CHROMOPHORE OF BACTERIORHODOPSIN IS CLOSER TO THE CYTOPLASMIC SURFACE OF PURPLE MEMBRANE

Fluorescence Energy Transfer on Oriented Membrane Sheets

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ABSTRACT Transmembrane location of the retinal chromophore, either native or reduced in situ to a fluorescent derivative, of the purple membrane of *Halobacterium halobium* was investigated with fluorescence energy transfer techniques. Single sheets of purple membrane, either native or reduced with borohydride, were adsorbed on polylysine-coated glass; the orientation, whether the exposed surfaces were cytoplasmic or extracellular, was controlled by adjusting the pH of the membrane suspension before the adsorption. On the exposed surface of the reduced membrane, a layer of cytochrome *c*, hemoglobin, or ferritin was deposited. The rate of excitation energy transfer from the fluorescent chromophore in the membrane to the colored protein was greater when the protein was on the cytoplasmic surface of the membrane than when it was on the extracellular surface. Analysis in which uniform distribution of the protein on the surface was assumed showed that the reduced chromophore is situated at a depth of <1.5 nm from the cytoplasmic surface. The location of the native retinal chromophore was examined by depositing a small amount of tris(2,2′-bipyridyl)ruthenium(II) complex on the native membrane adsorbed on the glass. Energy transfer from the luminescent complex to the retinal chromophore was more efficient on the cytoplasmic surface than on the extracellular surface, suggesting that the native chromophore is also on the cytoplasmic side. From these and previous results we conclude that the chromophore, whether native or reduced, of bacteriorhodopsin is located at a depth of 1.0 ± 0.3 nm from the cytoplasmic surface of purple membrane.

INTRODUCTION

Purple membrane of *Halobacterium halobium* is a two-dimensional crystalline array of a protein bacteriorhodopsin with lipids filling the inter-protein spaces. Bacteriorhodopsin functions as a light-driven proton pump: Light absorption by the retinal chromophore in the protein initiates a photocycle, during which protons are actively transported across the membrane from inside the cell to the external medium. Mechanism of this molecular machine has been a subject of intensive studies (for recent reviews, see, e.g., Stoeckenius and Bogomolni, 1982; Dencher, 1983; Stoeckenius, 1985). Full understanding of the molecular mechanism, however, still awaits key experiments.

Elucidation of the structure of bacteriorhodopsin is of vital importance to the understanding of the mechanism. The amino acid sequence has been determined (Ovchinnikov et al., 1979; Khorana et al., 1979). Electron microscopy has shown that the protein consists of seven rod-shaped masses, presumably α-helices, all running nearly perpendicularly to the surface of purple membrane (Henderson and Unwin, 1975; Hayward and Stroud, 1981; Agard and Stroud, 1982). Amino acid residues, however, are not yet resolved in the three-dimensional structure. Even the position of the retinal chromophore has not been established completely.

Determination of the disposition of retinal in bacteriorhodopsin is of twofold importance. First, the chromophore is the site of primary events in the pumping cycle. Second, locating retinal helps establish correspondence between the known amino acid sequence and the three-dimensional structural data, since retinal is known to be bound to lysine 216 in the sequence (Bayley et al., 1981).

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Kouyama et al. (1981*b*) have studied the in-plane geometry of the retinal chromophore in purple membrane by a "crystallographic" analysis of fluorescence energy transfer. The most probable location and orientation proposed by these authors have been supported by neutron diffraction studies (Jubb et al., 1984; Seiff et al., 1985). In a recent neutron diffraction study (Seiff et al., 1986) the position of the ring portion of the chromophore has also been resolved in the plane of membrane. Fluorescence energy transfer studies have also shown that the chromophore is situated at a depth of 1.0 ± 0.3 nm from a surface of purple membrane (Kouyama et al., 1983; Tsetlin et al., 1983; Kometani et al., 1987). The membrane has a thickness of ~4.5 nm (Henderson, 1975; Agard and Stroud, 1982). The remaining problem is therefore to decide which of the two surfaces the chromophore is closer to.

Here we have tackled this last problem using again fluorescence energy transfer techniques. Following Fisher (Fisher et al., 1977, 1978; Fisher, 1982), we applied single purple membrane sheets on a glass surface so that either the cytoplasmic or extracellular surface of the membrane was exposed preferentially. Dye or colored protein molecules were then put on the exposed surface of the membrane sheets. Measurement of the rate of energy transfer, which is sensitive to intermolecular distances of the order of 1–10 nm (Förster, 1965; Stryer, 1978), between the retinal chromophore in the membrane and the external molecules on the surface indicated that the chromophore is closer to the cytoplasmic surface of the membrane.

