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Fluorescence Energy Transfer Studies of Transmembrane Location of Retinal in Purple Membrane

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A diffusion-enhanced energy transfer technique was employed for the determination of transmembrane location of the retinal chromophore in the purple membrane. Theoretical considerations showed that the rate of energy transfer from an energy donor embedded within a membrane to acceptors dissolved in solvent could be described by an analytical function of the distance a of closest approach between the donor and acceptor, if the "rapid-diffusion limit" was attained. The criterion for this limit was given by the relation: $(R_0)^6 \ll 20 D \tau_D a^4$, where R_0 is the characteristic distance of energy transfer, D is the diffusion coefficient of the acceptor and τ_D is the fluorescence lifetime of the donor in the absence of acceptor. By photo-reduction of the purple membrane with sodium borohydride, the retinal chromophore was converted to a highly fluorescent derivative, which showed a broad emission band in the visible region. From analysis of the fluorescence decay curves of the photo-reduced purple membrane in the presence of various concentrations of cobalt-ethylenediamine tetraacetate (Co-EDTA: energy acceptor), the depth of the chromophore from the membrane surface was estimated to be 8 ($\pm$3) Å.

This result was supported by investigations of energy transfer processes in a system where the native purple membranes and the photo-reduced membranes were stacked in parallel; the energy acceptor in this system was the native retinal chromophore.

1. Introduction

The purple membrane of *Halobacterium halobium* contains a single species of protein, bacteriorhodopsin, to which a retinal chromophore is bound *via* a protonated Schiff base linkage. Bacteriorhodopsin acts as a light-driven proton pump; light excitation of the chromophore initiates a photochemical reaction, by which protons are actively translocated from the cytoplasm to the outside of the cell and thereby a proton gradient is formed across the membrane (Stoeckenius *et al.*, 1979).

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Bacteriorhodopsin consists of a single polypeptide chain with 248 amino acid residues (Ovchinnikhov *et al.*, 1979; Khorana *et al.*, 1979; Dunn *et al.*, 1981) and, according to the recent reports (Lemke & Oesterhelt, 1981*b*; Mullen *et al.*, 1981; Bayley *et al.*, 1981), lysine 216 is the retinal binding residue. A three-dimensional model of the protein structure has been constructed by Henderson & Unwin (1975); the protein consists of seven α-helices about 40 Å long and 10 Å apart, each of which spans the membrane almost perpendicularly. It has been shown from various spectroscopic data that the retinal chromophore is very tightly buried in the membrane and slightly tilted out of the membrane plane (Razi-Naqvi *et al.*, 1973; Kouyama *et al.*, 1981*a*; Heyn *et al.*, 1977; Bogomolni *et al.*, 1977; Korenstein & Hess, 1978; Kimura *et al.*, 1981). Fluorescence energy transfer or neutron diffraction studies (Kouyama *et al.*, 1981*b*; King *et al.*, 1980) have shown that the chromophore is located near the centre of the protein.

There remains ambiguity with respect to the transmembrane location of the retinal chromophore. According to the neutron diffraction study of the purple membrane with ^{2}H-retinal (King *et al.*, 1979), the β-ionone ring portion of the retinal is situated centrally in the membrane; while the analyses of the flash-induced electric signal of the purple membrane suggested that the Schiff base portion was situated near the membrane surface (Drachev *et al.*, 1978; Keszthelyi & Ormos, 1980). The objective of the present work is to measure the transmembrane location of the retinal chromophore by using fluorescence energy transfer techniques.

It is well-known that the retinal chromophore is converted to a highly fluorescent derivative upon reduction of the purple membrane with sodium borohydride. The fluorescence decay of this derivative was investigated in the presence of cobalt-ethylenediamine tetraacetate (Co-EDTA) diffusing in solution, and analysed on the basis of a theory of diffusion-enhanced fluorescence energy transfer. The result of the analysis showed that the transmembrane location of the fluorescent retinal derivative was 8 (±3) Å below a membrane surface. The result was supported by those obtained from another experiment.

2. Materials and Methods

(a) *Sample preparations*

Purple membrane fragments of *H. halobium* were prepared according to Oesterhelt & Stoeckenius (1974).

Purple membranes in which all the retinal chromophores were converted to fluorescent derivatives were prepared as described (Peters *et al.*, 1976; Kouyama *et al.*, 1981*b*). Purified purple membranes were reduced with 1·0% (w/v) $NaBH_4$ (pH 8·9) at 0°C under visible light illumination from a 700 W projector. Reduced membranes were washed with cold water, and then irradiated with near ultraviolet light (408 nm) from a 500 W Hg lamp. The irradiation was continued at 0°C under nitrogen flow until the characteristic absorption spectrum developed fully.

Cobalt-ethylenediamine tetraacetate (Co-EDTA) was purchased from Dojin Co., Ltd. A concentrated solution of Co-EDTA (pH 8·9) was stocked at 4°C and used within 1 month.

A stacked sample of the native and/or photo-reduced purple membranes was obtained by drying the aqueous solution on a quartz plate *in vacuo* (Henderson, 1975).

(b) *Fluorometry*

Fluorescence decay kinetics were measured with a single-photoelectron counting apparatus (Kinosita *et al.*, 1981). The nanosecond light pulses were provided by a free-running discharge in H_2 gas (1200 kPa). The excitation wavelength was selected at 381 nm with a bandwidth of 8 nm. Fluorescence emission from a thermostatically controlled sample was measured with Hamamatsu R943-02 photomultiplier tubes, through cut-off filters (Fuji-Film SC-46) that transmitted light above 460 nm. In order to minimize the inner filter effect on excitation and emission lights, a micro-cuvette ($4 \times 4 \times 40\ mm^3$) was used as a sample container. Experimental decay curves $S^{ex}(t)$ of total fluorescence intensity were computed from the 2 principal components of polarized fluorescence decay. The fluorescence anisotropy decay curves were also computed, to check the immobilization of the fluorescent derivatives of retinal in the membrane. The experimental decay curve $S^{ex}(t)$ corresponds to the convolution product:

$$S^{ex}(t) = \int_0^t g(T)s(t-T)\mathrm{d}T, \tag{1}$$

where $s(t)$ is the fluorescence intensity that would be observed after an infinitely short excitation; $g(t)$ is the apparatus response function, which was approximated by the distribution function of light intensity of the flash measured at 505 nm. Single-exponential analyses of the fluorescence intensity decay curve were made by a least-squares method (Grinvald & Steinberg, 1974). For non-single-exponential decays, the initial time regions of the experimental curves were analysed as described (Kouyama *et al.*, 1981*b*).

