TRANSMEMBRANE LOCATION OF RETINAL IN PURPLE MEMBRANE

Fluorescence Energy Transfer in Maximally Packed Donor–Acceptor Systems

TADAATSU KOMETANI, KAZUHIKO KINOSITA, JR., TAIJI FURUNO, TSUTOMU KOUYAMA, AND AKIRA IKEGAMI

The Institute of Physical and Chemical Research, Hirosawa 2-1, Wako-shi, Saitama 351-01, Japan

ABSTRACT Transmembrane location of the retinal chromophore in the purple membrane of *Halobacterium halobium* was investigated in three different systems in which excitation energy transfer between the chromophore and external dye molecules condensed on the membrane surfaces was observed. In system *ii,* the energy donor was the retinal chromophore converted in situ to a fluorescent derivative. The fluorescent membranes were embedded in solid cobalt-EDTA, which served as energy acceptors. System *iii* was similar to system *ii,* except that the acceptors were tris(2,2′-bipyridyl)ruthenium(II) complex in solid form. The positively charged ruthenium complex had a radius of 0.7 nm, whereas the cobalt complex in system *ii* was smaller (radius ~0.4 nm) and negatively charged. System *iv* was stacked sheets of native purple membrane with interspersed ruthenium complex; energy transfer from the luminescent ruthenuim complex to the native retinal chromophore was observed. The energy transfer rates in these three systems, and in two additional systems already described (Kouyama, T., K. Kinosita, Jr., and A. Ikegami, 1983, *J. Mol. Biol.,* 165:91–107), were all consistent with a location of the retinal chromophore at a depth of 1.0 ± 0.3 nm from a surface of the purple membrane. All the analyses in the present work involved an assumption that contacts between the external dye molecules and membrane surfaces were maximal; the depth values obtained cannot be underestimates. The chromophore therefore must be outside the middle one-third of the thickness, ~4.5 nm, of the purple membrane.

INTRODUCTION

Bacteriorhodopsin, the sole protein constituent of the purple membrane of *Halobacterium halobium,* functions as a light-driven proton pump. The molecular mechanism of the pump action has been the object of intensive studies (for reviews, see, e.g., Stoeckenius and Bogomolni, 1982; Dencher, 1983; Stoeckenius, 1985).

Knowledge about the structure of bacteriorhodopsin is essential for the understanding of the mechanism. The amino acid sequence is known (Ovchinnikov et al., 1979; Khorana et al., 1979). Retinal, the chromophore of the protein, is bound at lysine 216 via a Schiff base linkage (Lemke and Oesterhelt, 1981; Mullen et al., 1981; Bayley et al., 1981; Katre et al., 1981). In the purple membrane, bacteriorhodopsin molecules form a two-dimensional crystalline lattice (Blaurock and Stoeckenius, 1971). The three-dimensional structure of bacteriorhodopsin has been revealed in an electron microscopic study by Henderson and Unwin (1975). According to their model, the protein consists of seven α-helices running nearly perpendicularly to the surface of the membrane. The retinal chromophore, however, could not be resolved in the electron density map or in subsequent images at higher resolutions (Hayward and Stroud, 1981; Agard and Stroud, 1982).

Determination of the disposition of retinal in bacteriorhodopsin is of twofold importance. First, the chromophore is the site of the primary event(s) in the pumping cycle. Isomerization of retinal from all-*trans* to 13-*cis* occurs early in the cycle (Pettei et al., 1977; Tsuda et al., 1980; Braiman and Mathies, 1982; Hsieh et al., 1983) and the Schiff base proton is released at an intermediate stage (Lewis et al., 1974; Terner et al., 1979). Second, locating retinal helps establish correspondence between the known amino acid sequence and the three-dimensional structural data, since the binding site in the sequence has already been identified.

Fluorescence energy transfer (Förster, 1965; Stryer, 1978) is a useful tool for studies of the chromophore disposition. Kouyama et al. (1981*b*) have determined the in-plane geometry of retinal in the purple membrane by a "crystallographic" analysis of energy transfer. The most probable location and orientation suggested in the study have been supported by recent neutron diffraction studies (Jubb et al., 1984; Seiff et al., 1985). In a subsequent study by Kouyama et al. (1983), which we refer to as paper I, the transmembrane location of retinal was investigated with

Dr. Kometani's present address is Tsukuba Kenkyuusho, Sanyo Denki Co., Ltd., Takanodai 2, Yatabe-cho, Tsukuba-gun, Ibaraki 305, Japan.

energy transfer techniques. The study suggested that the chromophore was situated at a depth much less than the thickness of the purple membrane. A fluorescence energy transfer study by Tsetlin et al. (1983) also pointed to a similar conclusion. These results, however, are not consistent with an earlier neutron diffraction study (King et al., 1979), which has indicated a central location. A cross-linking study by Huang et al. (1982) has also suggested a central location.

In paper I Kouyama et al. attempted to determine the "distance of closest approach" (Thomas et al., 1978) between donors in the membrane (the retinal chromophore converted in situ to a fluorescent derivative) and acceptors external to the membrane. The analyses involved an assumption, among others, that the distribution of acceptors in the external space was uniform. Thus one could argue that the actual distribution might have been distorted and have yielded an erroneously small depth value for the location of the chromophore. The analyses by Tsetlin et al. (1983) also depended on assumptions. Here we report a novel approach that can set an upper limit to the depth value: We attempted to push external dye molecules, by drying, against the membrane surfaces. In the analyses we assumed a priori that the maximal contact between the dye molecules and membrane surfaces was realized. Such an analysis may overestimate, but never underestimate, the depth value. We investigated three independent systems in which the chromophore acted both as a donor and as an acceptor. The altogether five different systems, including the two in paper I, have given a consistent answer that the retinal chromophore is at a depth of 1.0 ± 0.3 nm from a surface of the purple membrane. Careful examination of the assumptions involved in the analyses leads us to conclude that the chromophore must be outside the middle one-third of the transmembrane section.

