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INFLUENCE OF MEMBRANE LIPIDS ON THE PHOTOCHEMISTRY OF BACTERIORHODOPSIN IN THE PURPLE MEMBRANE OF *HALOBACTERIUM HALOBIUM*

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Summary

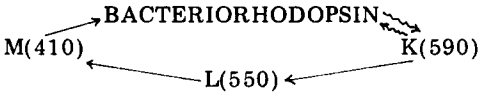
Purple membrane fragments from *Halobacterium halobium* were reconstituted with the native lipids replaced by dipalmitoyl phosphatidylcholine and by egg lecithin. In parallel studies the temperature dependence of bacteriorhodopsin phototransient lifetime and absorption dichroism and of in situ lipid microviscosity were determined; the former two by, respectively, conventional and polarization flash photometry, and the latter by observation of emission depolarization of an embedded fluorescent dye, 1,6-diphenyl-1,3,5-hexatriene. Discontinuities in lipid microviscosity profiles in native and egg lecithin purple membrane were reflected in both the photochemical cycle frequency and bacteriorhodopsin chromophore rotational mobility. The influence exerted by membrane-lipid viscosity appears to be a secondary effect, and points to the bacteriorhodopsin chromophoric group being situated in the protein interior.

Introduction

The purple membrane assembled by *Halobacterium halobium* when grown in the light under reduced oxygen tension contains a single protein, bacteriorhodopsin [1]. The sole biological function of this specialized region of the cell membrane is to serve as a proton pump, maintaining a higher pH in the cytoplasm than in the cell medium [2—5]. The influx of protons under the influence of this ion gradient is coupled energetically to ATP synthesis in the organism [5]. In this manner the light-driven pump energizes phosphorylation, and affords a means for survival for the cell during the period until conditions are restored which enable the organism to resume respiration.

On light absorption, bacteriorhodopsin enters into a photochemical cycle

involving a sequence of at least four intermediates [6—11]. The first of these, K(590) *, is produced in a photochemically reversible step. Of the three subsequent phototransients, L(550), M(410) and O(660), which are formed irreversibly in thermal steps, the former two may be fitted into an unbranched cycle:



O(660), occupying a terminal position in the cycle with M(410), is placed between the latter and bacteriorhodopsin by one group [9], but has been suggested to occupy a separate pathway by us [12]. Recent work has identified discontinuities in Arrhenius plots of the rates of formation and decay of L(550), M(410) and O(660) at about 30°C. This phenomenon may also be observed in the light cycle rates for intact cells (Sherman, W.V., unpublished results). It is intriguing to speculate on whether this discontinuity, existing at a physiological temperature for the organism, may serve any important function.

Bacteriorhodopsin constitutes 75%, by weight, of purple membrane [1] and is organized in a two-dimensional crystal lattice [13,14]. The membrane lipids are confined to interstitial regions between the protein lattice points. Chignell and Chignell, using a lipid-soluble spin label, have also identified a membrane discontinuity at 30°C [15]. However, these authors favor the view that this reflects a primary change in bacteriorhodopsin which indirectly affects the ordering of the interstitial lipid region. However, we believe that the role of membrane lipids in the photochemical cycle of bacteriorhodopsin in purple membrane is of sufficient importance to warrant further study to confirm the primary cause of the discontinuity in the photochemical cycle kinetics. In the present study we have used two synthetic purple membranes in which the native lipid has been replaced by dipalmitoyl phosphatidylcholine and by egg lecithin and relate the decay kinetics and time-dependent absorption dichroism of the terminal phototransient M(410) to in situ lipid microviscosity estimated by emission dichroism of an embedded fluorophore, 1,6-diphenyl-1,3,5-hexatriene.

Experimental

Materials. The primary source of purple membrane was *H. halobium* strain M_1 grown under low oxygen tension in the light. The preparation of native purple membrane fragments was as described previously [6]. Purple membrane fragments with the native lipid replaced by dipalmitoyl phosphatidylcholine or egg lecithin were prepared by Dr. Evert P. Bakker. Approximately 7 mg of native purple membrane was suspended in 3 ml of 150 mM KCl containing 30 mg of lipid (dipalmitoyl phosphatidylcholine or egg lecithin) and 120 mg sodium cholate. After incubating at room temperature for 1 h, the mixture was

* Phototransients are presented alphanumerically by a letter representing the species chronology in the sequence and a number indicating its optical absorption maximum in nm.

layered on a 20—60% linear sucrose gradient and centrifuged for 16 h at 100 000 × *g*. The purple band was then separated and dialyzed for 24 h versus 150 mM KCl. The product was then harvested by centrifugation, washed twice and resuspended in phosphate buffer at neutral pH. The product was shown by thin layer chromatography to be 90%+ free of native lipids.

