The Transverse Location of the Retinal Chromophore in the Purple Membrane by Diffusion-enhanced Energy Transfer

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We have used fluorescence energy transfer in the rapid-diffusion limit (RDL) to estimate the trans-membrane depth of retinal in the purple membrane (PM). Chelates of Tb(III) are excellent energy donors for the retinal chromophore of PM, having a maximum Förster energy transfer of approximately 62 Å (assuming a donor quantum yield of 1). Energy transfer rates were measured from the time-resolved emission kinetics of the donor. The distance of closest approach between chelates and the chromophore was estimated by simulating RDL energy-transfer rate constants according to geometric models of either PM sheets or membrane vesicles. The apparent rate constant for RDL energy transfer between Tb(III)HED3A and retinal in PM sheets is \(1.5 \times 10^6 \text{ M}^{-1} \text{s}^{-1}\), corresponding to a depth of approximately 10 ± 2 Å for the retinal chromophore.

Cell envelope vesicles (CEVs) from Halobacterium halobium were studied by using RDL energy transfer to assess the proximity of retinal to either the extracellular or intracellular face of the PM. The estimated depth of retinal from the extravesicular face of the PM is 10 ± 3 Å, based on the RDL energy-transfer rate constant.

Energy-transfer levels to retinal in the PM were estimated by an indirect method with energy donors trapped in the inner-aqueous space of CEVs. The rate constants derived for this arrangement are too low to be consistent with the shortest depth of retinal deduced for PM sheets. Thus, the intravesicular face of CEVs, corresponding to the cytoplasmic face of cells, is the more distant surface from the chromophore of bacteriorhodopsin.

1. Introduction

Bacteriorhodopsin (BR)†‡ is a light-driven proton pump, found in the plasma membrane of Halobacterium halobium. It is possibly the simplest biological light-energy transducer yet discovered. Among the membrane-bound ion-pumps that have been isolated, BR is important both because its structure has been determined at near-atomic resolution (Henderson & Unwin, 1975; Henderson et al., 1986) and because it has played a special role in illustrating chemiosmotic synthesis of ATP (Racker & Stoeckenius, 1974). The availability of the complete sequence of BR (Ovchinnikov et al., 1979; Khorana et al., 1979) and of a 7 Å electron density map of its integral membrane structure (Henderson & Unwin, 1975) greatly enriches our understanding of this system. Additional knowledge about light-dependent isomerization of retinal (e.g. see Smith et al., 1985) and light-driven changes in protonation of the protein (Lozier et al., 1976) have led to detailed speculation on the mechanism of light-driven proton translocation (e.g. see Schulten et al., 1984). Comprehensive reviews covering the structure and
function of BR have appeared (Stoeckenius et al., 1979; Stoeckenius & Bogomolni, 1982).

Bacteriorhodopsin is isolated in the purple membrane (PM) fraction of the *H. halobium* cell envelope. Purple membrane is an ordered, two-dimensional array of bacteriorhodopsin (BR) molecules interspersed with phospholipids, apparently contiguous and coplanar with the inner cell membrane. BR is the sole protein constituent of purified PM and, as for rhodopsins of eukaryotes, one mole of retinal is bound per mole of BR in the membrane. A PM unit cell is composed of three identical BR polypeptides in centrosymmetric trimmer arrangement and approximately 30 phospholipids in bilayer structure. The purple color of PM is due to the visible chromophore of retinylidene—opsin, which absorbs maximally at 586 nm. This is far to the red of other protonated Schiff bases that are not bound to opsin-like proteins. The retinylidene chromophore bound to the protein by a Schiff base linkage at Lys216, serves as the locus of primary energy storage in BR and is very likely to provide the trigger for light-driven proton transport.

One class of molecular models describing the mechanism of proton translocation involves proton movement between weakly acidic groups in BR, possibly including carboxylates, tyrosinates and the Schiff base (Gerwert et al., 1989). However, no specific path has been established for the protons that are pumped through BR, partly owing to the absence of high-resolution structural information. To define better the relation of structure and function in this energy-transducing protein, the transmembrane position of the retinal chromophore and of amino acids essential for light-driven transport must be determined.

The trans-membrane location of retinal in the PM has been studied previously by several methods, resulting in an apparent discrepancy (King et al., 1979; Kouyama et al., 1981, 1983; Tsetlin et al., 1983; Nabiev et al., 1985; Hasselbacher & Dewey, 1986; Hasselbacher et al., 1986; Otomo et al., 1988). All measurements indicate that in the ground-state of BR, the retinal chromophore is inaccessible to direct contact with solvent. However, there are quantitative differences among measurements to determine how deeply buried the retinal chromophore may be. Some fluorescence energy-transfer results (Hasselbacher & Dewey, 1986; Hasselbacher et al., 1986) and a study by profile neutron diffraction (King et al., 1979) places the retinal chromophore near the center of the 45 Å thick membrane. Other measurements either by fluorescence energy transfer (Tsetlin et al., 1983; Kouyama et al., 1983; Komeyama et al., 1987; Otomo et al., 1988) or by surface-enhanced resonance Raman spectroscopy (Nabiev et al., 1985) are more consistent with the chromophore being near a surface of the PM, i.e. 10 Å or less from the aqueous phase. Part of the disagreement among these studies results from different definitions of the retinal chromophore and from the unique preparations utilized in some of the experiments (see below). Since it is rarely possible to study a biological system in its native state, the depths of retinal obtained using non-native preparations of PM were previously accepted.

In the present study we seek to resolve uncertainty about the trans-membrane location of retinal by measuring energy transfer in the rapid-diffusion limit (RDL) from aqueous chelates of Tb(III) to the native chromophore of the PM. Rapid-diffusion energy transfer was first established as a method for measuring the depth of chromophores using dyes and model membrane systems (Thomas et al., 1978) and was subsequently used to measure the depth of chromophores in both soluble proteins (Yeh & Mcarces, 1980) and membrane proteins (Thomas & Stryer, 1982). In the latter study, the distance of closest approach between Tb(III)DPA$^{3-}$ and the retinal chromophore of rod outer segment disc membranes was measured to be 28 Å from the extradiscal membrane surface and 22 Å from the intradiscal membrane surface. BR was studied previously by rapid diffusion energy transfer after covalent modification and photoconversion of retinal to create a fluorescent donor (Kouyama et al., 1983). These experiments argued strongly for proximity of the retinal-based fluorophore to a surface of the PM. However, since irreversible destruction of retinal’s visible chromophore occurred before the measurement, the depth of retinal in native BR remains an open question.

Rapid-diffusion energy transfer is applied here to measure energy transfer rate constants to the retinal chromophore in either PM sheets or cell-envelope vesicles (CEVs) from *H. halobium*. While PM sheets present both the cytoplasmic and periplasmic faces of BR to aqueous donors, the periplasmic face is primarily exposed to donors added outside CEVs (Lanyi, 1969). Thus, it should be possible to establish not only the depth of the chromophore, but also its disposition relative to either the periplasmic or cytoplasmic membrane surface. The specificity of energy transfer to the retinal chromophore is evaluated by reconstituting the chromophore of BR with all-trans retinal from the hydroxylamine-bleached apo protein, bacteriorhodopsin, in either the PM or CEVs. To complement measurements from outside CEVs, experiments are also performed for donors trapped inside CEVs, since these trapped donors are exposed primarily to the cytoplasmic face of the PM.

### 2. Materials and Methods

(a) Chemicals

Terbium chloride (TbCl$_3$·6H$_2$O, 99.9% purity relative to other lanthanides) was purchased from Alfa Products (Morton Thiokol, Inc., Danvers, MA). The chelators HED3A, EDTA and DPA were purchased from Aldrich Chemical Company [Milwaukee, WI], EGTA and Mops were purchased in the free and forms from Sigma Chemical Company (St Louis, MO). All-trans retinal (<95% other isomers) and hydroxylamine hydrochloride were purchased from Sigma. Ethyl and methyl alcohol were...
either spectral grade or HPLC grade and were purchased from either Fischer Scientific Company (Springfield, NJ) or from Mallinkrodt (St Louis, MO). Other chemicals were from local suppliers and were of the highest purity available.

Tb(III) chelates were prepared at approximately 50 mM-Tb. pH 7.0 (NaOH) from concentrated stock solutions of TbCl₃ and the designated chelator. The ratio of chelator to Tb was greater than or equal to 1:2 for all chelates except Tb(III)DPA⁻⁻, for which a chelator to metal ratio of 5:1 was maintained.

**All-trans** retinal was dissolved in methanol at the desired concentration assuming a formula weight of 284.42 g/mol. Solutions of retinal were handled at 4°C in brown glass vials under dim red light, and were prepared freshly as needed. Solutions were stored on ice and in complete darkness for 3 days at the most. **All-trans** retinal solutions were standardized by dilution into ethanol, using ε₃₂₅ nm = 43.4 mM⁻¹ cm⁻¹ (Mukohata & Sugiyama, 1982). NH₂OH·HCl solutions were prepared freshly before use at 4°C (pH 3.5), assuming a formula weight of 65.05 g/mol.