THEORETICAL

A. Excitation Energy Transfer via the Förster Mechanism

In a system containing N donors and M acceptors, the intensity $F(t)$ of donor fluorescence after a pulsed excitation at time $t = 0$ is given by

$$F(t) = \frac{1}{N}\sum_{i=1}^{N} \exp\left[-\left(\frac{1}{\tau_D} + \sum_{j=1}^{M} k_{tr}^{(ij)}\right)t\right], \quad (1)$$

where τ_D is the excited-state lifetime of the donor in the absence of acceptors, and $k_{tr}^{(ij)}$ is the rate of energy transfer from donor i to acceptor j. If we assume the Förster mechanism (Förster, 1965), k_{tr} is given by

$$k_{tr} = (\kappa^2/\tau_D)\,(R_0/R)^6. \quad (2)$$

Here κ^2 is the orientation factor defined by

$$\kappa^2 = [(\mathbf{E}_D \cdot \mathbf{A}_A) - 3(\mathbf{E}_D \cdot \mathbf{R})(\mathbf{A}_A \cdot \mathbf{R})/\mathbf{R}^2]^2, \quad (3)$$

where $\mathbf{E}_D$ and $\mathbf{A}_A$ are unit vectors along the emission transition moment of the donor and the absorption transition moment of the acceptor, respectively, and $\mathbf{R}$ is the vector connecting the donor and acceptor. The value of κ^2 ranges between 0 and 4. The constant R_0 in Eq. 2 represents the efficiency of the resonance interaction, and is given by

$$R_0^6 = 8.785 \cdot 10^{-25} n^{-4} Q_D \int f_D(\lambda)\epsilon_A(\lambda)\lambda^4\, d\lambda / \int f_D(\lambda)\, d\lambda, \quad (4)$$

where n is the refractive index of the medium, Q_D is the quantum yield of the donor emission in the absence of acceptors, f_D is the quantum emission spectrum of the donor, ϵ_A is the extinction coefficient (in M^{-1} cm^{-1}) of the acceptor at wavelength λ (in cm), and the unit of R_0 is centimeter. Note that our R_0 slightly differs from the common definition (Stryer, 1978) in which κ^2 is included.

The intensity decay $F(t)$ in Eq. 1 is generally non-exponential, since the sum of transfer rates ($\Sigma_j k_{tr}^{(ij)}$) is different for different donors. The simplest way of analysis, then, is to look at the initial rate of the (normalized) decay where all the transfer rates make unbiased contributions:

$$\begin{aligned} k_d(0) = k_d(t)_{t\sim 0} &\equiv -\left[\frac{1}{F(t)}\frac{dF(t)}{dt}\right]_{t\sim 0} \\ &= \frac{1}{\tau_D} + \frac{1}{N}\sum_{i=1}^{N}\sum_{j=1}^{M} k_{tr}^{(ij)} \\ &= \frac{1}{\tau_D}\left[1 + R_0^6 \cdot \frac{1}{N}\sum_{i=1}^{N}\sum_{j=1}^{M}\left(\frac{\kappa^2}{R^6}\right)^{(ij)}\right]. \end{aligned} \quad (5)$$

Under certain conditions, e.g., at the rapid diffusion limit (Thomas et al., 1978; Kouyama et al., 1983; Kinosita et al., 1987), the normalized decay rate $k_d(t)$ becomes practically time independent, resulting in an exponential decay. Otherwise $k_d(t)$ is a decreasing function of time t. (Diffusion of donors and/or acceptors, whether rapid or slow, does not affect $k_d(0)$.)

B. Energy Transfer from a Donor to Acceptors in a Plane(s)

Consider a donor at a height of z above a plane. The plane contains acceptors distributed either regularly or randomly. We calculate the initial decay rate $k_d(0)$ for an ensemble of N such pairs of a donor and acceptor plane, all with the same donor height of z. We make the following assumptions: (*a*) The Förster mechanism. (*b*) No correlation exists between the donor position (and orientation) and the acceptor distribution in the plane. (*c*) Orientation of donor is random (applicable to system *iv* in Results), or (*d*) orientations of acceptors are random (systems *ii* and *iii* in Results).

Under the above assumptions, the average over donors in Eq. 5, i.e., $(1/N)\,\Sigma_i$, can be replaced by an integral:

$$\frac{1}{N}\sum_{i=1}^{N}\left(\frac{\kappa^2}{R^6}\right)^{(ij)} = \left[\int \frac{\langle\kappa^2\rangle_\Omega}{(x^2+y^2+z^2)^3}\,dx\,dy\right] \Big/ \left(\int dx\,dy\right), \quad (6)$$

where (x, y, z) are the Cartesian components of the separation vector $\mathbf{R}$, and $\langle\kappa^2\rangle_\Omega$ is the average over the orientation of donor (c) or of acceptors (d):

$$\langle\kappa^2\rangle_\Omega = \frac{1}{3} + \frac{(\mu \cdot \mathbf{R})^2}{R^2}. \quad (7)$$

Here μ stands for $\mathbf{A}_A$ for the case c and $\mathbf{E}_D$ for d. Performing integration in Eq. 6 and inserting the result into Eq. 5 we obtain

$$k_d(0) = \frac{1}{\tau_D}\left[1 + \frac{\pi}{4A} \cdot \frac{R_0^6}{z^4}(1 + \cos^2\Theta)\right], \quad (8)$$

where Θ is the angle between μ and the normal to the plane, and A is the area per acceptor. If the value of Θ is not unique, $\cos^2\Theta$ in Eq. 8 should be replaced with its average value (⅓ for random orientations).

If each donor is associated with multiple acceptor planes, all parallel to each other and meeting assumptions *a–c* or *d*, then the initial decay rate $k_d(0)$ is given by a modification of Eq. 8 in which the second term is now a summation over planes with different z's. (Only those planes with z comparable to or smaller than R_0 are taken into account.) In particular if the donor is between a pair of parallel planes, one at a distance of z and the other at z', then the rate is given by

$$k_d(0) = \frac{1}{\tau_D}\left[1 + \frac{\pi}{4A} \cdot R_0^6\left(\frac{1}{z^4} + \frac{1}{z'^4}\right)(1 + \cos^2\Theta)\right]. \quad (9)$$

C. Energy Transfer from a Donor to Acceptors beyond a Boundary Plane(s)

If acceptors are distributed beyond a boundary plane that is at a distance of z from the donor, and if (*e*) the acceptor distribution is random along the direction normal to the plane, then the initial decay rate is given simply by integration of Eq. 8:

$$k_d(0) = \frac{1}{\tau_D}\left[1 + \frac{\pi}{4} \cdot R_0^6 C(1 + \cos^2\Theta)\int_z^\infty \frac{dz}{z^4}\right]$$
$$= \frac{1}{\tau_D}\left[1 + \frac{\pi}{12} \cdot \frac{R_0^6}{z^3} C(1 + \cos^2\Theta)\right], \quad (10)$$

where C is the number of acceptor molecules per unit volume.

If the donor is between a pair of parallel boundary planes, at distances z and z', beyond which acceptors are distributed at a concentration C, then the rate is given by

$$k_d(0) = \frac{1}{\tau_D}\left[1 + \frac{\pi}{12} \cdot R_0^6 C\left(\frac{1}{z^3} + \frac{1}{z'^3}\right)(1 + \cos^2\Theta)\right]. \quad (11)$$

Both theoretical and experimental aspects of excitation energy transfer in layered donor–acceptor systems have been developed by Kuhn and co-workers. The present study is an extention of the pioneering work reviewed in Kuhn et al. (1972).