Dipalmitoyl phosphatidylcholine (Sigma) was used as received, and egg lecithin (Sigma) was purified by column chromatography. Diphenylhexatriene (Aldrich, puriss.) was the gift of Dr. Meir Shinitzky of the Weizmann Institute.

Phototransient kinetics and dichroism. The conventional flash-photometric system incorporating a temperature-controlled sample cuvette was described previously [6,16]. Photomultiplier output was recorded by a Tektronix 564 B storage oscilloscope equipped with camera attachment. For dichroism studies, Polaroid type HNP'B polarizing filters were used. One pair of polarizers, with polarizing axes vertical, were interposed between the flash lamps and the sample cuvette. Corning 3486 530 nm cutoff filters were also placed between the flash lamps and polarizers. A third polarizer was placed between the analyzing lamp and the sample. Photomultiplier response was then recorded with the polarizer in the analyzing train successively with its axis vertical (parallel to the excitation-light polarizer) and then horizontal (perpendicular to the excitation-light polarizer). For a diagram, see ref. 17. Time-dependent sample absorbance at 410 nm, $A_{\parallel}(t)$ and $A_{\perp}(t)$, were recorded after succesive flashes with the analyzing polarizer axes respectively vertical and horizontal.

The theory of time-dependent polarized emission or absorption for a transient chromophore possessing rotational mobility has been developed by Weber [18,19], Jablonski [20] and Albrecht [21]. Using the notation of the latter for the experimental configuration used by us (i.e., plane polarized light in the vertical plane; viewing axis perpendicular to exciting axis)

$$A_{\parallel}(t) = [5/3 + (1 - \epsilon)\,\beta \exp(-t/\tau_{\mathrm{rot}})]\,P(t) \tag{1}$$

$$A_{\perp}(t) = [5/3 - 1/2(1 - \epsilon)\,\beta \exp(-t/\tau_{\mathrm{rot}})]\,P(t) \tag{2}$$

where β is a function of the optical transition probability with respect to the three orthogonal laboratory axes for light absorption by bacteriorhodopsin and the phototransient being viewed; ϵ is a randomisation factor ($0 < \epsilon < 1$) which takes into account depolarizing effects, including experimental artefacts, which are not directly due to phototransient rotation, and finally, τ_{rot} is the rotation lifetime (= reciprocal of the rate constant for rotation, θ). $P(t)$ represents the time-dependent population of the phototransient being viewed.

Defining a function $R(t) = [A_{\parallel}(t) - A_{\perp}(t)]/[A_{\parallel}(t) + 2A_{\perp}(t)]$, which is analogous to anisotropy used for fluorescence polarization, gives

$$R(t) = 3/10(1 - \epsilon)\,\beta \exp(-\theta t) \tag{3}$$

thus, absorption anisotropy, $R(t)$, is independent of the decay kinetics of the species being viewed. The only limitation that the phototransient decay kinetics imposes on measurements of anisotropy is that the viewable lifetime be sufficient for observing significant changes in $R(t)$; i.e. phototransient decay rate constant, $k \not\ll \theta$.

For a rotating sphere of radius a embedded in a homogeneous medium of

viscosity η [22]

$$\theta = 3kT/4\pi a^3\eta \tag{4}$$

where k is Boltzmann's constant and T the temperature in K. This would be a reasonable equation to apply to a globular protein molecule embedded in a lipid bilayer membrane. Significant electrostatic interaction between protein molecules (as for bacteriorhodopsin [14] see Discussion) would preclude the use of this equation for estimating the dimensions of the membrane-bound protein molecule as would, of course, protrusion outside the hydrophobic membrane interior. Purple membrane fragments are disc-like bodies [1,2]. The membrane is about 45 Å thick and the disc radius about 0.5 μm. If tumbling of purple membrane fragments in the aqueous medium, viscosity η_ω, is the controlling factor affecting time-dependent depolarization, then the best shape approximation is that of an oblate ellipsoid with axes $a = b >> c$ for which we get [22]

$$\theta = 9kT/8a^3\eta_\omega \tag{5}$$

For purple membrane fragments in water at room temperature (25°C), $\theta = 41\ s^{-1}$ and the rotation lifetime, $\tau_{rot} = 24$ ms.