All buffered solutions included 20 mM-Mops, pH 7.0 (NaOH), except as noted. Solutions containing NaCl were prepared from stock solutions of saturated NaCl, which were standardized by their density. Tb chelate solutions were always prepared to be isoionic with the suspensions of PM sheets or CEVs with which they were mixed.

(b) **Purple membrane and cell-envelope vesicle preparations**

*H. halobium* cells from all strains were grown according to Oesterhelt & Stoeckenius (1975). PM was isolated as described by Oesterhelt & Stoeckenius (1974) and was either used immediately or frozen in liquid nitrogen until use. For energy transfer studies, PM was thawed on ice and washed into 20 mM-Mops, pH 7.0. CEVs were prepared according to published procedures (Groma et al., 1984) and suspended in 4 mM-NaCl/Mops buffer (4 mM-NaCl, 20 mM-Mops, pH 7.0) for energy transfer measurements. CEVs, like *H. halobium* cells, display hypersensitivity to reduction of the external salt concentration (Stoeckenius & Rowen, 1976). They were therefore suspended in solutions of ionic strength 3 mM or greater at all times.

**H. halobium** strains JW1 (hop⁺, ret⁺, rub⁺) JW3 (hop⁺, ret⁺, rub⁻), and JW5 (hop⁺, ret⁻, rub⁻) were kindly provided by H. Weber (Weber & Bogomolni, 1981). Strain JW3 is an overproducer of BR and is also deficient in lycopene (Weber & Bogomolni, 1981). H. halo-...
tured sample and a bleached sample of identical opsin concentration.

(iii) Orientation of CEVs

The orientation of membranes in CEVs was checked by measuring the NADH dependent menadione reductase activity before and after disruption of CEV membranes. The NADH-menadione reductase activity of H. halobium cells is localized on the inner surface of the cytoplasmic membrane and serves as a simple marker for the net orientation and leakiness of either cells or CEVs (Lanyi, 1969). Thus, the difference between marker enzyme activity before and after vesicle solubilization is proportional to the fraction of outside-out vesicles (Lanyi, 1969; Groma et al., 1984). Each preparation studied by energy transfer had 90%, or more of the menadione reductase activity accessible only after detergent treatment of CEVs (data not shown). This orientation corresponds to the orientation of the enzyme in intact cells.

(iv) The diameter-distribution of CEVs

Electron microscopy was performed to determine the distribution of diameters of the CEV population. Negative staining was carried out with uranyl acetate essentially as described by Stoeckenius & Rowen (1967). CEVs in 4 M-NaCl were cross-linked with glutaraldehyde, quenched by addition of glycine (in 4 M-NaCl) to 0.1 M, diluted to a final NaCl concentration of 0.1 M and finally diluted into a solution of 0.1% uranyl acetate. After incubation in the heavy-metal stain for 1 to 10 min, CEVs were sedimented in an Eppendorf microcentrifuge (5 min, full speed). Samples of 10 μl were dropped onto a glow-discharged, Formvar-coated copper grid (200 mesh; Polysciences, Inc., St Louis) and imaged at 60 kV on a Philips 201C electron microscope. The average vesicle size was 740 nm, as measured from negative images of the CEVs. A rough assessment of the size distribution is shown in the inset to Figure 10 (below).

(d) Fluorescence energy transfer

(i) Theoretical

Models used to calculate RDL energy transfer rate constants are shown in Fig. 1. The model for PM sheets (Fig. 1a) is identical to that of Kouyama et al. (1983). It emphasizes the thickness of the sheet (T), the depth of the chromophore from either aqueous face (d1 or d2) and the relative orientations of donor and acceptor transition moments. The ensemble-average energy transfer rate (kRDL) is obtained by averaging the Förster transfer rate over all possible donor positions and orientations (Thomas et al., 1978; Thomas & Stryer, 1982). For PM sheets, the result is:

$$k_{RDL} = \frac{\pi \rho_A R_0^6}{6\nu_0} \left[ \frac{1}{a_{\text{PM}}} + \frac{1}{(T - a_{\text{PM}})} \right]$$

where ρA is the concentration of acceptors (chromophores) in the membrane (in molecules/cm²) and R0 is the characteristic distance, defined by Förster, at which the efficiency of energy transfer is 50%. (Stryer, 1978). The orientation factor, K2, is included in the estimation of R0. If θ is defined as the angle of the acceptor transition moment to the membrane normal and it is assumed that Tb chelates rotate rapidly and isotropically in solution, then the ensemble-average value of K2 (derived by Kouyama et al., 1983) is (1 + cos²θ)/2 or 0.56 ± 0.02 for PM sheets. This analysis assumes that retinal lies at 70° ± 2° in the membrane normal (Heyn et al., 1977; Kimura et al., 1984). To solve for aPM, the value of T is first taken to be 45 Å (Henderson, 1975). For values of aPM less than about T/3, the transfer rate is essentially independent of T. However, small values of T and aPM > T/3 will combine to increase the calculated energy-transfer rate constant from donors at the more distant membrane face. Under these assumptions, the value of T must be carefully chosen in
the membrane sheet model if we seek to measure $a_{PM}$ (see Fig. 5, below).

For donors outside a spherical shell containing acceptors (Fig. 1(b)), the RDL energy transfer rate is given by (Thomas & Stryer, 1982):

$$k_{T}^{RDL} = \frac{4\pi a_{OUT} R_{B}^{3}}{3a_{IN}^{3}} \left[ 2 - \left( \frac{a_{OUT}}{a_{OUT} + b} \right)^{3} \right]^{-1},$$

(2)

where $a_{OUT}$ is the distance of closest approach between donors and acceptors and $b$ is the distance from the center of a vesicle to the chromophores in the wall of the vesicle. Energy transfer from outside vesicles is quite insensitive to $b$ for $b \gg a_{OUT}$, as is the case for CEVs. The ensemble-average value of $K^{2}$ for donors outside CEVs is assumed to be the same as it is from one side of a PM sheet.

For donors in the inner-aqueous space of a spherical membrane vesicle, the RDL transfer rate is given by Thomas & Stryer (1982) (with all common variables defined as above):

$$k_{T}^{RDL} = \frac{3n a_{IN} R_{B}^{3}}{2a_{IN}^{3}(b - a_{IN})^{3}} \left[ \frac{2}{b} \left( \frac{1}{a_{IN}} - \frac{(b - a_{IN})^{2}}{2a_{IN}} \right) \right]$$

(3)

The surface density ($\phi_{A}$) rather than the concentration of acceptors ($\rho_{A}$) determines the transfer rate for this geometry, and $a_{IN}$ is the distance of closest approach between donors in vesicles and an acceptor buried in the wall of the vesicle. Also, it should be noted that the vesicle radius ($b$) is an important determinant of the transfer rate with donors inside vesicles. For this case, the value of $K^{2}$ is assumed to be 2/3 with an error of less than 10% in the value of $a_{IN}$ (Thomas & Stryer, 1982).

(ii) Experimental

(1) Luminescence decay kinetics

Fluorescence energy transfer rates were measured by recording the decay of Tb luminescence after excitation by a pulse of blue light (488 ± 1 nm) from a dye laser. The instrument used was essentially the same as the one described by Ludescher & Thomas (1988), except for modifications necessary to detect Tb luminescence. The 546 nm emission line of Tb was selected using a 1 cm interference filters (546 ± 10 nm; 546 ± 25 nm; Optical Thin Films, North Conway, NH) to absorb scattered light. Individual emitted photons were resolved on the microsecond time-scale using a gated photomultiplier tube (model R928P; Hamamatsu Corp.). The tube was maintained at 1000 V except for approximately 100 ns after each laser pulse, when the tube was gated to 200 V. The gating circuit was designed and constructed by Mr. R. Bennett (unpublished). After discrimination and amplification, impulses were counted and dumped to a microcomputer using a multi-channel scaler (model 3221 MCS; LeCroy Instruments, NY).

Luminescence decay data were analyzed by a nonlinear least-squares procedure as described (Eads et al., 1984), to obtain a fit to either 1 or 2 exponential components with a constant background. Deconvolution of the excitation pulse was not necessary due to its short duration (about 20 ns) compared with the lifetimes measured (Thomas & Stryer, 1982). A weighted difference between the data and the best-fit curve was calculated according to Grinvald & Steinberg (1974).

Initial rates of donor luminescence decay were estimated in 2 steps: first, the entire decay profile was analyzed into 2 exponential components plus a constant background, as described above; then the analytical derivative of the best-fit curve was calculated at zero time. For a decay that can be represented by sum of two exponentials, the initial decay rate $F(0)$ is given by:

$$F(0) = A_{1}/\tau_{1} + A_{2}/\tau_{2},$$

where $A_{1}$ and $A_{2}$ represent the normalized amplitudes, and $\tau_{1}$ and $\tau_{2}$ are the lifetimes of the optimal biexponential fit curve.