MATERIALS AND METHODS

Preparation of Purple Membrane and Its Fluorescent Derivative

Halobacterium halobium, strain JW3 or R_1M_1, was grown and purple membrane was isolated according to an established procedure (Oesterhelt and Stoeckenius, 1974). The two strains did not show noticeable differences in the results below. Purified membranes were stored at −80°C in 50% (wt/vol) sucrose. Before use they were washed several times with water.

The retinal chromophore in the purple membrane was reduced, in situ, with borohydride and then further converted with ultraviolet light as described by Kouyama et al. (1981*b*). The product, which we refer to as "reduced membranes," showed a multi-peaked absorption spectrum in the near ultraviolet region and fluoresced strongly upon excitation in the absorption band.

Oriented Adsorption of Membrane Sheets on a Cover Glass

Both native and reduced purple membranes were adsorbed on the surface of polylysine-coated cover glasses by the method of Fisher (1982) with some modifications. According to Fisher et al. (1977), membranes suspended at neutral pH (≈7) are adsorbed predominantly with the cytoplasmic surface opposing the glass surface whereas at acidic pH (≈3) the extracellular surface faces the glass. Here we refer to the former as "neutral" preparation and the latter "acidic."

Membranes, either native or reduced, were suspended in water at ~5 mg/ml. Water used in the present work was doubly distilled and passed through a NANO pure II system (Sybron/Barnstead Co., Boston, MA). Further distillation in a Pyrex-glass system did not alter the results. The membrane suspension was passed through a column of cation-exchange resin (Dowex 50W-X8, mesh 200–400, obtained from Muromachi Kagaku Kogyo Kaisha, Ltd., Chuo-ku, Tokyo, washed with HCl and water). The column treatment converted the membranes into an acidic form, as evidenced by the blue color in the case of native purple membrane, without aggregation (the so-called deionized blue membrane: Kimura et al., 1984; Chang et al., 1985). For the "acidic" preparation the eluent was used without further treatment. Addition of citrate (pH 3) up to 0.5 mM did not alter the results. For the "neutral" preparation the eluent was titrated with NaOH to pH 7. Alternatively the pH was raised with 5 mM phosphate or Hepes (*N*-2-hydroxyethylpiperazine-*N*-2-ethenesulfonic acid) buffer (pH 7), without affecting the results. The final concentration of the membranes was ~2 mg/ml.

Cover glasses (11 × 22 mm^2) were cleaned and coated with poly-L-lysine (molecular weight 1,000–4,000, Sigma Chemical Co., St. Louis, MO) as described (Fisher, 1982). The cover glasses were used immediately after the coating. On each surface of a coated cover glass 20 μl of a membrane suspension was applied and the cover glass was gyrated for 30 s. The suspension was then washed away with water. In order to remove excess membranes, the treated cover glass held in a small glass container was sonicated in a bath sonicator (Laboratory Supplies Co., Inc., Hicksville, NY). The sonication was repeated until the amount adsorbed became less than one half the amount required to cover the entire glass surfaces uniformly with one layer of membrane sheets. The sonicated cover glasses were washed with water by overflowing the container and stored in water. The membranes did not show any tendency to come off.

Estimation of the Amount of Membranes Adsorbed

Absorption spectrum of native or reduced purple membranes adsorbed on a cover glass was recorded on a Shimadzu UV-3000 spectrophotometer (Shimadzu Seisakusho, Ltd., Kyoto, Japan). The cover glass was held in a cuvette filled with water; a clean cover glass in water served as a blank. To estimate the amount adsorbed, we assumed that a cover glass with both surfaces uniformly covered with one layer of membranes would give an absorbance of ~0.024 at 560 nm for native purple membranes and 0.020 at 382 nm for reduced membranes. The absorbance values are based on the extinction coefficients for suspension and the orientation of the chromophore with respect to the membrane plane (Kouyama et al., 1981*b*), and the unit cell dimension (Henderson, 1975).

In the case of reduced membranes, routine estimation was made by fluorescence measurement: The cover glass in a cuvette filled with water was excited at 382 nm in a Hitachi 650–60 spectrofluorometer (Hitachi, Ltd., Tokyo) and the emission spectrum was recorded over 400–600 nm. Correspondence between the fluorescence intensity and the amount adsorbed was established by the absorption measurement above.