Fluorescence studies with embedded diphenylhexatriene. The procedure followed for incorporating diphenylhexatriene into purple membrane was adapted from that of Shinitzky and Inbar [23]. 2 mM diphenylhexatriene in tetrahydrofuran (0.1 ml) was injected into 100 ml of rapidly stirred water. The mixture was then purged with nitrogen at 50°C for 15 min to remove tetrahydrofuran and 1 ml mixed with 1 ml of purple membrane in 0.1 M phosphate buffer ($A_{570} \approx 1$). The suspension was then diluted to 5 ml and incubated at 50°C for 30 min. Fluorescent emission spectra were determined with a Perkin-Elmer/Hitachi spectrofluorimeter. Lifetime measurements were performed by Drs. Elisha Haas and Ari Gafni of the Chemical Physics Dept., W.I.S. [24]. Fluorescent polarization measurements were performed on an instrument described by Teichberg and Shinitzky [25].

Rotational depolarization of a fluorophore is described by the Perrin equation [26]:

$$(r_0/r - 1)^{-1} = V\eta/kT\tau \tag{6}$$

where r_0 and r are, respectively, the limiting and measured fluorescence anisotropies, V the effective rotational volume of the fluorophore and τ its emission lifetime. The viscosity of virtually all fluids decreases exponentially with temperature according to the empirical equation

$$\eta = A\ \exp(E/RT) \tag{7}$$

Since it may be assumed that the product $T\tau$ on the right-hand side of Eqn. 6 is constant with temperature over the temperature range of the study, we can write Eqn. 7 in its differential form:

$$\frac{d\ \log\eta}{d(1/T)} = \frac{d\ \log(r_0/r - 1)^{-1}}{d(1/T)} = \frac{E}{2.303\ R} \tag{8}$$

Hence, a plot of $\log(r_0/r - 1)^{-1}$ versus $1/T$ will yield the activation energy for

viscous flow, E, of the medium in which the fluorophore diphenylhexatriene is embedded. Any discontinuity in the Arrhenius plot will be indicative of a melting phenomenon.

Results

In contrast to native purple membrane, in which the decay of M(410) back to bacteriorhodopsin follows first order kinetics, purple membrane incorporating dipalmitoyl phosphatidylcholine and egg lecithin did not exhibit simple first order decay. Subsequent to an initial rapid decay, which was comparable to that for native purple membrane, the synthetic membranes crossed over to a slower decay rate. In order to compare the light cycle frequency for each of the three membranes we therefore use the reciprocal of the time required to decay to half the maximum absorbance, $\tau_{1/2}$. The results are presented as an Arrhenius plot in Fig. 1. As pointed out previously, native purple membrane is characterized by a discontinuity at about 30°C. The low-temperature and high-temperature regions yield respectively $E = 18$ kcal · mol^{-1} and 9.1 kcal · mol^{-1}. Purple membrane incorporating egg lecithin lacks any clear discontinuity and gives $E = 5.1$ kcal · mol^{-1}. Purple membrane incorporating dipalmitoyl phosphatidylcholine exhibits a complex Arrhenius plot with two clear breaks centered at about 25 and 40°C. The absence of extended linear regions precludes estimation of accurate values for E, although below 20°C the latter appears to approximate that for egg lecithin membrane.