Steady-excitation fluorometry was done with the same optical system as described above, except that a xenon lamp was used as the excitation source and a Jobin Yvon monochromator was placed before the photomultiplier tube. Emission spectra were corrected as described (Kouyama *et al.*, 1981*b*).

3. Theoretical

The effect of diffusion on resonance energy transfer by Förster's mechanism has been theoretically and experimentally investigated by many workers (Kurskii & Selivanenko, 1960; Samson, 1962; Elkana *et al.*, 1968; Dale & Eisinger, 1975). In general, the efficiency of resonance energy transfer is influenced by translational and rotational diffusion in a very complicated manner. Thomas *et al.* (1978) have explored a convenient method of analysing the diffusion-enhanced excitation energy transfer, by which trans-membrane location of a chromophore can be estimated easily. They made use of the fact that enhancement of the efficiency of energy transfer by diffusion is saturated in the rapid-diffusion limit, where the transfer efficiency is given as an analytical function of the distance of closest approach between the donor and acceptor molecules. They have shown that this limit can be attained if $D\tau_D \gg s^2$, where D is the sum of the diffusion coefficients of the donor and acceptor molecules, τ_D is the excitation lifetime of the donor and s is the mean distance between the donor and acceptor molecules. Thus they prepared a special compound with an exceedingly long excitation lifetime (~ 1 ms). If typical fluorescence probes (with excitation lifetime about 10^{-8} s) are used, the above criterion will not be satisfied. However, we have noticed that, at least for the system where acceptor molecules diffusing in solution receive excitation energy of a donor embedded within a membrane only *via* the resonance transfer mechanism, another criterion for the "rapid-diffusion limit" should be adopted. A theoretical consideration of this point is presented below.

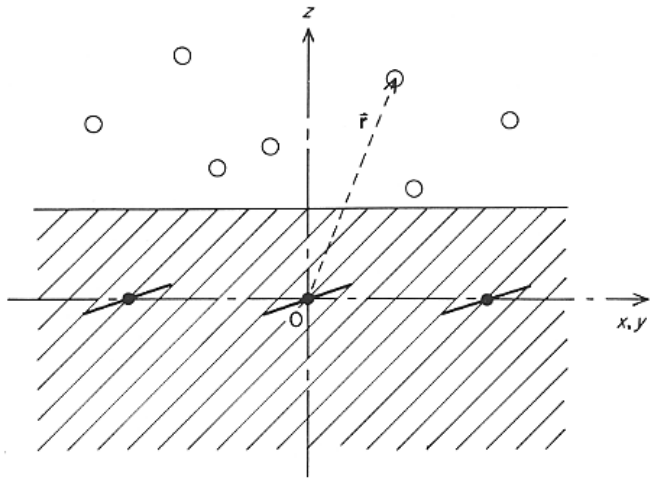

FIG. 1. Resonance energy transfer from a fluorescent chromophore (●) embedded within a membrane (indicated by oblique lines) to acceptor molecules (○) diffusing in solution.

(a) *Effect of diffusion on resonance energy transfer*

Let us consider the system in which energy donors are embedded within a membrane and acceptors are dissolved in aqueous solution (Fig. 1). We shall here confine our interest to the case where the donors are completely immobilized and the rotational diffusion of the acceptors is much faster than the deactivation rate of the excited donor.

A comprehensive investigation of the effect of translational diffusion on energy transfer was carried out by Steinberg & Katchalski (1968). According to their theory, the number $m(t)$ of excited donor molecules at time t is expressed as follows:

$$m(t) = m(0) \exp \{-t/\tau_D - \epsilon(t)\}, \tag{2}$$

where $m(0)$ denotes the number of excited donors at time zero and $\epsilon(t)$ is given by:

$$\epsilon(t) = \int \{c_0 - c(\vec{r}, t)\} d\vec{r}, \tag{3}$$

where c_0 is the concentration of acceptors and $c(\vec{r}, t)$ denotes the distribution of acceptors that have not received the excitation energy from the donor at the origin. The function $c(\vec{r}, t)$ satisfies the differential equation:

$$\frac{\delta}{\delta t} c(\vec{r}, t) - D\nabla^2 c(\vec{r}, t) = -k(\vec{r}) c(\vec{r}, t) \tag{4}$$

subject to the boundary conditions:

$$c(\vec{r}, 0) = c_0 \qquad \text{at } z \geq a \tag{5a}$$

and

$$\frac{\delta}{\delta z} c(\vec{r}, t) = 0 \qquad \text{at } z = a. \tag{5b}$$

In equation (4), D is the diffusion coefficient of the acceptor and $k(\vec{\mathbf{r}})$ is the rate of energy transfer between the donor and acceptor separated by $\vec{\mathbf{r}}$. The boundary condition (5b) is different from that used by Steinberg & Katchalski (1968); i.e. $c(\vec{\mathbf{r}}, t) = 0$ at $z = a$. The latter boundary condition was introduced because they assumed a complete quenching of an excited donor at the instance of collision between the donor and an acceptor (donors were assumed to diffuse freely in solution). On the other hand, it is assumed here that acceptors could affect the excitation lifetime of the donor only *via* the resonance energy transfer process.

According to Förster's theory (Förster, 1965), $k(\vec{\mathbf{r}})$ in equation (4) is given by:

$$k(\vec{\mathbf{r}}) = \left(\frac{R_0}{|\vec{\mathbf{r}}|}\right)^6 \kappa^2 \tau_D^{-1}, \tag{6}$$

where κ is the orientation factor and R_0 is the characteristic distance of energy transfer defined as follows:

$$R_0^6 = 8{\cdot}785 \times 10^{-25} QJ/n^4, \tag{7}$$

where Q is the fluorescence quantum yield of the donor in the absence of acceptors, J is the overlap integral between absorption spectrum of the acceptor and fluorescence emission spectrum of the donor, and n is the refractive index of the medium.