MATERIALS AND METHODS

Sample Preparations

Purple membranes of *Halobacterium halobium*, strain R_1M_1, were prepared according to Oesterhelt and Stoeckenius (1974).

The retinal chromophore in the purple membrane was converted to a fluorescent derivative as described (Peters et al., 1976; Schreckenbach et al., 1977; Kouyama et al., 1981*b*). Purified purple membranes were reduced with 1.0% (wt/vol) $NaBH_4$ (pH 8.9) at 0°C under illumination with orange light (500–600 nm) from a 700-W projector. After the purple color disappeared, the membranes were washed with cold water. The reduced membranes in water were then exposed at 0°C under nitrogen flow to ultraviolet light (408 nm) from a 500-W Hg lamp until the characteristic absorption spectrum developed fully.

Cobalt(II)-EDTA (Co-EDTA) was purchased from Dojin Co., Ltd. (Kengun-cho, Kumamoto, Japan) and a fresh solution (500 mM, pH 8.9) was made. Tris-(2,2'-bipyridyl)ruthenium(II) complex $[Ru(bpy)_3Cl_2]$ was synthesized and purified by Dr. Masao Kaneko at the Institute of Physical and Chemical Research. It was dissolved in water at 5 mM.

Samples for fluorescence measurements were prepared by mixing a concentrated aqueous suspension of purple membranes, either native or reduced, with a solution containing desired substances; the final concentration of bacteriorhodopsin was 0.02–0.1 mM. A drop of the mixture was dried on a 11 × 22-mm coverglass slowly under reduced pressure and then in vacuo. In some experiments, the mixture was briefly sonicated with a bath sonicator (Laboratory Supplies Co., Inc., Hicksville, NY) before application to the coverglass. The sonication, intended to ensure complete mixing, did not affect the results. The mixture of reduced membranes and excess ruthenium complex was dried quickly in vacuo at about 50°C to minimize segregation of the two substances.

Fluorescence Measurements

Decay of fluorescence intensity after pulsed excitation was measured with a single-photon-counting apparatus (Kinosita et al., 1981). The excitation source was a free-running discharge lamp filled with high-pressure hydrogen. The coverglass was mounted in the sample chamber so that the excitation beam fell on the dried purple membrane film with an angle of incidence of 45°. Emission at a right angle to the incident beam was detected through the rear surface of the coverglass. In most experiments the detector was an R943-02 photomultiplier tube (Hamamatsu Photonics Co., Ltd., Hamamatsu, Shizuoka, Japan) operated at −20°C. Fast decays were measured with a two-stage multichannel-plate photomultiplier tube (R1564U-01 kindly lent by Hamamatsu Photonics), which gave a measured lamp profile with a full width at half maximum of 0.8 ns. For the measurement of the fluorescence of reduced purple membranes, the excitation monochromator was set at 382 nm with a bandpass of 8 or 12 nm, and two Hoya U-360 filters (Hoya Glass Co., Ltd., Tokyo) were used to reduce stray light. Emission between 485 and 520 nm was selected by a broad-band interference filter (Koshin Kogaku Co., Ltd., Kanagawa, Japan), two Fuji-Film SC46 filters (Fuji-Film Co., Ltd., Tokyo), and two Hoya C-500 filters. The ruthenium complex was excited at 450 nm with a bandpass of 12 nm. A short-pass filter with a cut-off of 480 nm (Ditric Optics Inc., Marlboro, MA), two Hoya B-460 filters, and a Hoya C-500 filter were placed in the excitation beam. Emission above 600 nm was observed through two Fuji-Film SC56 filters and a Fuji-Film SC60 filter. Time constants characterizing the emission decay were determined with a least-square deconvolution program.

Emission spectra were measured with the same apparatus operated as a photon-counting spectrofluorometer. Excitation source was a 150-W xenon lamp. A Jobin Yvon (Longjumeau, France) monochromator was placed before the photomultiplier tube, and the spectra were corrected with a standard tungsten lamp as a calibration source.

Quantum yield of ruthenium emission was estimated with rhodamine B in ethylene glycol as a standard. Quantum yield of the standard was assumed to be 0.89 (Weber and Teale, 1957). Refractive index of ethylene glycol was taken as 1.43.

All fluorescence measurements were made at 20°C.

Estimation of R_0

The characteristic distance, R_0, for energy transfer was calculated from Eq. 4. The extinction coefficients, $\epsilon(\lambda)$, of the dark-adapted purple membrane and Co-EDTA were taken from Kouyama et al. (1981*b*). The extinction coefficient of the ruthenium complex in dried film was assumed to be the same as that in solution (ϵ[456 nm] = 13,600 M^{-1} cm^{-1} [Kurimura et al., 1982]), since the excitation spectra were similar to each other. The refractive index, n, in Eq. 4 was assumed to be 1.4.

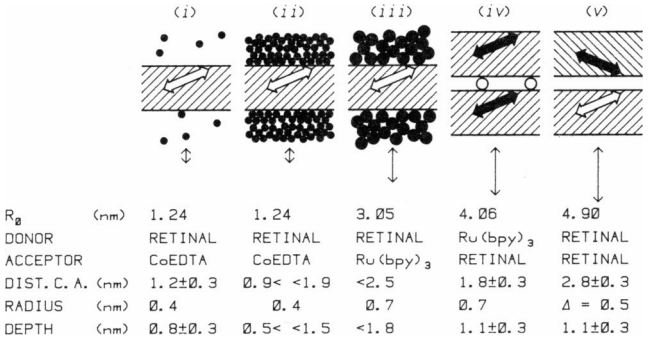
Reliable estimation of the quantum yield, Q_D, could be made only in suspension systems. We estimated Q_D in dry systems by assuming the proportionality between Q_D and the lifetime τ_D; the latter was measured accurately in both systems. For the emission from the reduced purple membranes in suspension, Q_D was determined to be 0.24 and τ_D 21.0 ns (Kouyama et al., 1981*b*). Measured τ_D in dried membranes of 19.7 ns (see Results) thus led to an estimated Q_D of 0.23 for the reduced chromophore in dried film. For ruthenium emission, Q_D in air-saturated aqueous solution, estimated as described above, was 0.051, and τ_D was found to be 410 ns (data not shown). In contrasts, τ_D of the ruthenium complex interspersed in between the reduced purple membranes, where energy acceptors were absent, was 1,250 ns (see Results). Thus Q_D of the ruthenium complex in membrane stack was assumed to be 0.16. The longer lifetime in dried membranes may be due to protection from oxygen quenching, since the lifetime in aqueous solution also became longer upon deaeration.

X-ray Measurement

Diffraction from the purple membranes dried on the coverglass was measured with an x-ray camera equipped with a position-sensitive detector (Furuno et al., 1983). The coverglass was mounted on a second guard slit of the x-ray camera. To avoid the reflection of the first-order diffraction at the glass surface, the glancing angle of the incident x-ray was adjusted to ~1°.