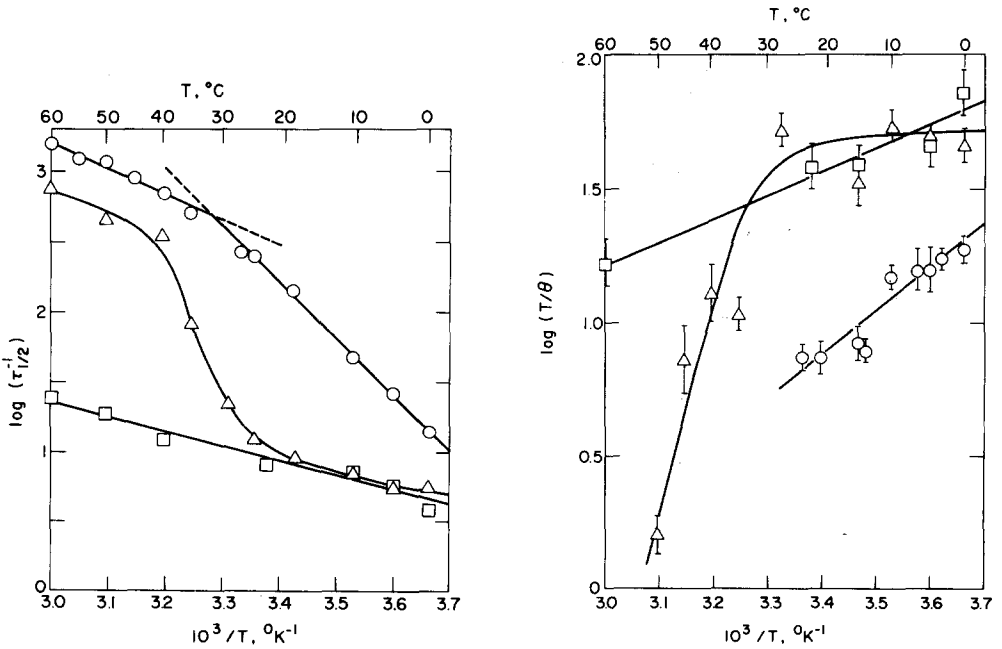


Fig. 1. Arrhenius plot of reciprocal half-lives ($\tau_{1/2}^{-1}$) for decay of M(410). ○, native membrane; △, dipalmitoyl phosphatidylcholine; □, egg lecithin.

Fig. 2. Arrhenius plot of the viscosity proportional function T/θ for dipalmitoyl phosphatidylcholine (△), egg lecithin (□) and native membrane (○).

The time dependent decay of absorption anisotropy for M(410) conformed to linear semi-log plots, as predicted by Eqns. 4 and 7, with correlation coefficients of 0.95 or better for all three purple membrane samples over the temperature ranges covered in Fig. 2. Meaningful measurements on the native purple membrane samples were not feasible above approx. 25°C owing to the short lifetimes of M(410). (Note that the activation energy for phototransient decay is 18 kcal · mol^{-1}, while for rotational relaxation it is 7.4 kcal · mol^{-1}.)

A detailed study of absorption anisotropy of native purple membrane was made by us previously [17]. We concluded that since the energy of activation differed significantly from that for medium-limited tumbling of purple membrane fragments, internal rotation within the membrane contributes to at least part of the observed kinetic depolarization. E for water viscosity calculated from data between 10 and 40°C from the Handbook of Physics and Chemistry is 4.2 kcal · mol^{-1}; that for native purple membrane is 7.4 ± 0.3 kcal · mol^{-1}. Rotational mobility of the chromophore within native purple membrane was confirmed by us by observing depolarization from samples in which membrane fragments were immobilized in polyacrylamide gels [17]. Here $E = 13 \pm 0.05$ kcal · mol^{-1}. For the synthetic membranes, Fig. 2 yields for egg lecithin $E = 4.0 \pm 0.1$ kcal · mol^{-1}; and for the quasi-linear region between 0 and 20°C for dipalmitoyl phosphatidylcholine about 0.3 kcal · mol^{-1}. Over the range for which measurements were possible on native purple membrane, the rotational mobility of the chromophore in both synthetic membranes is, in each case, significantly smaller.

The emission spectrum of diphenylhexatriene embedded in native purple membrane and the two synthetic membranes showed the same general characteristics as described previously for the membrane-bound fluorophore [23]. Lifetime measurements give a mean lifetime of 5 ns (Fig. 3), which is a little less than half that exhibited by protein-free synthetic membranes in general, 11 ns [27]. The experimental data give a best fit to two first-order decay curves with lifetimes of 7 ns and 0.2 ns. Thus, tentatively, in native purple membrane embedded diphenylhexatriene would seem to exist in two domains: one characteristic of non-proteinous liposomes, the other, while essentially characteristic of lipid, susceptible to energy transfer to protein. Interestingly, Chen et al., in a recent study, have observed two emission lifetimes (5.5 and 10.3 ns) for diphenylhexatriene embedded in protein-free dimyristoyl phosphatidylcholine vesicles [31]. Emission from diphenylhexatriene in contact with or embedded in bacteriorhodopsin is precluded owing to contact quenching that would result from overlap between the emission band of diphenylhexatriene and bacteriorhodopsin absorption.