(2) Trapping chelates inside CEVs

In order to load CEVs with Tb chelates, the osmotic shock method of Lanyi & MacDonald (1979) was used. A vesicle suspension (150 µl) in 4 M-NaCl/Mops buffer (4 M-NaCl, 20 mM-Mops, pH 7.0) was osmotically shocked by rapid dilution (10 to 20 ml final volume) into ice-cold shocking buffer (3 M-NaCl, 20 mM-Mops, 1 mM-Tb(III)X, pH 7.0, where X is one of the chelators studied). CEVs were allowed to equilibrate and reseal on ice for up to 12 h. Loaded vesicles were recovered by centrifugation (15 min, 15,000 revs/min, Sorvall SS-34 rotor) and untrapped chelate was removed by washing in 3 M-NaCl/Mops buffer (3 M-NaCl, 20 mM-Mops, pH 7.0).

A variety of evidence indicates that Tb(III) chelates are trapped inside CEVs by this procedure. Primary evidence for trapping is the observation of leakage. After washing, ressealed CEVs in 3 M-NaCl/Mops buffer 3 times, luminescence decay kinetics are invariant upon further dilution or washing. The donors that remain associated with CEVs exhibit short lifetimes, consistent with energy transfer. However, unlike tightly "bound" ligands, donors gradually recover a characteristic, unquenched decay profile with time after trapping. When vesicles are rewarshed after "leakage" has occurred, the short lifetimes indicative of energy transfer are again observed. This procedure may be repeated until donors are depleted.

For studies with Tb(III)DPA$_{3}^{3-}$ and bleached CEVs, the inaccessibility of Tb to EGTA provides an additional criterion for trapping. When DPA ligates Tb$^{3+}$, Tb luminescence is strongly enhanced under near-ultraviolet (u.v.) excitation (e.g. 308 nm). EGTA, however, can be added to solutions of Tb(III)DPA$_{3}^{3-}$ in such a way that DPA ligands are displaced and the intensity enhancement is abolished. Preliminary experiments using u.v. excitation with Tb(III)DPA$_{3}^{3-}$ to detect the intensity enhancement showed that 3 washes of a loaded CEV preparation in 3 M-NaCl/Mops buffer are sufficient to remove the ECTA-accessible Tb population.

A further criterion for trapping is the effect of vesicle disruption on Tb chelate luminescence kinetics. Energy transfer is measured before and after disruption of loaded vesicles in each trapping experiment. CEVs may be disrupted by any of several methods yielding the same result: loss of energy transfer. Methods of disruption include detergent solubilization (Lanyi, 1971), severe osmotic shock treatment (Stoeckenius & Rowen, 1967) and freeze–thaw treatment. Energy transfer is effectively abolished by each of these procedures, as judged by: (1) a change from multi-exponential to monoexponential decay kinetics; and (2) recovery of the unquenched lifetime of the donor (data not shown). A small amount of energy transfer still occurs after disruption, and is proportional to the concentration of BR.
3. Results

(a) Rₜ, for Förster energy transfer

The overlap of BR absorbance with Tb chelate emission was used to calculate an overlap integral of 4·3·(±0·1)·10⁻¹⁴ cm² m⁻¹, according to equation (2) of Thomas et al. (1978). Using values of ϑ = 70±3°, as described above, estimating the value of the refractive index as 1·4±0·07, and assuming that Q, the donor quantum yield, equals unity, we obtain Rₜ = 61·7±1·8 Å. The value of Rₜ for each chelate is evaluated according to:

\[ Rₜ = R₀(Q^{1/6}) \Lambda, \]  

(4)

where the apparent quantum yield, Q, is defined as \( \tau_D / \tau_R \); \( \tau_D \) is the measured donor lifetime in the absence of acceptors, and \( \tau_R \), the radiative lifetime of the donor, is 4·76 ms (Stein & Wurzberg, 1975).

For the donors used below with BR as an acceptor: Rₜ = 47±2 Å (Tb(III)HED3A); 49±2 Å (Tb(III)EDTA⁻) and 53±2 Å (Tb(III)DPAPA⁻). The Rₜ values for Tb donors and reconstituted PM are assumed to be identical to those for native PM, based on the apparent identity of their visible absorption spectra (Rehorek & Heyn, 1979).

(b) Attainment of the RDL

The RDL of fluorescence energy transfer will commonly be attained with long-lived Tb donors in solution (Thomas et al., 1978). For quantitative evaluation of attainment of the RDL, two criteria have been proposed (Thomas et al., 1978; Kinosita et al., 1987). Both are examined below.

A general criterion for the RDL, which applies to all donor–acceptor systems, is:

\[ 6DΔr > s², \]  

(5)

where D is the sum of donor and acceptor diffusion coefficients, \( Δr \) is the donor lifetime in the absence of acceptors and \( s² \) is the mean square distance between acceptors (Thomas et al., 1978). On the basis of inequality (5), the RDL should be attained for homogeneous suspensions of donors and acceptors if \( Δr \) is one millisecond or longer and if the acceptor concentration is 1 µM or higher (Meares & Rice, 1981). These criteria are fulfilled for all experiments below with PM sheets and with donors outside CEVs, assuming that the acceptor distributions are homogeneous. Trapped donors are discussed below.

Alternative criteria for the RDL apply to specific geometric arrangements of donors and acceptors (Kouyama et al., 1983; Kinosita et al., 1987). For an infinite membrane sheet with a buried acceptor at a depth, \( a_{PM} \), from the surface and at a fixed angle, \( ϑ \), with respect to the membrane normal (Fig. 1(a)), the RDL should be attained if:

\[ D\tau_D \left( a_{PM} / R_{BR} \right) > 0·0490 + 0·0729 \cos² \theta. \]  

(6)

For Tb(III) and retinal in the PM, inequality (6) is always satisfied by a factor of 100 or more, assuming that \( D > 5 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}, \) \( τ > 1 \text{ ms}, \) \( a_{PM} \geq 10 \text{ Å}, \) \( R_{BR} \leq 55 \text{ Å} \) and \( ϑ > 67°. \) If the RDL is attained, then donors displaying single-exponential luminescence in the absence of acceptors will also display monoexponential emission kinetics in the presence of acceptors.

When donors are localized outside a spherical vesicle of radius \( b, \) with acceptors buried in the vesicle wall so that a distance of closest approach, \( a_{out} \), exists between donors and acceptors, the RDL is predicted to occur if inequality (7) is satisfied (Kinosita et al., 1987):

\[ \frac{D\tau_D}{a_{out}} \geq \left[ \frac{1}{\delta} \left( \frac{δ}{(δ+1)} \right)^{1/2} \right] \left( \frac{2(3\delta^2-1)}{(δ+1)^3} \right) \]  

(7)

\( δ, \) defined as the ratio \( (b-a_{out})/b, \) is nearly unity for CEVs because the vesicle radius is much greater than the membrane thickness for all vesicles. The maximum value of the right-hand side of inequality (7) is thus 0·333 for CEVs, while the value of the left-hand side always exceeds 20 for BR and Tb(III) donors, assuming that \( D > 5 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}, \) \( τ > 1 \text{ ms}, \) \( a_{out} \geq 10 \text{ Å}, \) \( R_{BR} \leq 55 \text{ Å} \) and \( b \leq 1000 \text{ nm} \). Inequality (7) should always be satisfied by a factor of 90 or more and the RDL should be attained for Tb donors outside CEVs by this criterion.

For energy donors trapped inside hollow vesicles with acceptors in the wall at a depth, \( a_{IN} \), from the inner aqueous space, satisfaction of the inequality:

\[ \frac{D\tau_D \left( a_{IN}/b \right)}{a_{IN}} \geq \frac{2π\sigma_{2H}a_{IN}b}{9\delta s^4} \left( \frac{3\delta^2-1}{(δ+1)^3} \right) \]  

(8)

should lead to the RDL (Kinosita et al., 1987). \( D, τ, a_{IN}, R_{BR} \) and \( δ \) in inequality (8) are defined as above, but \( Δr \) has been redefined as \( δ = (b+a_{out})/b \) in (8) (Kinosita et al., 1987). By this criterion, we expect that the RDL will be attained for Tb(III) donors trapped in many but not all H. halobium CEVs, with \( R_{CO} \) and \( τ \) chosen as above and \( a_{2H} = 6·97 \text{ mol cm}^{-2}. \) For large CEVs in our population (≥1000 nm), with high \( R_{CO} \) values (≥50 Å), and \( a_{IN} \) unknown over the range 10 to 35 Å, the RDL may not be attained. In this case, the ensemble-average energy transfer rate of trapped donors can be evaluated only from the initial rate of donor luminescence decay (Kinosita et al., 1987). Since donor luminescence may not be exponential when the RDL is not attained, ensemble-average rates for trapped donors are evaluated from the initial rates of luminescence decay as described above.