Papain Treatment and Electrophoresis

Cover glasses with adsorbed membranes were held in a rack and immersed in 100 ml of a solution containing 20 mM phosphate buffer (pH 6.3), 0.3 mM ethylenediaminetetra-acetate, 1.7 mM cysteine, and 0.1 mg of papain (aus Carica papaya, Boehringer Mannheim Yamanouchi, Ltd., Tokyo). After incubation at 37°C for 10–15 min, the container was overflowed with water for 10–15 min. Adsorbed membranes were then scraped off the two surfaces of the cover glass into 20 μl of a solution containing 300 mM tris(hydroxy-methyl)aminomethane HCl (pH 8.8), 3.4% (wt/vol) sodium lauryl sulfate, 20% (wt/vol) sucrose, and 5% (vol/vol) β-mercaptoethanol. Membranes from two to three cover glasses were collected into the same solution and subjected to polyacrylamide gel electrophoresis (Laemmli, 1970).

Electron Microscopy

A cover glass coated with native or reduced purple membrane sheets was air-dried, after excess water was blotted off with filter paper, by holding

the glass vertically with forceps for 1–3 min at 25°C. The specimen was rotary shadowed with platinum/carbon at an elevation angle of 20° using an electron beam gun of a freeze-etching apparatus (JFD-7000, JEOL Co., Ltd., Akishima, Tokyo). The replica was examined with a JEOL 100-CX electron microscope at 100 kV.

Application of Ruthenium Complex on the Adsorbed Membranes

A cover glass on which native purple membrane sheets were adsorbed was soaked in a solution of tris(2,2'-bipyridyl)ruthenium(II) complex (chloride salt, 1 mM in water) for a few seconds. Excess solution was blotted off with filter paper. The cover glass was then submerged in water several times until the amount of ruthenium complex adsorbed was reduced to several moles per mole of bacteriorhodopsin. The amount adsorbed was estimated by comparing the luminescence intensity of the ruthenium-treated cover glass in water with the intensity of a ruthenium solution of known concentration in a 2 × 10-mm cuvette held at the same position as the cover glass (Hitachi 650–60 spectrofluorometer, excitation at 455 nm, emission between 500 and 700 nm). Control experiment in which polylysine-coated cover glasses without adsorbed membranes were treated in the same manner showed that the positively-charged ruthenium complex was not attached to the positively-charged polylysine surface: The luminescence intensity dropped to a negligible value after the second wash. On the cover glass with membranes, therefore, the ruthenium complex was bound predominantly on the membrane surfaces. In order to ensure contact between the complex and membrane, the cover glass was dried under reduced pressure.

Application of Proteins on the Adsorbed Membranes

50 μl of an aqueous solution of cytochrome *c* (Sigma, from horse heart), hemoglobin (Sigma, bovine), or ferritin (Boehringer Mannheim Yamanouchi) at a concentration in the order of 1 mg/ml was spread uniformly on a cover glass on which the reduced membranes had been adsorbed. The cover glass was dried under reduced pressure and the other surface was coated with the protein in the same manner. Absorption spectra of the dried cover glasses showed that cytochrome *c* was in the oxidized form (Margoliash and Schejter, 1966) and hemoglobin in the met-form (Antonini and Brunori, 1971). The amount of protein adsorbed was determined from the absorption spectra.

Time-Resolved Fluorometry

Decay of luminescence 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 cover glass was mounted in the sample chamber so that the excitation beam made an angle of incidence of 45° with the surface of the glass. Emission at a right angle to the incident beam was detected through the rear surface of the cover glass. For the measurement of the ruthenium emission, the excitation monochromator was set at 450 nm with a bandpass of 12 nm; a short-pass filter with a cut-off at 480 nm (Ditric Optics, Inc., Malboro, MA), a Fuji-Film BPB-45 filter (Fuji Photo Film, Ltd., Tokyo), and two Hoya C-500 filters (Hoya, Ltd., Akishima, Tokyo) were used to reduce stray light. Emission above 560 nm was observed through two Fuji SC-54 filters and a Fuji SC-56 filter. For the reduced membranes, excitation was at 382 nm with a bandpass of 12 nm; a Corning 7-54 filter (Asahi Glass, Ltd., Tokyo), two Toshiba UV-D33S filters (Toshiba Kasei, Ltd., Tokyo), two Hoya C-500 filters were placed in the excitation beam. Emission above 460 nm was observed through a Fuji SC-38 filter, two Fuji SC-42 filters, and a Fuji SC-46 filter. Time constants characterizing the emission decay were determined with a least-square deconvolution program. All fluorescence measurements were made at 20°C.

