# Fluorescence Quenching and Electron Spin Resonance Study of Percolation in a Two-Phase Lipid Bilayer Containing Bacteriorhodopsin

Barbora Piknová,* Derek Marsh,# Thomas E. Thompson*
*Biochemistry Department, University of Virginia, Charlottesville, Virginia 22908 USA, and #Max-Planck-Institut für Biophysikalische Chemie, D-37077 Göttingen, Germany

ABSTRACT The effect of bacteriorhodopsin (BR) on the percolation properties of dimyristoylphosphatidylcholine/distearoylphosphatidylcholine bilayers was examined by studying the quenching of a lipid-bound fluorophore by a lipid-bound quencher, and by spin-spin interactions of a nitroxide-labeled lipid using electron spin resonance (ESR). At the low concentrations of BR used, differential scanning calorimetry showed that although the transition enthalpy was reduced in a concentration-dependent manner by incorporation of BR, the solidus and fluidus phase boundaries and overall shape of the heat capacity profiles were essentially unchanged. However, fluorescence quenching and spin-label ESR data showed that the domain topology, as reflected in the percolation properties, is strongly affected by the protein. In contrast to our previous fluorescence data for the pure lipid mixtures, quenching in the coexistence region is independent of the fluid phase fraction when BR is present. In addition, the percolation threshold estimated by spin-label ESR is shifted in the presence of BR to a higher gel phase fraction at a given lipid composition. Both the fluorescence quenching and spin-label ESR data, together with the results of earlier simulations, strongly suggest that the fluid phase domains are substantially larger and/or less ramified in the presence of BR than in its absence. We have previously reported a similar effect of a transmembrane peptide, pOmpA (*Escherichia coli* outer membrane protein A signal peptide), on fluid domain connectivity in binary phosphatidylcholine mixtures.

## INTRODUCTION

It is well established that the membranes of many cells are divided into permanent or transient domains of different composition. These domains may be observed by physical and chemical methods such as fluorescence digital imaging microscopy (Rodgers and Glaser, 1993; Luang et al., 1995; Pagano and Sleight, 1985), NMR (Selinsky and Yeagle, 1985), electron spin resonance (ESR) (Needham et al., 1985), study of the lipid metabolism (D'Souza et al., 1983; Josseleau-Petit and Kepes, 1982), and lipid cross-linking (de Bony et al., 1989).

Many questions concerning the origin and physiological function of domain structure are, however, still unanswered. There is some evidence that membrane proteins as well as cytoskeletal proteins play an important role in domain formation, but considerable evidence also supports a role for membrane lipids (for review see Welti and Glaser, 1994; Boggs, 1987). It has been suggested and shown theoretically that the domain structure and its topological properties (connection or disconnection) may be an important element in the regulation of membrane-localized biochemical reactions (Thompson et al., 1992, 1995; Melo et al., 1992).

In our previous work we have investigated the effect of connection and disconnection of the fluid phase on the fluorescence quenching of diphenylhexatriene-labeled phosphatidylcholine (DPH-PC) by spin-labeled phosphatidylcholine (SL-DMPC) in two-phase bilayers formed from dimyristoylphosphatidylcholine/distearoylphosphatidylcholine (DMPC/DSPC) binary mixtures (Piknová et al., 1996). The reduction of quenching observed in the systems with a disconnected fluid phase was found to be a function of the fluid phase fraction, $X_{fluid}$. This result was attributed to the heterogeneous distribution of a relatively small number of fluorophores and quenchers over the large number of small fluid-phase domains. The results of computer simulations showed that the reduction in quenching was correlated with the occupancy of the disconnected domains and, at constant phase composition, that the size of fluid-phase domains in the region of two-phase coexistence was proportional to the fluid-phase fraction, $X_{fluid}$, as shown previously for interlipid spin-spin interactions by Sankaram and co-workers (1992).

In the present work we extend our studies to a lipid-protein system. Because proteins constitute a major part of the cell membranes, it is important to understand their effect on the lipid organization in the membrane. DMPC/DSPC two-phase, two-component lipid mixtures with bacteriorhodopsin incorporated as the membrane protein are used at various BR/PC molar ratios. The influence of bacteriorhodopsin on the lipid phase transition is studied by differential scanning calorimetry (DSC), and the aggregation state of the protein is characterized by circular dichroism (CD). The DSC results show that bacteriorhodopsin does not change the macroscopic characteristics of the lipid system significantly; that is, the DMPC/DSPC phase diagram was found to be unchanged up to 1 mol% of incorporated protein.

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Address reprint requests to Dr. Thomas E. Thompson, Department of Biochemistry, University of Virginia, School of Medicine, Box 440, Charlottesville, VA 22908. Tel.: 804-924-2651; Fax: 804-924-5069; E-mail: tet@virginia.edu.



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However, the presence of even a trace amount of bacteriorhodopsin causes significant changes in the lipid domain structure, as deduced from fluorescence quenching and spin-label ESR data. The fluid phase lipid domains detected in the presence of protein are substantially larger than those in its absence. A similar result was obtained in an ESR study of interlipid spin-spin interactions in two-phase, two-component lipid mixtures incorporating a small membrane peptide, pOmpA (*Escherichia coli* outer membrane protein A signal peptide) (Sankaram et al., 1994).

## MATERIALS AND METHODS

### Chemicals

Dimyristoylphosphatidylcholine (DMPC), distearoylphosphatidylcholine (DSPC), and 1-palmitoyl-[2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl] ethyl]-carbonyl-*sn*-glycero-3-phosphocholine (DPH-PC) were purchased from Avanti Polar Lipids (Alabaster, AL) and were used without further purification. 1-Myristoyl-2-[13-(4,4-dimethyloxazolidine-*N*-oxyl)]myristoyl-*sn*-glycero-3-phosphocholine (SL-DMPC) was synthesized as previously described (Marsh and Watts, 1982). Bacteriorhodopsin (BR) was purchased from Sigma Chemical Co. (St. Louis, MO) and was used without further purification.