Let $H(\vec{\mathbf{r}}, t; \vec{\mathbf{r}}', t')$ be the solution of the differential equation (8) subject to the boundary conditions (5a) and (5b):

$$\frac{\delta}{\delta t} H(\vec{\mathbf{r}}, t; \vec{\mathbf{r}}', t') + D\nabla^2 H(\vec{\mathbf{r}}, t; \vec{\mathbf{r}}', t') = -\delta(t-t')\delta(\vec{\mathbf{r}}-\vec{\mathbf{r}}'). \tag{8}$$

($H(\vec{\mathbf{r}}, t; \vec{\mathbf{r}}', t') = G(\vec{\mathbf{r}}, -t; \vec{\mathbf{r}}', -t')$, where $G(\vec{\mathbf{r}}, t; \vec{\mathbf{r}}', t')$ is the Green's function for equation (4).) From an extended Green's theorem, $c(\vec{\mathbf{r}}, t)$ can be expressed as follows:

$$c(\vec{\mathbf{r}}, t) = c_0 - \int_0^t \mathrm{d}t' \int_{z'>a} \mathrm{d}\vec{\mathbf{r}}'\ k(\vec{\mathbf{r}}')c(\vec{\mathbf{r}}', t')H(\vec{\mathbf{r}}', t'; \vec{\mathbf{r}}, t). \tag{9}$$

Inserting this into equation (3) and noting that

$$\int_{z>a} H(\vec{\mathbf{r}}'\ t'; \vec{\mathbf{r}}, t)\ \mathrm{d}\vec{\mathbf{r}} = 1,$$

we obtain:

$$\epsilon(t) = \int_0^t \mathrm{d}t' \int_{z'>a} \mathrm{d}\vec{\mathbf{r}}'\ k(\vec{\mathbf{r}}')c(\vec{\mathbf{r}}', t'). \tag{10}$$

(b) *An approximation for* ϵ(t)

In general, $\epsilon(t)$ can not be expressed in an analytical form. Under certain conditions, however, $\epsilon(t)$ can be approximated by a linear function of time. This is the case when the second term at the right-hand side of equation (9) is small

compared to c_0, or when the following relation is satisfied:

$$I(\vec{\mathbf{r}}, t) = \int_0^t \mathrm{d}t' \int_{z'>a} \mathrm{d}\vec{\mathbf{r}}'\, k(\vec{\mathbf{r}}') H(\vec{\mathbf{r}}', t'; \vec{\mathbf{r}}, t) \ll 1. \tag{11}$$

This relation defines the criteria for the "rapid-diffusion limit". In this limit, $\epsilon(t)$ is approximated by:

$$\epsilon(t) = \left\{ c_0 \int_{z>a} k(\vec{\mathbf{r}})\, \mathrm{d}\vec{\mathbf{r}} \right\} t. \tag{12}$$

First, we shall calculate the integral $\int k(\vec{\mathbf{r}})\, \mathrm{d}\vec{\mathbf{r}}$ in equation (12), and then discuss the experimental conditions under which the above approximation is valid.

(c) *Fluorescence lifetime in the presence of rapidly diffusing acceptors*

The orientation factor κ^2 in equation (6) should be regarded as a function of the co-ordinate $\vec{\mathbf{r}}$. That is, κ^2 is given as follows:

$$\kappa^2 = \langle \{ (\vec{\mathbf{e}}_A \vec{\mathbf{e}}_D) - 3(\vec{\mathbf{e}}_A \vec{\mathbf{r}})(\vec{\mathbf{e}}_D \vec{\mathbf{r}})/|\vec{\mathbf{r}}|^2 \}^2 \rangle_{\Omega_A}, \tag{13}$$

where $\vec{\mathbf{e}}_A$ and $\vec{\mathbf{e}}_D$ are the unit vectors of the absorption dipole moment of the acceptor and of the emission dipole moment of the donor, respectively; $\langle\ \rangle_{\Omega_A}$ denotes the average with respect to the orientation of the acceptor, which is assumed to rotate rapidly. Let $\vec{\mathbf{e}}_D$ be fixed within the xz plane and let θ_D be the angle between $\vec{\mathbf{e}}_D$ and $\vec{\mathbf{e}}_z = (0, 0, 1)$; see Figure 1. Then equation (13) is reduced to:

$$\kappa^2 = \frac{1}{3} + \frac{(z \cos\theta_D + x \sin\theta_D)^2}{(x^2+y^2+z^2)}. \tag{14}$$

From equations (6) and (14), we obtain:

$$\int_{z>a} k(\vec{\mathbf{r}})\, \mathrm{d}\vec{\mathbf{r}} = R_0^6 \tau_D^{-1} \int_{-\infty}^{\infty} \mathrm{d}x \int_{-\infty}^{\infty} \mathrm{d}y \int_a^{\infty} \mathrm{d}z \left\{ \frac{1}{3(x^2+y^2+z^2)^3} + \frac{(z\cos\theta_D + x\sin\theta_D)^2}{(x^2+y^2+z^2)^4} \right\} \tag{15}$$

By using the formulae

$$\int_{-\infty}^{\infty} (1+x^2)^{-k}\, \mathrm{d}x = \pi(2k-3)!!/(2k-2)!!,$$

$$\int_{-\infty}^{\infty} x^2 (1+x^2)^{-k}\, \mathrm{d}x = \pi(2k-5)!!/(2k-2)!!$$

and

$$\int_{-\infty}^{\infty} (1+y^2)^{-k-1/2}\, \mathrm{d}y = 2(2k-2)!!/(2k-1)!!,$$

we obtain:

$$\int_{z>a} k(\vec{\mathbf{r}})\, \mathrm{d}\vec{\mathbf{r}} = \frac{\pi}{12} \frac{R_0^6}{a^3 \tau_D} (1 + \cos^2\theta_D). \tag{16}$$

From equations (2), (12) and (16), one can see that the decay constant τ of the excited donor in the presence of rapidly diffusing acceptors is given by:

$$\frac{1}{\tau} = \frac{1}{\tau_D}\left\{1 + \frac{\pi}{12}\frac{R_0^6}{a^3}(1+\cos^2\theta_D)c_0\right\}. \tag{17}$$

If energy transfer to acceptors on the other side of the membrane is also taken into account, a^{-3} in the above equation should be replaced by $a^{-3}+(L-a)^{-3}$; where L is the membrane thickness.

