

Influence of the Intrinsic Membrane Protein Bacteriorhodopsin on Gel-Phase Domain Topology in Two-Component Phase-Separated Bilayers

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ABSTRACT We have investigated the effect of the intrinsic membrane protein bacteriorhodopsin of *Halobacterium halobium* on the lateral organization of the lipid phase structure in the coexistence region of an equimolar mixture of dimyristoylphosphatidylcholine and distearoylphosphatidylcholine. The fluorescence recovery after photobleaching (FRAP) technique was used to monitor the diffusion of both a lipid analog (*N*-(7-nitrobenzo-2,3-diazol-4-yl)-dimyristoylphosphatidylethanolamine, NBD-DMPE) and fluorescein-labeled bacteriorhodopsin (FI-BR). In the presence of bacteriorhodopsin, the mobile fractions of the two fluorescent probes display a shift of the percolation threshold toward lower temperatures (larger gel-phase fractions), independent of the protein concentration, from 43°C (without bacteriorhodopsin) to 39°C and 41°C for NBD-DMPE and FI-BR, respectively. Moreover, in the presence of bacteriorhodopsin, the gel-phase domains are much less efficient in restricting the diffusion of both probes than they are in the absence of the protein in the two-phase coexistence region. Bacteriorhodopsin itself, however, obstructs diffusion of NBD-DMPE and FI-BR to about the same extent in the fluid phase of the two-phase region as it does in the homogeneous fluid phase. These observations suggest that 1) the protein induces the formation of much larger and/or more centrosymmetrical gel-phase domains than those formed in its absence, and 2) bacteriorhodopsin partitions almost equally between the coexisting fluid and gel phases. Although the molecular mechanisms involved are not clear, this phenomenon is fully consistent with the effect of the transmembrane peptide pOmpA of *Escherichia coli* investigated by electron spin resonance in the same lipid system.

INTRODUCTION

There is considerable evidence to suggest that many biological membranes have an in-plane domain structure and that this structure may play a role in regulating physiological functions (Thompson et al., 1995). Even though the physical origins of these domains are not clear, colocalizations of certain lipids and membrane proteins have been demonstrated in a variety of cellular systems (Luan et al., 1995; Rogers and Glaser, 1993). The lateral organization adopted by a lipid mixture in the two-phase coexistence region has been extensively studied over the past few years (Almeida et al., 1992; Schram et al., 1996; Vaz et al., 1989), but little is known about the influence of a transmembrane protein on the lipid-phase domain topology. However, intrinsic proteins, present in high proportion in biomembranes, are likely to play a role in lateral domain formation and organization, at least through nonspecific lipid-protein interactions such as the local increase in the acyl chain order at the protein/lipid interface (Jähnig, 1981; Sperotto and Mouritsen, 1991).

In the study reported here, we have investigated the influence of an intrinsic membrane protein, bacteriorhodopsin of *Halobacterium halobium*, on the phase topology in two-component two-phase phosphatidylcholine bilayers, by

using fluorescence recovery after photobleaching (FRAP). This method, which measures under the microscope the long-range (few microns) translational diffusion of membrane components, has been applied successfully to elucidate the lateral organization in heterogeneous model membranes (Vaz, 1992). Both the diffusion coefficient (*D*) and the mobile fraction (*M*) deduced from a fluorescence recovery can be related to the topology of the obstacles to diffusion (lipid gel-phase domains or intrinsic membrane proteins). The mobile fraction depends mainly on the obstacle shape and thus is a measure of the percolation threshold, the obstacle concentration at which the fluid phase becomes disconnected (Stauffer and Aharony, 1992; Vaz et al., 1989). The diffusion coefficient depends on both the size and shape of the obstacles at a given area fraction (*c*); obstacles of smaller size and of more ramified topology are the most efficient in restricting diffusion (Saxton, 1992; Schram et al., 1996).

We have measured the diffusion of a fluorescent lipid analog (*N*-(7-nitrobenzo-2,3-diazol-4-yl)-dimyristoylphosphatidylethanolamine, NBD-DMPE) and of fluorescein-labeled bacteriorhodopsin (FI-BR) in an equimolar mixture of dimyristoylphosphatidylcholine and distearoylphosphatidylcholine (DMPC/DSPC) with increasing amounts of bacteriorhodopsin incorporated. Although the presence of the protein lowers the transition enthalpy of the lipid mixture, bacteriorhodopsin does not significantly modify the onset and completion temperatures of the phase diagram of DMPC/DSPC up to a protein/lipid molar ratio of 1/100 (Piknova et al., *Biophys. J.* In press). In the absence of protein, the mobile fractions of NBD-DMPE in the equimolar mixture of DMPC/DSPC display a percolation threshold

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at 43°C. When bacteriorhodopsin is present at a protein/phosphatidylcholine (BR/PC) molar ratio varying from 1/400 to 1/100, both NBD-DMPE and FI-BR display a percolation threshold at lower temperatures, 39°C and 41°C, respectively, which is essentially independent of bacteriorhodopsin concentration. For both probes, the restriction of diffusion due to the gel-phase domains is much lower than in the absence of the protein. In fact, in the two-phase coexistence region, the obstruction effect of bacteriorhodopsin is not significantly different from that observed in a homogeneous fluid phase, for both NBD-DMPE and FI-BR probes.

These observations strongly suggest that 1) bacteriorhodopsin induces a reorganization of the gel phase toward more compact and/or more centrosymmetrical domains in a concentration-independent manner, and 2) bacteriorhodopsin partitions almost equally between the coexisting fluid and gel phases. The influence of bacteriorhodopsin on the gel-phase domain topology is fully consistent with the reported effect of the transmembrane peptide pOmpA of *Escherichia coli* investigated in the same lipid system by electron spin resonance (ESR) (Sankaram et al., 1994). This suggests that rather nonspecific lipid/transmembrane protein interactions are involved.