### Fluorescence quenching experiments

Large unilamellar vesicles made from DMPC and DSPC at DMPC/DSPC mixtures with molar ratios of 2/8, 3/7, 4/6, 1/1, 6/4, 7/3, and 8/2 were prepared by extrusion of multilamellar liposomes as described previously (Piknová et al., 1996). The required amounts of DMPC and DSPC (usually ~0.2 mg total per sample) were dissolved in 500 μl of chloroform along with fluorophore (DPH-PC, at a molar ratio DPH-PC/PC of 0.002) and quencher (SL-DMPC, at a molar ratio SL-DMPC/PC of 0, 1/12,500, 1/5000, 1/2500, 1/1250, 1/500, 1/250, 1/125, 1/50, 1/25, or 1/12). The chloroform was removed on a rotary vacuum evaporator to create a thin lipid film, which was then dried overnight under high vacuum. BR was incorporated into the liposomes using a modification of the spontaneous insertion method described by Scotto and Zakim (1988). Bacteriorhodopsin/phosphatidylcholine (BR/PC) molar ratios of 1/5000 and 1/1000 were used. To make the proteoliposome suspension, the required amount of bacteriorhodopsin solution in buffer (10 mM HEPES, 10 mM NaCl, pH 7.2, for a total volume of 4 ml) was added at 10°C above the phase transition temperature of the lipid mixture. The sample was then incubated at this temperature for 2 h to achieve complete lipid hydration. The resulting suspension of lipid multilayers was rapidly frozen in liquid nitrogen and thawed at room temperature three times, then extruded through a Multipore filter (100-nm-diameter pores). The extruder and the lipid sample were kept at 10°C above the phase transition of the lipid mixture at all times. Samples were prepared in duplicate. Liposomes were annealed for 24 h before use in experiments. At room temperature, quasielastic light scattering showed a single population of liposomes with an average diameter of 150 ± 40 nm. As reported in the Results and Discussion, the CD spectrum determined at 38°C and 55°C showed only the band characteristic of the BR monomer. No negative band, characteristic of nonincorporated purple membranes, was observed. We estimate from the purity of the Sigma bacteriorhodopsin preparation, as determined by spectroscopy and by sample weight, that ~7 moles of endogenous lipid accompanied each BR molecule. This agrees with the 8–11 lipids, reported by Scotto and Zakim (1988), for membranes prepared the same way. The lipid content was determined by the method of Bartlett (1959), and the protein concentration was determined spectroscopically, using $\epsilon_{368mn}$ = 63,000 $M^{-1}$ $cm^{-1}$ (Stoeckenius et al., 1979).

The absorbance of each sample was adjusted to be less than 0.06, to eliminate possible artifacts from light scattering and internal filter effects. All fluorescence spectra were recorded on an SLM/8000C Aminco spectrofluorimeter (Urbana, IL). The excitation wavelength was 350 nm, and emission spectra were recorded from 360 to 650 nm. Spectra were corrected for instrument response, and integral intensities were used in all cases. For each DMPC/DSPC mixture, the cooling scan started 10–15°C above the fluidus and ended at 20°C. Temperatures were changed in 2.5°C steps, with an equilibration time at each temperature of 15 min. The temperature control was accurate to ±0.1°C. The complete set of different lipid mixtures used and temperatures examined is given in Fig. 1.

### Circular dichroism

Large unilamellar vesicles formed from an equimolar mixture of DMPC/DSPC with BR/PC at a molar ratio of 1/100 were prepared by the same procedure as described above. The total amount of lipid was 10 mg per sample in a sample volume of 3 ml.

Circular dichroism spectra were recorded on a Jasco J-720 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan), using a thermostated cylindrical quartz cuvette with an optical path length of 5 mm. Spectra were recorded between 390 and 710 nm; 10 scans were accumulated to improve the signal-to-noise ratio.

### Differential scanning calorimetry

To form the multilayers, the general procedure described above for fluorescence quenching experiments was followed up to the extrusion step. The lipid concentrations were usually 10 mg per sample in a total sample volume of 1.6 ml. Samples without bacteriorhodopsin and with a BR/PC molar ratio of 1/500 were prepared for the following DMPC/DSPC molar ratios: 0, 9/1, 8/2, 7/3, 6/4, 1/1, 4/6, 3/7, 2/8, 1/9, and 1. For a BR/PC molar ratio of 1/100, only the following DMPC/DSPC molar ratios were examined: 0, 7/3, 1/1, 3/7, and 1. The lipid and protein compositions were determined as described above.

Thermograms were obtained using a Microcal MC-2 calorimeter (Microcal, Northampton, MA) at a scanning rate of 90°C/h. The baseline was determined with both cells filled with the buffer. Only heating scans were recorded, and each sample was scanned at least four times. The first scan was rejected, and the remaining scans were treated using Microcal Origin

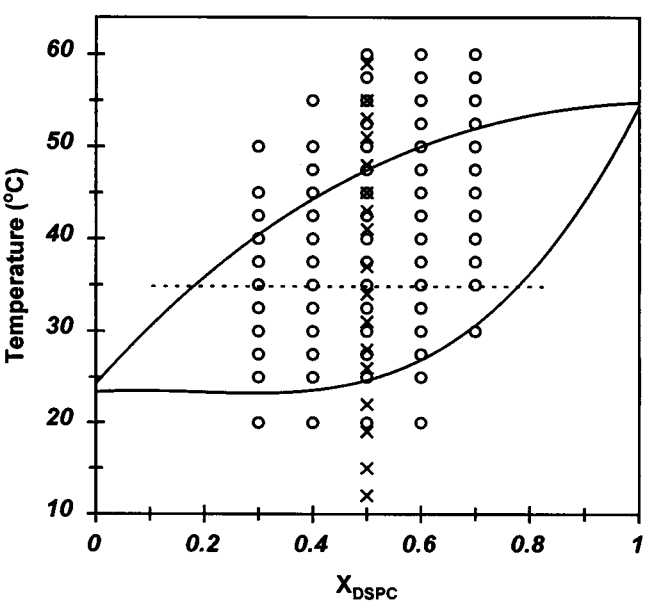


FIGURE 1 Summary of the experimental data. Each point in the phase diagram represents a point at which the fluorescence (○) or ESR (×) spectrum was recorded. Data shown in Fig. 5 *A–C* lie on the dashed line.

software to subtract the baseline and to obtain values of the enthalpy of transformation, $\Delta H_{trans}$. The temperatures of the beginning and the completion of the phase separation, $T_1$ and $T_2$, respectively, were determined as the crossing points of the tangent at mid-height and the baseline, as shown in the inset of Fig. 2. As a control experiment, bacteriorhodopsin was loaded into the measuring cell, and the thermogram was recorded between 2°C and 97°C. No transition was found in this region at scanning rates of 90°C/h or 20°C/h.