(d) *Experimental condition for the rapid-diffusion limit*

It is not difficult to show that, for a given value of t, $I(\vec{\mathbf{r}}, t)$ in equation (11) has a maximum value at $\vec{\mathbf{r}} = \vec{\mathbf{a}} = (0, 0, a)$. Thus the relation $I(\vec{\mathbf{a}}, t) \ll 1$ gives a sufficient criterion for the rapid diffusion limit. By using the formula $H(\vec{\mathbf{r}}', t'; \vec{\mathbf{a}}, t) = 2\{4\pi D(t-t')\}^{-3/2} \exp\{-[x'^2+y'^2+(z'-a)^2]/4D(t-t')\}$ (for $t' < t$), we obtain an explicit expression of $I(\vec{\mathbf{a}}, t)$:

$$I(\vec{\mathbf{a}}, t) = \tau_D^{-1} R_0^6 \int_0^t dt' \int_{-\infty}^{\infty} dx' \int_{-\infty}^{\infty} dy' \int_a^{\infty} dz' \frac{\kappa^2}{(x'^2+y'^2+z'^2)^3} \times \frac{2}{\{4\pi D(t-t')\}^{3/2}} \exp\{-[x'^2+y'^2+(z'-a)^2]/4D(t-t')\}. \tag{18}$$

By substitutions of $a/\sqrt{4D(t-t')} = v$, $x'/a = \xi$, $y'/a = \eta$ and $(z'-a)/a = \zeta$, the above equation is reduced to:

$$I(\vec{\mathbf{a}}, t) = \frac{R_0^6}{\pi^{3/2} D \tau_D a^4} \chi(a/\sqrt{4Dt}), \tag{19}$$

where

$$\chi(u) = \int_u^{\infty} dv \int_{-\infty}^{\infty} d\xi \int_{-\infty}^{\infty} d\eta \int_0^{\infty} d\zeta \frac{\exp\{-(\xi^2+\eta^2+\zeta^2)v^2\}}{\{\xi^2+\eta^2+(1+\zeta)^2\}^3} \kappa^2. \tag{20}$$

It is not difficult to show that $\chi(u) < \chi(0) = \pi^{3/2}\{11/10 - 3\pi/16 + (37\pi/16 - 35/6)\cos^2\theta_D\}/12$. Thus we obtain the following relation:

$$I(\vec{\mathbf{a}}, t) < I(\vec{\mathbf{a}}, \infty) = \frac{R_0^6}{12\, D\, \tau_D a^4}\left\{\frac{11}{10} - \frac{3\pi}{16} + \left(\frac{37\pi}{16} - \frac{35}{6}\right)\cos^2\theta_D\right\} \sim (0{\cdot}043 + 0{\cdot}119\cos^2\theta_D)\frac{R_0^6}{D\,\tau_D a^4}. \tag{21}$$

From the above discussion, it can be understood that $\epsilon(t)$, at any time, will be safely given by equations (12) and (16) if the donor-acceptor pair satisfies the following relation (the θ_D value is assumed to be close to 90°):

$$R_0^6 \ll 20 D \tau_D a^4. \tag{22}$$

Thus, a donor-acceptor pair with a small R_0 value is appropriate for the experimental determination of the a value (or transmembrane location of the donor), when the a value is small.

Another theoretical consideration similar to that above has shown that, if the relation $a^2 \ll D\tau_D$ is satisfied, $\epsilon(t)$ can also be approximated with a linear function of time. In this case, however, $\epsilon(t)$ is not necessarily expressed by equation (12); if the relation (22) is not satisfied, the coefficient $c_0 \int k(\vec{r})\, d\vec{r}$ appearing in equation (12) should be replaced by another coefficient that could not be expressed in a simple form.

The criterion for the rapid-diffusion limit shown by equation (22) is apparently different from that derived by Thomas *et al.* (1978). Their criterion was suggested from a dependence of the energy transfer efficiency on the diffusion coefficient, which was calculated by a computer analysis of the differential equation (4) with a fixed R_0 value. It seems unlikely that the criterion derived by Thomas *et al.* can be applied to another donor-acceptor pair with a different R_0 value.

4. Results

(a) *Characteristic distance* R_0 *for the energy transfer between the fluorescent retinal derivative and cobalt-ethylenediamine tetraacetate (Co-EDTA)*

An aqueous solution of Co-EDTA (pH 8·9) showed a weak absorption band ($\epsilon_{max} = 18\ cm^{-1}\ M^{-1}$) in the visible region, where the fluorescent retinal derivative in the photo-reduced and then ultraviolet light-converted purple membrane showed a broad emission band with a maximum at 500 nm (Fig. 2). From the overlap integral (J) between the two spectra in Figure 2, the characteristic distance R_0 for the energy transfer between the above donor-acceptor pair was estimated to be 12·6 (±0·5) Å; in this estimation (eqn (7)), a fluorescence lifetime of 20·4 nanoseconds and a fluorescence quantum yield of 0·23 (at 20°C) were used, and the refractive index n was assumed to be 1·4 (Kouyama *et al.*, 1981*b*). For $D = 5 \times 10^{-6}$ cm^2 per second (diffusion coefficient of Co-EDTA in aqueous