(c) Energy transfer between Tb(III) chelates and PM sheets

(i) Strategy and principles

The rate constant for RDL energy transfer from Tb(III)HED3A to BR in PM sheets is determined from the variation in the lifetime (τ) of Tb(III)
luminescence as the concentration of BR is varied. The concentration of BR is varied either by adding PM to an aqueous donor solution to the desired concentration or by adding sub-stoichiometric amounts of all-trans retinal to a suspension of NH₄OH-bleached PM. The rate of donor-luminescence decay, \( k = 1/\tau \), depends on both the intrinsic donor decay rate, \( k_o \), and on the apparent second-order energy-transfer rate constant, \( k_{2,\text{RDL}} \) (Wensel et al., 1986):

\[
k = k_o + k_{2,\text{RDL}} [\text{BR}] \text{ s}^{-1}.
\]

\( k_o \) may be defined generally as the background decay rate of the donor, including all processes that contribute to de-excitation of the donor except energy transfer to BR. \( k_o \) is simply the reciprocal of the donor lifetime in the absence of acceptors (\( \tau_o \)) if no background energy transfer occurs, but may be the rate of donor decay with apomembranes present if non-retinyl energy transfer occurs; \( k_{2,\text{RDL}} \) is generally determined as the slope of a plot of \( k \) versus the concentration of BR.

(ii) The transfer rate constant for Tb(III)HED3A

When Tb(III)HED3A is mixed with a suspension of native PM sheets at \([\text{BR}] = 0.1 \text{ mM}\), \( k_{\text{Tb(III)HED3A}} \) increases by approximately 15%. Time-resolved emission kinetics of Tb(III)HED3A either with or without PM are shown in Figure 2 (points) along with single-exponential calculated curves (continu-

\[\dagger\] Note that \( k_{2,\text{RDL}} \) is actually a second-order rate constant, since the full rate equation for the decay of \( \text{Tb}^* \) shows terms proportional to both \([\text{Tb}^*]\) and \([\text{BR}]\). While the light intensity measured is proportional to the excited-state concentration, \([\text{Tb}^*]\), the measured rate constant is apparently first-order in BR, and is not a function of \([\text{BR}]\).

\[\dagger\]

Figure 2. Luminescence emission kinetics of Tb(III)HED3A with or without PM sheets. Tb(III)HED3A emission intensity was counted in 5-µs intervals after each flash of 488 nm light until the emission reached background levels (≈5 ms) (points: data; continuous lines: best-fit curves). Upper trace, Tb(III)HED3A in the absence of membranes; middle, Tb(III)HED3A in the presence of 0.1 mM-BR; lower, Tb(III)HED3A in the presence of 0.025 mM-BR (raw data have been omitted for clarity; see residual in inset). Inset: weighted residuals for decay data and fit curves in a, b and c (top to bottom) were calculated according to Grinvald & Steinberg (1974). The time-scale for the residuals is the same as that for raw decays. The vertical scale for the residuals ranges from −5 to 5 for each residual plotted, and tick marks thus represent 5 residual units each.
Figure 3. (a) Energy transfer rates between Tb(III)HED3A and retinal in PM sheets. Six independent experiments are summarized in this plot, yielding the apparent energy-transfer rate from neutral Tb(III)HED3A to PM sheets (open squares). The linear least-squares fit of these data to a line is shown, indicating that the PM exhibits approximately first-order quenching kinetics, as expected for energy transfer in the rapid-diffusion limit. The slope of the line is the apparent second-order rate constant for energy transfer ($k_2 = 1.9(\pm 0.1) \times 10^6$), and the intercept corresponds to $k_0 = 1040$ s$^{-1}$. These data reflect the total energy-transfer rate between Tb(III)HED3A and purple membranes, including both retinal-specific and possible non-specific terms. (b) Addition of all-trans retinal to reconstitute NH$_2$OH-bleached PM. Identical, 45 µl samples of concentrated, bleached PM (≈ 0.1 mM-opsin) were transferred to separate microcuvettes and 5 µl of either Tb(III)HED3A or 20 mM Mops was added to the cell. The Tb and Mops samples were placed in the sample and reference positions of the spectrophotometer, respectively. After recording the instrument baseline from 750 to 300 nm, the Tb sample was removed and its emission lifetime ($\tau_T$) was measured. Step-wise reconstitution of the Tb sample was then carried out by adding sub-microliter samples of all-trans retinal under dim red light. Reconstitution was allowed to proceed in the dark until no further change in absorbance at 531 nm could be detected. The emission lifetime of Tb(III)HED3A was measured after retinal-binding to opsin had reached equilibrium. The concentration of BR was determined from the difference in optical density at 568 nm between reconstituted PM and a bleached membrane preparation at the same opsin concentration, measured after light-adaption of PM in the laser beam. The data were fit to a line by a linear least-squares procedure, yielding an energy transfer rate constant of $1.47(\pm 0.06) \times 10^6$ M$^{-1}$ s$^{-1}$ and $k_0 = 1210(\pm 90)$ s$^{-1}$. 
A plot of the modified residual for each fit is included in the inset to the Figure. The modified residual for each dataset is flat, with approximately equal weight distributed above and below zero. Thus, a single-exponential rate law adequately describes each of the luminescence decays. Single-exponential decays observed in the presence of PM are consistent with attainment of the RDL.

As the concentration of BR is varied between 0.4 mM and 20 μM by dilution of PM sheets, $k_{\text{TRb(III)HED3A}}$ varies essentially linearly with the BR concentration. The luminescence decay rates of Tb(III)HED3A (Fig. 3(a)) are fit to a straight line by a least-squares procedure, yielding $k_{2}\text{RDL} = 1.87(\pm 0.10) \times 10^6 \text{ M}^{-1} \text{s}^{-1}$, according to equation (9). The magnitude of this value is consistent with dipole-dipole energy transfer between Tb(III) and retinal in PM, but is nearly an order of magnitude less than would be expected for either collisional quenching or electron-exchange energy transfer from Tb(III) (Lakowicz, 1983; Stryer et al., 1982). The distance of closest approach between Tb and retinal would be approximately 13 ± 1 Å, based on this rate constant (see Fig. 5, below).

The apparent rapid-diffusion energy transfer rate to retinal in PM is insensitive to Tb(III)HED3A concentration over the range tested (20 μM to 50 mM), and the apparent distance of closest approach does not change significantly (Fig. 4). At each Tb concentration tested, the observed transfer rate is consistent with a distance of closest approach between Tb(III)HED3A and retinal of 13 ± 2 Å. Ionic strength (from 1 mM to 2 M) is also without significant effect on the distance of closest approach between this neutral chelate and the chromophore of BR (data not shown). For evaluation of either the salt or the Tb concentration dependence of energy transfer, simple mixtures of PM, Tb(III)HED3A and NaCl in Mops buffer have been employed.

In order to check the specificity of energy transfer from Tb(III)HED3A to the retinal chromophore in BR, energy transfer was measured as bleached PM was reconstituted with all-trans retinal (Fig. 3(b)). All-trans retinal binds specifically and quantitatively to bacterioopsin in bleached membranes, reforming the protonated Schiff base and red-shifted retinal chromophore of BR (Rehorek & Heyn, 1979). Retinal in methanol was added sub-stoichiometrically as described in Materials and Methods, maintaining the concentration of membranes essentially constant. Methanol alone had no effect on the lifetime of Tb(III)HED3A at the concentrations used. Luminescence decay rates derived from a stepwise reconstitution of bleached membranes (Fig. 3(b)) illustrate the linear relation between emission decay rate and chromophore concentration under these conditions. Since the transfer rate is directly proportional to the chromophore concentration, equation (9) is used to estimate $k_{2}\text{RDL}$, yielding a retinal-specific energy-transfer rate constant. From three independent reconstitution experiments, the average value of $k_{2}\text{RDL}$ is

![Figure 4](image_url)

Figure 4. Rapid-diffusion limit energy transfer from Tb(III)HED3A to PM sheets as the concentration of Tb(III)-HED3A is varied. The apparent distance of closest approach between Tb(III)HED3A and the retinal chromophore of BR was estimated, varying the Tb(III)HED3A concentration over nearly 3 orders of magnitude. Energy transfer was measured as the difference between luminescence decay rates for the donor in the presence and in the absence of PM at a BR concentration of 0.056 mM. The distance of closest approach was determined independently for each Tb concentration by calculating the apparent energy-transfer rate constant, $k_{2}\text{RDL}$, as the ratio: $(k_{2}-k_{0})/[\text{BR}]$ (i.e. from a single point). Energy-transfer rate constants were matched to the most-probable energy-transfer simulation shown in Fig. 5. The horizontal line drawn at 13.4 Å represents the average value of $\alpha_{RDL}$ determined from these data.
1.47(±0.00) × 10^6 s^{-1}, corresponding to a
distance of closest approach of 14±1 Å between
Tb(III)HED3A and retinal. Attainment of the RDL
is confirmed at each step of the reconstitution,
on the basis of monoexponential luminescence kinetics
of the donors.