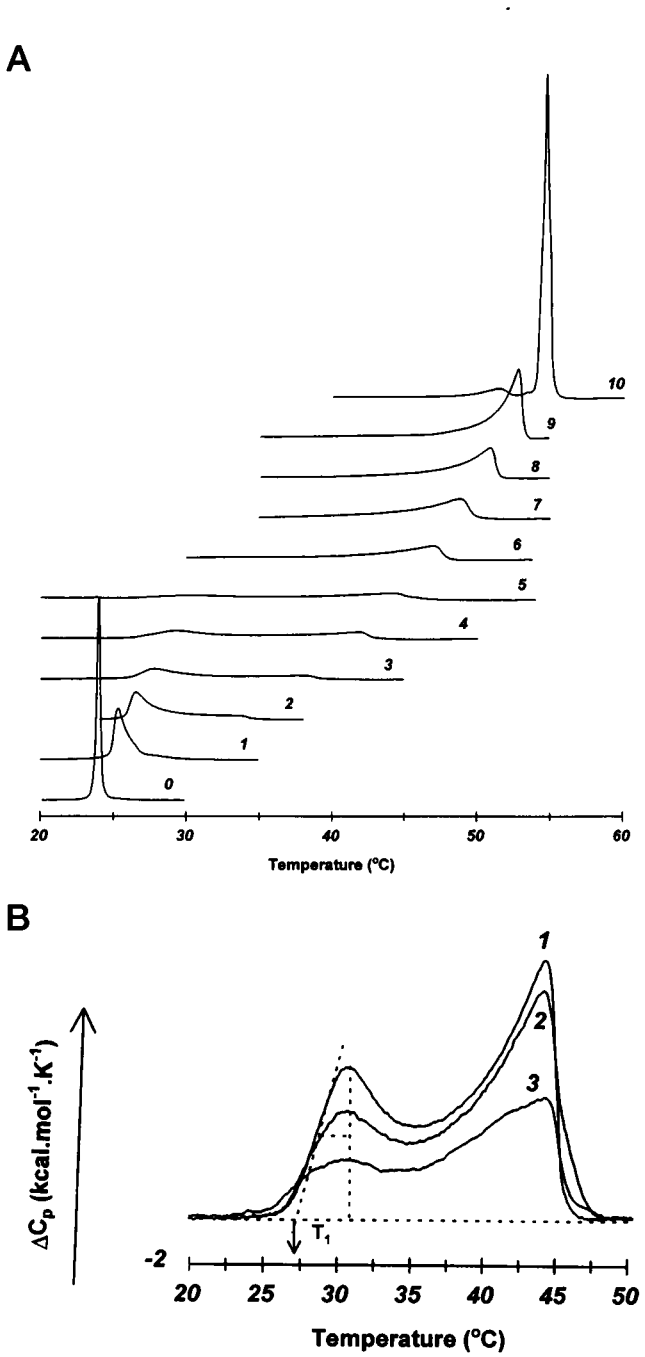


FIGURE 2 Calorimetric heating scans obtained for DMPC/DSPC binary mixtures in the presence of bacteriorhodopsin at a BR/PC molar ratio of 1/500. Composition of DMPC/DSPC mixtures (mol/mol): DMPC (*curve 0*), 9/1 (*curve 1*), 8/2 (*curve 2*), 7/3 (*curve 3*), 6/4 (*curve 4*), 5/5 (*curve 5*), 6/4 (*curve 6*), 7/3 (*curve 7*), 8/2 (*curve 8*), 9/1 (*curve 9*), DSPC (*curve 10*). (*Inset*) Heating scans obtained for an equimolar mixture of DMPC/DSPC in the absence of BR (*curve 1*) and for BR/PC molar ratios of 1/500 (*curve 2*) and 1/100 (*curve 3*). The dashed lines on curve 1 show how the phase boundaries were determined.

## Electron spin resonance

To form the multilayers, the same procedure was used as described for DSC experiments, except that spin-labeled PC was also incorporated. The total lipid amount was usually 1 mg per sample in a total sample volume of 80 $\mu$l. Equimolar mixtures of DMPC/DSPC without bacteriorhodopsin and with a BR/PC molar ratio of 1/5000 were examined. Samples with and without bacteriorhodopsin were prepared at the following SL-DMPC/total PC molar ratios: 1/5000, 1/1000, 1/200, 1/100, 1/67, 1/50, 1/40, 1/33, 1/29, 1/25, 1/20, and 1/17. The lipid and protein compositions were determined as described above. Samples were prepared in triplicate.

ESR spectra were recorded on a 9-GHz Varian Century Line series spectrometer (Varian Associates, Palo Alto, CA). The temperature of the sample was controlled by a thermostated nitrogen gas flow regulation system. The sealed 80-$\mu$l quartz capillary containing the sample was placed in a standard 5-mm NMR tube filled with silicone oil to ensure thermal stability. The temperature was recorded directly in the oil with a thin-wire thermocouple. Data were recorded during the heating scans, with a temperature step of 2°C and an equilibration time at each temperature of 15 min. The temperatures used are given in Fig. 1. The EPR data Acquisition System, version 2.41A software (P. D. Morse, II, and Scientific Software Services, Bloomington, IL), with a resolution of 0.1 G, was used for the collection of data. The peak-to-peak linewidth, $\Delta H^{pp}$, and the intensity ratio, $R$, were determined from digitized spectra, as illustrated in the inset of Fig. 6.

