been observed in $(Ca^{2+}$-$Mg^{2+})$ATPase,[27] immunoglobulins.[28] In the case that the above three motions are independent of one another and the segmental motion is much faster than rotation of the whole protein molecule, $r(t)$

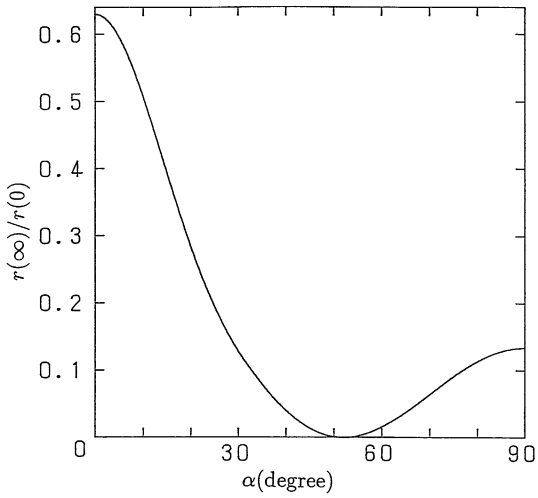

Fig. 5. α dependence of the time-independent residual anisotropy ratio in "wobbling under rotational effect" model when $\theta_c=31.2°$.

may be written as:

$$r(t)=r_p(t)r_s(t)r_r(t), \tag{54}$$

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 the whole protein rotation.

A large loss of anisotropy due to $r_p(t)$ has been observed for *N*-(1-anilinonaphth-4-yl) maleimide (ANM) bound to cytochrome oxidase,[24] eosin-maleimide bound to band 3 in erythrocytes and eosin-maleimide bound to ADP/ATP translocator in mitochondria. The rapid decay of $r_p(t)$ was also observed for eosin-maleimide and eosin-iodoacetamide bound to (Ca^{2+}-Mg^{2+})ATPase in sarcoplasmic reticulum while not observed for ANM and 5-[[(iodoacetamido)ethyl]amino] naphthalene-1-sulfonate (IAEDANS) bound to it.[29] For (Ca^{2+}-Mg^{2+})ATPase, independent segmental flexibility of part of the protein is also found. The wobbling of the hydrophilic head was estimated to be $\phi=60$ nsec for ANM bound segment and $\phi=200$ nsec for IAEDANS bound segment, which is more rapid than the rotation of the ATPase molecule with $\phi=40\,\mu$sec.[29] All of these examples may well be described by eq. (54), since each motion occurs in the different time range.

However, for (Ca^{2+}-Mg^{2+})ATPase, there is still a possibility of the slow segmental flexible motion with $\phi=1\sim40\,\mu$sec, though not yet found due to the experimental difficulties. Furthermore, for other proteins such as cytochrome b_5, glycophorin A, oligosaccharide or NADPH-cytochrome *P*-450 reductase, the time range of whole protein rotations might be comparable to flexible motion. To analyze the experimental data of these proteins, eq. (54) is not applicable and one should exploit the theory which is developed in the present work. It should be noted that even in the $t\to\infty$ case, our results (eqs. (15), (31), (37)) do not coincide with eq. (54). This implies that our model incorporates new coupling effects which is absent when we consider wobbling or rotation independently.

Acknowledgments

The authors would like to express their appreciation to Professor Kazuhiko Kinosita for useful discussions. We also thank Dr. Toru Miyazawa for helpful comments.

Appendix A

In this Appendix we briefly review the results for "wobbling-in-cone" model. The wobbling diffusion coefficient is denoted by D_w. By employing the notation $x_c=\cos\theta_c$, Kinosita *et al.*[11] obtained

$$\frac{r(\infty)}{r(0)}=\left[\frac{1}{2}\cos\theta_c(1+\cos\theta_c)\right]^2=\left[\frac{1}{2}x_c(1+x_c)\right]^2=A_\infty, \tag{A·1}$$

$$\frac{r(t)}{r(0)}=\sum_{i=1}^{\infty}A_i\exp(-D_w t/\sigma_i), \tag{A·2}$$

where A_i and σ_i are constants that depend only on θ_c, but cannot be expressed as an analytical function of it. They performed numerical calculation, and displayed these functional forms graphically. Furthermore, they presented a useful approximate expression for $r(t)/r(0)$ with monoexponential damping,

$$\left(\frac{r(t)}{r(0)}\right)_{approx}=A_\infty+(1-A_\infty)\exp(-t/\phi), \tag{A·3}$$

where ϕ is an effective relaxation time given by

$$D_w\phi(1-A_\infty)=\sum_{i\neq\infty}A_i\sigma_i. \tag{A·4}$$

As was pointed out by Lipari *et al.*,[13] this approximation is based on the next relation;

$$\int_0^\infty \left[\left(\frac{r(t)}{r(0)}\right)_{\text{approx}} - A_\infty\right] \mathrm{d}t = \int_0^\infty \left[\frac{r(t)}{r(0)} - A_\infty\right] \mathrm{d}t. \tag{A·5}$$

They further derived an exact analytic expression for the functional dependence of ϕ on x_c,

$$D_w\phi(1-A_\infty) = -\frac{x_c^2(1+x_c)^2}{2(1-x_c)}\left(\log\frac{1+x_c}{2} + \frac{1-x_c}{2}\right) + \frac{1}{24}(1-x_c)(6+8x_c-x_c^2-12x_c^3-7x_c^4)$$
$$= f(x_c). \tag{A·6}$$

Once A_∞ and ϕ are obtained from curve fitting of experimental data, we can calculate θ_c (see eq. (A·1)) and hence D_w from eq. (A·6).