RESULTS

Oriented Adsorption of Purple Membrane Sheets on Polylysine-Coated Glass

According to Fisher et al. (1977), purple membranes adsorbed at neutral pH have the predominant orientation in which the extracellular surface is exposed in the solution phase, whereas the cytoplasmic surface is exposed at acidic pH. To assess the degree of orientation in our preparation we treated our samples with papain. Since the membranes did not show any tendency to come off the glass surface during the papain treatment in the large volume of solution, we assume in the following that the membranes did not reorient during the treatment. Papain is known to cleave 17 amino acid residues off the carboxyl terminus of bacteriorhodopsin (Renthal et al., 1979). The carboxyl terminus is on the cytoplasmic side of purple membrane (Ovchinnikov et al., 1979).

Fig. 1 shows the gel electrophoresis patterns of native and reduced membranes treated with papain on the cover glass. In membranes adsorbed at acidic pH the digestion was almost complete, indicating that most of the membranes had their cytoplasmic surface exposed. In the neutral preparation, on the other hand, more than one half of bacteriorhodopsin molecules remained undigested, i.e., more than half of the cytoplasmic surfaces were concealed. Densitometry on several different preparations (native and reduced) showed that the degree of digestion was 85 ± 5% in acidic preparations and 45 ± 10% in neutral preparations. The tendency is in accord with the results of Fisher et al. (1977), although the degree of orientation in our neutral preparations is apparently poorer.

The orientation was monitored also by electron microscopy (Fig. 2). The membranes in the acidic preparation (Fig. 2 *b*) were mostly smooth and pitted, with the remaining few cracked. In the neutral preparation (Fig. 2 *a*) the number of cracked membranes was slightly greater than that of smooth ones. The populations of the cracked membranes were consistent with the results of papain

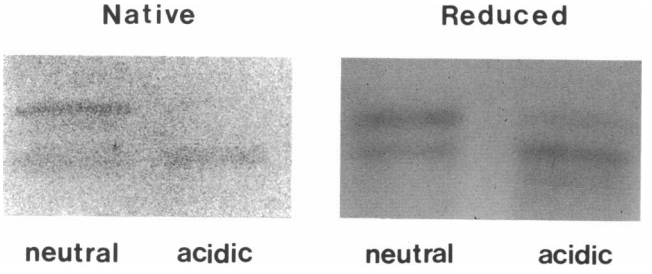

FIGURE 1 Sodium-lauryl-sulfate polyacrylamide-gel electrophoresis patterns of purple membrane adsorbed on polylysine-coated glass and treated with papain. Upper bands, undigested bacteriorhodopsin; lower bands, papain-digested bacteriorhodopsin. Native (*left pair*) or reduced and then ultraviolet converted (*right pair*) purple membrane sheets were adsorbed on the glass at neutral or acidic pH. The glass was then immersed in a papain solution. Adsorbed membranes were then scraped off the glass and subjected to electrophoresis.