The distance of closest approach (a_{pm}) between
Tb(III) donors and the retinal chromophore of PM
is estimated above by comparing the measured
value of k_{2,RDL} with values of k_{2,RDL} calculated using
equation (1) (see Fig. 5). The most probable value of
a_{pm} is derived from the central curve, representing
the most probable parameters of the sheet model
discussed above. The range of error is estimated
using "extreme value" simulations, shown in Figure
5 on either side of the most-probable curve. Uncertainty
in a_{pm} is due to both: (1) random error in the
experimental result (vertical error bound); and
(2) imperfect estimation of parameters in the
membrane-sheet model (represented by the outer
simulated curves). Since calculated rate constants
for all the curves shown increase steeply for a_{pm}
≤10 Å, a strong inference from these data is that
no contact occurs between Tb donors in solution
and the retinal chromophore of BR.

Using a_{pm} = 14±1 Å, as shown in Figure 5, the
depth of retinal in PM is estimated to be 10±2 Å
by subtracting the van der Waals' radius of
Tb(III)HED3A (4 Å; Wensel et al., 1987) from a_{pm}.
We assume a maximum uncertainty of 1 Å in the
chelate radius.

The effect of membrane thickness on the RDL
estimate of a_{pm} was estimated using the simulation
shown in the inset to Figure 5. While fixing k_{2,RDL}
at the measured value, T is varied and the distance of
closest approach to retinal is evaluated. It is evident
from the lower line of the plot that as thickness is
decreased to 29 Å in the model, the distance of
closest approach (i.e. from the nearest face of PM)
increases by only 1 Å. At the same time, the

Figure 5. Simulation of rapid-diffusion limit energy transfer between aqueous Tb(III)HED3A and retinal in PM
sheets. Most-probable or extreme-value energy-transfer simulations are based on eqn (1), above. The most probable
value of the distance of closest approach is 15.8 Å, shown as the intersection of the mean energy-transfer rate constant
with the most-probable simulation (central curve). The box shown represents estimated uncertainty in the distance of
closest approach between Tb(III)HED3A donors and retinal, based on both model-dependent errors (upper and lower
curves) and experimental uncertainty (vertical dimension of box). The left side of the box touching the minimum energy-
transfer curve represents a lower limit on the distance of closest approach (12.9 Å), while the right side of the box,
touching the maximum energy-transfer curve represents an upper limit (19.7 Å) on a_{pm}. Inset, the effect of membrane
thickness on the estimated distance of closest approach. Using eqn (1) to calculate energy-transfer rates, the membrane
thickness was varied while requiring that the experimental value of k_{2,RDL} match the result. The distance of closest
approach between Tb(III)HED3A and retinal was varied to obtain this result, as shown. The depth of retinal was
obtained by subtracting the radius of Tb(III)HED3A (4 Å) from the best value of a_{pm} obtained. Thus all values of a_{pm}
shown in the Figure (lower curve) are consistent with k_{2,RDL} = 1.5(±0.1) × 10^6 s^{-1}, assuming the membrane thickness
shown. The upper curve (− + − + −) represents the estimated depth of retinal from the opposite face of the PM.
apparent distance from the opposite face would vary from 35 Å to 18 Å. For a 45 Å membrane, more than 95% of the reported energy transfer rate constant to PM sheets occurs from the closest face, ensuring that the total energy transfer is insensitive to membrane thickness.

(iii) Energy transfer rates and chromophore depth at high ionic strength and with charged donors

The effective depth of the retinal chromophore in PM sheets has been further assessed by measuring energy transfer from charged Tb(III) chelates to BR in high ionic strength suspensions of PM. At 2 to 4 M NaCl, the repulsion of negatively charged donors by the membrane potential should be largely screened by mobile ions, and the transfer rate should reflect the distance of closest approach between donors and retinal. In 4 M NaCl, the rate of energy transfer from uncharged Tb(III)HED3A to retinal in PM ($k_{RDL} = 2.2 \times 10^6$) is essentially the same as the value at low ionic strength ($1.87 \times 10^6$), corresponding to $a_{PM} \approx 12$ Å between Tb(III)HED3A and retinal. The energy transfer rates between either Tb(III)EDTA$^-$ or Tb(III)DPA$_3^{3-}$ and the chromophore of BR are comparable to but slightly lower than $k_{RDL}^{RDL}$ for Tb(III)HED3A at high ionic strength. Although the rate constants between negatively charged donors and PM sheets depend strongly on ionic strength (R. Leder & D. D. Thomas, unpublished results), measurements at high ionic strength ($\geq 2$ m-NaCl) are consistent with the depth of retinal derived above. For the uni-negative donor, Tb(III)EDTA$^-$, $k_{RDL}^{RDL} = 1.3 \times 10^6$ and $a_{PM} = 14 \pm 2$ Å (based on simple dilution of PM), while for Tb(III)DPA$_3^{3-}$, the estimated distance of closest approach is 17 Å, based on a transfer rate constant of $0.83 \times 10^6$ (data not shown). By subtracting the estimated radius of either chelate (4 Å for Tb(III)EDTA$^-$; 6.4 Å for Tb(III)DPA$_3^{3-}$; Donato & Martin, 1975), a depth of approximately 10 Å for retinal is derived using either donor. These results quantitatively confirm the conclusion that retinal lies near a surface of PM, supporting the evidence cited above using a neutral chelate. A detailed report of the salt dependence of energy transfer will be published elsewhere.

(d) Energy transfer with donors external to CEVs

(i) The transfer rate constant for Tb(III)HED3A

Experiments with PM sheets, as described above, allow Tb chelates to approach both aqueous surfaces of the PM. However, when Tb(III)HED3A

![Figure 6](image_url)

Figure 6. Emission kinetics of Tb(III)HED3A outside cell-envelope vesicles. Addition of purple-membrane vesicles to a solution of Tb(III)HED3A increases the rate of Tb luminescence decay due to energy transfer. Upper trace, Tb(III)HED3A; middle trace, Tb(III)HED3A with ~91 mM-BR; lower trace, Tb(III)HED3A in the presence of ~92 mM-BR. Decay kinetics are very nearly single-exponential, with reduced $\chi^2$ values equal to 0.95, 1.15 and 1.13 for the upper, middle and lower traces, respectively (continuous lines, raw data; smooth lines, the least-squares calculated curves). Inset: modified residuals for upper, middle and lower traces, calculated as in Fig. 5, are shown to be nearly linear and nearly randomly distributed about zero.
is added externally to CEVs, the extravesicular (periplasmic) face of the PM is selectively exposed. As predicted for the RDL, Tb(III)HED3A luminescence decays homogeneously in the presence of CEVs (Fig. 6). At a concentration of 0.1 mM-BR (20 mg CEV protein/ml), the luminescence decay rate of Tb(III)HED3A increases by 11% from its value in 4 M-NaCl/Mops buffer. This increase is nearly the same as that observed with PM sheets at 0.1 mM-BR, either at low ionic strength (15%), or at 2 M-NaCl (18%). As ρBR is varied by isoionic dilution of CEVs, the luminescence decay rate decreases nearly linearly (data not shown), yielding the energy transfer rate constant, \( k_{2}^{RDL} \), \( = 1.54(\pm 0.21) \times 10^6 \text{ M}^{-1} \text{s}^{-1} \) (the average of six independent measurements). This rate constant corresponds to a closest-approach distance of 14 ± 2 Å between Tb(III)HED3A and retinal from the extravesicular PM face (see Fig. 8, below) or a depth of 10 ± 3 Å for retinal.

The specificity of energy transfer from Tb(III)-HED3A to the retinal chromophore of BR in CEVs was estimated by reconstituting NH2OH-bleached vesicles with all-trans retinal. As in studies of PM sheets, the luminescence decay rate of Tb(III)-HED3A was measured as a function of ρBR (Fig. 7). As step-wise reconstitution proceeds, the luminescence decay rate (and hence the energy transfer rate) varies directly with ρBR (ΔΛ285), as expected for a simple RDL system. The average value of \( k_{2}^{RDL} \) for Tb(III)HED3A outside reconstituted CEVs is \( 1.9(\pm 0.3) \times 10^6 \text{ M}^{-1} \text{s}^{-1} \), as determined from three independent experiments. Calculated values of \( k_{2}^{RDL} \) based on equation (9), are compared in Figure 8 with the measured value to obtain \( a_{OUT} = 13 \pm 2 \text{ Å} \), the apparent distance of closest approach of Tb(III)HED3A to retinal from the periplasmic face of PM.