## RESULTS AND DISCUSSION

### Bacteriorhodopsin in DMPC/DSPC bilayers: system characterization

#### *Calorimetry experiments*

A set of thermograms for the DMPC/DSPC mixtures obtained with a BR/PC molar ratio of 1/500 is plotted in Fig. 2. The inset in this figure compares the thermograms obtained for an equimolar DMPC/DSPC mixture without protein and with BR/PC molar ratios of 1/500 and 1/100. It is clear that the presence of the protein does not significantly change the shape of thermograms. This observation suggests that bacteriorhodopsin does not interact preferentially with either lipid in the DMPC/DSPC mixture. The temperatures of the onset and completion of the lipid phase separation, $T_1$ and $T_2$, and the transformation enthalpies, $\Delta H_{trans}$, are summarized in Table 1, together with the corresponding values obtained for the pure lipid system. The values of $T_1$ and $T_2$ for the lipid systems without BR were used to construct the phase diagram plotted in Fig. 3, which is in good agreement with that obtained by DSC (van Dijck et al., 1977). For comparison, the data obtained for the protein-containing lipid mixtures at BR/PC molar ratios of 1/500 and 1/100 are plotted in the same figure. As clearly seen from the data in Fig. 3, the presence of bacteriorhodopsin up to 1 mol% does not significantly change the positions of the solidus and fluidus in the DMPC/DSPC phase diagram. This suggests that the protein molecules are distributed equally between the fluid and gel phases. A similar conclusion, based on a fluorescence recovery after photobleaching

**TABLE 1 Temperatures of onset and completion of the phase conversion ($T_1$ and $T_2$, respectively) in different DMPC/DSPC mixtures derived from the DSC experiments for systems in the absence and in the presence of the membrane protein bacteriorhodopsin**

| | DMPC/DSPC | | | BR/PC 1/500 | | | BR/PC 1/100 | | |
|---|---|---|---|---|---|---|---|---|---|
| $X_{DSPC}$ | $T_1$ (°C) | $T_2$ (°C) | $\Delta H_{trans}$ (kcal/mol) | $T_1$ (°C) | $T_2$ (°C) | $\Delta H_{trans}$ (kcal/mol) | $T_1$ (°C) | $T_2$ (°C) | $\Delta H_{trans}$ (kcal/mol) |
| 0 | 23.5 | 24.8 | 6.6 | 23.3 | 24.8 | 5.6 | 23.3 | 24.5 | 4.6 |
| 0.1 | 23.5 | 30 | 6.5 | 23 | 30 | 5.5 | | | |
| 0.2 | 23 | 34.5 | 7.07 | 23 | 35.5 | 6.1 | | | |
| 0.3 | 23 | 42 | 8.65 | 23 | 42 | 7.4 | 25 | 39 | 6.1 |
| 0.4 | 23 | 45 | 8.23 | 25.5 | 45 | 6.7 | | | |
| 0.5 | 27 | 46 | 8.63 | 26.5 | 48 | 7.4 | 25 | 46.5 | 6.1 |
| 0.6 | 27 | 51 | 8.61 | 27 | 49 | 7.3 | | | |
| 0.7 | 28 | 51.5 | 9.54 | 28 | 51.5 | 8.2 | 31 | 54 | 6.7 |
| 0.8 | 37 | 53.5 | 9.28 | 37 | 52 | 7.9 | | | |
| 0.9 | 45 | 54 | 9.96 | 45 | 53.5 | 8.5 | | | |
| 1 | 54 | 55 | 11.39 | 53.8 | 55.5 | 9.8 | 52.2 | 54.2 | 7.7 |

$\Delta H_{trans}$ is the transition enthalpy for the complete phase conversion.

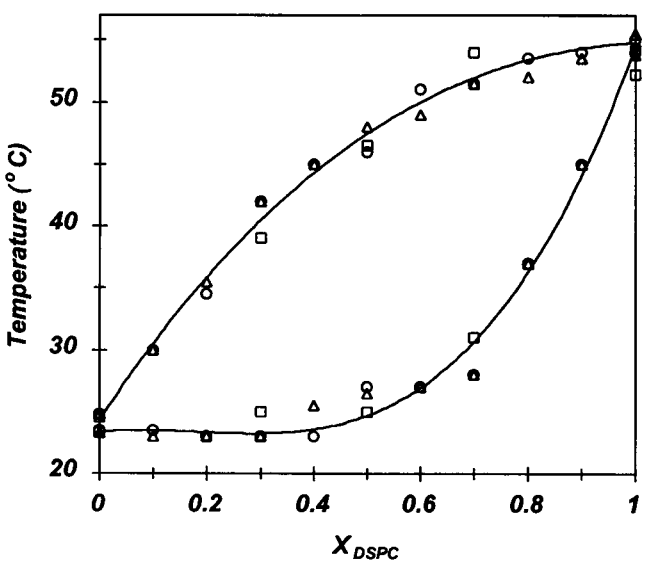


FIGURE 3 Phase diagram obtained from DSC data for DMPC/DSPC mixtures at bacteriorhodopsin/phosphatidylcholine molar ratios of 0 (○), 1/500 (△), and 1/100 (□). Continuous lines are third-order polynomial fits of the points for DMPC/DSPC mixtures without BR.

(FRAP) investigation of the same system, has recently been reported (Schram and Thompson, 1997).

We found, however, that the values of the transition enthalpy, $\Delta H_{trans}$, in the presence of protein are lower than those in lipid mixtures without protein. Assuming that the difference in the $\Delta H_{trans}$ values is due to the fraction of lipids around the bacteriorhodopsin that do not participate in the lipid phase transition, we can estimate the number of the lipid molecules perturbed by one protein to be 20–40. This is in reasonable agreement with the values of 19 and 25 lipids removed from the transition as found by DSC in 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and DMPC bilayers by Alonso and co-workers (1982).

### *Circular dichroism*

Bacteriorhodopsin molecules form trimers in the purple membranes of *Halobacterium halobium*. The trimeric form has a characteristic two-component CD spectrum centered close to the absorption maximum, with one positive and one negative band that result from the exciton coupling of the retinals contained in each of the participating protein molecules (Cassim, 1992). It has been shown that when bacteriorhodopsin molecules are dispersed as monomers in a lipid bilayer, only one broad positive peak persists in the CD spectrum (Cherry et al., 1978; Dencher et al., 1983). The large difference between the CD spectra of the two bacteriorhodopsin forms allows an estimation to be made of the aggregation state of the protein.