RESULTS

Fig. 1 summarizes the basic diagrams and results of the present experiments (systems *ii, iii,* and *iv*) together with those reported earlier in paper I (*i* and *v*). Below we describe the details of the present results, and then compare the five systems in Discussion. Validity of the assumptions involved in the analyses of the five systems is examined in Discussion.



		(i)	(ii)	(iii)	(iv)	(v)
R_0	(nm)	1.24	1.24	3.05	4.06	4.90
DONOR		RETINAL	RETINAL	RETINAL	$Ru(bpy)_3$	RETINAL
ACCEPTOR		CoEDTA	CoEDTA	$Ru(bpy)_3$	RETINAL	RETINAL
DIST. C. A.	(nm)	1.2±0.3	0.9< <1.9	<2.5	1.8±0.3	2.8±0.3
RADIUS	(nm)	0.4	0.4	0.7	0.7	Δ = 0.5
DEPTH	(nm)	0.8±0.3	0.5< <1.5	<1.8	1.1±0.3	1.1±0.3

FIGURE 1 Summary of the energy transfer experiments for the determination of the transmembrane location of the chromophore. The shaded area denotes a transmembrane section of a purple membrane sheet. The solid arrow represents the native chromophore, retinylidene-lysine, and the open arrow the reduced chromophore, which is retroretinyl-lysine (Peters et al., 1976; Schreckenbach et al., 1977). Vertical arrows indicate the size of R_0 compared with the thickness of the membrane.

System *ii:* Energy Transfer from the Reduced Chromophore to Co-EDTA

The reduction of the purple membrane by $NaBH_4$, followed by exposure to ultraviolet light, converts the retinal chromophore in bacteriorhodopsin into a highly fluorescent derivative. The conversion is made in situ, with no indication of drastic changes in the chromophore disposition, protein structure, or the lattice structure (Stoeckenius et al., 1979; Kouyama et al., 1981*a, b;* Tsetlin et al., 1983).

The curve labeled $-Co$ in Fig. 2 shows the fluorescence intensity decay of the reduced membranes embedded in Na-EDTA in dry form (mother solution, EDTA titrated with NaOH to pH 8.9). The decay was exponential and characterized by a lifetime τ_D of 19.7 ns. Dry mixture of the membrane and Ca-EDTA gave the same result. When the surrounding Na-EDTA or Ca-EDTA was replaced with Co-EDTA, whose absorption spectrum fully overlapped with the emission spectrum of the reduced chromophore, the fluorescence decay was clearly accelerated (curve $+Co$) as a result of energy transfer.

Since the acceptors were in large excess (the molar ratio of Co-EDTA to bacteriorhodopsin = 10^4), this system is represented by diagram *ii* in Fig. 1 and corresponds to case *C* in the Theoretical section. As is seen in Fig. 2, the fluorescence decay in this system was almost exponential despite the presence of acceptors distributed randomly. Such is expected when the distance of closest approach z between the donor and acceptor is greater than R_0 and the

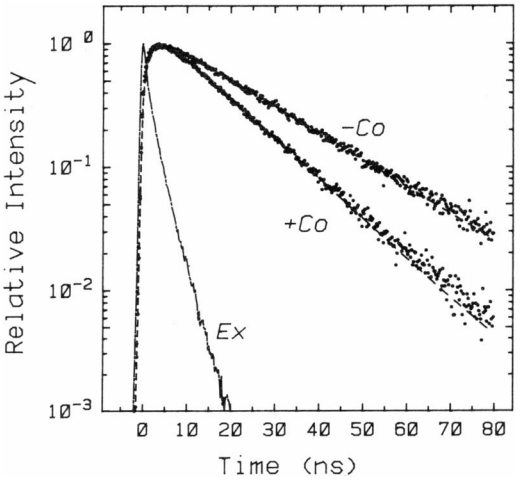


FIGURE 2 Fluorescence decay of the reduced purple membranes embedded in solid Na-EDTA ($-Co$) or in solid Co-EDTA ($+Co$). *Dots,* experimental points; *dashed lines,* best-fit single-exponential approximation. *Ex,* profile of the excitation light pulse. EDTA/bacteriorhodopsin molar ratio, 10^4.

separation between acceptors is smaller than z. In this case, many acceptors contribute a small fraction to the sum $\Sigma_j k_{tr}^{(ij)}$ in Eq. 1, none of them being dominant; the sum is therefore practically the same for all donors i. Since the radius of Co-EDTA, estimated from a space-filling model, is only 0.4 nm and R_0 of 1.24 nm is smaller than the thickness of the membrane of ~4.5 nm (Agard and Stroud, 1982), this system is not far off the above situation unless the donor, the reduced chromophore, is situated immediately below a surface.

The apparent fluorescence lifetime τ in the presence of Co-EDTA was 13.6 ± 0.4 ns over Co-EDTA/bacteriorhodopsin ratios of $(1 \sim 4) \times 10^4$. Equating $1/\tau$ with the initial decay rate $k_d(0)$, we estimated the distance of closest approach z from Eq. 11 under the assumptions *a–e*. The term $1/z'^3$ in Eq. 11 was assigned to the (minor) contribution from acceptors on the distal surface of the membrane: $z' = L + 2a - z$ where L (=4.5 nm) is the thickness of the membrane and a (=0.4 nm) is the radius of Co-EDTA. The tilt angle Θ of the reduced chromophore has been estimated to be 67° (Kouyama et al., 1981*b*). The acceptor concentration C in the dry film, however, could not be determined experimentally. We therefore set a higher limit of 2.4 molecules/nm^3, a value for a crystal of NaCoEDTA · $2H_2O$ (Weakliem and Hoard, 1959). A lower limit may be set at 500 mM = 0.3 molecules/nm^3, the concentration in the mother solution. Thus we finally obtain 0.9 nm $< z <$ 1.9 nm. Subtraction of the radius of Co-EDTA, 0.4 nm, yields a conclusion that the chromophore is situated at a depth between 0.5 and 1.5 nm from a surface of the membrane.

Note that the actual depth value is unlikely to be close to the limiting values. The lower limit of 0.5 nm corresonds to C = 500 mM, which is obviously too low. The nearly exponential decay in Fig. 2 also renders small z values unlikely. In contrast, the higher limit of 1.5 nm requires the formation of microcrystals of Co-EDTA and at the same time contact of all membrane surfaces with the crystal. Crystal formation, however, will generally lead to segregation of, and/or interspacing between, the membranes and acceptors.

System *iii:* Energy Transfer from the Reduced Chromophore to $Ru(bpy)_3Cl_2$

The experiment and analysis in this system were basically similar to those in system *ii* above. Dry films with the molar ratio of Ru to bacteriorhodopsin of 2×10^2 were examined; at higher ratios, emission from the ruthenium complex interfered with the measurement of the fluorescence from the reduced chromophore.