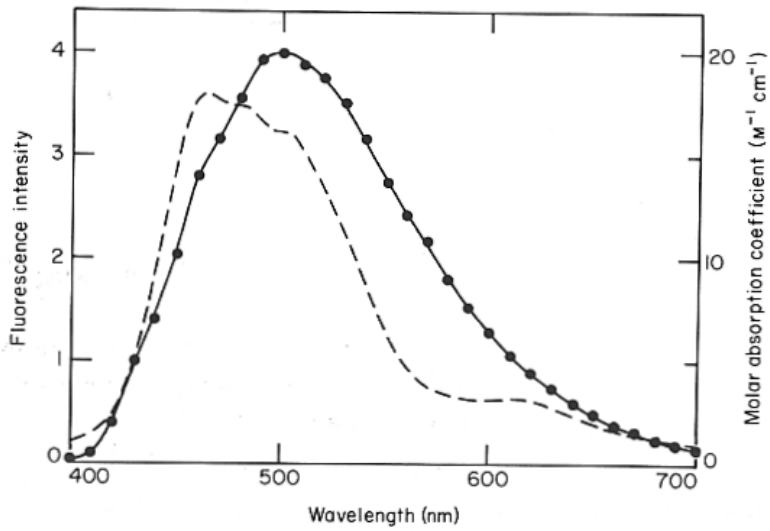

FIG. 2. The fluorescence emission spectrum (●) of the reduced and then ultraviolet light-converted purple membrane and the absorption spectrum (broken line) of Co-EDTA solution (pH 8·9).

solution at 20°C; Lapple, 1974) and $\tau_D = 20{\cdot}4$ nanoseconds, the relation (22) holds for a values larger than about 4 Å. On the other hand, the radius of Co-EDTA itself is a few ångström units. Therefore, the rapid-diffusion approximation is valid, unless the energy donor is exposed to the solvent (the absence of dynamic quenching of fluorescence by KI (Kouyama *et al.*, 1981*a*) suggests that the reduced chromophore cannot be at the membrane surface).

(b) *Fluorescence lifetime of the retinal derivative in the presence of Co-EDTA*

Fluorescence decay curves of the fluorescent retinal derivative in the reduced and then ultraviolet light-converted purple membrane were investigated in aqueous solutions in which various concentrations (0 to 0·25 M) of Co-EDTA were dissolved. The pH of the solutions was adjusted to 8·9 by the addition of 10 mM-borate buffer, to avoid aggregation of the membrane at high ionic strengths. At pH 8·9, the fluorescence decay curves observed in the presence of Co-EDTA could be described well with a single-exponential function. In Figure 3, the fluorescence lifetimes calculated are shown as a function of the concentration of Co-EDTA. With increasing concentration of Co-EDTA up to 0·24 M, the fluorescence lifetime decreased almost linearly from 20·4 to 18·6 nanoseconds.

The data shown in Figure 3 were obtained under solvent conditions in which sodium EDTA was present as well as Co-EDTA, except for that shown by △, which was obtained without any EDTA complex present. The large amount of sodium EDTA dissolved ($\sim 0{\cdot}25$ M) could reduce the concentration of free Co^{2+} far below the protein concentration (~ 1 μM). The effect of such a small amount of free Co^{2+} on the fluorescence lifetime was expected to be negligible, partly because the purple membrane was shown to have no specific binding site for divalent cations (A. Ikegami *et al.*, unpublished results), and partly because the

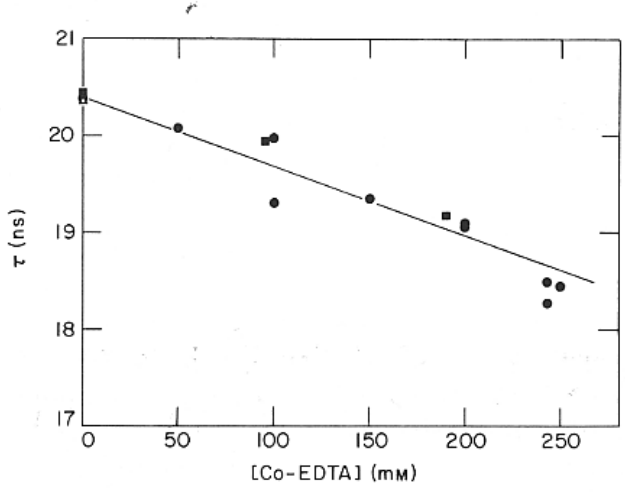

FIG. 3. The fluorescence lifetime of the retinal derivative (donor) as a function of the concentration of Co-EDTA (acceptor) dissolved in solution. The fluorescence decay curves $S^{ex}(t)$ were observed above 460 nm after excitation at 381 nm. The lifetimes were evaluated by single-exponential analyses of the experimental curves $S^{ex}(t)$. Solvent conditions: 0·24 M-EDTA complexes with divalent cations (cobalt or calcium), 0·24 M-Na-EDTA, and 0 M (●) or 1 M (■) NaCl, at pH 8·9 and at 20°C; except for the datum shown by △, which was obtained in the absence of any EDTA complex.

extinction coefficient of free Co^{2+} is much smaller in the visible region ($\epsilon_{max} = 5\ cm^{-1}\ M^{-1}$) than that of Co-EDTA. In fact, addition of a small amount of $CoCl_2$ ($\sim 0{\cdot}1$ mM) to an aqueous solution of the reduced and then ultraviolet light-converted purple membrane did not induce any detectable change in the fluorescence decay curve.

Calcium EDTA, which has no absorption band in the visible region, was also added to cancel out any possible influence of Co-EDTA on the fluorescence lifetime *via* any mechanisms other than the resonance energy transfer mechanism. The concentration of calcium EDTA was adjusted so that the total amount of EDTA complexes with divalent cations was the same for all the samples. Since calcium EDTA alone did not affect the fluorescence lifetime at all, we could say that the decrease in the fluorescence lifetime with increasing concentration of Co-EDTA (Fig. 3) was due only to the resonance energy transfer between the retinal derivative and Co-EDTA. One might consider the possibility that a static energy transfer to the Co-EDTA adsorbed on the membrane surface was dominant. In this respect, we observed that the apparent efficiency of energy transfer became considerably lower when aggregation of the membranes became so significant as to be visualized (especially at a low pH), indicating that the apparent concentration of Co-EDTA was lower in the intermembrane spacing among the aggregated membranes than in the bulk of solution. It is therefore unlikely that the static energy transfer mentioned above contributed to the decrease in fluorescence lifetime observed upon addition of Co-EDTA.