Fig. 4 shows the CD spectra for bacteriorhodopsin incorporated into an equimolar DMPC/DSPC mixture at a BR/PC molar ratio of 1/100 at three temperatures corresponding to the different phase states of the bilayer: 20°C (*curve 1*, gel phase), 35°C (*curve 2*, region of the coexistence of fluid and gel phases, $X_{fluid} = 0.44$), and 55°C (*curve 3*, fluid phase). All spectra show a large positive peak centered at 542–545 nm, with no negative band for the samples at 38°C and 55°C. At 20°C there is a small residual negative band at 525 nm. For comparison, the CD spectrum of purple membranes at 20°C with the same protein concentration as that used in the DMPC/DSPC liposomes is also shown in Fig. 4 (*curve 4*). The degree of aggregation can be roughly estimated by comparison of the experimental curve with curves obtained as a linear combination of spectra corresponding to the aggregated (*curve 4*) and monomeric (*curve 3*) states. Such a comparison shows that bacteriorhodopsin remains monomeric in the fluid phase, and in the fluid/gel phase coexistence region (55°C and 38°C). About 75% of the protein is monomeric in the gel phase (20°C). This is in contrast to findings in one-component lipid systems (DMPC or DPPC), in which the monomeric form of bacteriorhodopsin was found only above the phase transition (Cherry et al., 1978; Dencher et al., 1983). Pre-

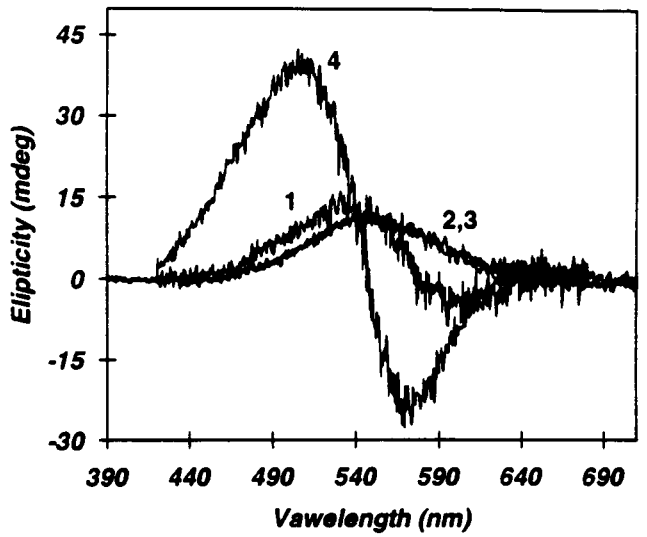


FIGURE 4 Circular dichroism spectra of bacteriorhodopsin incorporated in an equimolar DMPC/DSPC mixture at a BR/PC molar ratio of 1/100 at different temperatures: 20°C (*curve 1*), 38°C (*curve 2*), and 55°C (*curve 3*). Curve 4 is the CD spectrum of bacteriorhodopsin in purple membranes at 20°C.

sumably in the present lipid mixtures, an optimum hydrophobic match with BR is achieved, resulting in monomeric dispersion over the temperature interval studied.

## Effect of bacteriorhodopsin on the lateral organization of PC bilayers

### *Fluorescence quenching experiments*

Using DPH-PC as fluorophore and SL-DMPC as quencher, data were obtained at constant total system composition as a function of temperature for the various BR/(DMPC/DSPC) mixtures. As in the previous study (Piknová et al., 1996), analysis of the data was carried out at constant temperature because then the compositions of the coexisting gel and fluid phases are constant, with only the fractions of both phases varying. Fig. 5, *B* and *C*, shows the sets of quenching data obtained at 35°C for BR/PC molar ratios of 1/5000 and 1/1000. Qualitatively, the same behavior was observed at all of the temperatures examined. For comparison, Fig. 5 *A* shows the quenching data at 35°C in the absence of bacteriorhodopsin obtained under the same experimental conditions (Piknová et al., 1996).

Comparison of the fluorescence quenching curves at different concentrations of bacteriorhodopsin with those in the absence of protein leads to two conclusions: 1) The protein suppresses the well-defined separation of the quenching curves obtained at different fluid phase fractions, $X_{fluid}$, that was observed in the DMPC/DSPC lipid mixtures without protein (Fig. 5 *A*; Piknová et al., 1996). For a given BR/PC molar ratio at a given temperature, the individual fluorescence quenching curves obtained at different constant values of $X_{fluid}$ are very similar (Fig. 5, *B* and *C*). 2) The values