MATERIALS AND METHODS

Chemicals

DMPC, DSPC, and NBD-DMPE were purchased from Avanti Polar Lipids (Alabaster, AL). Bacteriorhodopsin, in the form of purple membranes from *H. halobium*, was purchased from Sigma Chemical Co. (St. Louis, MO). As judged by the absorption of the retinal ($\epsilon = 63,000 \text{ l} \cdot \text{cm}^{-1} \cdot \text{mol}^{-1}$ at 570 nm, Stoeckenius et al., 1979), the purple membranes contain 83% (weight) bacteriorhodopsin; that is, each protein is associated with approximately seven endogenous lipids (Kates et al., 1982). This is taken into account in calculating the bacteriorhodopsin area fraction, assuming a molecular area of 75 \AA^2 for each endogenous lipid molecule (Blaurock, 1975). 5-Carboxyfluorescein-succinimidyl ester was purchased from Molecular Probes (Eugene, OR).

Multilayers

Supported multilayers of an equimolar mixture of DMPC/DSPC containing various amounts of bacteriorhodopsin were prepared using the protocol of Schram and co-workers (1994). Bacteriorhodopsin was incorporated into preformed lipid vesicle suspensions with the spontaneous insertion method described by Scotto and Zakim (1988).

Chloroform solutions of DMPC, DSPC, and NBD-DMPE (at a molar ratio of phosphatidylcholine/fluorescent probe of 5/1000) were mixed and incubated for 1 h at 60°C. The chloroform was evaporated under a nitrogen stream and then for 1 h under high vacuum. After 1 ml of distilled water preheated at 60°C was added, the sample was vortexed until complete dissolution of the lipid deposit and incubated 1 h at 60°C in a thermostatted bath. The vesicle suspension was then sonicated in a bath sonicator for 15 min at 60°C, and the desired amount of bacteriorhodopsin (suspension of purple membranes in distilled water) was added. After another 30 s of sonication, the sample was incubated overnight at 60°C.

The volume of the resulting proteoliposome suspension was then reduced to approximately 20 μl by evaporating the water under 25 mmHg at 60°C, and the suspension was deposited on a microscope slide and dried for 2 h under a nitrogen stream, and then under high vacuum for 1 h.

Multilayers were rehydrated for 7 h at 60°C in a water-saturated atmosphere before adding 70 μl of a preheated buffer: NaCl (10 mM), Na_2HPO_4 (5 mM), NaH_2PO_4 (5 mM), EDTA (10^{-5} M), pH 7. The deposit was then covered with a coverslip and incubated for 1 h at 60°C, and the preparation was sealed with Teflon grease (Baxter Scientific Products, McGaw Park, IL) to prevent dehydration. Samples were kept at 60°C in a water-saturated atmosphere for 2 days before measurements, ensuring further dispersion of the bacteriorhodopsin in the supported multilayers.

Labeling of bacteriorhodopsin

5-Carboxyfluorescein-succinimidyl ester was reacted with purple membrane suspensions following the method of Heberle and Dencher (1992). Briefly, the bacteriorhodopsin was dissolved at 1 mg/ml in 0.1 M NaHCO_3 (pH 8.5), and 125 $\mu\text{l}/\text{mg}$ of protein of fluorescein-succinimidyl ester freshly dissolved in anhydrous *N,N*-dimethyl-formamide at 10 mg/ml was added. After 6 h at ambient temperature under gentle stirring, purple membranes were washed four times by centrifugation and resuspension in 0.1 M NaHCO_3 (pH 8.5), left overnight at 4°C, then washed again three times in KCl (150 mM), and resuspended in distilled water. Labeling of the purple membranes was checked by absorption ($\lambda_{\text{max}} = 495 \text{ nm}$) and fluorescence spectroscopy ($\lambda_{\text{em}} = 520 \text{ nm}$) of the fluorescein chromophore. The experimental conditions used have been reported to yield 0.7–1 fluorescein molecule bound per bacteriorhodopsin molecule, with the fluorescein forming an amide bound with the lysine 129 at the extramembrane surface of the bacteriorhodopsin (Heberle and Dencher, 1992). Note that even though a fluorescence transfer from the fluorescein to the retinal is likely to occur because of spectral overlap, we had no detection problem under the FRAP microscope when using a fluorescein-labeled bacteriorhodopsin/phosphatidylcholine (FI-BR/PC) molar ratio of 1/400.

FRAP experiments

FRAP experiments were carried out on an ACAS 470 workstation (Meridian Instruments, Okemos, MI) as previously described (Schram and Thompson, 1995). Unless indicated, each diffusion coefficient, D , and fractional recovery, M , reported is the average of 40 recoveries recorded on four different samples. The temperature of the sample was regulated by a Peltier stage unit. Multilayers were preincubated at the highest temperature of the scan for 1 h, then cooled down in steps of 3°C, allowing 30 min of equilibration at each temperature.

RESULTS

Influence of the bacteriorhodopsin on the phase transition of DMPC/DSPC mixtures

Bacteriorhodopsin of *H. halobium* is a single polypeptide of 278 amino acids, 70% of which are hydrophobic. The protein contains seven transmembrane α -helices and has very small extramembranous domains (Khorana et al., 1979). In the purple membranes, bacteriorhodopsin is organized in a two-dimensional hexagonal lattice of protein trimers, each monomer having an elliptical transmembrane footprint with an axis of $25 \times 35 \text{ \AA}$ (Henderson and Unwin, 1975). The aggregation state of the bacteriorhodopsin has been investigated in a variety of lipids (Lewis and Engelman, 1983a), and it can reasonably be assumed that in a liquid-crystalline lipid bilayer, the protein remains dispersed at protein/lipid molar ratios up to 1/100 (Gulik-Krzywicki et al., 1987; Heyn et al., 1981). In calculating the

protein area fraction, the transmembrane area of the bacteriorhodopsin approximates to a circle of radius 15 Å.

