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THE TRANSVERSE LOCATION OF TRYPTOPHAN RESIDUES IN THE PURPLE MEMBRANES OF *HALOBACTERIUM HALOBIUM* STUDIED BY FLUORESCENCE QUENCHING AND ENERGY TRANSFER

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Fluorescence quenching by a series of spin-labelled fatty acids is used to map the transverse disposition of tryptophan residues in bacteriorhodopsin (the sole protein in the purple membranes of *Halobacterium halobium*). A new method of data analysis is employed which takes into account differences in the uptake of the quenchers into the membrane. Energy transfer from tryptophan to a set of *n*-(9-anthroyloxy) fatty acids is used as a second technique to confirm the transverse map of tryptophan residues revealed by the quenching experiments. The relative efficiencies of quenching and energy transfer obtained experimentally are compared with those predicted on the basis of current models of bacteriorhodopsin structure. Most of the tryptophan fluorescence is located near the surface of the purple membrane. When the retinal chromophore of bacteriorhodopsin is removed, tryptophan residues deep in the membrane become fluorescent. These results indicate that the deeper residues transfer their energy to retinal in the native membrane. The retinal moiety is therefore located deep within the membrane rather than at the membrane surface.

Introduction

Bacteriorhodopsin is arranged in the purple membrane of the halophilic bacterium *Halobacterium halobium* in a precise two-dimensional hexagonal lattice as revealed by X-ray diffraction measurements [1,2]. The three-dimensional structure has been obtained from electron micrographs, X-ray diffraction and neutron diffraction experiments [3,4]. The retinal chromophore is attached via a Schiff base linkage to Lys 216 [5,6] although the possibility of migration of retinal between several lysine residues has been entertained [7]. The absorption of light by the retinal initiates a complex photocycle which is coupled to the extrusion of protons from the cell. The proton motive force thus generated is used in the synthesis of ATP [8].

The polypeptide chain of bacteriorhodopsin traverses the membrane in seven α-helical segments which are arranged in a crescent when viewed from above the plane of the membrane. With knowledge of the amino acid sequence and the peptide bonds which are accessible to proteases, it is possible to construct models which display the approximate transverse positions of the individual amino acid residues [4,9]. This paper is concerned with the transverse position of tryptophan residues in bacteriorhodopsin and with the proximity of these residues to the retinal chromophore. Tryptophan residues are believed to stabilize the α-helical structure in bacteriorhodopsin [31] and interact with retinal during its photochemical cycle [10]. We describe fluorescence quenching and resonance energy transfer experiments which measure the transverse distribution of

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tryptophan residues in the membrane and reveal the transverse position of the retinal itself.

Fluorescence quenching methods have been used successfully to determine the transverse position of fluorophores and quenchers in phospholipid bilayers [11–13] in micelles [14] and in biological membranes [15]. Resonance energy transfer has also been used to determine the depth of fluorophores in serum lipoproteins [16] and in reconstituted membranes [11,17]. In this study we make use of probes which have a paramagnetic quenching moiety (doxyl group) or an acceptor chromophore (anthroyloxy group) attached to various positions along the acyl chain of a fatty acid. They therefore locate at a graded series of depths in the membrane and are capable of sensing the transverse distribution of tryptophan fluorescence within the membrane.

ESR data indicate that 12-doxylstearate can distribute between the aqueous and lipid phases of a membrane suspension. When dilute suspensions of non-fluid membranes with a high probe/lipid ratio are used, a significant fraction of the probe may remain in the aqueous phase [18]. The series of *n*-doxylstearates may differ in their distribution between the two phases. Such differences can lead to significant artefacts when attempting to use the doxyl stearates as quenchers to determine the transverse position of a fluorophore in bilayers [32]. We have therefore used the procedure of Encinas and Lissi [19] to correct the quenching data for differences in uptake between the series of *n*-doxylstearates so that conclusions can be drawn about the transverse organisation of the tryptophan residues of bacteriorhodopsin in the purple membranes of *H. halobium*.