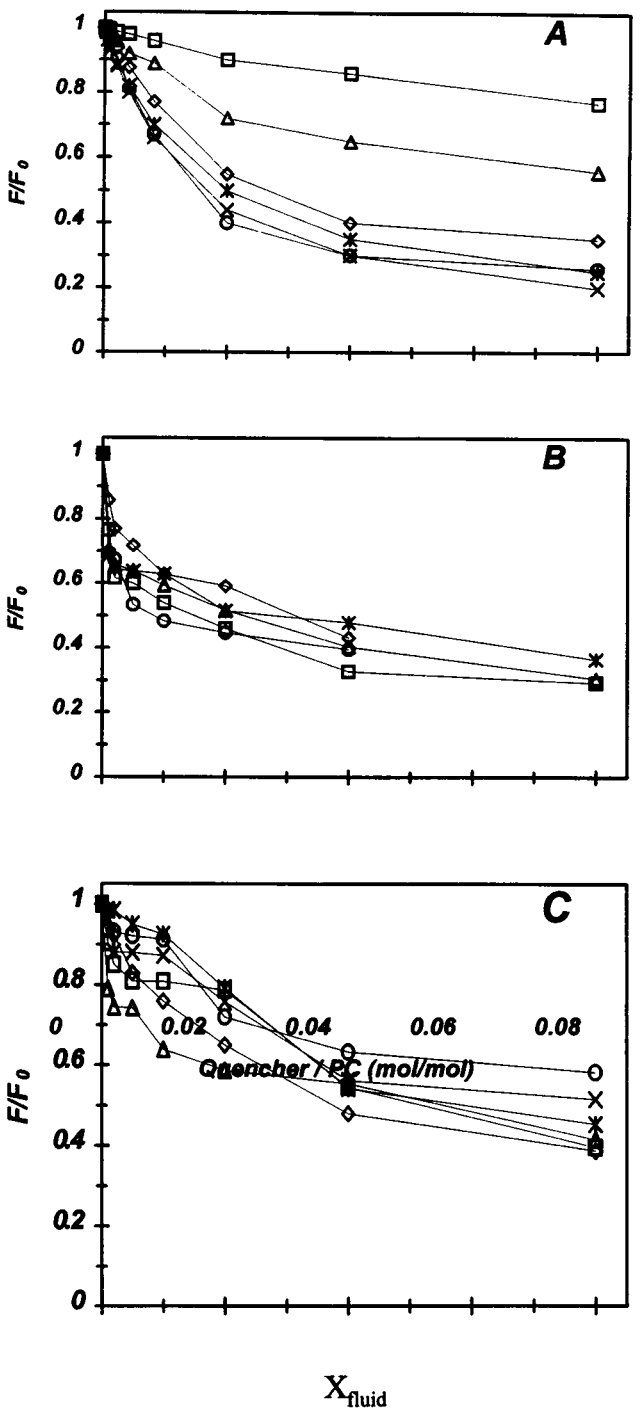


FIGURE 5 Fluorescence quenching of DPH-PC by SL-DMPC in large unilamellar DMPC/DSPC vesicles at 35°C for different fractions of the fluid phase, $X_{fluid}$. The fluorophore/total PC molar ratio was kept constant at 1/500 in all cases. $F$ is the fluorescence intensity with quencher, $F_0$ that without quencher. Bacteriorhodopsin/total PC molar ratios: 0 (*A*, replotted from Piknová et al., 1996), 1/5000 (*B*), and 1/1000 (*C*). $X_{fluid}$: 0 (*), 0.17 (□), 0.38 (△), 0.61 (×), 0.85 (◇), and 1 (○).

of the quenching efficiency, $F/F_0$, at both BR/PC ratios lie close to the range of values for $F/F_0$ found in the lipid all-gel and all-fluid systems without the protein (compare Fig. 5 *A* with Fig. 5, *B* and *C*).

The observation that the fluorescence quenching in the BR-containing bilayers does not depend on the fluid phase fraction, as it does in the lipid systems without BR, can be understood in terms of two possible effects of BR on the lipid domain topology. The first of these is an increase in the average fluid domain size; the second is an increase in the compactness (decrease in the ramifications) of the fluid domains.

Computer simulations reported in one of our earlier papers (Piknová et al., 1996) have shown that a decrease in fluorescence quenching can be caused by the inhomogeneous distribution of a small number of quencher and fluorophore molecules among compact, closed compartments. With the size of the system held constant, this decrease in quenching increases as the size of compartments decreases. Simulations at fluorophore and quencher concentrations similar to those used in this study show that the effect of compartmentalization on fluorescence quenching becomes too small to be detected when the compartment size is greater than ~500 lipid molecules (Piknová et al., 1996). Under these conditions the quencher and fluorophore molecules behave as if they were homogeneously distributed among the compartments. Thus it is possible that the absence of an effect of compartmentalization of the fluid phase on quenching in the protein-containing systems is due to the fact that BR increases the size of the individual lipid domains.

It is possible, however, that the absence of an effect on quenching is due to a decrease in the degree of the ramification of fluid domain shape. Because the fluorescence quenching we observed is static, quenching depends upon nearest-neighbor configurations on a nanosecond time scale. On this time scale, a single, highly ramified domain can be effectively subdivided by the time limits of lateral diffusion and appear in the fluorescence quenching experiments to behave as a set of smaller domains. Thus it is possible that the effect of BR is to cause the fluid domains to become, on average, less ramified, but of the same size. It is possible, of course, that BR affects both the size and shape of the fluid domains.

### *ESR experiments*

It was found previously that the positions of the percolation threshold could be estimated in DMPC/DSPC mixtures, both in the absence and in the presence of the small membrane peptide pOmpA, by using ESR spin-label linewidth measurements (Sankaram et al., 1992, 1994). Estimation of the percolation threshold was obtained from the temperature dependence of the peak-to-peak linewidth, $\Delta H^{pp}$, of one of the hyperfine manifolds in the ESR spectra of a specific spin-labeled lipid. The temperature dependence characteristically shows three clear discontinuities at temperatures corresponding to the solidus, percolation threshold, and fluidus in the phase diagram. Discontinuities observed at the solidus and fluidus arise from the sensitivity of the ESR linewidths to rotational motion, whereas the change at the percolation threshold corresponds, at least partially, to the effects of compartmentation on the interlipid spin-spin interactions. Additionally, a diagnostic intensity ratio, $R$, was defined that reflected the sensitivity of the ESR lineshapes to compartmentation and domain size (Sankaram et al., 1992, 1994).

ESR spectra obtained from the equimolar DMPC/DSPC mixture in the absence (*left column*) and in presence (*right column*) of bacteriorhodopsin at a BR/PC molar ratio of 1/5000 are given in Fig. 6 for six different temperatures of measurement. The way in which the peak-to-peak linewidth, $\Delta H^{pp}$, and diagnostic intensity ratio, $R$, were determined from the spectra of the SL-DMPC spin label is illustrated in the inset of Fig. 6. The complete set of temperatures at which the ESR spectra were recorded is given in Fig. 1. The temperatures chosen in Fig. 6 correspond to