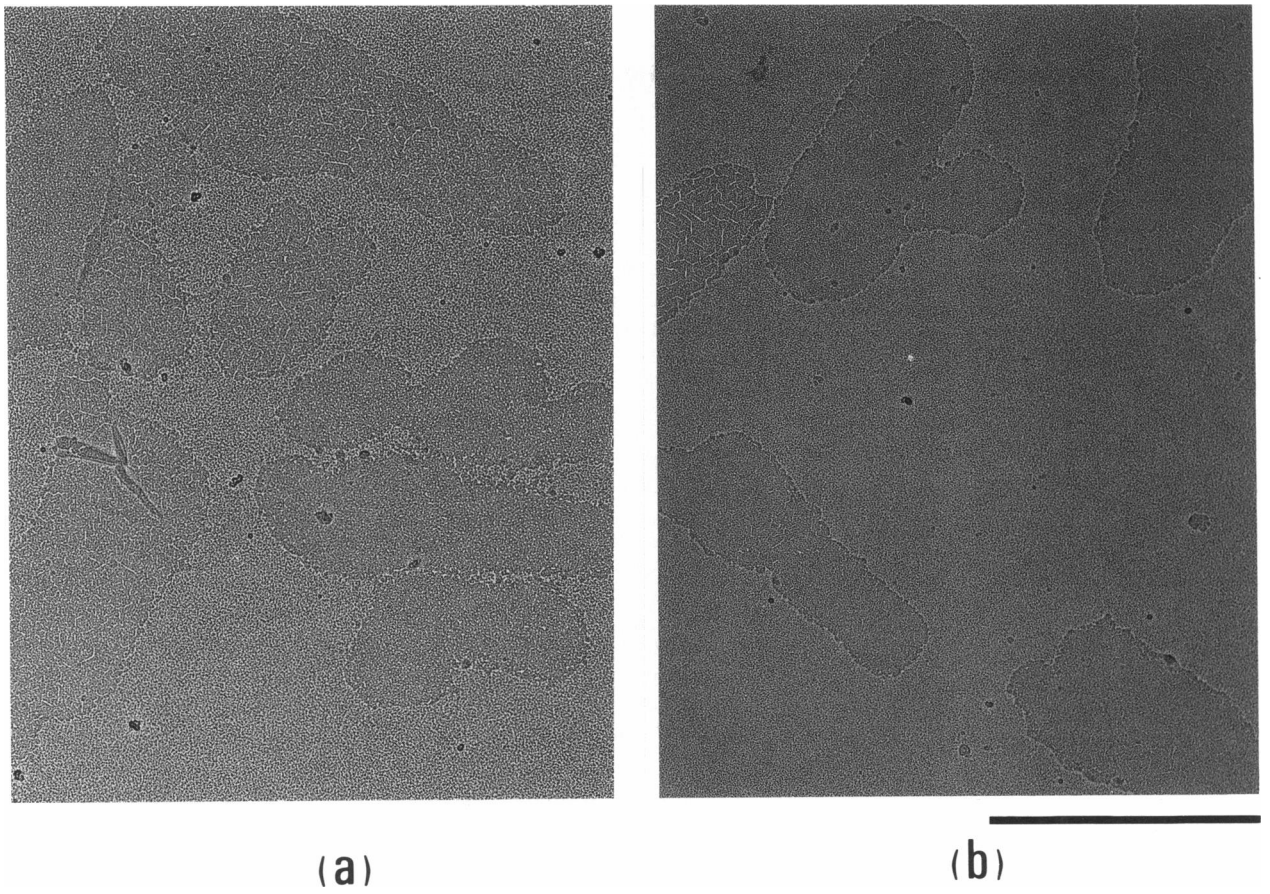

FIGURE 2 Electron micrographs of rotary shadowed purple membrane sheets adsorbed on polylysine-coated cover glass at neutral pH (*a*) and at acidic pH (*b*). Bar indicates 1 μm; original magnification ×10,000.

digestion above, if the cracked surface represented the extracellular surface as indicated by Fisher et al. (1977). In some cases the membranes were all cracked both in the acidic and neutral preparations. We could, however, distinguish two populations of membranes characterized by different types of cracks. Here again the ratio between the two populations was consistent with the papain results. The orientation of the adsorbed membrane sheets was controlled.

Energy Transfer from Reduced Chromophore to External Acceptors

The oriented adsorption of membrane sheets allowed us to design an experiment diagrammed in Fig. 3: We deposited cytochrome *c* on the exposed surface of the reduced purple membrane sheets adsorbed on the glass. Measurement of the rate of excitation energy transfer from the fluorescent chromophore (donor) in the membrane to the cytochrome *c* molecules (acceptor) would tell us which of the two surfaces the chromophore is closer to.

We chose the heme protein as the acceptor because the large protein molecule would not penetrate into the membrane-glass interface: Only the exposed surfaces, i.e., the membrane surfaces accessible to papain, would be covered by the cytochrome *c* molecules. The protein molecules were packed on the membrane by drying (not to the extent of complete dehydration). The resultant protein layer appeared homogeneous except at the edge where it was thicker. Absorption measurement showed that the layer was more than 10 molecules thick.

In the absence of the acceptors the reduced purple membrane adsorbed on the polylysine-coated glass fluoresced with a fluorescence lifetime of 20 ± 0.5 ns, which is comparable with the lifetime of 19.7 ns found for the sheets stacked parallel on a glass surface (Kometani et al., 1987). The neutral and acidic preparations did not show notice-

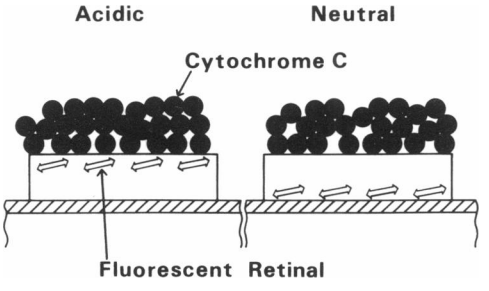

FIGURE 3 A diagram showing the principle of the energy transfer experiment.

able difference. Compared in Fig. 4 are the fluorescence decays of the neutral and acidic preparations in the presence of the overlaid cytochrome *c* molecules. The decay for the membranes adsorbed at acidic pH is clearly faster, indicating a higher rate of energy transfer. Since the membrane surfaces touching the cytochrome *c* were mostly cytoplasmic in the acidic preparation, the result suggests that the reduced chromophore is located closer to the cytoplasmic surface of the membrane. In the neutral preparation about one half of the donors (the fluorescent chromophore) faced the acceptors through the extracellular surface. The efficiency of energy transfer was therefore smaller.