(ii) Uncertainty in the depth of retinal

The argument applied to estimate the uncertainty in \( a_{PM} \) also applies to errors in \( a_{OUT} \). The calculated values of \( k_{2}^{RDL} \) in Figure 8 represent the most probable (central curve), the minimum (lower curve), or the maximum (upper curve) energy transfer for a given distance of closest approach, consistent with the assumptions of the vesicle model. The most probable value of \( a_{OUT} \) is determined by matching the mean measured value of \( k_{2}^{RDL} \) with the simulated curve from the most-probable parameter set. For each extreme-value curve, all parameter values are simultaneously chosen so as to either minimize or maximize \( k_{2}^{RDL} \). For example, to maximize \( k_{2}^{RDL} \) at all values of \( a_{OUT} \), the refractive index is assumed to be 1.333, its lowest physically reasonable value (the refractive index of water), while the value of \( K^2 \) (0.58) is the largest value consistent with measurements of retinal’s orientation (cited above). The estimated depth of retinal from the extravesicular surface of H. halobium CEVs is 9 ± 3 Å, as determined by subtracting the donor radius from \( a_{OUT} \).

![Figure 7. Addition of all-trans retinal to bleached cell-envelope vesicles leads to retinal-specific energy transfer. 5 μl of 10 mM-Tb(III)HED3A, in 4 M-NaCl was added to a 45 μl sample of bleached vesicles (in 4 M-NaCl/Mops buffer) containing 0.1 mM-bacteriopsin. After baseline absorbance and luminescence kinetics were measured, sub-microliter samples of all-trans retinal were added to reconstitute the chromophore of BR. Changes in absorbance and the luminescence kinetics of Tb were measured as described in the legend to Fig. 3 for PM sheets. The energy-transfer rate constant was calculated from the slope of the quenching curve as shown. An energy transfer rate constant of 1.9(±0.3) \times 10^6 \text{ M}^{-1} \text{s}^{-1} \) was estimated as the average of 3 independent reconstitution experiments.](image-url)
(e) Energy-transfer measurements with trapped donors

(i) Strategy

The results described above localize retinal to the outer leaflet of the PM, but do not directly determine retinal’s depth from the cytoplasmic face of the PM. If the thickness of PM at retinal is 45 Å, then a depth of 35 Å from the cytoplasmic face of PM is implied by the retinal-specific transfer rate. However, other values of the membrane thickness are also consistent with the energy-transfer data (Fig. 5, inset).

To measure energy transfer selectively from the cytoplasmic surface of PM, Tb(III) donors were first trapped in the inner-aqueous space of CEVs. Then, after washing donors away from the external aqueous space, the luminescence kinetics of the remaining trapped donors were recorded. Energy transfer was estimated from the difference between the apparent initial rates of donor decay in the presence and in the absence of the chromophore by either of two methods, as described below.

(ii) Hydroxylamine bleaching

By analogy with equation (9), the ensemble-average luminescence decay rate for donors trapped in CEVs, $\langle k \rangle$, should follow the equation:

$$\langle k \rangle = k_0 + k_{2\text{RDL}} [\sigma_{\text{R}R}]$$

where $k_0$ is as defined above and $k_{2\text{RDL}}$ is the ensemble-average or RDL energy-transfer rate constant. The units of $k_{2\text{RDL}}$ (cm$^2$ [mol BR]$^{-1}$ s$^{-1}$) are like those of a first-order rate constant that varies with the surface density of BR ($\sigma_{\text{BR}}$) rather than with its concentration in solution ($\rho_{\text{BR}}$).

CEVs were bleached either fully or partially, using procedures described in Materials and Methods, and donors were then trapped inside by a brief, mild osmotic shock: dilution from 4 M to 3 M ionic strength. Although we expected to observe some decrease in the energy-transfer rate due to bleaching of the chromophore, no decrease in the energy-transfer rate from Tb(III)DPAS$^3$– trapped inside CEVs could be detected from either initial decay rates or biexponential analysis of the decays (data not shown). The error associated with the initial rate measurement (approximately 10%) is large due to the non-exponential character of the decay curves. Allowing for the error, however, the apparent insensitivity of trapped Tb(III)DPAS$^3$– to the state of retinal indicates that the transfer rate constant is less than $8.4 \times 10^{12}$ cm$^2$ M$^{-1}$ s$^{-1}$ (based on the measured value of $\sigma_{\text{BR}}$), consistent with a distance of closest approach greater than or equal to 32 Å between the negative chelate and retinal at the inner CEV surface.

(iii) CEVs from cells that lack BR

Further experiments addressing the energy transfer rate to retinal from inside CEVs were performed by comparing luminescence of trapped
donors in CEVs from *H. halobium* strains JW1, JW3 and JW5 (see Materials and Methods). $a_{N}$ is estimated from the net energy transfer to BR, calculated as the difference in initial rates between donors trapped in CEVs from different strains (using the model of Fig. 1(b)). The apparent distances of closest approach that have been derived are between 17 Å (Tb(III)HED3A) and 19 Å (Tb(III)EDTA⁻) (Table 1). After the radii of the chelates are subtracted, comparison of strains would suggest that retinal is 13 to 15 Å from the intravesicular face of PM. Although this distance, combined with $a_{PM}$ or $a_{OUT}$, is too short to validate a uniform, 45 Å value for the membrane thickness, it is at the same time too long to be equated with the depth of retinal from the nearest surface of PM, $a_{PM}$. The error bounds for these estimates were obtained by matching the measured values of $k_{RDL}$ to extreme-value simulations, as shown in Figure 9.

Additional energy-transfer estimates were made by reconstituting BR in CEVs from *H. halobium* JW5 with all-trans retinal. These membranes contain small but significant levels of bacterioopsin, and the retinal chromophore of BR formed by addition of all-trans retinal is apparently identical with that of native BR (Weber & Bogomolni, 1981). As the BR absorbance develops after retinal addition, a small increase in the initial rate of luminescence decay of trapped Tb(III)EDTA⁻ also occurs (see Table 1). Using the estimated surface density of bacterioopsin in JW5 CEVs (22% of that in JW3), and a simulation similar to that of Figure 9, $a_{N}$ is found to be 17 Å (Tb(III)EDTA⁻). In agreement with energy transfer derived from comparisons between strains, reconstitution data are consistent with an intravesicular depth for retinal larger than the depth derived for PM sheets.

4. Discussion

(a) Summary

The purpose of this energy-transfer study is to determine the depth and possible asymmetry of retinal in the trans-membrane dimension of BR.
Using aqueous chelates of Tb(III) as rapid-diffusion energy donors and the retinal chromophore of BR in either PM sheets of *H. halobium* CEVs as energy acceptors, the distance between retinal and the nearest aqueous surface has been estimated to be 10 Å. Since energy transfer in the rapid-diffusion limit was measured in aqueous suspensions with unmodified PM preparations, possible artifacts deriving from either covalent modification or detergent treatment of membranes are avoided. Because CEVs largely preserve the membrane asymmetry of unmodified PM preparations, possible artifacts deriving from either covalent modification or detergent treatment of membranes are avoided. Because CEVs largely preserve the membrane asymmetry of unmodified PM preparations, possible artifacts deriving from either covalent modification or detergent treatment of membranes are avoided. Because CEVs largely preserve the membrane asymmetry of unmodified PM preparations, possible artifacts deriving from either covalent modification or detergent treatment of membranes are avoided.

(b) Interpretation of energy transfer between donors and PM sheets

Two alternative procedures, dilution and reconstitution, were used above to vary the concentration of BR in order to evaluate energy transfer between Tb chelates and PM sheets. Dilution of the PM suspension was most convenient, but did not control for the possibility that non-retinyl acceptors such as carotenoids may have co-purified with PM (Braiman & Mathies, 1982). However, since the results obtained with these methods were similar, the systematic error associated with the dilution method should be limited to 20%/ in the rate constant, or 1 Å in the distance of closest approach. The slightly higher rate constant observed in dilution experiments may be due to a small population of carotenoids near a surface of the PM and/or to a systematic underestimate of BR concentrations in PM by conventional spectrophotometry. Such an underestimate would occur due to overcorrection for light-scattering.

Since the Tb(III)HED3A concentration could be varied without significantly altering our estimate of the \( \Phi_{PM} \) it seems unlikely that donors bind to PM in any way that affects energy transfer. Binding of Tb(III)HED3A to almost any position on the PM could result in donors with high probability of energy transfer, since most sites on the membrane are expected to be within 47 Å (\( R_0 \)) of retinal. However, it is clear that in experiments done with fewer than one donor per BR (e.g. 20 \( \mu \text{M-Tb(III)} \)-HED3A, 50 \( \mu \text{M-BR} \)), the membrane lattice could not be saturated with bound donors. As the donor : acceptor ratio increases from this value, any binding that can occur should increase, presumably increasing the ensemble-average energy-transfer efficiency. However, no effect on the apparent distance of closest approach was evident as Tb(III)-HED3A was increased to nearly 50 \( \mu \text{M} \). Additional results negating the importance of binding were obtained in an equilibrium dialysis experiment with 1 mM-Tb(III)HED3A and approximately 80 \( \mu \text{M-BR} \). At these concentrations, which are typical of energy-transfer experiments, no binding was detected.