Appendix B

We present here a brief summary of procedure to obtain the analytic expression relating the diffusion coefficient to the effective relaxation time. ϕ_I is defined by eq. (18). Substituting eq. (18) into eq. (19), we have

$$\phi_I(1-B_\infty) = \int_0^\infty \left[\frac{r(t)}{r(0)} - B_\infty\right] \mathrm{d}t. \tag{B·1}$$

By a procedure similar to the one in ref. 13), we can show that ϕ_I is expressed as a sum of five terms:

$$\phi_I(1-B_\infty) = \sum_{m=-2}^{2} \phi_m, \tag{B·2}$$

where

$$\phi_m = (-1)^m \left[\frac{(2-m)!}{(2+m)!}\right]^{1/2} \int [P_2^m(\cos\theta)\, \mathrm{e}^{-im\varphi} - \delta_{m0}\langle P_2\rangle] T_{2m}(\mu) w^s(\mu)\, \mathrm{d}\mu, \tag{B·3}$$

for $m \geq 0$ and $\phi_{-m} = \phi_m$. $P_2^m(\cos\theta)$ is the associated Legendre polynomial, δ_{m0} the Kronecker delta, $w^s(\mu)$ and $\langle P_2\rangle$ are given by eqs. (14) and (27), respectively. $T_{2m}(\mu)$ satisfies the following differential equation:

$$D\nabla_\mu^2 T_{2m}(\mu) = (-1)^{m+1}\left[\frac{(2-m)!}{(2+m)!}\right]^{1/2} P_2^m(\cos\theta)\, \mathrm{e}^{im\varphi} + \delta_{m0}\langle P_2\rangle. \tag{B·4}$$

T_{2m} is subjected to the boundary conditions eq. (12). Letting $x = \cos\theta$, the solution of eq. (B·4) for $m = 0$, 1, 2 are given as follows;

$$DT_{20} = \frac{x^2}{4} + c_1 \log(1-x) + c_2 \log(1+x), \tag{B·5}$$

$$DT_{21} = \frac{-\mathrm{e}^{i\varphi}}{2\sqrt{6}}\left[x(1-x^2)^{1/2} + c_3\left(\frac{1-x}{1+x}\right)^{1/2} + c_4\left(\frac{1+x}{1-x}\right)^{1/2}\right], \tag{B·6}$$

$$DT_{22} = \frac{\mathrm{e}^{2i\varphi}}{4\sqrt{6}}\left[(1-x^2) + c_5\left(\frac{1-x}{1+x}\right) + c_6\left(\frac{1+x}{1-x}\right)\right]. \tag{B·7}$$

c_1 to c_6 are given by eqs. (21) to (26), respectively. Evaluating the integrals in eq. (B·3), we get eq. (20) in the text.

Appendix C

The problem of rotational diffusion of a nonlinear chromophore fixed rigidly in a protein that

rotates about the normal to the plane of the membrane was treated firstly by Kawato *et al.*[21] Simple expressions for $r(t)/r(0)$ and $r(\infty)/r(0)$ can be obtained for linear and circularly symmetric chromophores. Using the rotational diffusion constant D_r, they are given as follows;

$$\frac{r(t)}{r(0)}=3\sin^2\alpha\cos^2\alpha e^{-D_r t}+\frac{3}{4}\sin^4\alpha e^{-4D_r t}+\frac{1}{4}(1-3\cos^2\alpha)^2, \quad (C\cdot 1)$$

$$\frac{r(\infty)}{r(0)}=\left[\frac{1}{2}(1-3\cos^2\alpha)\right]^2. \quad (C\cdot 2)$$

In the above, α is the fixed angle between membrane normal $\boldsymbol{n}$ and $\boldsymbol{\mu}$ (linear chromophore) or between $\boldsymbol{n}$ and normal to the plane of the chromophore (circularly symmetric chromophore).

If we put $\theta_c=0$ keeping α constant, $x_1=x_2=\cos\alpha$. To obtain the approximated monoexponential expression for eq. (C·1) (similar to (18)), we employ the relation (19). This expression is characterized by one effective relaxation time ϕ_r which is given by

$$D_r\phi_r(1-R_\infty)=\frac{3}{16}(1+14x_1^2-15x_1^4), \quad (C\cdot 3)$$

where

$$R_\infty=\left[\frac{1}{2}(1-3\cos^2\alpha)\right]^2=\left[\frac{1}{2}(1-3x_1^2)\right]^2. \quad (C\cdot 4)$$

On the other hand, eq. (20) reduces to

$$D\phi_I(1-R_\infty)=\frac{3}{16}(1-x_1^2)(1+14x_1^2-15x_1^4), \quad (C\cdot 5)$$

in this limit. Physically speaking, D and D_r should be related by

$$D=(1-x_1^2)D_r. \quad (C\cdot 6)$$

Therefore (C·5) is consistent with (C·3).

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