(c) *Transmembrane location of the fluorescent retinal derivative*

From the analysis of the data shown in Figure 3 using equation (17), the distance a of closest approach between the retinal derivative and Co-EDTA was calculated to be 12·4 (± 3) Å. In this analysis, the values of θ_D and R_0^6 in equation (17) were assumed to be $\theta_D = 67\ (\pm 11)^\circ$ (Kouyama *et al.*, 1981*b*), and $R_0^6 = 4\ (\pm 1) \times 10^6$ Å^6. The slope in Figure 3 was calculated as $\Delta\tau/c_0 = 0{\cdot}0078 \pm 0{\cdot}0015$ ns/mM at 95% confidence level from the linear regression analysis. The a value thus estimated is considerably greater than the critical distance a_{min} discussed above, indicating that the present system satisfies the condition for the approximation used in the derivation of equation (17).

The filled squares and circles in Figure 3 show the data that were obtained in the absence and presence of 1 M-NaCl, respectively. Since the efficiency of energy transfer was almost independent of whether the large amount of NaCl was present, we need not pay any special attention to the electrostatic interaction between the negatively charged surface of the purple membrane and Co-EDTA (negatively charged). Then, the distance a can be approximated by the sum of the radius of Co-EDTA (about 4 Å) and the depth of the chromophore from the membrane surface. That is, the retinal derivative in the reduced and then ultraviolet light-converted purple membrane was estimated to be located about 8 Å below the membrane surface. (If the electrostatic interaction (repulsion) is taken into account, it may be estimated that the chromophore is located closer to the membrane surface than 8 Å.)

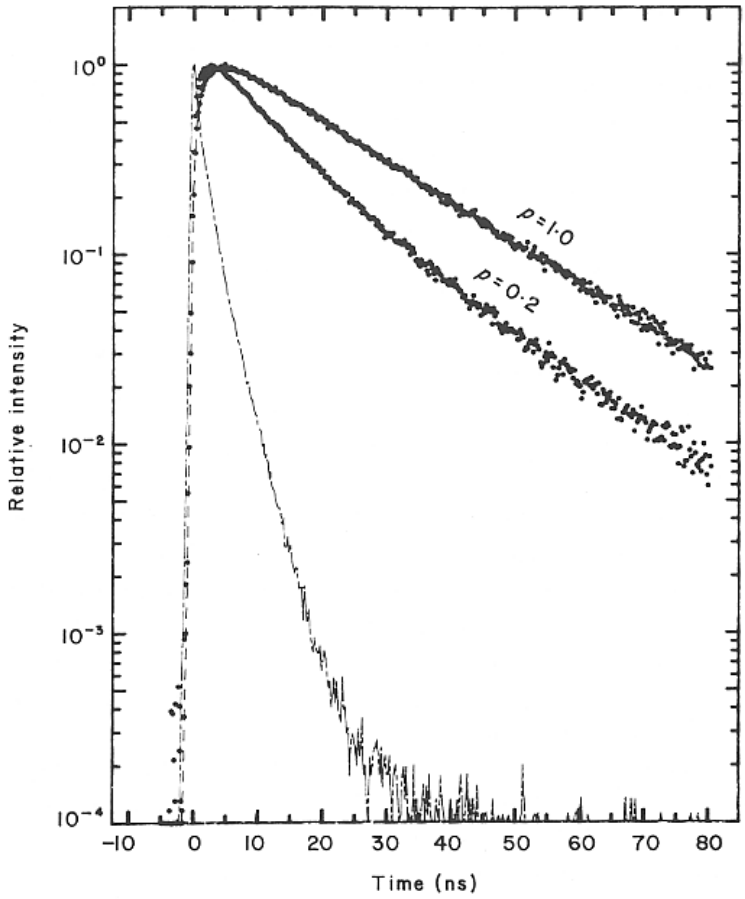

FIG. 4. Experimental fluorescence decay curves (filled circles) of the retinal derivatives in the stacked samples of the native and reduced purple membranes; the fraction of the reduced purple membrane is indicated by p. The chain line shows the response function $g(t)$. The fluorescence decay curve at $p = 1$ (i.e. in the absence of the native purple membrane) could be described by a single-exponential function with a decay constant of 19·8 ns (at 20°C). Excitation was at 381 nm and emission above 460 nm was collected together.

(d) *Transmembrane location of the native chromophore*

In order to confirm the above result, we investigated energy transfer processes in the system where the native purple membranes (containing energy acceptors) and the reduced and then ultraviolet light-converted purple membranes (containing energy donors) were stacked in parallel. The stacked sample was obtained by drying an aqueous solution containing the two types of membranes on a quartz plate *in vacuo*. Typical examples of the fluorescence decay curves observed for the stacked samples containing various fractions p of the reduced membranes are shown in Figure 4. The fluorescence decay curve observed at $p = 1$ (i.e. for the sample containing only the reduced and then converted membranes) could be described well with a single-exponential function. Whereas the decay curves observed at $p \neq 1$ (i.e. in the presence of the native purple membranes) deviated greatly from a single-exponential decay. At small values of p, the decay components with short decay constants developed significantly.

In the Appendix, a theoretical consideration is given concerning the fluorescence decay kinetics that would be expected if the native purple membranes and the modified membranes are stacked in a completely random way. In this case, the average rate of deactivation of excited donor is given as a linear function of $(a_A+a_D+\Delta)^{-4}$; where a_A and a_D are the depths of the native retinal and its fluorescent derivative, respectively, from the membrane surface; Δ is the intermembrane spacing (eqn (29)). From the analysis of the initial time region of the experimental fluorescence decay curve (using the method described by Kouyama *et al.*, 1981*b*), the value of $a_A+a_D+\Delta$ was estimated to be between 26 Å and 31 Å. The repeating distance of the dried membrane obtained by the X-ray diffraction measurements was $d = 50{\cdot}5$ ($\pm 1{\cdot}5$) Å for seven mixtures of native and photo-reduced membranes (T. Furuno, personal communication). A similar value has been reported for the native membrane (Henderson, 1975). By subtracting the thickness of the bacteriorhodopsin molecule, 45 Å (Agard & Stroud, 1982), the Δ value is obtained as 5·5 ($\pm 1{\cdot}5$) Å. Thus the transmembrane location of the retinal chromophore, $(a_A+a_D)/2$, was estimated to be $9 \sim 14$ Å, on the assumption that the chromophore did not move upon photo-reduction.