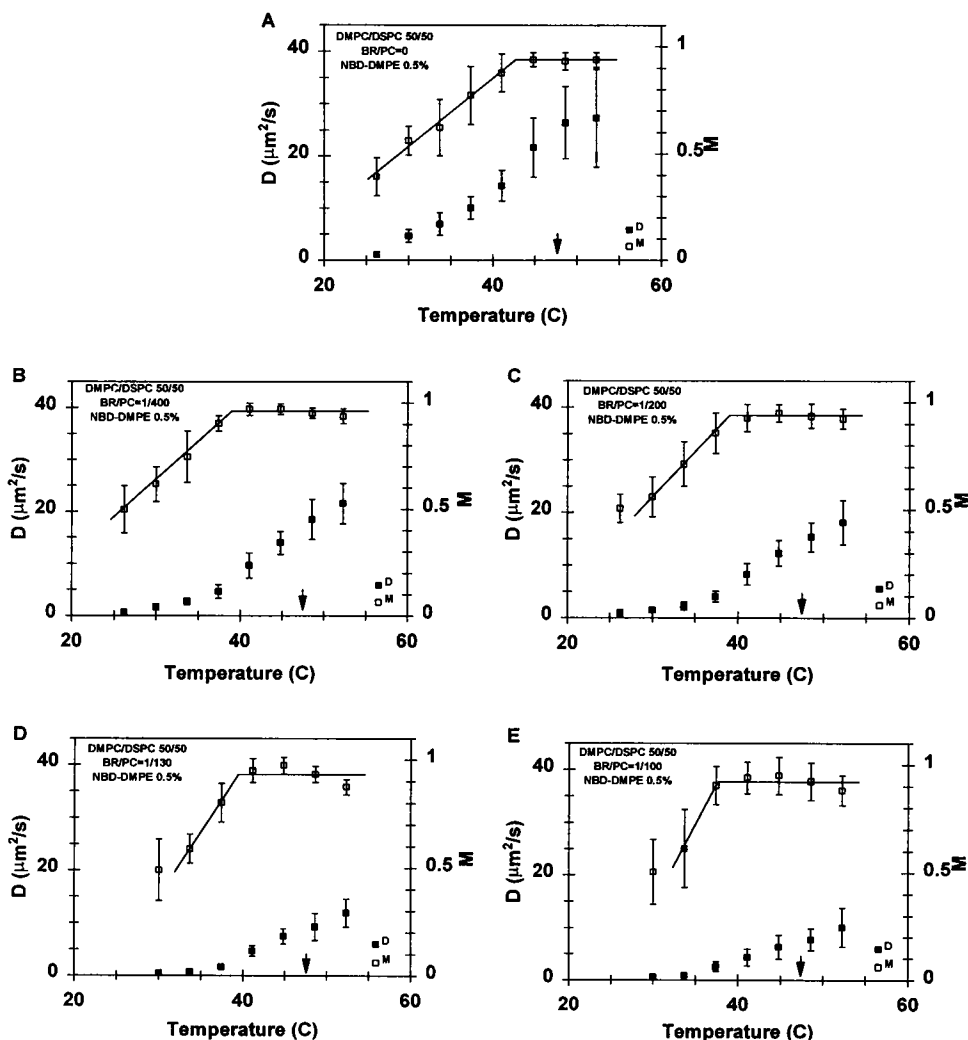
The influence of the bacteriorhodopsin on the lipid mixture DMPC/DSPC has been investigated recently by differential scanning calorimetry (Piknova, et al., *Biophys. J.* In press). At a protein/phosphatidylcholine molar ratio between 1/500 and 1/100, bacteriorhodopsin does not significantly modify the onset and completion temperatures of the phase transition of DMPC/DSPC (Piknova et al., *Biophys. J.* In press), suggesting that it does not interact specifically with either DMPC or DSPC. Note that the phase diagram of DMPC/DSPC obtained by differential scanning calorimetry (Piknova et al., *Biophys. J.* In press; van Dijck et al., 1977) is significantly different from the one deduced by small-angle neutron scattering by Knoll and co-workers (1981), particularly in the position of the solidus. To remain consistent with previous percolation studies based on the phase diagram obtained by neutron scattering (Sankaram et al., 1992, 1994; Vaz et al., 1989), we will consider only the DMPC/DSPC phase diagram obtained by Knoll and co-workers (1981). Taking into account the phase diagram obtained by differential scanning calorimetry (van Dijck et

al., 1977) increases the fraction of gel phase present at low temperatures, but does not modify the conclusions drawn in this study.

Diffusion of NBD-DMPE

The diffusion coefficients and mobile fractions of the NBD-DMPE probe obtained by FRAP in multilayers of an equimolar mixture of DMPC/DSPC incorporating various amounts of bacteriorhodopsin are plotted versus the temperature in Fig. 1. Above the fluidus line ($T = 47.4^\circ\text{C}$ for DMPC/DSPC = 50/50), the protein has a marked effect on the lipid diffusion coefficient: a molar ratio of 1/100 of bacteriorhodopsin decreases the diffusion coefficient by a factor of almost 2.5 (Fig. 1, A and E), consistent with the results obtained in DMPC (Peters and Cherry, 1982) and in egg yolk phosphatidylcholine (egg PC) (Schram et al., 1994) multilayers. The protein also has a large effect on the lipid diffusion coefficient in the two-phase coexistence region (Fig. 1, B–E), in which, because the mobile fraction remains close to 100%, even when a significant amount of

FIGURE 1 Diffusion coefficients (■) and mobile fractions (□) of NBD-DMPE obtained by FRAP in multilayers of DMPC/DSPC (50/50 molar) incorporating a molar ratio of bacteriorhodopsin to phosphatidylcholine (BR/PC) of 0 (A), 1/400 (B), 1/200 (C), 1/130 (D), and 1/100 (E). Each point is the average of 40 recoveries recorded on four samples, with the standard deviations. Continuous lines are guidelines drawn through the mobile fractions. The arrow indicates the position of the fluidus (47.4°C) for an equimolar mixture of pure DMPC/DSPC (Knoll et al., 1981).



gel phase is present, NBD-DMPE reports only the diffusion occurring in the liquid-crystalline phase (Schram et al., 1996; Vaz et al., 1989).