The photocycle of bacteriorhodopsin can be initiated by excitation at 290 nm and thus there is strong evidence that tryptophan can transfer its fluorescence energy to retinal in the native protein. Indeed, it has been proposed that 5 or 6 out of the total of 7 tryptophan residues are involved in this internal transfer process [20]. Quenching and energy transfer experiments should reveal the transverse location of the tryptophan residue(s) which is/are not involved in the internal transfer to retinal. Bleaching of the retinal should allow those residues previously quenched by internal transfer to be available for quenching by the *n*-doxylstearic acid probes and for energy transfer to alternative acceptors such as the *n*-(9-anthroyloxy) fatty acids. The experiments described below were designed to test this hypothesis.

Theory

Quenching of a membrane-bound fluorophore by a quencher which is capable of distributing between the aqueous and membrane phases is dependent on the intramembranous quencher concentration regardless of the mechanism by which the fluorescence is quenched (i.e. static or dynamic). The procedure of Encinas and Lissi [19] can be used to determine equilibrium constants which describe such partition thus enabling the intramembranous concentration of quencher to be determined. An equilibrium distribution constant (K_{eq}) can be defined:

$$K_{eq} = \frac{[Q_L]}{[Q_A][\text{Memb}]} \tag{1}$$

where $[Q_L]$ and $[Q_A]$ are the concentrations of quencher in the lipid and aqueous phases expressed with respect to the total volume of the system, respectively, and [Memb] is the concentration of membranes. Quenching decreases as the concentration of membrane increases and the quencher becomes diluted in the lipid phase. Nevertheless, at several membrane concentrations a given level of quenching corresponds to the same average number of quenchers in the membrane ($\bar{n}$). It can be shown that at constant $\bar{n}$ [19]:

$$[Q_T] = \frac{\bar{n}}{K_{eq}} + \bar{n}\,[\text{Memb}] \tag{2}$$

where $[Q_T]$ is the total concentration of quencher. A plot of $[Q_T]$ versus [Memb] allows determination of K_{eq} and $\bar{n}$. The concentration of bacteriorhodopsin membranes can be expressed as the number of moles of membrane fragments per dm^3 (i.e., number of membrane fragments/(Avogadro's number × volume)) in order to facilitate analysis of the quenching data by the above method. The number of membrane fragments was calculated using the following constants [21]: density of purple membrane = 1.18 g/cm^3; protein/

lipid = 3 : 1 (w/w) (to convert mass of bacteriorhodopsin to total membrane volume); diameter and thickness of disc-shaped membrane fragments = 0.5 μm and 45 Å, respectively (to calculate the average volume of a single membrane fragment). The number of fragments is given by the total membrane volume divided by the volume of a single fragment.

Materials and Methods

Chemicals . *n*-Doxyl stearic acids ($n = 5, 7, 12, 16$) were purchased from Molecular Probes (Junction City, OR). *n*-(9-Anthroyloxy) fatty acids ($n = 2, 6, 9, 12$) were prepared by anhydride synthesis from the corresponding *n*-hydroxy fatty acids and anthracene-9-carboxylic acid [22]. All other chemicals were of analytical grade.

Methods

Bacteriorhodopsin was isolated as previously described [23]. Retinal was removed by photobleaching with orange light in the presence of hydroxylamine [24] followed by three extractions with heptane to remove residual retinaloxime. Removal was quantitative as shown by the complete loss of the α- and β-absorption bands. This treatment did not change the molecular weight of the polypeptide or the density of the membrane as demonstrated by SDS-polyacrylamide gel electrophoresis and sucrose density gradient centrifugation, respectively.