Representative results for room temperature (25°C) are summarized in the last column of Table I. Viscosities listed for dipalmitoyl phosphatidylcholine and egg lecithin are consistent with literature values (see Table I, footnote) which supports the contention that the fluorophore is probing essentially the same environment as in experiments with protein-free synthetic liposomes. However, our lower energy of activation for fusion indicates some difference; possibly reflecting the rather small interstitial regions between the two-dimensional crystalline lattice of bacteriorhodopsin available for lipids.

The fluorescence polarization data for native and relipidated purple mem-

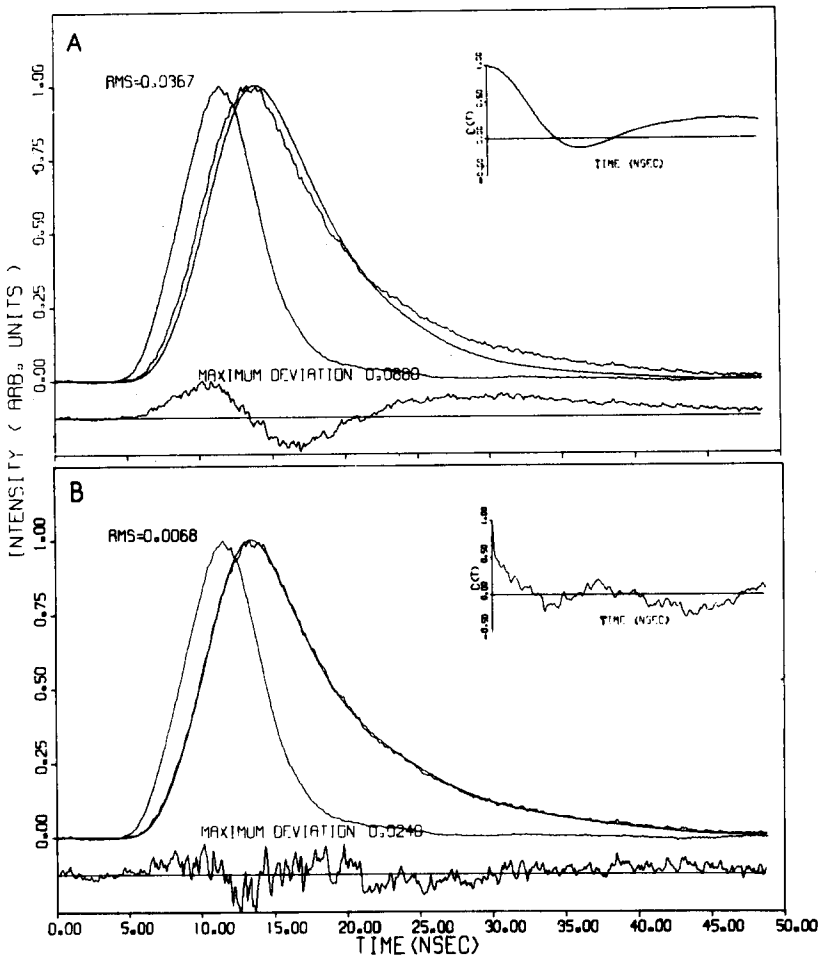


Fig. 3. Time dependence of fluorescence emission from diphenylhexatriene in native membrane. A. The lifetime is calculated by the best fit to a single exponential (τ = 4.556 ns). B. Lifetimes are calculated by the best fit to two exponentials (τ_1 = 0.169 ns, τ_2 = 7.326 ns). See ref. 24 for definition of terms.

brane fragments are presented in Fig. 4 as Arrhenius plots of the Perrin function $(r_0/r - 1)^{-1}$. The three curves exhibit the same general characteristics as those for M(410) kinetics (Fig. 1), and also, within the limitations of measurement, as those for M(410) dichroism (Fig. 2). Specifically, the discontinuity in the fluorescence results for native purple membrane appears at about 24°C, slightly lower than the kinetic discontinuity temperature, approx. 30°C. The melting range for dipalmitoyl phosphatidylcholine commences at about the same temperature for fluorescence as for M(410) kinetics (approx. 25°C) and the curves exhibit their maximum slope at almost coincident points (approx. 40° and 35°C, respectively). The scatter in M(410) dichroism results for dipalmitoyl phosphatidylcholine (Fig. 2) precludes an exact identification of the temperature for onset of the first break, but it approximates to the temperature for M(410) kinetics and fluorescence. Again, the maximum curve slope is also at a similar temperature. The close resemblance between the