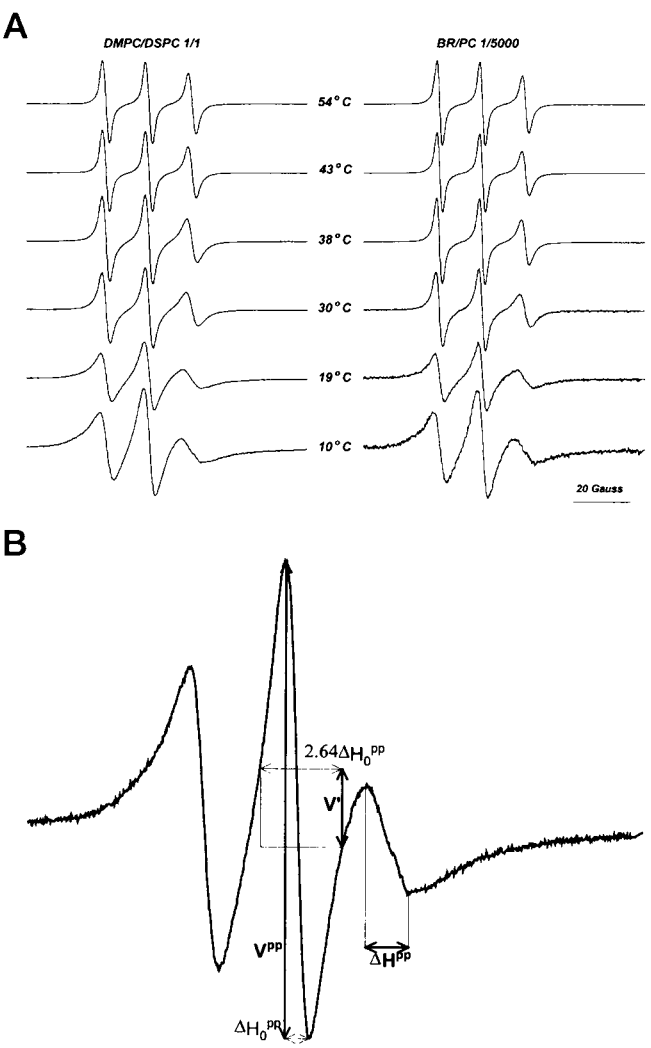


FIGURE 6 ESR spectra recorded at different temperatures for an equimolar DMPC/DSPC mixture in the absence (*left column*) and in the presence (*right column*) of bacteriorhodopsin at a BR/PC molar ratio of 1/5000. The SL-DMPC/PC molar ratio was 1/100 in all cases. (*Inset*) Determination of values of the peak-to-peak linewidth, $\Delta H^{pp}$, of the high-field ESR line and the intensity ratio, $R = V'/2V^{pp}$, of the central ESR line.

the gel phase (10°C and 17°C), to the coexistence of the gel and fluid phases (30°C, 38°C, 43°C), and to the fluid phase (54°C), according to the boundaries in the phase diagram determined by DSC (Fig. 3). From comparison of the ESR spectra recorded for the gel or fluid phases in the presence and absence of bacteriorhodopsin, it appears that the protein does not affect the overall shape of the ESR spectra appreciably in the single-phase, homogeneous systems. Even if there are 20–40 boundary lipids per protein (cf. above), their fractional population is almost negligible at the low bacteriorhodopsin concentrations that are used here. Consequently, the intensity of this protein-induced, motionally restricted component in the ESR spectrum (Marsh, 1985) is well below the detection level. The major differences between the ESR spectra in the presence and absence of bacteriorhodopsin therefore can be attributed to changes in the phase structure of the bulk lipid caused by the presence of the protein.

The influence of bacteriorhodopsin on the shape of the ESR spectrum is seen qualitatively in the data recorded at 38°C (Fig. 6). The equimolar DMPC/DSPC mixture in the absence of bacteriorhodopsin is in the two-phase coexistence region at 38°C, and the fluid phase is disconnected, as demonstrated by both FRAP (Vaz et al., 1989) and spin-label ESR experiments (Sankaram et al., 1992). As seen in the ESR spectra measured at 38°C, there is still broadening in the outer wings of the spectrum recorded in the absence of bacteriorhodopsin, whereas the ESR spectrum recorded in the presence of bacteriorhodopsin is very similar to that recorded for the fluid phase. This clearly suggests that bacteriorhodopsin affects the domain topology in the two-phase regions.

The temperature dependence of the high-field linewidth, $\Delta H^{PP}$, is given in Fig. 7 for the equimolar DMPC/DSPC mixture in the absence and in the presence of bacteriorhodopsin at a BR/PC molar ratio of 1/5000. From this figure it is seen that the discontinuity corresponding to the percolation threshold occurs at 41.5°C in the absence of protein. This discontinuity, less well defined upon incorporation of bacteriorhodopsin, is shifted to a somewhat lower temperature of ~38°C. Such a shift in the percolation threshold to lower temperatures was also observed for the pOmpA peptide in mixed lipid membranes by Sankaram and co-workers (1994).

The dependence on spin-label concentration of the diagnostic intensity ratio, $R$, for the central hyperfine line in the ESR spectra of SL-DMPC in an equimolar DMPC/DSPC mixture with a BR/PC ratio of 1/5000 mol/mol is given in Fig. 8. Temperatures at which the spectra were recorded correspond to the gel (23°C), fluid (55°C), and coexistence (35°C, 45°C) regions. At low spin-label concentration, the ratios are relatively low, close to the value of $R_G = 0.067$ expected for a pure Gaussian lineshape (Bales, 1989). Comparison with the considerably higher values obtained previously for DMPC/DSPC mixtures without protein (Sankaram et al., 1992) suggests that the incorporation of BR leads