As a control we prepared a system in which the acceptors resided on both sides of the membrane: The reduced membrane sheets were embedded in a thick layer of packed cytochrome *c* molecules by drying a mixture of the membrane suspension and excess cytochrome *c* on a clean cover glass. The decay of fluorescence in this system (Fig. 5) was similar to, or slightly faster than, the decay for the acidic preparation shown in Fig. 4. Donors in this system faced acceptors both on the cytoplasmic and extracellular surfaces. Owing to the steep distance-dependence of the rate of energy transfer (see below), however, the acceptors on the distal surface are far less efficient than the proximal ones unless the donors are exactly at the center of the membrane. Thus the result above is consistent with our interpretation that the cytoplasmic surface is proximal and the extracellular surface distal to the reduced chromophore.

Below we attempt a quantitative analysis. The initial rate, $k_d(0)$, of fluorescence decay in the present system is given by (Kometani et al., 1987)

$$k_d(0) = \frac{1}{\tau_D}\left[1 + \frac{\pi}{12} \cdot R_0^6 C\left(\frac{f_c}{z_c^3} + \frac{f_e}{z_e^3}\right)(1 + \cos^2 \Phi)\right], \quad (1)$$

where z_c and z_e are the distance of closest approach between the fluorescent donor in the membrane and the acceptors on the cytoplasmic and extracellular surfaces, respectively, f_c and f_e are the fraction of donors that face the acceptors through the cytoplasmic and extracellular surfaces, respectively, τ_D is the fluorescence lifetime of the donor in the absence of acceptors, C is the concentration of the acceptors, Φ is the angle between the transition moment of the donor and the membrane normal, and R_0 is the critical distance of energy transfer given by

$$R_0^6 = 8.785 \cdot 10^{-25} n^{-4} Q_D J, \quad (2)$$

where n is the refractive index of the medium, Q_D is the quantum yield of the donor fluorescence in the absence of acceptors, J is the overlap integral (in $M^{-1}cm^3$) between the fluorescence spectrum of the donor and the absorption spectrum of the acceptor, and the unit of R_0 is cm. (Our R_0 slightly differs from the common definition [Stryer, 1978] in which the so-called orientation factor is included). The assumptions in deriving Eq. 1 are (a) the Förster mechanism (Förster, 1965) of excitation energy transfer, (*b*) uniform distribution of acceptors on the membrane, and (*c*) random orientations of acceptors.

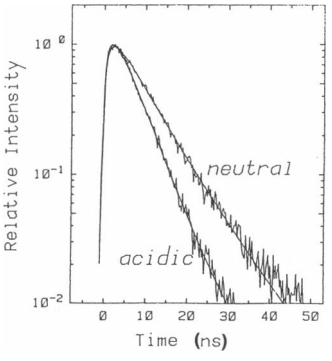

FIGURE 4 Fluorescence decays of reduced and then ultraviolet converted purple membrane sheets adsorbed on polylysine-coated glass at indicated pH and covered with a layer of cytochrome *c*. Zigzag lines, observed; smooth lines, the best-fit two-exponential approximations. Time 0 refers to the peak of the excitation light pulse, which had a measured full width at half maximum of 2 ns.

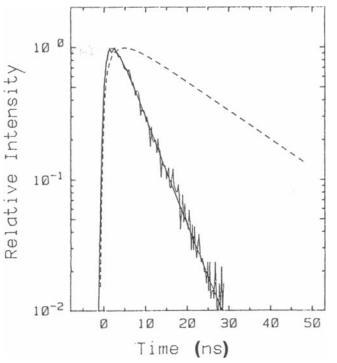

FIGURE 5 Fluorescence decay of reduced and then ultraviolet converted purple membrane sheets dispersed in a layer of cytochrome *c*. Molar ratio of cytochrome *c* to bacteriorhodopsin = 50. Zigzag line, observed; smooth solid line, the best-fit two-exponential approximation. Dashed line represents the best-fit exponential approximation to the decay in the absence of cytochrome *c*.