Fluorescence was measured with a Hitachi-Perkin Elmer MPF3 spectrofluorometer equipped with a thermostated cell block. Excitation and emission wavelengths for the quenching of tryptophan fluorescence in bacteriorhodopsin membranes were 286 nm and 320 nm, respectively; these same wavelengths were used for the measurement of energy transfer by the quenching of donor (tryptophan) fluorescence. Quenchers and acceptors were added as 5 μl aliquots of methanolic stock solutions. No time dependence of the fluorescence signal was observed after quencher (*n*-doxylstearic acid) addition, indicating that uptake was very rapid. One hour was allowed, however, for uptake of the acceptors (*n*-(9-anthroyloxy) fatty acid) before the fluorescence was recorded. The efficiencies of quenching (Q) and of energy transfer (E) are expressed according to the equations $Q = (I_0/I) - 1$ and $E = 1 - (I/I_0)$, where I and I_0 are the intensities in the presence and absence of the quencher or acceptor.

Fluorescence lifetimes were measured by the technique of time-correlated single-photon counting using a pulsed flash lamp system from Applied Photophysics. The excitation wavelength of 290 nm was selected by a grating monochromator and the emission above 305 nm was selected with a Schott UG305 cutoff filter. The fluorescence decays were fitted to single or double exponential functions by nonlinear least squares fitting procedures. Goodness-of-fit was assessed by the magnitude of the reduced X^2 value and by inspection of the weighted residuals plot. Further details of the technique are described elsewhere [25,26].

The relative concentrations of the spin labelled fatty acids (1.5 to 2.5 mM in methanol) were determined by EPR spectroscopy from the peak-to-trough distances of the first derivative spectra. Concentrations of the anthroyloxy fatty acids (0.5 mM) were determined by absorption using the extinction coefficients reported by Thulborn and Sawyer [22]. The monomer molecular weight of bacteriorhodopsin was taken a 26 500 and protein concentrations were determined using an extinction coefficient of 63 000 $M^{-1} \cdot cm^{-1}$ at 570 nm. Total protein was determined by the method of Lowry et al. [27].

Results

Quenching and energy transfer in the native membrane

Stern-Volmer plots for the quenching of tryptophan fluorescence of bacteriorhodopsin by *n*-doxylstearic acid (*n*-NS) probes are shown in Fig. 1. The relative quenching efficiencies are in the order of 5-NS > 7-NS > 16-NS > 12-NS. This information by itself does not assist in defining the transverse positions of the tryptophan residues in the purple membrane since the data are plotted against the total concentration of quencher rather than against the concentration in the membrane phase. Correction for the distribution of quencher between aqueous and membrane compartments was made by using the procedure of Encinas and Lissi [19]. Stern-Volmer plots for the quenching of

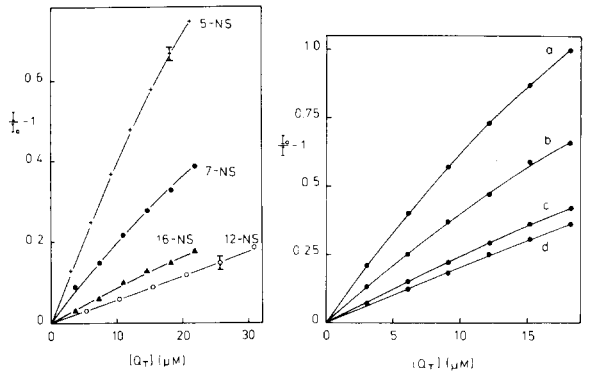

Fig. 1. Stern-Volmer plots for the quenching of tryptophan fluorescence of purple membranes by *n*-doxylstearic acid (*n*-NS). Bacteriorhodopsin concentration, 120 μg/ml in 50 mM phosphate (pH 7.0). Error bars show the range of values obtained in duplicate experiments.

Fig. 2. Stern-Volmer plots for the quenching of tryptophan fluorescence by 5-doxylstearic acid at various bacteriorhodopsin concentrations. (a) 57 μg/ml, (b) 116 μg/ml, (c) 230 μg/ml, (d) 290 μg/ml.