Following the method of Vaz and co-workers (1989), we determined a percolation point, corresponding to the beginning of a compartmentalization of the diffusion plane, as the point at which the mobile fraction starts to drop below its value in the all-fluid phase. In pure DMPC/DSPC (50/50), the percolation point is located at 43°C (Fig. 1 A), in good agreement with previous FRAP (Vaz et al., 1989) and ESR (Sankaram et al., 1992) investigations. However, when bacteriorhodopsin is present, the percolation point drops to 39°C for BR/PC = 1/400 and remains at essentially the same temperature when the protein concentration is increased, at 38°C for BR/PC = 1/100 (Fig. 2, B–E). Assuming molecular areas of 63 Å² (Lewis and Engelman, 1983b) and 45 Å² (Wiener et al., 1989) for a phosphatidylcholine molecule in the fluid and gel phases, respectively, independent of their acyl chain length, we calculated that the percolation of NBD-DMPE occurs at a gel-phase area fraction of 28% without the protein and 43% for bacteriorhodopsin concentrations ranging from 1/400 to 1/100. This shift of the percolation threshold toward a higher gel-phase area fraction corresponds to a lower efficiency of the gel-phase domains in compartmentalizing the diffusion plane.

In the two-phase coexistence region, the diffusion is restricted by two factors, the gel-phase lipid domains and the bacteriorhodopsin molecules present in the liquid crystalline phase. To separate the effect of both, the diffusion coefficients were normalized in two different ways. In Fig. 2 A, diffusion coefficients of NBD-DMPE at various BR concentrations were normalized relative to the values obtained with this probe in 1-palmitoyl-2-oleoyl-phosphatidylcholine multilayers at the same temperature (data not shown) to give the normalized diffusion coefficient, $D^* = D/D_0$, where D is the lateral diffusion coefficient and D_0 is the normalizing 1-palmitoyl-2-oleoyl-phosphatidylcholine value. In this figure, D^* is plotted versus the gel phase area fractions to show the restriction of diffusion due to the gel-phase domains. In Fig. 2 B, the same NBD-DMPE diffusion coefficients, normalized relative to the diffusion coefficients obtained in DMPC/DSPC (50/50 mol) in the absence of bacteriorhodopsin, $D(\text{BR})/D(\text{BR} = 0)$, are plotted versus the area fraction of protein. This shows the effect of bacteriorhodopsin obstruction on lipid diffusion, because the protein does not significantly modify the onset and completion temperatures of the DMPC/DSPC phase transition (Fig. 1).

Comparison of the data in Fig. 2 A with those in Fig. 2 B consistently shows that, at the same area fraction, bacteriorhodopsin is more efficient in restricting the diffusion than are the gel-phase domains (note the differences in the abscissa scale between Fig. 2 A and Fig. 2 B). In fact, the obstruction effect caused by the bacteriorhodopsin is not significantly different from that measured in a homogeneous fluid phase of egg PC (Schram et al., 1994), despite the gel-phase area fraction varying from 0 to 0.36 (Fig. 2 B,

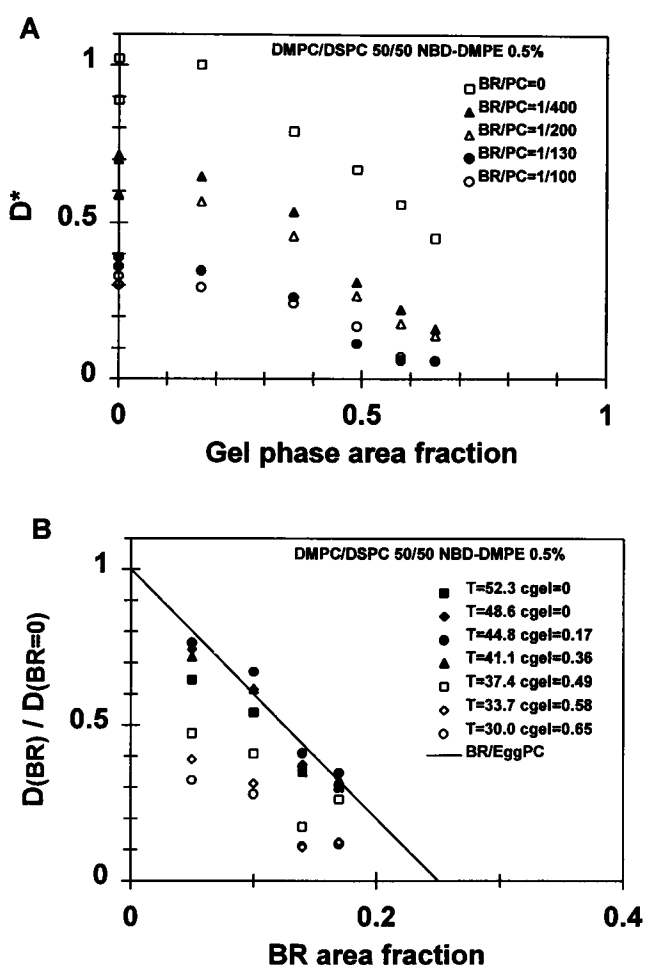


FIGURE 2 Normalized diffusion coefficients of NBD-DMPE in DMPC/DSPC (50/50 molar) multilayers incorporating various amounts of bacteriorhodopsin, plotted versus the gel phase area fraction (A) and the bacteriorhodopsin area fraction (B). See text for calculation of D^* (A) and $D(\text{BR})/D(\text{BR} = 0)$ (B). In A, the molar ratios BR/PC are BR/PC = 0 (□), BR/PC = 1/400 (▲), BR/PC = 1/200 (△), BR/PC = 1/130 (●), and BR/PC = 1/100 (○). In B, the temperatures (T) and corresponding gel phase area fractions (c_{gel}) are $T = 52.3^\circ\text{C}$, $c_{\text{gel}} = 0$ (■); $T = 48.6^\circ\text{C}$, $c_{\text{gel}} = 0$ (◆); $T = 44.8^\circ\text{C}$, $c_{\text{gel}} = 0.17$ (●); $T = 41.1^\circ\text{C}$, $c_{\text{gel}} = 0.36$ (△); $T = 37.4^\circ\text{C}$, $c_{\text{gel}} = 0.49$ (□); $T = 33.7^\circ\text{C}$, $c_{\text{gel}} = 0.58$ (◇); $T = 30.0^\circ\text{C}$, $c_{\text{gel}} = 0.65$ (○). Closed symbols in B correspond to temperatures above the percolation threshold of NBD-DMPE (39°C). The straight line in B represents the diffusion coefficients obtained for NBD-PC in egg PC/BR multilayers (Schram et al., 1994).