Typical data are shown in Fig. 3. Since the extinction coefficient of $Ru(bpy)_3Cl_2$ (maximal at 450 nm) was much higher than that of Co-EDTA, R_0 in this system was larger and was 3.05 nm. The fluorescence decay was therefore faster and required at least two exponential terms for an adequate fit (curve $+Ru$ in Fig. 3).

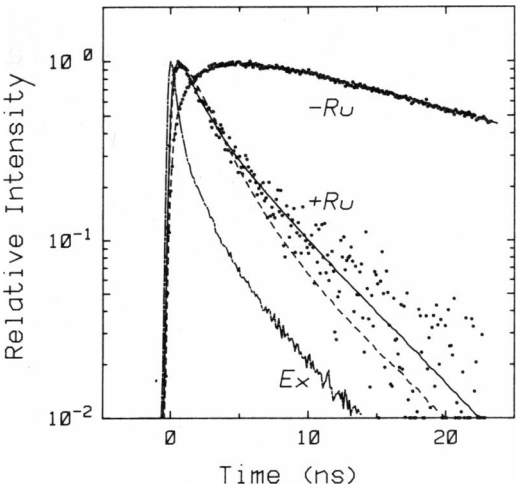


FIGURE 3 Fluorescence decay of the reduced purple membranes in the absence ($-Ru$) and presence ($+Ru$) of solid $Ru(bpy)_3Cl_2$. *Dots*, experimental; *dashed lines* ($-Ru$ and $+Ru$), best-fit single-exponential approximation; *solid line* ($+Ru$), best-fit double-exponential approximation. *Ex*, excitation light pulse. Ruthenium/bacteriorhodopsin molar ratio was 2×10^2 for +Ru.

The initial rate of the decay estimated from the two-exponential approximation was 1.4 ± 0.2 ns^{-1} for the samples examined. An upper limit of C is estimated at 1.2 molecules/nm^3 from crystal data on a hexafluorophosphoate salt (Rillema et al., 1979). A space-filling model showed that the radius of the ruthenium complex is ~0.7 nm. Introducing these values into Eq. 11 (z' = 4.5 nm + 1.4 nm − z) we obtained an upper limit of the distance of closest approach z of 2.5 nm. The chromophore should be within 1.8 nm from a surface. This upper limit for the depth is slightly higher than that in system *ii* above. Probable explanations are the rather low acceptor/donor ratio and the tendency toward segregation of the membranes and the ruthenium complex during the drying procedure (solubility of $Ru[bpy]_3Cl_2$ in water was below 50 mM). Lower limit could not be set in this system.

System *iv:* Energy Transfer from $Ru(bpy)_3Cl_2$ to Native Chromophore

The ruthenium complex embedded in between the reduced purple membranes showed an intense emission around 620 nm upon excitation at 450 nm. The emission decay was exponential with a lifetime τ_D of 1,250 ns (curve *Red* in Fig. 4 *A*). Since the emission spectrum overlaps with the absorption spectrum of dark-adapted native purple membranes, energy transfer from the ruthenium complex to the retinal chromophore is expected in a mixture of the complex and native membranes. In fact we observed a marked acceleration of the emission decay, as shown in curve *Nat* in Fig. 4 *A*.

The nature of the ruthenium luminescence, whether fluorescence or phosphorescence, appears controversial. Here we simply assume that the interaction between the excited ruthenium complex and the retinal chromophore is

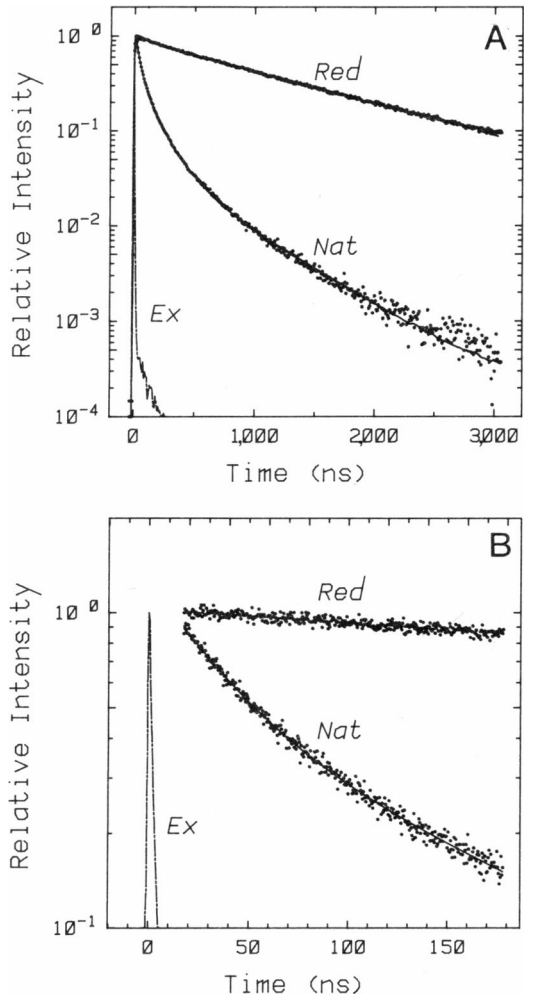


FIGURE 4 Luminescence decay of the ruthenium complex in between native (*Nat*) or reduced (*Red*) purple membrane sheets. (*A*) Entire decay; (*B*) initial portion. *Dots*, experimental; *dashed lines* (*Red*), best-fit single exponential approximation; *solid lines* (*Nat*), best-fit triple- (*A*) or double- (*B*) exponential approximation. *Ex*, excitation light pulse. Ruthenium/bacteriorhodopsin molar ratio, 1.

of the Förster type and that the decay kinetics is given by Eqs. 1–4. According to Harrigan et al. (1973), the emission of the ruthenium complex at room temperature is predominantly from an excited singlet state, i.e., fluorescence.

To locate every donor, the ruthenium complex, immediately on the membrane surface, as diagrammed in Fig. 1 *iv*, samples with a ruthenium/bacteriorhodopsin molar ratio between 0.5 to 2 were prepared. X-ray measurements showed that the purple membranes were stacked parallel upon drying, as has been reported by Henderson (1975).

The emission decay in this system deviated markedly from exponential, as is seen in Fig. 4, due presumably to a random distribution of the donors along the surface of the membrane. The initial 200 ns portion of the decay, however, could be adequately fit with two exponential terms as shown in Fig. 4 *B*. (Data at 0–20 ns were discarded, since the filter combination used did not block the scattered stray light completely. The stray light, however, was weak and did not distort the emission decay of the ruthenium complex beyond 20 ns.) For samples with a ruthenium/bacteriorhodopsin ratio between 0.5 and 2, the initial decay rate $k_d(0)$, calculated from the two-exponential approximation, was within 0.022 ± 0.002 ns^{-1}. A sample with a ratio of 10 gave $k_d(0)$ of 0.017 ns^{-1}.