The above estimation was also based on the assumption of random stacking of the membranes (assumption A-1 $\sim$ 3 in the Appendix). With respect to assumptions A−2 and A−3 (see the Appendix), the X-ray diffraction data obtained from the dried specimens of purple membranes have suggested that there is no tendency for interlocking of the adjacent membrane sheets or for pairing of the membranes (Henderson, 1975). There remains ambiguity with respect to assumption A−1. Its influence on the estimation of $a_A+a_D+\Delta$ values could be minimized by analysing the experimental data obtained at very small values of p, where the average rate of deactivation of the excited donor, γ, is relatively insensitive as to whether the magnitude of intermembrane interaction between the two types of membranes is (slightly) different. Though it seems that the above estimation is slightly deeper, there is no difficulty in estimating that the depth of the chromophore from the membrane surface is 8($\pm$)3 Å from the experiment using Co-EDTA.

5. Discussion

(a) *Isomerization of the retinal chromophore*

The fluorescence intensity (at 530 nm) of the fluorescent retinal derivative in the dried specimens of a mixture of native and reduced purple membranes ($p = 0{\cdot}12$) was independent, within experimental error, of whether the native purple membrane was dark-adapted or light-adapted. In this experiment, the light-adapted purple membrane was obtained by irradiating a dried specimen that had been equilibrated at almost 100% relative humidity (Korenstein & Hess, 1978). It has been suggested that the light-adapted purple membrane contains only all-*trans* retinal, while the dark-adapted purple membrane contains an approximately equimolar mixture of all-*trans* and 13-*cis* retinals (Sperling *et al.*, 1977). The present observation suggests that the isomerization of retinal does not accompany a large change in the transmembrane location of the chromophore.

(Note that there is only a slight difference between the characteristic distances of energy transfer from the fluorescent retinal derivative for the two retinal isomers (Kouyama *et al.*, 1981*b*).) This result is consistent with the observation that the isomerization does not accompany a large change in the direction of the retinal with reference to the membrane normal (Bogomolni *et al.*, 1977; Kimura *et al.*, 1981). It should be added, however, that a small displacement of the retinal upon light–dark adaptation of the purple membrane might be detected by a more accurate analysis of the experiment described above.

(b) *Folding of the polypeptide chain of bacteriorhodopsin*

Several attempts have been made to fit the amino acid sequence of bacteriorhodopsin to the three-dimensional density map of the protein. Based on the limited proteolysis experiments, Ovchinnikov *et al.* (1979) proposed a model of the folding of the polypeptide chain in which the Lys216 residue (retinal binding site) is situated near the centre of the membrane. Engelman *et al.* (1980) proposed another model based on the criteria of connectivity of non-helical link regions, charge neutralization and total scattering density per helix. In the latter model, transmembrane location of Lys216 was changed slightly toward the cytoplasmic surface. The present estimation of transmembrane location of the retinal chromophore is consistent with the model proposed by Engelman *et al.* (1980) if the tilt angle ($\sim 20°$) of the retinal chromophore with respect to the membrane plane is taken into consideration; it is also possible that transmembrane location of the main body of the chromophore deviates, by a few ångström units, from that of the α-carbon of the lysine residue. Support for the present estimation comes from the suggestion that Tyr26, which is situated about 10 Å below the cytoplasmic surface in both models, participates in the chromophoric structure *via* a hydrogen bridge (Lemke & Oesterhelt, 1981*a*). On the other hand, the neutron diffraction study of the purple membrane with ^{2}H-retinal (King *et al.*, 1979) has suggested that the retinal is located 17 Å below the membrane surface. There remains an apparent discrepancy between their estimation and ours. This discrepancy may come partly from a difference in definition of the membrane surface; the membrane surface used in our estimation is a border beyond which Co-EDTA is not accessible, while their's is defined from the transmembrane profile of neutron scattering density.

APPENDIX

Here we shall consider energy transfer processes in the system where the native purple membranes (containing energy acceptors) and the reduced (and then ultraviolet light-converted) purple membranes (containing energy donors) are stacked in parallel (Fig. 5). In order to avoid complicated discussions, we shall confine our interest to the case where the native and reduced membranes are stacked in a completely random way: i.e. assumption A−1, the magnitude of intermembrane interaction is not different between the two types of membranes;