tryptophan fluorescence by 5-doxylstearic acid at several membrane concentrations are shown in Fig. 2. Plotting these data according to Eqn. 2 yields a straight line (fig. 3) of slope $\bar{n}$ and ordinate intercept $\bar{n}/K_{eq}$. Values of K_{eq} decreased slightly with increasing $\bar{n}$ (Fig. 4) suggesting that the accumulation of surface charge might inhibit the uptake of successive molecules of quencher. To compare the quenching results obtained with native and bleached membranes, an average value of K_{eq} was used to calculate $\bar{n}$ for given values of $[Q_T]$ and [Memb] using Eqn. 2. This approach results in a Stern-Volmer plot which is corrected for differences in the equilibrium distribution of quenchers between aqueous and lipid compartments. The corrected plot (Fig. 5a) shows that the quenching efficiency decreases progressively in going from 5-doxylstearic acid to 12-doxylstearic acid indicating that most of the tryptophan fluorescence in the native membrane is located close to the membrane surface.

A more rigorous approach, which took into account the variation of K_{eq} with $\bar{n}$, resulted in data similar to that shown in Fig. 5a to within experimental error.

The transverse disposition of fluorescent tryptophan residues was also studied using energy transfer techniques. The transfer from tryptophan to the series of *n*-anthroyloxy fatty acids ($n = 2, 6, 9, 12$) was examined as a function of acceptor concentration. Transfer efficiency increases as the surface density of acceptors increases. Moreover, the results in Fig. 6a show that the transfer efficiency decreases as the acceptor is moved deeper into the membrane suggesting that the average donor-acceptor distance is smaller when the acceptor is nearer the membrane surface than the membrane interior, that is, that the fluorescing tryp-

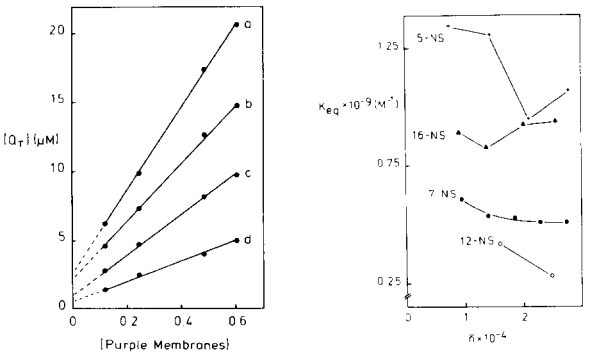

Fig. 3. Data in Fig. 2 re-plotted as $[Q_T]$ versus mol of purple membrane fragments per dm^3 at various $((I_0/I)-1)$ values. (a) 0.4, (b) 0.3, (c) 0.2, (d) 0.1.

Fig. 4. K_{eq} values for the association of *n*-doxylstearic acid (*n*-NS) probes with purple membranes as a function of the average number of quenchers per membrane fragment ($\bar{n}$).

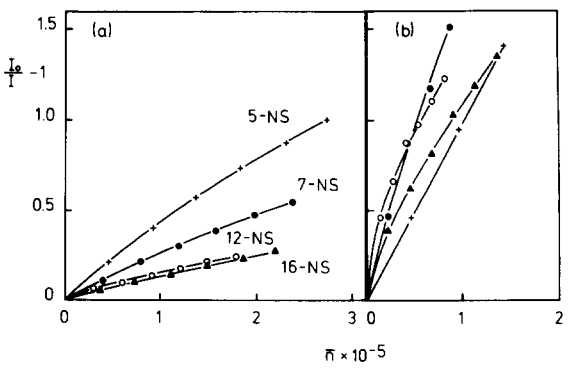

Fig. 5. Stern-Volmer plots for the quenching of tryptophan fluorescence as a function of $\bar{n}$. (a) Purple membranes. (b) Bleached membranes.

tophan residues are those closer to the membrane surface.

In both the quenching and energy transfer experiments discussed above (Figs. 5a and 6a) the differences between the probes are well resolved and are in the same relative order.