Case *B* in the Theoretical section is expected to apply to this system. Since the donors were sandwiched between two membranes, three situations are to be considered: Both of the two membrane surfaces touching a donor were proximal to the retinal chromophore, one was proximal and the other distal (as in Fig. 1, *iv*), or both were distal. Averaging the three situations with statistical weights of 1:2:1 yields Eq. 9 with z for the proximal surface and $z' = 4.5\ nm + 1.4\ nm - z$ for the distal surface. The membrane area per bacteriorhodopsin A in Eq. 9 has been determined to be 11 nm^2 (Unwin and Henderson, 1975). The tilt angle Θ is again set at 67°, which is within the experimental values for native chromophore obtained by Heyn et al. (1977). Thus we obtain $z = 1.8$ nm for the distance between the donor and the proximal acceptor plane. The retinal chromophore in the native purple membrane is thus situated at a depth of 1.1 nm from a surface. The experimental error in this number is estimated to be well within 0.3 nm, corresponding to a combined error in R_0^6 $(1 + \cos^2\Theta)$ and $k_d(0)$ of more than twofold.

DISCUSSION

As is seen in the bottom row of Fig. 1, the results with systems *ii–iv* in the present study as well as with systems *i* and *v* in paper I are all consistent with a transmembrane location of the retinal chromophore at a depth of 1.0 ± 0.3 nm from a surface of the purple membrane. Note that the upper limits obtained in systems *ii* and *iii* correspond to the ideal (unrealistic) situation where all membrane surfaces are in direct contact with crystalline acceptors. The chromophore is closer to one of the surfaces of the membrane with a thickness of ~4.5 nm. Below we discuss the characteristics of the five systems and the validity of the assumptions made in the analyses. The major point is whether the depth value could be an underestimate.

In system *i*, the donor was the reduced chromophore and the acceptor was Co-EDTA in solution. The rapid diffusion limit was attained in this system: The diffusion of acceptor molecules was rapid enough to average out the distribuiton of acceptors around each donor before significant energy transfer had occurred. All donors were thus equivalent, resulting in an exponential emission decay. The determination of the initial decay rate $k_d(0)$ was therefore simple, and the depth determined from Eq. 10 (the term $1/z'^3$ in Eq. 11 turned out to be negligible) was 0.8 ± 0.3 nm.

Assumptions *b* and *e*, uniform distribution of acceptors, were involved in the analysis of system *i*. If the local concentration of Co-EDTA near the membrane surface, particularly in the vicinity of the chromophore site, was

higher than the bulk concentration, the obtained depth of 0.8 nm is an underestimate. Thus we designed the experiments with systems *ii* and *iii*, where the acceptors, Co-EDTA or $Ru(bpy)_3Cl_2$, were condensed on the membrane surface. The upper limits of the depth value in these systems, 1.5 nm in *ii* and 1.8 nm in *iii*, do not depend on whether the assumptions *b* and *e* were actually met by these systems, since the limits correspond to an ideal situation of maximal packing. Also note that Co-EDTA was negatively charged, whereas the ruthenium complex was positive; the interaction of these complexes with the membrane surface should be different from each other.

In the above three systems, the location was determined for the reduced chromophore. Native retinal chromophore was examined in system *iv*, which again indicated an off-central location.

Since the number of the donors, the ruthenium complex, per bacteriorhodopsin was rather small in system *iv*, the assumption *b* may be questioned: The donors might have been bound on the proximal membrane surface at a site(s) immediately above the chromophore where the energy transfer would be most efficient; then the depth value of 1.1 nm could be an underestimate. We therefore made numerical evaluation of Eq. 5 for all possible locations of the donor, assuming the most-probable in-plane disposition of the chromophore determined previously (Kouyama et al., 1981*b*). The results showed that the depth value cannot be greater than 1.4 nm as long as we maintain the assumption *c*, random orientation of the donor (see below). If all donors had adopted the same particular disposition (orientation and position with respect to bacteriorhodopsin) that would maximize the energy transfer efficiency, a very unlikely assumption, the depth could be 1.8 nm. Note, however, that the experimental decay rate at the donor/bacteriorhodopsin ratio of 10, when inserted into Eq. 9, gives a depth of 1.2 nm. The 10 donors cannot occupy the same site.

Now we examine the assumptions *c* or *d*, random orientation of the metal complexes, for systems *i* through *iv*. The assumptions have been introduced merely to facilitate the evaluation of the orientation factor κ^2 in Eq. 5. Rigorous randomness is not required since a change in the averaged orientation factor, $(1 + \cos^2\Theta)/2$ in Eqs. 9–11, by more than a factor of two does not alter the depth value beyond the experimental error quoted in Fig. 1. In particular, violation of the assumptions cannot lead to a large upward revision of the depth value (e.g., see the paragraph above and also Stryer [1978]). Both Co-EDTA and $Ru(bpy)_3Cl_2$ are globular. The latter, in particular, is almost spherical with the ruthenium ion exactly at the center. It is thus inconceivable that a unique orientation had prevailed in the systems where the molar ratio of metal complex to bacteriorhodopsin was >1.

Analysis in the four systems above tacitly assumed that the membrane surfaces are flat and featureless. If there were a depression above or below the chromophore, the "depth" we obtained would be an underestimate. For systems *ii–iv* a remote possibility also exists that the metal complexes might have penetrated below the surface of the membrane during the drying procedure. (The possibility that free Co^{2+} unassociated with EDTA had acted as acceptor was denied for system *i* by the addition of $CoCl_2$ [Kouyama et al., 1983].) Although x-ray (Henderson, 1975) and neutron (Zaccai and Gilmore, 1979) diffraction studies on membrane stacks obtained by drying have already denied the presence of large depressions, we sought for direct evidence in system *v*. A parallel stack of reduced and native purple membranes were prepared and the intermembrane energy transfer from the reduced chromophore to native retinal was analyzed. The system corresponds basically to case *B* in the Theoretical section. The vertical distance z between the chromophore planes for a pair of membranes with the proximal surfaces opposed against each other was estimated to be 2.8 ± 0.3 nm. X-ray measurements on the sample showed a stacking repeat of 5.0 nm; subtraction of the assumed membrane thickness of ~4.5 nm suggested a thickness Δ of intermembrane space of 0.5 nm. The chromophore depth was therefore calculated as $(z - \Delta)/2 = 1.1 \pm 0.3$ nm. Below we examine whether this value obtained in paper I could have been an underestimate.