straight line and closed symbols). Because the protein is very efficient in restricting the diffusion coefficient of NBD-DMPE, a change in the bacteriorhodopsin concentration in the fluid phase caused by the partitioning of the protein between fluid and gel phase should be easily detected. Fig. 2 B is thus a strong indication that the actual bacteriorhodopsin concentration in the fluid phase of a phase-separated DMPC/DSPC is nearly identical to the global BR/PC ratio. This can be accounted for by an almost equal partitioning of the protein between the fluid and gel phases. It is noteworthy that the relative diffusion coefficients in Fig. 2 A display a significant decrease between the

temperature of 41.1°C and 37.4°C (*full and open symbols*), consistent with the percolation threshold temperature of 39°C deduced from the mobile fractions.

Diffusion of fluorescein-labeled bacteriorhodopsin

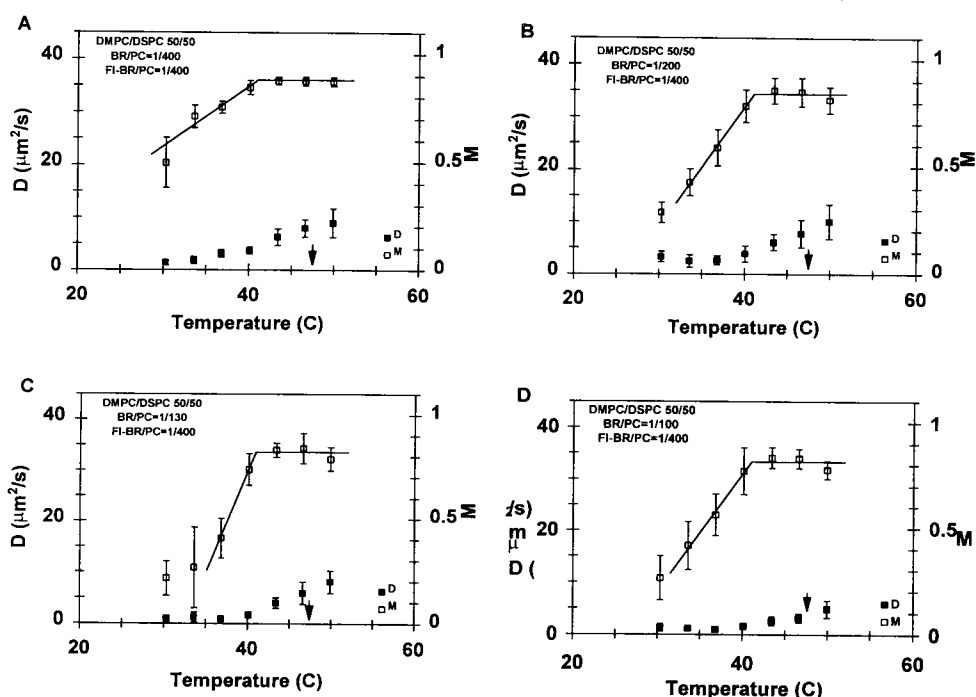
The diffusion coefficients and mobile fractions obtained with fluorescein-labeled bacteriorhodopsin (FI-BR) are plotted in Fig. 3 versus the temperature. These data were obtained with a FI-BR/PC molar ratio of 1/400, with the total BR/PC ratio varying between 1/400 and 1/100 (i.e., in Fig. 3 A, 100% of the bacteriorhodopsin present is the fluorescein-labeled protein). The mobile fractions display a percolation threshold at 41°C (corresponding to a gel-phase area fraction of 36%) almost independent of the bacteriorhodopsin area fraction (Fig. 3). The difference in the percolating gel-phase area fraction between FI-BR (36%) and NBD-DMPE (43% in the presence of BR) is probably attributable to the larger transmembrane area of the protein compared to a phosphatidylcholine analog; in an expanding two-dimensional percolating network, one expects larger molecules to be trapped before small ones. Note that even though the mobile fractions above the fluidus (*arrows in Fig. 3*) decrease slightly at higher concentrations of bacteriorhodopsin (from 0.89 at BR/PC = 1/400 to 0.81 at BR/PC = 1/100), a significant fraction of gel phase (36%) can exist without a noticeable immobilization of the labeled protein, as was observed for NBD-DMPE. This indicates that only FI-BR present in the fluid phase is detected by FRAP. As expected, the diffusion coefficients of FI-BR are much lower than those of NBD-DMPE (Figs. 1 and 3), because of the larger transmembrane area of the bacterio-

rhodopsin (Vaz et al., 1984). Increasing the concentration of bacteriorhodopsin from BR/PC = 1/400 to 1/100 restricts its own diffusion by a factor of almost 2 above the fluidus line, and in the two-phase coexistence region (Fig. 3, A and D).

To normalize the diffusion coefficients obtained with FI-BR, the diffusion of the labeled protein was measured in homogeneous fluid-phase DMPC at different temperatures. Results obtained with a FI-BR/DMPC = 1/200 are in good agreement with the data obtained by Peters and Cherry (1982) with an eosin-BR/DMPC molar ratio of 1/210 (Fig. 4). However, the increase of the diffusion coefficient between FI-BR/PC = 1/200 and 1/400 (Fig. 4) corresponds to a self-obstruction effect of the diffusion of the protein. Because of the large transmembrane area of the bacteriorhodopsin, a molar ratio FI-BR/PC = 1/400 used in the DMPC/DSPC samples corresponds to a protein area fraction of 5%, at which the self-obstruction effect already decreases the diffusion coefficient by approximately 5% (Abney et al., 1989; Pink et al., 1986). Assuming that between 0 and 10% area fraction, the self-obstructed diffusion coefficient can be approximated with a straight line, we extrapolated our data at 0% protein area fraction with a simple linear regression of the linear fits obtained on the Arrhenius plot for FI-BR/PC = 1/200 and 1/400. This value was used to normalize the diffusion coefficients of FI-BR in DMPC/DSPC in the form of $D^* = D/D_0$, where D_0 is the extrapolated diffusion coefficient at a given temperature (*dashed line in Fig. 4*).