Quenching and energy transfer studies in the bleached membrane

Fig. 5b shows a corrected Stern-Volmer plot for the *n*-doxylstearic acid quenchers in bleached membranes. This plot was derived from quenching data at a single membrane concentration (64 μg protein/ml). Higher membrane concentrations could not be examined because of aggregation of membrane fragments. In calculating values of $\bar{n}$, it was assumed that the values of K_{eq} describing the distribution of quenchers between aqueous and membrane compartments were the same as for the unbleached membrane. The transverse profile of tryptophan fluorescence was different to that for the native membrane. The quenching efficiency was greatest for 7- and 12-doxylstearic acid and least for 16- and 5-doxylstearic acid. An important feature of Fig. 5 is that the differences in quenching efficiency between the doxylstearates are smaller in the bleached membrane than in the native membrane. Quenching was also generallv more effective in the bleached than in the native membrane.

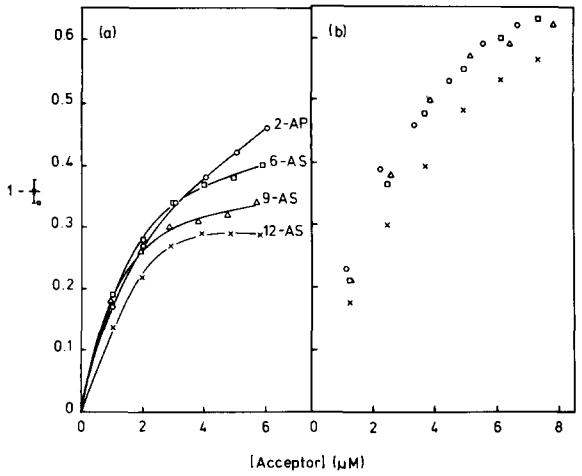

Fig. 6. Energy transfer from tryptophan to *n*-(9-anthroyloxy) fatty acid (*n*-AF) in (a) purple membranes and (b) bleached membranes. Bacteriorhodopsin concentration, 60 μg/ml. Transfer efficiency is measured by donor quenching. Fatty acids: P, palmitic acid; S, stearic acid.

The corresponding energy transfer data for the bleached membrane are shown in Fig. 6b. The efficiency of energy transfer was similar for 2-, 6- and 9-(9-anthroyloxy) fatty acid, and somewhat lower for 12-(9-anthroyloxy) fatty acid. The pattern is significantly different from that found with native membranes (Fig. 6a) where there were well defined differences between the various acceptors.

To summarize, a significant feature of Figs. 5 and 6 is that the differences between probes are poorly resolved in the bleached membrane compared to the clear resolution seen in the natural membrane.

Tryptophan lifetimes in bacteriorhodopsin

The decay of tryptophan fluorescence in bacteriorhodopsin could not be fitted to a single exponential function but was satisfactorily fitted to a double exponential function. The fluorescence lifetimes were 0.58 ± 0.06 ns and 4.82 ± 0.60 ns ($\chi^2 = 1.32$) with the shorter-lived component contributing 93% of the total emission. These values are in good agreement with those obtained by Kalisky et al. [20]. In the bleached membrane the majority of the fluorescence (> 80%) emitted with a lifetime of 2.06 ± 0.06 ns, the remainder being a longer-lived component.

Discussion

The models of Ovchinnikov [9] and Engelman et al. [4] for the structure of bacteriorhodopsin show that the seven tryptophan residues are distributed fairly uniformly in the transverse plane of the external leaflet of the membrane. The depths of the α-carbon atoms of these residues below the membrane surface are summarized in Table I. These distances are only approximate, and the position of the indole side chain itself may deviate from these values depending on rotation about the C_α-C_β and C_β-C_γ bonds of the side chain.

The quenching data for the native membrane (Fig. 5a) suggest that the fluorescing tryptophan residues are near the membrane surface. This pattern of quenching determined experimentally may be compared with that predicted by a simple model

based on the following assumptions. We assume that the main determinant of the quenching efficiency is the transverse displacement of the tryptophan residue from the transverse position of the quencher. To determine the latter, we have assumed that the doxyl group of 12-doxylstearic acid is located 15 Å below the membrane surface, and that the position of the doxyl group on the other quenchers (5-, 7- and 16-doxylstearic acid) can be determined by simple proportion. Finally, we assume that the quantum yields of the tryptophan residues in the retinal-free membrane are roughly equivalent. The lateral proximity of the quencher to the protein is also an important consideration. Most of the lipid present in the bacteriorhodopsin membrane can be classified as boundary lipid. The molar ratio of lipid to protein is approx. 11 : 1, and there is barely sufficient lipid present to form a single layer around each bacteriorhodopsin trimer, an observation which probably accounts for the rigidity of purple membranes and their lack of a transverse fluidity gradient [28,29]. We therefore expect that the lateral approach of the doxylstearic acid quencher to the protein is close and probably within the single layer of lipid next to the protein surface.