An extra assumption in the above analysis was that the two kinds of membranes were mixed randomly in the stack, parallel and antiparallel being undiscriminated. Any deviation from the assumption, however, would lead to an overestimated depth value, except for an unlikely tendency of pairing of the two kinds of membranes with the proximal surfaces opposed against each other. Also, any intermembrane spaces greater than Δ above left in the sample, as a result of imperfect stacking, would lead to an overestimated depth value. (This last statement applies to system *iv* as well.)

Corresponding to the assumptions *b* and *e*, we assumed in the analysis of system *v* in paper I that neither translational nor rotational correlations existed between adjacent membranes. To assess the consequence of this assumption, we again evaluated Eq. 5 numerically, assuming the most-probable in-plane disposition of the chromophores. The results showed that the depth value cannot exceed 1.5 nm ($z < 3.5$ nm) for any arrangement of the membranes in the stack, even in the complete absence of intermembrane spaces greater than Δ. Thus, system *v* also denies the central location and gives a depth value consistent with the other four systems. The small depth values obtained with the metal complexes are not due to the presence of depressions that accommodate the complexes.

Finally we examine the assumption *a*, the Förster mechanism. The strongest argument in support of the mechanism is the consistency of the answers in the five quite different systems (Fig. 1, *bottom row*). The efficiency of the assumed resonance interaction, as represented by R_0^6, varied by orders of magnitude among these systems, yet

the final answers were similar to each other. Second is that compounds related to the acceptors but lacking the required spectral overlap failed to accelerate the emission decay (Na-EDTA or Ca-EDTA versus Co-EDTA in systems *i* and *ii*, the reduced chromophore versus native chromophore in systems *iv* and *v*). Third, other interactions that may lead to deactivation of a donor, such as the exchange interaction (Meares et al., 1981), are generally characterized by a much steeper distance dependence and are short-ranged, requiring a close contact between the donor and acceptor. The chromophore in the purple membrane, however, appears to be unexposed to the aqueous phase. For example, quenchers such as I^- or Cs^+ in the external solution failed to quench the fluorescence of the reduced chromophore (Kouyama et al., 1981*a*; Tsetlin et al., 1983). The reduction of the retinal chromophore by borohydride does not proceed unless light activates the photochemical reaction of bacteriorhodopsin (Peters et al., 1976; Schreckenbach et al., 1977).

The discussion above shows that our conclusion does not depend on particular assumptions. The center of the polyene chain part, along which the transition moment lies, of the retinal chromophore is located at a depth of 1.0 ± 0.3 nm from a surface of the purple membrane. Tsetlin et al. (1983) have also obtained a similar depth value from the analysis of energy transfer from the reduced chromophore to lanthanide or cobalt ions on the membrane surface. The chromophore must be outside the middle one-third of the transmembrane section.

We thank Dr. M. Kaneko for the gift of $Ru(bpy)_3Cl_2$.

This work was supported by special coordination funds for the promotion of science and technology and a grant for "Solar-Energy-Photosynthesis" given by the Agency of Science and Technology of Japan, and by Grants-in-Aid from Ministry of Education, Science and Culture of Japan.

Received for publication 13 February 1987 and in final form 26 May 1987.

REFERENCES

Agard, D. A., and R. M. Stroud. 1982. Linking regions between helices in bacteriorhodopsin revealed. *Biophys. J.* 37:589–602.

Bayley, H., K.-S. Huang, R. Radhakrishnan, A. H. Ross, Y. Takagaki, and H. G. Khorana. 1981. Site of attachment of retinal in bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA.* 78:2225–2229.

Blaurock, A. E., and W. Stoeckenius. 1971. Structure of the purple membrane. *Nature New Biol.* 233:152–155.

Braiman, M., and R. Mathies. 1982. Resonance Raman spectra of bacteriorhodopsin's primary photoproduct: evidence for distorted 13-*cis* retinal chromophore. *Proc. Natl. Acad. Sci. USA.* 79:403–407.

Dencher, N. A. 1983. Yearly review. The five retinal-protein pigments of halobacteria: bacteriorhodopsin, halorhodopsin, P565, P370, and slow-cycling rhodopsin. *Photochem. Photobiol.* 38:753–776.

Förster, T. 1965. Delocalized excitation and excitation transfer. *In* Modern Quantum Chemistry. O. Sinanoglu, editor. Academic Press, Inc., New York. 93–137.

Furuno, T., A. Ikegami, H. Kihara, M. Yoshida, and Y. Kagawa. 1983. Small-angle X-ray scattering study of adenosine triphosphatase from the thermophilic bacterium PS3. *J. Mol. Biol.* 170:137–153.

Harrigan, R. W., G. D. Hager, and G. A. Crosby, 1973. Evidence for multiple-state emission from ruthenium(II) complexes. *Chem. Phys. Lett.* 21:487–490.

Hayward, S. B., and R. M. Stroud. 1981. Projected structure of purple membrane determined to 3.7 Å resolution by low temperature electron microscopy. *J. Mol. Biol.* 151:491–517.

Henderson, R. 1975. The structure of the purple membrane from *Halobacterium halobium:* analysis of the X-ray diffraction pattern. *J. Mol. Biol.* 93:123–138.

Henderson, R., and P. N. T. Unwin. 1975. Three-dimensional model of purple membrane obtained by electron microscopy. *Nature (Lond.).* 257:28–32.

Heyn, M. P., R. J. Cherry, and U. Müller. 1977. Transient linear dichroism studies on bacteriorhodopsin: determination of the orientation of the 568 nm all-*trans* retinal chromophore. *J. Mol. Biol.* 117:607–620.

Hsieh, C.-L., M. A. El-Sayed, M. Nicol, M. Nagumo, and J.-H. Lee. 1983. Time-resolved resonance Raman spectroscopy of the bacteriorhodopsin photocycle on the picosecond and nanosecond time scales. *Photochem. Photobiol.* 38:83–94.

Huang, K.-S., R. Radhakrishnan, H. Bayley, and H. G. Khorana. 1982. Orientation of retinal in bacteriorhodopsin as studied by cross-linking using a photosensitive analog of retinal. *J. Biol. Chem.* 257:13616–13623.

Jubb, J. S., D. L. Worcester, H. L. Crespi, and G. Zaccai. 1984. Retinal location in purple membrane of *Halobacterium halobium:* a neutron diffraction study of membranes labelled in vivo with deuterated retinal. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:1455–1461.

Katre, N. V., P. K. Wolber, W. Stoeckenius, and R. M. Stroud. 1981. Attachment site(s) of retinal in bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA.* 78:4068–4072.

Khorana, H. G., G. E. Gerber, W. C. Herlihy, C. P. Gray, R. J. Anderegg, K. Nihei, and K. Biemann. 1979. Amino acid sequence of bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA.* 76:5046–5050.