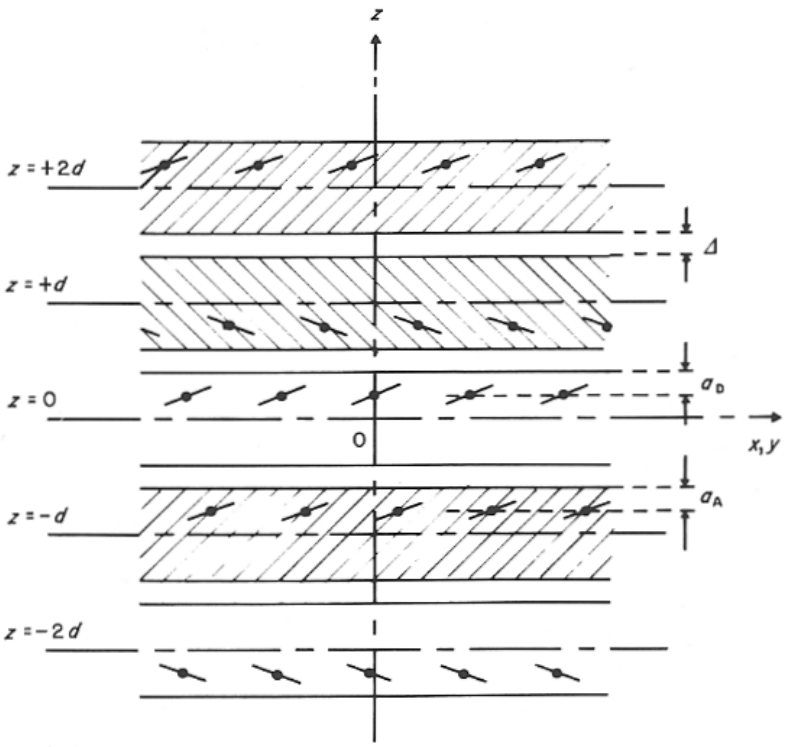

FIG. 5. An example of the possible distribution of the retinal chromophores (●) in the stacked sample of the native purple membranes (shown with oblique lines) and the reduced and then ultraviolet light-converted purple membranes. The centre of each membrane was assumed to be at $z = z_i = id$, where d is the spacing of the stacking and $i = 0, \pm 1, \pm 2, \ldots$. The distances a_A and a_D indicate the depths of the native retinal and of its fluorescent derivative, respectively, from the membrane surface; Δ indicates the inter-membrane spacing.

the probability of finding a reduced membrane with its centre at $z = z_i = id$ (Fig. 5) is equal to the fraction p of the reduced membrane, where d is the spacing of the stacking and $i = 0, \pm 1, \pm 2, \ldots$; assumption A−2, the magnitude of intermembrane interaction does not depend on the sidedness of the membrane; there is no tendency for pairing (Henderson, 1975) of the membranes; assumption A−3, there is no correlation in the orientation and displacement (in the membrane plane) between adjacent membrane sheets; there is no tendency for interlocking of adjacent membrane sheets.

Then the fluorescence decay (ensemble average) of a donor in the reduced membrane with its centre at $z = 0$ can be described by the following equation (Kouyama *et al.*, 1981*b*):

$$s(t) = \exp(-t/\tau_D) \prod_{i \neq 0} \left\{ p + (1-p) \left\langle \exp\left(- \sum_j k_{ij} t \right) \right\rangle \right\}. \tag{23}$$

Here, $\sum_j k_{ij}$ is the sum of the rates of energy transfer from the donor to all the acceptors that are contained in the native membrane at $z = z_i$; $\langle\ \rangle$ means the average over all the possible displacements and orientations (in the membrane plane) of the native membranes with reference to the reduced membrane at $z = 0$.

At times much shorter than τ_D, the fluorescence decay can be approximated as follows:

$$s(t) \simeq 1-\gamma t, \tag{24}$$

where γ is the average rate of deactivation of the excited donor, and is given by:

$$\gamma = (1-p)\sum_i \left\langle \sum_j k_{ij} \right\rangle + 1/\tau_D. \tag{25}$$

From assumptions A−1 and A−2,

$$\left\langle \sum_j k_{ij} \right\rangle$$

in the above equation is reduced to:

$$\sum_{i\neq 0} \left\langle \sum_j k_{ij} \right\rangle = \frac{R_0^6}{4\pi A \tau_D} \sum_{i\neq 0} \int_{-\infty}^{\infty} dx \int_{-\infty}^{\infty} dy \int_0^{2\pi} d\phi \{(\kappa_i^+)^2 [|(i-1)d + a_D + a_A + \Delta|^2 + x^2 + y^2]^{-3} + (\kappa_i^-)^2 [|i(d+a_D-a_A)|^2 + x^2 + y^2]^{-3}\}, \tag{26}$$

where A is one-third of the area of the unit cell of the two-dimensional lattice in the purple membrane ($A = 1{\cdot}1 \times 10^3$ Å^2, according to Unwin & Henderson (1975)); R_0 is the characteristic distance of energy transfer between the native and reduced retinals ($R_0 = 49$ Å; Kouyama *et al.*, 1981*b*); a_D and a_A are the depths of the donor and of the acceptor, respectively, from the membrane surface; Δ is the intermembrane spacing (Fig. 5); κ_i^+ and κ_i^- are the orientation factors, which are functions of variables x, y and ϕ. Since both the native retinal (acceptor) and fluorescent retinal derivative (donor) tilt only slightly from the membrane plane (Heyn *et al.*, 1977; Bogomolni *et al.*, 1977; Kimura *et al.*, 1981, Kouyama *et al.*, 1981*b*), these orientation factors can be approximated to be $\cos\phi$. (Note that the integrand in eqn (26) becomes significant only at small values of x and y.) Then equation (26) is reduced to:

$$\sum_{i\neq 0} \left\langle \sum_j k_{ij} \right\rangle \sim \frac{\pi R_0^6}{8A\tau_D} \sum_{i\neq 0} \{|(i-1)d + a_D + a_A + \Delta|^{-4} + |i(d+a_D-a_A)|^{-4}\}. \tag{27}$$

By substituting equation (27 into equation (25), we can see that γ is given as follows:

$$\gamma = \frac{1}{\tau_D}\left\{1 + (1-p)\frac{\pi R_0^6}{8A}[(a_D+a_A+\Delta)^{-4} + (d+a_D-a_A)^{-4} + (d-a_D+a_A)^{-4} + \ldots]\right\}. \tag{28}$$

When $(a_D+a_A+\Delta)^{-4} \gg (d+a_D-a_A)^{-4}$, the initial rate of deactivation of the excited donor, γ, is approximately given by:

$$\gamma = \frac{1}{\tau_D}\left\{1 + (1-p)\frac{\pi R_0^6}{8A}(a_D+a_A+\Delta)^{-4}\right\}. \tag{29}$$

The above discussion has not involved the effect of the mosaic spread of the membrane in the stacked sample. The possible open spaces in actual stacked

samples effectively reduce the probability of finding an acceptor in an adjacent membrane of the ideally stacked system, $1-p$. Thus $(1-p)$ in equation (29) should be replaced by $\beta(1-p)$, where $\beta(<1)$ is a correction factor. Although it is difficult to estimate the lower limit of β rigorously, the minimum value should be around 0·5, which corresponds roughly to a system where the open and occupied spaces in a mosaic spread are present to the same extent. Uncertainty in $\beta\,(0{\cdot}5 \sim 1)$ will not introduce a serious difficulty in the estimation of $a_A+a_D+\Delta$; the error should be less than $\pm 10\%$.

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