The energy transfer rates between negatively charged Tb(III) donors and PM sheets at high ionic strength are fully consistent with the depth for retinal derived using a neutral donor. Both Tb(III)EDTA\textsuperscript{−} and Tb(III)DPA\textsuperscript{3−} transfer energy to PM sheets in high salt at levels consistent with retinal being buried at 11 Å from the closest PM surface. Because the chelates studied are diverse in size, net charge and quantum yield, this agreement bolsters assumptions used in the simulation of rate constants, while confirming the shallow depth of retinal.

For energy-transfer studies above, the transmembrane position of BR's chromophore is assumed to be the depth of the center of retinal's transition dipole moment. However, the maximum error incurred by assuming that both donors and acceptors behave as point dipoles (as in Förster's theory) is probably limited to 2 or 3 Å, based on the following considerations. For the Tb chelates used above, the electron density of the \( ^{5}D_{4} \) excited-state, from which transfer occurs, is mainly confined to the metal atom, which is less than 1 Å in radius (see Richardson, 1982). The maximum error contributed by the donor is therefore very small. Retinal, while 15 Å in total length, spans only (sin 20°) 13 Å, or 4 Å in the transverse dimension of PM. If energy-transfer theory were to include the effect of finite-sized dipoles, a plausible assumption would be that segments of the finite-sized dipole accept energy independently. If this occurs for BR, then the distance of closest approach to the center of retinal would be at most 2 Å (half retinal's transverse span) greater than the distance inferred above.

<table>
<thead>
<tr>
<th>Table 1</th>
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<td>Distances of closest approach for Tb(III) chelates trapped in CEVs.</td>
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<table>
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<tr>
<th>Donor</th>
<th>JW3-JW5</th>
<th>JW3-JW1</th>
<th>(JW5 + retinal) - JW5</th>
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<tbody>
<tr>
<td>Tb(III)EDTA\textsuperscript{−}</td>
<td>n/d</td>
<td>17 ± 1</td>
<td>n/d</td>
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<tr>
<td>Tb(III)EDTA\textsuperscript{−}</td>
<td>19 ± 2</td>
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| Distance of closest approach between Tb(III) donors and the retinal chromophore of BR in *H. halobium* CEVs. The distance of closest approach between trapped donors and the retinal chromophore of BR was estimated in each experiment by matching the transfer rate constant to a simulation of energy transfer (as shown in Fig. 9) on the basis of trapped-donor geometry with acceptors arranged as shown in Fig. 1(b). The error bounds are estimated by matching the measured values of \( k_{n/d} R_{DL} \) to extreme-value simulations shown in Fig. 9. |

\* All distances in Å.
The distance of closest approach from Tb(III)-HED3A outside CEVs to retinal in BR is estimated to be 13 ± 2 Å. Much of the discussion of luminescence data obtained with PM sheets also pertains to experiments with donors outside CEVs. However, two simplifications are apparent for the external donor geometry: (1) the thickness of PM does not influence transfer rates; and (2) the probability that binding will affect energy transfer is reduced. The latter assertion may be rationalized since (a) one face of the PM is inaccessible; and (b) non-PM sites, represent a significant portion of the CEV surface area. If any tendency exists for donors to associate non-specifically with membranes, then the net level of interaction with PM and its chromophore would be reduced in CEVs.

The natural asymmetry of CEVs is crucial to measurements of \( a_{\text{OUT}} \), and experimental evidence for their outside-out orientation was originally presented by Stocekenuis & Rowen (1967). The high capacity of CEVs to produce and sustain a large, outside-positive, light-driven electrochemical potential gradient is further evidence for the strong asymmetry of PM in the CEV wall. However, it is possible to rule out significant errors in \( a_{\text{OUT}} \) due to partial misorientation of PM in the vesicles by analyzing energy-transfer measurements with PM sheets. How much energy transfer could occur to the misoriented fraction if 10% of PM in CEVs showed the cytoplasmic face at the extravesicular phase? If retinal were actually 10 Å from the cytoplasmic surface of PM rather than the periplasmic face, the maximum contribution of this fraction to the observed rate constant from donors outside CEVs would be 10% of the measured energy-transfer rate constant between Tb(III)-HED3A and PM sheets, or 0.15 \( \times 10^6 \ \text{M}^{-1} \ \text{s}^{-1} \). The remaining 90% of the transfer rate constant (1.33 \( \times 10^6 \ \text{M}^{-1} \ \text{s}^{-1} \)) would then be specifically due to donors near the periplasmic face of PM, and \( a_{\text{OUT}} \) would increase to 15 Å at the most (see Figs 5 and 8). This worst-case analysis would apply only if the depth of retinal from the cytoplasmic face were greater than 10 Å. If the depth of retinal from the cytoplasmic face were greater and the misoriented population were 10%, or less, the error would be less.

Energy transfer was measured by reconstituting bleached BR in CEVs to evaluate the degree of specificity of energy transfer for retinal in BR, and to facilitate measurement of the BR concentration by difference absorbance. Although CEVs may possess non-retinyl energy acceptors for Tb (see below), these acceptors apparently do not contribute to energy transfer from the external face of CEVs. Within a small margin of error (± 1 Å), the values of \( a_{\text{OUT}} \) estimated by dilution and by reconstitution were equal. However, as discussed below, non-retinyl energy acceptors in CEVs may contribute to the apparent energy transfer rate when donors are trapped inside CEVs.

(d) Interpretation of transfer from trapped donors

Although significant energy transfer occurs from Tb donors exposed to the intravesicular face of CEVs, suggesting a shallow depth for retinal on the cytoplasmic face of PM, control experiments with both bleached CEVs and chromophore-deficient CEVs show that a nearly equal amount of energy transfer occurs in these systems and cannot be due to the retinal chromophore of BR. Thus, retinal in BR is not the major energy acceptor at the intravesicular surface of CEVs (see below). In the control systems where energy-transfer rate constants could be estimated, either by reconstitution of JW5 or by quantitative comparison of either JW1 or JW5 with JW3 CEVs, \( a_{\text{OUT}} \) was estimated to be 16 to 21 Å. The latter estimate requires the assumption that the absence of BR's retinal chromophore in JW1 and JW CEVs is the only difference between these and JW3 CEVs that affects energy transfer. If, contrary to evidence presented above, \( a_{\text{OUT}} = 14 ± 1 \) Å (the sum of a 10 Å depth and a 4 Å chelate radius), then the predicted range of \( k_{\text{RDL}} \) would be as shown in the simulation of Figure 9 (box b). The experimental values of \( k_{\text{RDL}} \) (box a, Fig. 9) do not overlap with this range. Thus, estimation of \( a_{\text{OUT}} \) using BR-deficient mutants and bleached membranes supports the conclusion that retinal lies further from the cytoplasmic face of PM than from the periplasmic face, and that the depth estimated from measurements with PM sheets represents the depth from the extracellular or periplasmic face of PM.

The high level of background energy transfer observed with donors trapped inside CEVs is consistent with the possibility that non-retinyl acceptor(s) present in the CEV membrane are solvent-accessible near the intravesicular surface. Non-retinyl acceptors, including both carotenoid lipids (Kates, 1972) and cytochrome proteins (Lanyi, 1971), are expected to be present in CEVs. Furthermore, the heme chromophores of cytochromes are likely to be exposed at the cytoplasmic surface of CEVs, as they are in cells, poised to function in the electron-transport chain. If any heme chromophore is solvent-accessible at that surface, as it is in myoglobin (Wensel & Meares, 1983), then even surface densities undetectable by conventional spectrophotometry could lead to substantial energy transfer (R. Leder & D. Thomas, unpublished calculations). Carotenoids, too, could influence energy transfer significantly if they were exposed to donors at the intravesicular surface. However, there is no evidence that these molecules are preferentially localized to the cytoplasmic side of the membrane. Non-retinyl energy acceptors in CEVs have not yet been identified.

Because shorter distances and higher transfer rates would be more readily detectable above background, the apparent depth of retinal derived from
trapped-donor studies should represent a lower limit on the depth of retinal from the cytoplasmic face. If the trans-membrane segment of BR near retinal is at least 28 Å in length, a minimum value required to span the hydrocarbon phase of the membrane, then the depth of retinal from the cytoplasmic face of PM must be at least 18 Å (see below).