The observed decays could be fitted with two exponentials as is seen in Fig. 4. The initial decay rate, $k_d(0)$, calculated from the two-exponential approximation was 0.244 ± 0.015 ns^{-1} for three acidic preparations and 0.162 ± 0.012 ns^{-1} for three neutral preparations. Of the parameters in Eq. 1 the donor lifetime τ_D was 20 ns (see above), Φ has been estimated at 67° (Kouyama et al., 1981*b*), and R_0 was calculated to be 3.74 nm from estimated J of 5.41×10^{-14} M^{-1}cm^3, Q_D of 0.23 (Kometani et al., 1987), and assumed n of 1.41. The acceptor concentration C was unknown but its upper limit can be set at 1 mol/24 nm^3, the highest value among six mammalian cytochrome *c* crystals (Margoliash and Schejter, 1966). Using this upper limit and taking $f_c = 1 - f_e = 0.85$ for the acidic preparations and $f_c = 1 - f_e = 0.45$ for the neutral preparations we obtain z_c of 2.0 nm and z_e of 4.5 nm. If the actual concentration C was 0.5 times the upper limit (c.f. the lowest among the 6 crystals = 1 mol/36 nm^3), z_c and z_e would be $2.0 \times (0.5)^{1/3} = 1.6$ nm and $4.5 \times (0.5)^{1/3} = 3.6$ nm, respectively. Uncertainty in z_c comes mainly from that in C; the above value of 2.0 nm is the upper limit. The value of z_e is sensitive to, in addition to C, the uncertainty in f_c; z_e must be $>1.5z_c$ (corresponding to the choice of $f_c = 0.95$ for the acidic and $f_c = 0.35$ for the neutral preparations), but its upper limit cannot be set.

For the case of acceptors on both sides of the membrane (the system in Fig. 5), the initial decay rate, $k_d(0)$,

estimated from two-exponential approximation was 0.260 ± 0.010 ns^{-1} for cytochrome *c*/bacteriorhodopsin molar ratios between 10 and 100. In this case both f_c and f_e in Eq. 1 are equal to 1. If we use the upper limit for C of $1/24$ nm^3 and neglect the term z_e^{-3} ($z_c^{-3} \gg z_e^{-3}$), we obtain $z_e = 2.0$ nm.

The distance of closest approach for the cytoplasmic surface, z_c, is thus at most 2.0 nm and is probably smaller ($C < 1/24$ nm^3). This distance refers to that between the center of the emission transition moment of the reduced chromophore (the center of the polyene chain part) and the center of the transition moment of cytochrome *c* absorption (the center of the heme moiety). Since the heme center is ~0.5 nm below the protein surface (Dickerson et al., 1971), the reduced chromophore in the membrane must be at a depth <1.5 nm from the cytoplasmic surface.

On several samples hemoglobin and ferritin were also tested as the acceptors on the adsorbed membrane sheets. With both proteins the fluorescence decay of the reduced membrane was faster in the acidic preparation than in the neutral preparation, supporting the results with cytochrome *c*.

Energy Transfer from Ruthenium Complex to Native Retinal

Location of the native chromophore can be examined by a reversal of the experiment in Fig. 3, i.e., by measuring the rate of energy transfer from donor molecules on the membrane surface to the native chromophore as the acceptor. In this reverse case, however, the thickness of the external donor layer must be controlled precisely since otherwise the donor molecules high above the membrane surface would contribute an unpredictable amount of fluorescence. Also the donors should not reside on those portions of the glass surface which are unoccupied by the membranes. These requirements are fulfilled by selecting a donor for which the polylysine-coated glass surface is repulsive and by depositing only a small amount of the donor so that all donor molecules are in contact with the membrane surface.

We chose as the donor tris(2,2′-bipyridyl)ruthenium(II) complex, which is positively charged as the polylysine. The complex has a long luminescence lifetime, in the absence of acceptors, of 410 ns in aqueous solution and 1,250 ns when sandwiched in between reduced purple membrane sheets (Kometani et al., 1987). The retinal chromophore in native purple membrane has been shown to serve as an acceptor: when the ruthenium complex was sandwiched in between native purple membrane sheets the decay of ruthenium emission was greatly accelerated. Analysis of the rate of the energy transfer has indicated that the retinal chromophore was situated at a depth of 1.0 ± 0.3 nm from one or the other surface of the membrane (Kometani et al., 1987).

Here we placed the ruthenium complex on top of the adsorbed membrane sheets as described in Methods. Excess ruthenium was washed away so that the exposed membrane surfaces would be covered by less than one molecular layer of the complex. Fig. 6 shows that the decay of ruthenium emission was faster in the acidic preparation than in the neutral preparation, suggesting that the ruthenium complex on the cytoplasmic surface was closer to the native retinal chromophore than the complex on the extracellular surface. The initial decay rate $k_d(0)$, based on the two-exponential approximation, was 0.065 ± 0.009 ns^{-1} in four acidic and 0.050 ± 0.006 ns^{-1} in four neutral preparations. The amount of ruthenium complex in these samples varied more than twofold. No correlation was found between the decay rate and the amount of ruthenium adsorbed, suggesting that the donors were distributed randomly on the membrane surface.