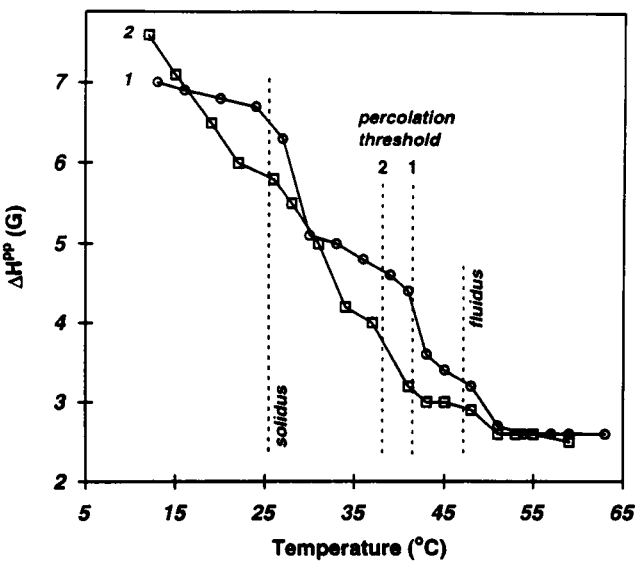


FIGURE 7 Temperature dependence of the peak-to-peak linewidth, $\Delta H^{PP}$, determined for the high-field ESR line in an equimolar DMPC/DSPC mixture in the absence (○, *curve 1*) and in the presence (□, *curve 2*) of bacteriorhodopsin at a BR/PC molar ratio of 1/5000. The SL-DMPC/PC molar ratio was 1/100. Vertical dashed lines represent the solidus, percolation threshold, and fluidus determined from ESR data according to the method used by Sankaram and co-workers (1992, 1994). The values of $\Delta H^{PP}$ were determined as illustrated in Fig. 6.

to considerable inhomogeneous broadening of the ESR spectra.

In the single-phase regions (either gel or fluid), increasing spin label concentration causes a progressive Lorentzian broadening of the lineshape, which increases the value of

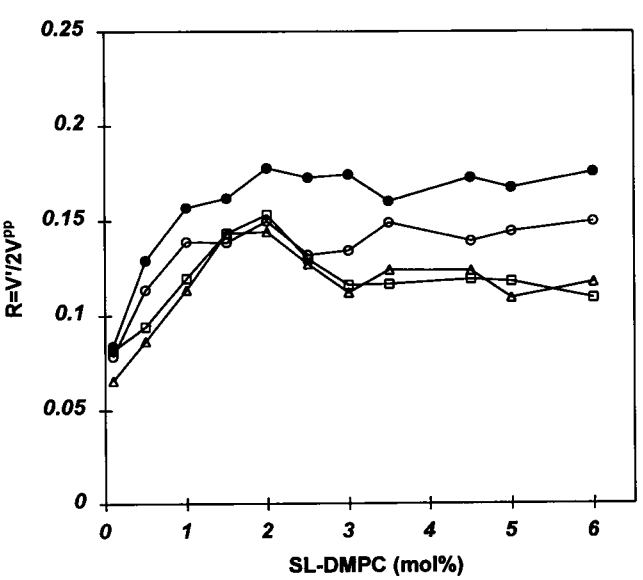


FIGURE 8 Dependence of the intensity ratio $R = V'/2V^{PP}$ of the central ESR line on the concentration of SL-DMPC for an equimolar DMPC/DSPC mixture with a BR/PC molar ratio of 1/5000 at different temperatures: 23°C (●), 35°C (○), 45°C ([trio), and 55°C (□). The intensity ratio was determined as shown in Fig. 6.

the intensity ratio (Bales, 1989; see Fig. 8). The limiting values achieved at high concentration are, however, less than that expected for a pure Lorentzian line, $R_L = 0.2125$ (see Bales, 1989), possibly because the original inhomogeneous broadening is not of true Gaussian character, but rather corresponds to the superposition of a small unresolved protein-interacting spectral component (cf. above). In the two-phase region, the dependence of the intensity ratio $R$ on spin label concentration is similar to that in the single fluid phase. There is little evidence for a well-defined maximum in the concentration dependence that is found in these lipid mixtures without protein and is characteristic of the presence of small confined lipid domains (cf. Sankaram et al., 1992). This similarity of behavior in the single and two-phase regions strongly resembles that observed in the fluorescence quenching experiments for samples containing BR (compare Figs. 5 and 8). It is also consistent with recent spectral simulations which suggest that the maximum in the spin-label concentration dependence of $R$ will be too small to be detected, with the present precision of measurement, for domains that contain more than 500 lipid molecules (Sankaram and Marsh, 1996). The spin-label ESR measurements, like the fluorescence quenching experiments, therefore indicate that incorporation of BR increases the size and/or decreases the ramifications of the fluid domains in DMPC/DSPC mixtures, and concomitantly lowers the fluid lipid fraction at which connectivity of these domains is achieved.

In a recent FRAP study, the fluid fraction at which connectivity occurs in DMPC/DSPC bilayers was found to be similarly decreased by the inclusion of BR at low concentrations in the bilayers. In this study it was concluded, in addition, that BR partitions equally between gel and fluid phases and that its effect is to increase the size and/or decrease the ramifications of the gel phase domains that hinder partial diffusion of the fluorescent probe confined to the fluid phase (Schram and Thompson, 1997).

## CONCLUSIONS

Both fluorescence quenching and spin-label ESR experiments reported here, as well as recent FRAP studies (Schram and Thompson, 1997), indicate that extremely low mole fractions of the integral protein bacteriorhodopsin have a very pronounced effect on the lipid domain formation in DMPC/DSPC mixed lipid membranes. This apparent long-range effect of the protein is undoubtedly related to the high intrinsic cooperativity of the lipid system in a region of lateral phase separation. Both the size of the fluid lipid domains and the mole fraction of gel phase lipid at which their connectivity is achieved are increased by the incorporation of bacteriorhodopsin. Whether this protein-linked property has the effect of poising biological membranes at a position close to that corresponding to lipid domain connectivity, with its attendant functional consequences (Thompson et al., 1992), will depend on the intrinsic tendency to phase separation of the component protein-free lipids. Whereas the protein-induced effects characterized here for bacteriorhodopsin may prove to be a typical property of transmembrane proteins (cf. Sankaram et al., 1994), two other possible scenarios are worthy of attention. First, any proteins that tend to partition preferentially into lipid gel phases (cf. Heimburg and Marsh, 1996) would be likely to have effects opposite those found here for bacteriorhodopsin. Second, proteins that might partition preferentially into interphase regions—presumably those with single transmembrane helical segments, such as the growth hormone receptors—may have diverse and wide-ranging effects on the shape of lipid domains and their connectivity. For these reasons, both the lipid and protein compositions of the specific biological membrane milieu are likely to be of critical importance.

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