(c) Depth results obtained by other techniques

Several empirical assessments of retinal's depth have appeared previously, utilizing diverse methods: profile neutron diffraction (King et al., 1979), fluorescence energy transfer (Tactin et al., 1983; Kouyama et al., 1983; Hasselbacher et al., 1986; Kometani et al., 1987; Otomo et al., 1988) and surface-enhanced resonance Raman spectroscopy (Nabiev et al., 1985).

The results of several prior energy transfer experiments utilizing either the PM or the photoconverted, NaBH₄-reduced derivative of BR (PCR-PM) are consistent with the evidence presented above for the shallow depth of retinal in PM sheets. Those measurements, including both a rapid-diffusion limit energy-transfer study (Kouyama et al., 1983) and several condensed-phase energy-transfer systems (Kometani et al., 1987) were interpreted to demonstrate that both retinal and the fluorescent retroretinyl group of PCR-PM are less than 15 Å from a surface of the PM.

The evidence shown above for retinal's disposition towards the extracellular face of PM is in harmony with the interpretations of prior surface-enhanced resonance Raman (SERS) measurements (Nabiev et al., 1985). In those experiments, the intensities of Schiff base vibrations were strongly enhanced when membranes or cells were dried to the surface of a silver electrode (Nabiev et al., 1985). Although the theory of distance dependence in SERS may not be as well-established as it is for fluorescence energy transfer, the observed enhancement was deemed possible only if the distance between the aldimine bond and the metal surface were less than 10 Å (Nabiev et al., 1985). Thus, our placement of retinal's transition moment at 10 Å from the extracellular surface complements the Raman-derived conclusion that the Schiff base of retinal lies near the exterior surface of cells.

Other published results support the idea that a large distance separates the cytoplasmic face of PM from retinal. With a fluorophore attached to the C terminus of BR on the cytoplasmic face of PM, the distance to retinal was estimated by energy transfer to be 35 Å or more (Marque et al., 1986). This result may be consistent with RDL energy transfer between donors trapped in CEVs and retinal in BR as described above if the C terminus of BR protrudes somewhat into the cytoplasmic aqueous phase. An alternative energy-transfer measurement involved a fluorescent donor covalently linked to BR at Lys41 and retinal as the energy acceptor. The author estimated that retinal was at least 29 Å from Lys41, a residue thought to lie at the cytoplasmic surface of PM (Renthal, 1981).

While numerous published results discussed above are consistent with the new evidence presented here, our conclusion from rapid-diffusion limit-energy transfer that retinal in native BR lies near the periplasmic face of PM is apparently at odds with the interpretations of Otomo et al. (1988). Using partially oriented films of either PCR-PM or PM on a glass coverslip, Otomo et al. measured fluorescence energy transfer either to or from a heterogeneous film of either donors or acceptors, presumably adsorbed to exposed membrane surfaces after drying. Unfortunately, both the donor molecules (tris(2,2'-bipyridyl)ruthenium(II)) and acceptor molecules (horse heart cytochrome c; hemoglobin) analyzed in detail by Otomo et al. were positively charged under conditions employed by those authors. Each system studied was therefore more likely to interact with membranes at the cytoplasmic face of PM than at the extracellular face, since the inner face possesses a strong negative charge. In particular, the cytoplasmic face of PM has been shown to have up to ten times the surface charge density of the extracellular face of PM (Renthal & Cha, 1984; Ehrenberg & Berezin, 1984). This probably led to selective adsorption of donors and acceptors at that surface, and might account for the differences in energy transfer observed. Experiments with uncharged donors and acceptors could reveal such a bias. Otomo et al. (1988) also failed to demonstrate directly the chromophore-specificity of observed changes in donor quantum yield measured throughout their study. In energy-transfer studies where the acceptor is non-fluorescent, other means must be used to demonstrate Förster interaction between the chromophore and fluorophore species under study. Fluorescence measurements with films of apo-cytochrome c and PCR-PM, for example, could reveal such an artifact.

At least two published studies have argued that retinal is deeply buried in PM, apparently placing retinal in the central one third of a 45 Å membrane (Hasselbacher et al., 1986; King et al., 1979). However, it may not be necessary to reconcile each of these with the evidence presented above. In the first case, it is difficult to compare the present results with the energy-transfer study of Hasselbacher et al. (1986), since detergent-solubilized BR was reconstituted into liposomes with fluorescent lipids for that study. We assume that substantial disruption of non-covalent PM structure must occur during this procedure, and it seems likely that some differences in BR structure would exist between this preparation and native PM. Perhaps a rapid-diffusion energy transfer measurement with Tb(III) donors and the BR preparation of Hasselbacher et al. (lacking fluorescent lipids) would reveal such differences.

Profile neutron diffraction studies utilizing hydroxyamine-bleached, retinal-reconstituted BR are also difficult to compare directly with rapid-diffusion energy-transfer results, mainly because
errors were apparently not well-determined in the
diffraction study. King et al. (1979) suggested that retinal is at least 17 Å from an aqueous surface, but
did not offer an error limit on this value. We also
note that the diffraction intensities of native PM
were not presented in that study for comparison
with those of the covalently modified membranes.

(f) Implications for the structure of BR

The most basic model for folding of BR into the
PM should identify the amino acids at which the
seven trans-membrane segments (A to G) begin and
end (Engelman et al., 1986). If segment G folds as a
perfect α-helix and if the center of retinal's transition
dipole moment lies 10 Å from the extracellular
face of the PM, as indicated by retinal-specific energy transfer from Tb(III)HED3A, then the most
likely candidates for amino acid residues lying at the
amino (periplasmic) and carboxyl (cytoplasmic)
boundaries of helix G (which bears retinal at
Lys216) can be inferred. First, the amino terminus
of helix G should be more than four residues
(Asp212) but fewer than 10 residues (Leu206) from
Lys216. This range of uncertainty includes consider-
ation of the absolute orientation of retinal and
assumes a pitch of 1.5 Å/residue, as expected for a
perfect α-helix. It also assumes that the side-chain
of Lys216 lies parallel to the membrane surface.

On the cytoplasmic face of the PM, Arg227, but
not Arg225, is accessible to proteolytic cleavage by
tryptsin (Walker et al., 1979). If helix G is a perfect
α-helix and is parallel to the membrane normal,
then: (1) the nine residues between Lys216 and
Arg225 span 13.5 Å towards the cytoplasm, prob-
able placing Arg225 at the interface between the
apolar membrane phase and the bulk aqueous
phase; and (2) the distance between Lys216 and
Leu206, at the periplasmic face of PM, would be
15 Å at the most. Thus a minimal, 28.5 Å hydrocar-
bon phase (Engelman et al., 1986) could contain
helix G. If helix G includes any elongating distortions,
as might be envisaged at residues Gly218 and
Gly220, or if the side-chain of Lys216 lies at any
angle other than 0° to the membrane surface, a
wider range of amino acids could be included among
the candidates for its boundaries.

The trans-membrane position of the β-ionone ring
of retinal may also be inferred from evidence pre-
presented above, although the position is not unique†.
If we consider the information obtained by photo-
chemical cross-linking methods with a diazirino-
phenyl analog of retinal (Huang et al., 1988), then
depths of Ser193 and Glu194 (located in the strand
of BR nearest to helix G in the primary structure)
should align with the ring portion of the chromo-
phore. In published models of BR, these residues are
between 4 and 7 Å from the periplasmic face of PM
(Huang et al., 1983; Engelman et al., 1986).
Assuming that retinal's transition moment is
10±2 Å from the periplasmic face, the depth of the
ring would be approximately the same as that of
these amino acid residues if the absolute orienta-
tion of retinal inclined from the Schiff base to the ionone
ring toward the periplasm.

(g) Conclusion

From measurements of the distance of closest
approach, we conclude that retinal in BR, like reti-
nal in bovine rhodopsin (Thomas & Stryer, 1982), is
buried with respect to both membrane surfaces. The
benefits derived from reduced access of solvent may
be increased efficiency of energy channeling from the
chromophore to the protein, or reduced probabi-
licity of chromophore oxidation. It would be interest-
ing to know which groups or atoms of PM serve as
local barriers, protecting the energy-transducing
center of BR from solvent. The moieties that steri-
cally isolate retinal from solvent represent possible
transducing elements in the protein structure. We
also conclude that retinal in BR, unlike retinal in
rhodopsin, is apparently located at a depth signifi-
cantly less than half the average thickness of the
membrane (10/45 Å) and closer to the periplasm
than to the cytoplasm of cells. Also unlike its dis-
tant cousin, rhodopsin, BR is thought to protrude
little or not at all from the membrane of which it is
an integral part (Henderson, 1975). Despite
increased knowledge about BR and rhodopsin, the
significance of structural differences between the
two proteins remains to be determined. It is likely
that the distinct functions of rhodopsin as a light-
signal transducer and of bacteriorhodopsin as a
light-driven proton pump will guide our under-
standing of these differences.

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