Table I summarizes the transverse deviations of each of the seven tryptophan residues from the doxyl group of the quenchers. In the native membrane where five or six of the tryptophan residues can transfer their energy to retinal [20], our experiments indicate that the fluorescing residues are those closest to the membrane surface, probably Trp 137 and/or 188. These residues have similar transverse positions in the membrane according to the Ovchinnikov-Engelman model, and therefore they have similar transverse displacements from the doxylstearic (NS) probes. If the probability of collisional interaction of fluorophore and quencher decreases with increasing transverse distance between them (Table I, lines 5 and 7) then we predict that the quenching efficiency is in the order 5-NS > 7-NS > 12-NS > 16-NS which is in excellent agreement with the experimental results (Fig. 5a).

With the bleached membrane, all seven tryptophan residues contribute to the protein fluorescence and the order of quenching efficiency may be related to the average displacements of the residues from the quenchers (Table I, line 8). For this situation the quenching efficiencies would be in the order 7-NS ≃ 12-NS ≃ 5-NS > 16-NS. The experimentally determined order is 7-NS ≃ 12-NS > 16-NS ≃ 5-NS (Fig. 5b), in reasonable agreement with theory except for the interchange of the

TABLE I

APPROXIMATE TRANSVERSE DISTANCES FROM TRYPTOPHAN RESIDUES TO FATTY ACID QUENCHERS AND ACCEPTORS IN MEMBRANES CONTAINING BACTERIORHODOPSIN

Line No.	Tryptophan residue	Helix [a]	Depth (Å) [b]	Transverse displacement of tryptophan [c]							
				Quenching				Energy transfer			
				5-NS (6.2)	7-NS (8.7)	12-NS (15.0)	16-NS (20.0)	2-AP (2.5)	6-AS (7.5)	9-AS (11.2)	12-AS (15.0)
1	10	A	9	−2.8	−0.3	6.0	11.0	−6.5	−1.5	2.2	6.0
2	12	A	13	−6.8	−4.3	2.0	7.0	−10.5	−5.5	−1.8	2.0
3	80	C	11	−4.8	−2.3	4.0	9.0	−8.5	−3.5	0.2	4.0
4	86	C	20	−13.8	−11.3	−5.0	0.0	−17.5	−12.5	−8.8	−5.0
5	137	E	7	−0.8	1.7	8.0	13.0	−4.5	−0.5	4.2	8.0
6	181	F	15	−8.8	−6.3	0.0	5.0	−12.5	−7.5	−3.8	0.0
7	188	F	7	−0.8	1.7	8.0	13.0	−4.5	−0.5	4.2	8.0
8			Mean	−5.5	−4.0	4.7	8.3	−9.2	−4.5	−3.6	4.7

[a] Helices labelled according to Engelman et al. [4].

[b] Approximate depths of tryptophan residues below membrane surface from the model of Ovchinnikov [9].

[c] Positive signs indicate that the tryptophan residues are closer to the membrane surface than are the probes; negative signs indicate that the tryptophan residues are deeper in the membrane than the probes.

5- and 16-doxylstearic acid probes. The model predicts that the quenching envelope (i.e. the difference in quenching efficiency between the most efficient and least efficient quencher) is narrower in the case of the bleached membrane than the natural membrane (Table I; line 8 compared to line 5 or 7). This effect is due to the fairly even transverse distribution of tryptophan residues across the membrane leaflet. The experimental results in Fig. 5 support this view. The differences in the quenching efficiency of the doxylstearic acid probes are smaller in the bleached membrane than the natural membrane, and may in some comparisons approach experimental error. The anomaly involving the 5- and 16-doxylstearic acid probes discussed above may be due in part to this phenomenon.