The normalized diffusion coefficients of FI-BR in DMPC/DSPC with various amounts of bacteriorhodopsin incorporated are plotted against the gel-phase area fraction (Fig. 5 A) and the bacteriorhodopsin area fraction (Fig. 5 B).

FIGURE 3 Diffusion coefficients (■) and mobile fractions (□) of fluorescein-labeled bacteriorhodopsin (FI-BR) obtained by FRAP in multilayers of DMPC/DSPC (50/50 molar) incorporating a molar ratio of bacteriorhodopsin to phosphatidylcholine (BR/PC) of 1/400 (A), 1/200 (B), 1/130 (C), and 1/100 (D). Note that in every case, the molar ratio BR/PC includes a molar ratio FI-BR/PC of 1/400. Each point is the average of 40 recoveries recorded on four samples, with the standard deviations. Continuous lines are guidelines drawn through the mobile fractions. The arrow indicates the position of the fluidus (47.4°C) for an equimolar mixture of pure DMPC/DSPC (Knoll et al., 1981).



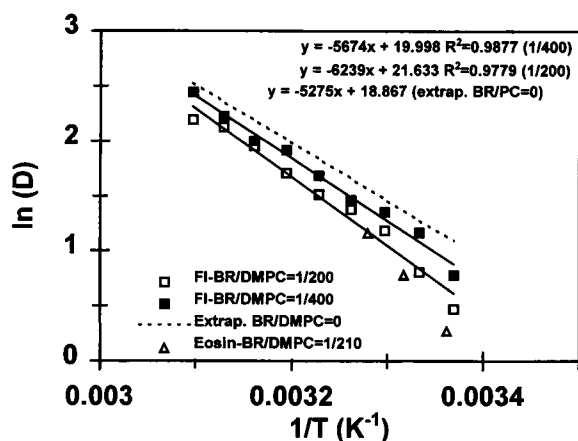


FIGURE 4 Arrhenius plots of the diffusion coefficients of fluorescein-labeled bacteriorhodopsin in DMPC multilayers with a BR/PC molar ratio of 1/200 (\square) and 1/400 (\blacksquare). Each point corresponds to the average of 20 recoveries recorded on two different samples. —, Linear regressions of the data points ($y = -5674x + 19.998$, $R^2 = 0.9877$ for BR/PC = 1/400, and $y = -6239x + 21.633$, $R^2 = 0.9779$ for BR/PC = 1/200); ---, their extrapolation at FI-BR/PC = 0 ($y = -5275x + 18.867$). \triangle , Diffusion coefficients of eosin-labeled bacteriorhodopsin in liquid-crystalline DMPC multilayers at BR/PC = 1/210 (Peters and Cherry, 1982).

This allows discrimination between the obstruction effect due to the gel-phase domains and to the bacteriorhodopsin molecules themselves, which are present in the fluid phase. Although the scattering of the data points is higher than with NBD-DMPE, the same trends are observed. The gel-phase domains are less efficient in restricting the diffusion of FI-BR than they are in restricting the diffusion of NBD-DMPE in absence of the protein (compare Figs. 5 A and 2 A), and the bacteriorhodopsin is very efficient in restricting its own diffusion (Fig. 5 B). Using the same approach as above, we extrapolated to 0% bacteriorhodopsin area fraction the diffusion coefficients of eosin-BR in DMPC obtained by Peters and Cherry (1982): the diffusion coefficients measured by these authors at temperatures above the phase transition of DMPC were plotted against the bacteriorhodopsin area fraction, and D_0 was extrapolated for every temperature (data not shown). The relative diffusion coefficients thus obtained at 28.4°C and 31.8°C plotted versus the protein area fraction fall on a common plot (*straight line* in Fig. 5 B). It is noteworthy that the self-obstruction effect of the bacteriorhodopsin in the homogeneous fluid phase extrapolated to 0% protein is almost identical to the obstruction effect of the protein on the lipid probe (compare *straight lines* in Figs. 2 B and 5 B).

As can be seen in Fig. 5 B, the diffusion coefficients of FI-BR in a homogeneous fluid phase extrapolated at 0% protein area fraction are not very different from the diffusion coefficients of FI-BR in DMPC/DSPC obtained at temperatures above the percolation threshold of the FI-BR (41°C or 36% gel phase area fraction), whereas the gel-phase area fraction varies between 0 and 0.27 (Fig. 5 B, *straight line and closed symbols*). The results obtained with the FI-BR probe are thus compatible with bacteriorhodopsin