DISCUSSION

The analyses of excitation energy transfer in five different systems (Kouyama et al., 1983; Kometani et al., 1987) have shown that the retinal chromophore, either native or reduced, is located at a depth of 1.0 ± 0.3 nm from a surface of purple membrane. The present work affords a sixth piece of evidence: The distance between the heme moiety of cytochrome *c* and the reduced chromophore is at most 2.0 nm, setting an upper limit of 1.5 nm for the depth value. The results with the large acceptor, cytochrome *c*, also strengthen the previous contention that the small depth value is not due to the presence of a depression on the membrane surface. The chromophore location is outside the middle one-third of the transmembrane section.

The results on the oriented reduced membranes further show that the cytoplasmic surface is the surface proximal to the reduced chromophore: the chromophore is within 1.5 nm from the cytoplasmic surface. This conclusion depends to some extent on the assumptions that the spatial and orientational distributions of cytochrome *c* on the membrane were uniform. Below we discuss the consequence of this assumption. The arrangement of cytochrome *c* that would maximize the efficiency of energy transfer is the one in which the exposed heme edge of a cytochrome *c* molecule touches the membrane surface at a point immediately above the reduced chromophore. In this case the transfer rate is given approximately by $(R_0/z)^6$ where z is

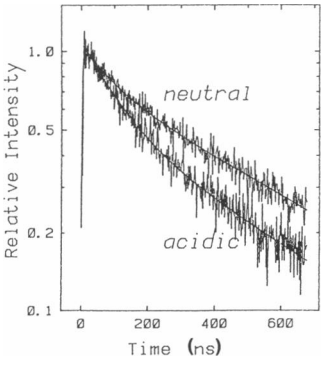

FIGURE 6 Fluorescence decays of tris(2,2′-bipyridyl)ruthenium(II) complex on the surface of native purple membrane sheets adsorbed on polylysine-coated glass at indicated pH. Zigzag lines, observed; smooth lines, the best-fit two-exponential approximations.

the distance between the chromophore and the closest heme. (Contribution from cytochrome *c* molecules other than the closest one is negligible since the size of the protein, $3.0 \times 3.4 \times 3.4$ nm^3 [Dickerson et al., 1971], is almost as large as R_0. The above expression in which the orientation factor is taken as unity is actually an overestimate by a factor of ~2, since the configuration that minimizes z is orientationally an unfavorable one). If, in membranes with their cytoplasmic surface exposed, every reduced chromophore had been associated with an acceptor cytochrome *c* in the above configuration, the observed transfer rates would predict $z = 2.9$ nm, or the depth from the cytoplasmic surface of 2.4 nm. This is the absolute maximum corresponding to an unrealistic situation and based on the overestimated orientation factor. Yet the value denies the chromophore position on the extracellular side. The reduced chromophore must be on the cytoplasmic side.

The results with the ruthenium complex suggest that the native chromophore is also on the cytoplasmic side. Here the evidence is not as solid as with the reduced chromophore, in view of the relatively large uncertainty in the decay rate. The previous studies (Kouyama et al., 1983; Kometani et al., 1987), however, have shown that both native and reduced chromophores are close to a membrane surface. If the native chromophore were on the extracellular side, a large conformational change of bacteriorhodopsin upon reduction would have to be postulated. Evidence to date disclaims this possibility (Stoeckenius et al., 1979; Kouyama et al., 1981*a*, 1981*b*; Tsetlin et al., 1983). We conclude that the native retinal chromophore is also on the cytoplasmic side.

Our conclusion is consistent with the one by Tsetlin et al. (1983) based on energy transfer experiments in systems different from ours. Nabiev et al. (1985), in contrast, have suggested, on the basis of surface-enhanced Raman spectroscopy, that the retinal Schiff base is located at a distance of 0.6–0.9 nm from the extracellular surface. We cannot explain the discrepancy. Finally we note that a location of the chromophore on the extracellular side seems inconsistent with current folding models of bacteriorhodopsin. In most models the chromophore binding site, Lys_{216}, is placed in the central part of an α-helical segment. On the carboxyl (at the same time cytoplasmic) side of the segment, beyond Leu_{224}, several charged residues including Arg_{225} are clustered (see, e.g., Stoeckenius and Bogomolni, 1982). Thus, if the chromophore were close to the extracellular surface, the charged residues would be inside the hydrophobic core of the membrane. This is energetically unfavorable.

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