A similar analysis can be carried out with respect to the energy transfer experiments. Here too we would expect the transfer distance to be dominated by the transverse rather than the lateral displacement of donor and acceptor. In the native membrane the experimentally determined order of transfer efficiency was 2-AF > 6-AF > 9-AF > 12-AF (Fig. 6a), whereas the theoretically determined order was 6-AF > 9-AF ≃ 2-AF > 12-AF (Table I, lines 5 and 7). There is therefore the suggestion that either the fluorescing tryptophan residues are closer to the membrane surface than predicted by the Ovchinnikov-Engelman model or that the 2-(9-anthroyloxy) fatty acid is deeper in the membrane than supposed. In the bleached membrane, however, there was little difference in transfer efficiency between probes (Fig. 6b) indicating that tryptophan residues have a fairly even transverse distribution in the membrane and can therefore transfer almost equally well to any acceptor regardless of the depth of that acceptor in the membrane. These results are therefore in agreement with the quenching data discussed above.

The relative transfer efficiencies to the various anthroyloxy fatty acid acceptors would be influenced by the surface density of acceptors and therefore by differences in the degree of uptake of the acceptors into the membrane, just as quenching order is affected by the uptake of quenchers. However, attempts to measure the uptake of the anthroyloxy probes into bacteriorhodopsin membranes, either by fluorescence methods or by centrifugal separation of membranes from free probe, proved unsuccessful due to technical difficulties. Nevertheless, the results are consistent with the quenching data discussed above.

We have also observed that removal of retinal from bacteriorhodopsin increases the lifetime of tryptophan approximately 3.5-fold and increases the steady-state fluorescence 8-fold indicating that a greater number of tryptophans are contributing to the fluorescence. The longer tryptophan lifetime found for the bleached membrane accounts at least in part for the more efficient quenching observed (compare Figs. 5a and 5b). When retinal is added to photobleached membranes the loss in tryptophan fluorescence is matched by a corresponding increase in the production of the photocycle intermediate M_{412} when excitation is at 280 nm (Smith and Ghiggino, unpublished results). The loss of fluorescence from the deep tryptophan residues when retinal is present implies that the retinal itself is close to the centre of the membrane as has also been suggested from studies of neutron diffraction [31], amino acid sequence [5], and photo-induced cross-linking [6].

Dynamic quenching of fluorophores in membranes is sensitive to the 'transverse fluidity gradient'. As the fluorescing and quenching moieties are moved deeper into a lipid bilayer they experience a more fluid environment and hence the bimolecular rate constant increases [12]. Quenching by a dynamic (collisional) mechanism is favoured when the solvent is of low viscosity and the fluorophore has a long lifetime, while viscous solvents and short lifetimes favour static quenching. The lifetimes of the bacteriorhodopsin tryptophans are short (< 1 ns) and difficult to measure accurately using photon counting methods. Identification of the quenching mechanism (static or dynamic) by measurement of lifetimes in the presence and absence of quencher is therefore difficult. Purple membranes are relatively 'rigid' [29] and there is little evidence that they possess a fluidity gradient [29]. Thus, it is probable that most of the quenching by the *n*-doxylstearic acid probes in bacteriorhodopsin membranes occurs by a static mechanism.

Perturbation of the membrane structure by the fatty acid probes remains a possibility in these experiments. However, we have not observed any

diminution of the photocycle activity as measured by the levels of the M_{412} intermediate even at the highest quencher concentrations used (13 mol% quencher: lipid) in this study. Thus perturbation as measured by this functional criterion is not evident.

In summary, the quenching and energy transfer experiments support the view that the fluorescing tryptophan residues in the native membrane are located near the membrane surface. Tryptophan residues deeper in the membrane transfer their fluorescence to the retinal chromophore which must itself be located deep within the membrane.

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