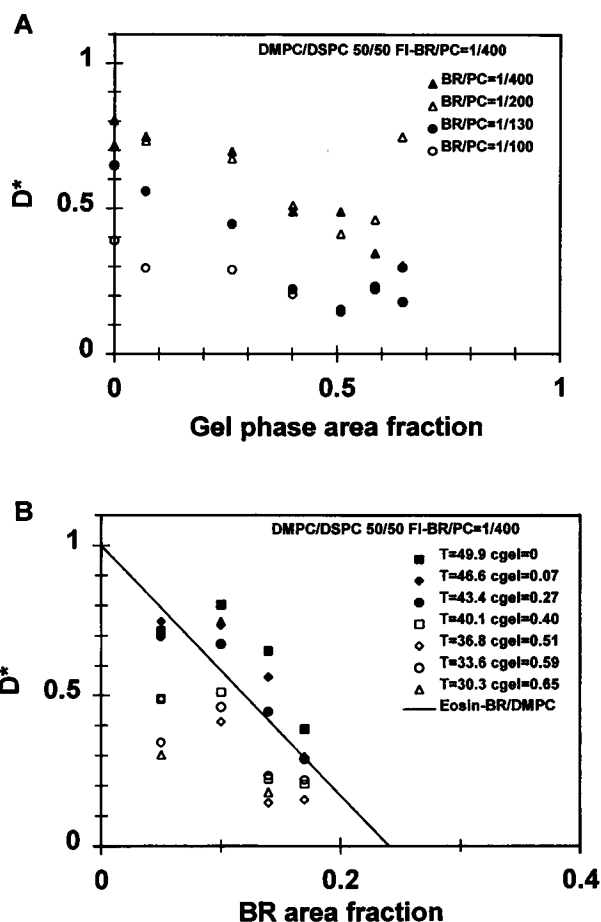


FIGURE 5 Normalized diffusion coefficients of fluorescein-labeled bacteriorhodopsin in DMPC/DSPC (50/50 molar) multilayers incorporating various amounts of bacteriorhodopsin, plotted versus the gel-phase area fraction (A) and the bacteriorhodopsin area fraction (B). See text for the calculation of D^* . In A, the molar ratios of BR/PC are BR/PC = 400 (\blacktriangle); BR/PC = 1/200 (\triangle); Br/PC = 1/130 (\bullet); and BR/PC = 1/100 (\circ). In B, the temperatures (T) and corresponding gel phase area fractions (c_{gel}) are $T = 49.9^\circ\text{C}$, $c_{gel} = 0$ (\blacksquare); $T = 46.6^\circ\text{C}$, $c_{gel} = 0.07$ (\blacklozenge); $T = 43.4^\circ\text{C}$, $c_{gel} = 0.27$ (\bullet); $T = 40.1^\circ\text{C}$, $c_{gel} = 0.40$ (\square); $T = 36.8^\circ\text{C}$, $c_{gel} = 0.51$ (\diamond); $T = 33.6^\circ\text{C}$, $c_{gel} = 0.59$ (\circ); $T = 30.3^\circ\text{C}$, $c_{gel} = 0.65$ (\triangle). Note that in every case, the molar ratio BR/PC includes a molar ratio FI-BR/PC of 1/400. Closed symbols in B correspond to temperatures above the percolation threshold of FI-BR (41°C). The straight line in B represents the diffusion coefficients obtained for eosin-labeled bacteriorhodopsin in DMPC multilayers extrapolated to 0% BR (Peters and Cherry, 1982).

partitioning almost equally between the coexisting fluid and gel phase, as already suggested by the lipid probe data. As previously observed with NBD-DMPE, the percolation threshold temperature of 41°C deduced from the mobile fractions is reflected by a significant decrease in the relative diffusion coefficients between 43.4°C and 40.1°C (Fig. 5 B, *full and open symbols*).

DISCUSSION

In this study we have investigated the effect of the intrinsic protein bacteriorhodopsin of *H. halobium* upon the gel-

phase domain topology in a phase-separated DMPC/DSPC equimolar mixture. The diffusion of both a phosphatidylcholine analog (NBD-DMPE) and a fluorescein-labeled bacteriorhodopsin (FI-BR) leads to essentially identical results: 1) Bacteriorhodopsin decreases the percolation threshold of the fluid phase from 43°C to 39°C and 41°C for NBD-DMPE and FI-BR, respectively, regardless of the protein concentration. 2) In the presence of bacteriorhodopsin, the gel-phase domains are far less efficient in restricting the diffusion than they are in the absence of the protein. 3) In the two-phase coexistence region, the obstruction effect due to the bacteriorhodopsin is not significantly different from that measured in a homogeneous fluid phase.

Note that although the percolation threshold temperatures are deduced from a fitting by eye of the average mobile fractions to two straight line segments, the standard deviations of M are fairly low (approximately $\pm 5\%$ and $\pm 10\%$ above and below the percolation threshold, respectively), and the fit is very good in almost every case (Figs. 1 and 3). Moreover, the same temperatures (39°C and 41°C for NBD-DMPE and FI-BR, respectively) are consistently found all across the bacteriorhodopsin concentration range investigated. The observed 2°C difference between the percolation point of NBD-DMPE and FI-BR is thus most likely to be meaningful, consistent with the effect of the diffusant size on the lateral diffusions deduced from Monte Carlo simulations (Saxton, 1993).

If the weak effect of the gel-phase area fraction on the diffusion coefficients of NBD-DMPE and FI-BR means that the gel-phase domains formed in the presence of bacteriorhodopsin are less efficient in restricting lateral diffusion, the shift of the percolation threshold toward lower temperatures corresponds to a lower efficiency of the gel-phase domains in compartmentalizing the diffusion plane. Both phenomena can be accounted for by a modification of the gel-phase domain topology toward larger and/or more centrosymmetrical structures; larger and more centrosymmetrical obstacles are less efficient in restricting lateral diffusion and percolate at a higher area fraction than small and ramified ones (Saxton, 1992; Schram et al., 1994, 1996; Stauffer and Aharony, 1992). For NBD-DMPE, at temperatures above the percolation threshold ($T = 39^\circ\text{C}$ or gel phase area fraction = 0.43), a maximum of 36% gel phase area fraction decreases the diffusion coefficient by an average 16% (Fig. 2). Assuming circular gel-phase domains, this corresponds to a domain size of approximately 400 lipid molecules (Schram et al., 1994). Note that for an equimolar mixture of DMPC/DSPC, FRAP data clearly suggest that the gel-phase domains are ramified to a certain extent (Schram et al., 1996); that is, they are more efficient in restricting the diffusion than are circular obstacles of the same size. The above estimate thus corresponds to a minimum size for the gel-phase domains. Given the approximations made in obtaining the data points with FI-BR and their scatter (Fig. 5 A), we did not estimate a minimum gel-phase domain size, but clearly, the weak influence of the gel-phase area fraction upon D^* also strongly suggests larger and/or more cen-

trosymmetrical domains when bacteriorhodopsin is present in the bilayers.