King, G. I., W. Stoeckenius, H. L. Crespi, and B. P. Schoenborn. 1979. The location of retinal in the purple membrane profile by neutron diffraction. *J. Mol. Biol.* 130:395–404.

Kinosita, K., Jr., R. Kataoka, Y. Kimura, O. Gotoh, and A. Ikegami. 1981. Dynamic structure of biological membranes as probed by 1,6-diphenyl-1,3,5-hexatriene: a nanosecond fluorescence depolarization study. *Biochemistry.* 20:4270–4277.

Kinosita, K., Jr., T. Kouyama, and A. Ikegami. 1987. Criteria for the rapid diffusion limit of fluorescence energy transfer. *J. Chem. Phys.* 86:3273–3282.

Kouyama, T., Y. Kimura, K. Kinosita, Jr., and A. Ikegami. 1981*a*. Immobility of the chromophore in bacteriorhodopsin. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 124:100–104.

Kouyama, T., Y. Kimura, K. Kinosita, Jr., and A. Ikegami. 1981*b*. Location and orientation of the chromophore in bacteriorhodopsin. Analysis by fluorescence energy tranfer. *J. Mol. Biol.* 153:337–359.

Kouyama, T., K. Kinosita, Jr., and A. Ikegami. 1983. Fluorescence energy transfer studies of transmembrane location of retinal in purple membrane. *J. Mol. Biol.* 165:91–107.

Kuhn, H., D. Möbius, and H. Bücher. 1972. Spectroscopy of monolayer assemblies. *In* Physical Methods of Chemistry. Vol. I. Pt. IIIB. A. Weissberger and B. W. Rossiter, editors. Wiley-Interscience, New York. 577–702.

Kurimura, Y., M. Nagashima, K. Takato, E. Tsuchida, M. Kaneko, and A. Yamada. 1982. Photoredox reactions using ion-exchange resin-adsorbed $Ru(bpy)_3^{2+}$. Photosensitized reductions of methyl viologen and molecular oxygen using ion-exchange resin-adsorbed tris(2,2'-bipyridine)ruthenium(II). *J. Phys. Chem.* 86:2432–2437.

Lemke, H.-D., and D. Oesterhelt. 1981. Lysine 216 is a binding site of the retinal moiety in bacteriorhodopsin. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 128:255–260.

Lewis, A., J. Spoonhower, R. A. Bogomolni, R. H. Lozier, and W.

Stoeckenius. 1974. Tunable laser resonance Raman spectroscopy of bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA.* 71:4462–4466.

Meares, C. F., M. Y. Simon, and L. Stryer. 1981. Exchange interaction contribution to energy transfer between ions in the rapid-diffusion limit. *J. Am. Chem. Soc.* 103:1607–1609.

Mullen, E., A. H. Johnson, and M. Akhtar. 1981. The identification of Lys_{216} as the retinal binding residue in bacteriorhodopsin. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 130:187–193.

Oesterhelt, D., and W. Stoeckenius. 1974. Isolation of the cell membrane of *Halobacterium halobium* and its fragmentation into red and purple membrane. *Methods Enzymol.* 31:667–678.

Ovchinnikov, Y. A., N. G. Abdulaev, M. Y. Feigina, A. V. Kiselev, and N. A. Lobanov. 1979. The structural basis of the functioning of bacteriorhodopsin: an overview. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 100:219–224.

Peters, J., R. Peters, and W. Stoeckenius. 1976. A photosensitive product of sodium borohydride reduction of bacteriorhodopsin. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 61:128–134.

Pettei, M. J., A. P. Yudd, K. Nakanishi, R. Henselman, and W. Stoeckenius. 1977. Identification of retinal isomers from bacteriorhodopsin. *Biochemistry.* 16:1955–1959.

Rillema, D. P., D. S. Jones, and H. A. Levy. 1979. Structure of tris(2,2′-bipyridyl)ruthenium(II) hexafluorophosphate, $[Ru(bipy)_3][PF_6]_2$; X-ray crystallographic determination. *J. Chem. Soc. Chem. Comm.* 849–851.

Schreckenbach, T., B. Walckhoff, and D. Oesterhelt. 1977. Studies on the retinal-protein interaction in bacteriorhodopsin. *Eur. J. Biochem.* 76:499–511.

Seiff, F., I. Wallat, P. Ermann, and M. P. Heyn. 1985. A neutron diffraction study on the location of the polyene chain of retinal in bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA.* 82:3227–3231.

Stoeckenius, W. 1985. The rhodopsin-like pigments of halobacteria: light-energy and signal transducers in an archaebacterium. *Trends Biochem. Sci.* 10:483–486.

Stoeckenius, W., R. H. Lozier, and R. A. Bogomolni. 1979. Bacteriorhodopsin and the purple membrane of halobacteria. *Biochim. Biophys. Acta.* 505:215–278.

Stoeckenius, W., and R. A. Bogomolni. 1982. Bacteriorhodopsin and related pigments of halobacteria. *Annu. Rev. Biochem.* 51:587–616.

Stryer, L. 1978. Fluorescence energy transfer as a spectroscopic ruler. *Annu. Rev. Biochem.* 47:819–846.

Terner, J., C.-L. Hsieh, A. R. Burns, and M. A. El-Sayed. 1979. Time-resolved resonance Raman spectroscopy of intermediates of bacteriorhodopsin: the bK_{590} intermediate. *Proc. Natl. Acad. Sci. USA.* 76:3046–3050.

Thomas, D. D., W. F. Carlsen, and L. Stryer. 1978. Fluorescence energy transfer in the rapid-diffusion limit. *Proc. Natl. Acad. Sci. USA.* 75:5746–5750.

Tsetlin, V. I., V. I. Zakis, A. A. Aldashev, A. B. Kuryatov, G. V. Ovechkina, and V. L. Shnyrov. 1983. Topography of the retinyl-binding section in reduced bacteriorhodopsin derivatives. *Bioorg. Khim.* 9:1589–1605.

Tsuda, M., M. Glaccum, B. Nelson, and T. G. Ebrey. 1980. Light isomerized the chromophore of bacteriorhodopsin. *Nature (Lond.).* 287:351–353.

Unwin, P. N. T., and R. Henderson. 1975. Molecular structure determination by electron microscopy of unstained crystalline specimen. *J. Mol. Biol.* 94:425–440.

Weakliem, H. A., and J. L. Hoard. 1959. The structures of ammonium and rubidium ethylenediaminetetraacetatocobaltate(III). *J. Am. Chem. Soc.* 81:549–555.

Weber, G., and F. W. J. Teale. 1957. Determination of the absolute quantum yield of fluorescent solutions. *Trans. Faraday Soc.* 53:646–655.

Zaccai, G., and D. J. Gilmore. 1979. Areas of hydration in the purple membrane of *Halobacterium halobium:* a neutron diffraction study. *J. Mol. Biol.* 132:181–191.