TABLE I

REPRESENTATIVE RESULTS FOR DECAY KINETICS AND DICHROISM OF M(410) AND FOR FLUORESCENCE POLARIZATION OF DIPHENYLHEXATRIENE (DPH) IN NATIVE AND SYNTHETIC PURPLE MEMBRANE AT 25°C

Lipid content of purple membrane	Decay of M(410)		Dichroism of M(410)		Dichroism of DPH		
	$\tau_{1/2}$ (ms)	E (kcal · mol^{-1})	τ_{rot} (ms)	E (kcal · mol^{-1})	r	η (poise)	E (kcal · mol^{-1})
Native	4.0	18	$2.1 \cdot 10^1$	7.4	0.27	3.2 *	2.6
Dipalmitoyl phosphatidylcholine	$8.0 \cdot 10^1$	—	$1.4 \cdot 10^2$	—	0.27	3.2 **	—
Egg lecithin	$1.3 \cdot 10^2$	5.1	$7.3 \cdot 10^1$	4.0	0.18	1.1 ***	3.4 ***

* Corrected owing to recalculation of previously determined value [29].

** Compare with 10.5 poise (Cogan et al. [28] (Cogan et al. [28]);

*** Compare with η = 1.5 poise, E = 7.3 kcal · mol^{-1} (Cogan, et al. [28]); η = 0.57 poise, E = 6.9 kcal · mol^{-1} (Vanderkooi and Callis [30]); η = 0.81 poise, E = 9.3 kcal · mol^{-1} (Shinitzky and Barenholtz [27]).

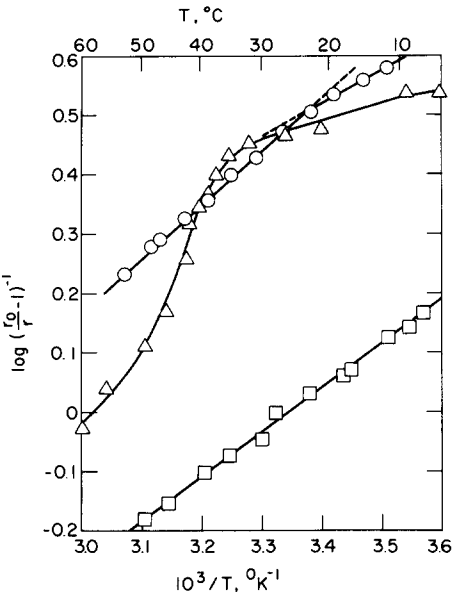


Fig. 4. Arrhenius plot of fluorescence polarization for diphenylhexatriene embedded in purple membrane. ○, Native membrane; △, dipalmitoyl phosphatidylcholine; □, egg lecithin.

Arrhenius profile of our dipalmitoyl phosphatidylcholine measurements with those for protein-free liposomes of this lipid [28] offers further confidence that these results reflect microviscosity of the membrane lipid.

Discussion

The general similarity of the temperature profiles for the photochemical cycle rate (Fig. 1) and M(410) chromophore rotational mobility (Fig. 2) with the rotational mobility of the lipid-embedded fluorophore diphenylhexatriene (Fig. 4) clearly shows that the first two phenomena are influenced by membrane lipid microviscosity. In addition to the locations of transition phenomena, there is also a general inverse correlation between both rate constants and microviscosity. While these tendencies are rather more qualitative than quantitative, this may be simply a consequence of disruption of the ordering of the lipid molecules occupying the interstitial regions of the protein crystal lattice as a result of the rather drastic alteration performed in lipid replacement. Clearly, however, the lipid molecules of purple membrane have a profound influence on the photochemical cycle kinetics and chromophore mobility. In regard to the latter, we previously presented evidence that this phenomenon does not involve rotation of whole bacteriorhodopsin molecules, but rather reflects internal conformational changes involving the retinylidene prosthetic group. The fact that over the temperature range 0—40° C the microviscosities of dipalmitoyl phosphatidylcholine and egg lecithin differ significantly, while protein rotational mobilities for synthetic purple membrane containing these lipids are similar, suggests that the influence exerted by membrane lipid is of a

secondary nature. Consequently, the bacteriorhodopsin prosthetic group is probably not in contact with the membrane lipid molecules.

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