The other result of this study is the equipartitioning of the bacteriorhodopsin between the coexisting fluid and gel phases, implied by the agreement between the obstruction effect of the protein above the fluid phase percolation threshold in DMPC/DSPC and that observed in a homogeneous fluid phase (*closed symbols and straight lines* in Figs. 2 B and 5 B). It is noteworthy that these results could also be accounted for by a different combination of gel-phase domain topology and bacteriorhodopsin partitioning, neither of which necessarily remains constant with the gel phase and protein area fractions. In particular, ESR (Sankaram et al., 1992), fluorescence quenching (Piknova et al., 1996), and FRAP (Schram et al., 1996) investigations consistently show that the size and shape of the fluid and gel phase domains in DMPC/DSPC vary across the phase diagram. However, the combination of both effects would have to compensate to yield the agreement in Figs. 2 B and 5 B. This seems quite unlikely, especially so given the consistency of the data obtained with both the lipid (NBD-DMPE) and protein (FI-BR) probes. Note that this equipartitioning of the bacteriorhodopsin is inconsistent with the plateau value of the mobile fractions of FI-BR from 0 to 36% gel-phase area fraction (Fig. 3). Similar to the situation occurring in a pure lipid (Heyn et al., 1981), bacteriorhodopsin molecules present in the DMPC/DSPC gel phase are most probably aggregated as trimers or higher oligomers. An enhanced intermolecular quenching of the fluorescein by the retinal of bacteriorhodopsin or an autoquenching of the fluorescein group may possibly occur in the aggregated state, thus making the protein in the gel phase undetectable by FRAP.

Although general considerations of free volume and packing dictate that a protein should partition strongly into the fluid phase, proteins such as erythrocyte band 3 display a high affinity for gel-phase DMPC (Morrow et al., 1986), probably because of hydrophobic matching constraints. Microscopic models suggest that hydrophobic thickness requirements are of prime importance for the protein lateral distribution (Sperotto and Mouritsen, 1993). In the case of bacteriorhodopsin, the energetic content due to the hydrophobic thickness adjustment between the protein and the surrounding lipid bilayer must be nearly equivalent in fluid and gel phases: the hydrophobic thickness of 30 Å of bacteriorhodopsin (Lewis and Engelman, 1983a) is intermediate between the hydrophobic thickness of a DMPC-rich fluid phase and a DSPC-rich gel phase (Lewis and Engelman, 1983a,b; Piknova et al., 1993). Moreover, in a pure lipid, the absence of a shift of the lipid heat capacity curves is indicative of an identical interaction energy of the protein with both the fluid and gel phases (Heimburg and Biltonen, 1996). Although this may not be strictly true in a lipid mixture, it is noteworthy that BR does not significantly modify the DMPC/DSPC phase diagram in the concentration range investigated here.

The increase in the gel-phase domain size caused by the presence of the bacteriorhodopsin is further supported by literature data: both the transmembrane peptide pOmpA of *E. coli* (Sankaram et al., 1994) and the bacteriorhodopsin investigated by fluorescence quenching and ESR (Piknova et al., *Biophys. J.* In press) has been reported to increase the fluid-phase domain size in DMPC/DSPC mixtures. Although both studies investigated this lipid mixture at temperatures below the percolation threshold when the gel phase is continuous, one can reasonably expect that the organizing effect of the protein (increase in the lipid phase domain) will be the same above the percolation point. Indeed, the reduction of the percolation temperature from 43°C to 39°C evidenced here for NBD-DMPE closely matches that deduced from ESR data with the pOmpA peptide (38.7°C; Sankaram et al., 1994).

Although the diffusion coefficients of both the NBD-DMPE and FI-BR probes suggest an equipartitioning of the bacteriorhodopsin between the coexisting fluid and gel phases, it is surprising that the shift of the percolation threshold toward larger gel-phase area fractions seems independent of the protein concentration between 1/400 and 1/100 (Figs. 1 and 3). A possible explanation is that the modification of the gel-phase domain topology is caused by a small saturating fraction of bacteriorhodopsin molecules that accumulates at the fluid/gel boundary. Beside the conformational disorder of the interfacial lipids, hydrophobic matching considerations provide the basis for such an accumulation: the hydrophobic thickness at the gel/fluid boundary, intermediate between those of the fluid and gel phases, is the most likely to satisfy the hydrophobic matching requirements of bacteriorhodopsin (Lewis and Engelman, 1983a,b). Accumulation at the gel/fluid interface is also demonstrated by microscopic model simulations when bacteriorhodopsin is modeled in a DLPC/DSPC mixture (Mouritsen et al., 1996).

However, the actual mechanisms by which the protein modifies the gel-phase domain topology are not clear. Because of the increase in the surrounding lipid acyl chain order caused by transmembrane proteins (Almeida et al., 1992; Heyn et al., 1981; Piknova et al., 1993; Rehorek et al., 1985; Schram et al., 1994), the accumulation of bacteriorhodopsin at the lipid-phase boundary probably stabilizes the interface and should tend to decrease the domain size. On the other hand, the two new interfaces created, fluid/BR and gel/BR, should tend to reduce the gel-phase domain perimeter, hence increasing its size and/or making it more centrosymmetrical. A balance of both phenomena is probably responsible for the modification of the gel-phase domain topology. Moreover, the agreement with the effect of the pOmpA peptide (Sankaram et al., 1994) suggests that rather nonspecific lipid/protein interactions are involved.

The present study, together with other approaches (Sankaram et al., 1994; Piknova et al., manuscript submitted for publication), clearly demonstrates that a transmembrane protein or single-helix peptide strongly modifies the lateral topology of phase-separated two-component bilayers in fa-

vor of larger and/or more centrosymmetrical lipid-phase domains. When a membrane protein displays a preferential partitioning between the fluid and gel phases, the phase properties of the lipid bilayer could play a crucial role in local protein segregation, which may be of considerable importance in many biological process.

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