J. Mol. Biol. (1983) **166**, 203–210

LETTERS TO THE EDITOR

Bacteriorhodopsin Remains Dispersed in Fluid Phospholipid Bilayers Over a Wide Range of Bilayer Thicknesses

We have used vesicles made from delipidated bacteriorhodopsin and synthetic lecithins to address the following questions. If the transmembrane dimension of a protein hydrophobic surface differs from the equilibrium thickness of its lipid bilayer environment, will protein monomers aggregate to decrease the protein–lipid contact surface area? If so, how large must the difference be to induce aggregation? Using lecithins with acyl chains from di-10 : 0 to di-24 : 1, the thickness of the bilayer hydrocarbon region above the lipid phase transition temperature (t_m) was varied from 14·5 Å less than to 7·5 Å more than the transmembrane dimension of the bacteriorhodopsin hydrophobic region. Bacteriorhodopsin remains dispersed when the surrounding bilayer hydrophobic region is 4 Å thicker or 10 Å thinner than the bacteriorhodopsin hydrophobic surface. Only the thin- (10 : 0) and thick- (24 : 1) bilayer samples showed any bacteriorhodopsin aggregation above t_m. Thus a surprisingly large difference between protein and lipid hydrophobic thicknesses can be accommodated without protein aggregation. The lipid bilayer can evidently sustain large local distortions with a small change in free energy.

Many functions of biological membranes are performed in specialized regions that contain subsets of the protein and/or lipid species within a single membrane; the mechanisms of formation and maintenance of such differentiated regions are poorly understood. Although it is generally recognized that hydrophobic interactions are key factors in membrane stability, their possible role in influencing the planar organization of membrane components has not been examined thoroughly. In particular, imperfect matching of protein and lipid bilayer surfaces may result in aggregation of transmembrane proteins and thus minimize exposure of hydrophobic surfaces to polar environments (Israelachvili, 1977).

We have explored the effect of lipid bilayer thickness on the planar organization of a transmembrane protein, using a fully defined model system in which the hydrophobic thicknesses of both protein and lipid are known. Reconstituted vesicles were made from delipidated bacteriorhodopsin, for which the structure is known to 7 Å resolution (Henderson & Unwin, 1975); and diacyl phosphatidylcholines of several acyl chain lengths, for which the thickness of the bilayer has been determined by X-ray diffraction (Lewis & Engelman, 1983). The planar organization of bacteriorhodopsin in such vesicles was determined by freeze-fracture studies of vesicles that were rapidly frozen from a defined temperature above the lipid phase transition temperature (t_m). The finding that bacteriorhodopsin is monomeric above t_m in vesicles containing excess di-14 : 0 or di-16 : 0 phosphatidylcholine (Cherry *et al.*, 1978) indicates that, although bacteriorhodopsin can crystallize under appropriate conditions, it does not do so

0022-2836/83/140203-08 $03.00/0

spontaneously in all environments. In addition, complete removal of the native purple membrane lipids from bacteriorhodopsin is possible (Huang *et al.*, 1980), so that a pure model system containing only a specific lipid and bacteriorhodopsin can be made.

The best fit of the amino acid sequence of bacteriorhodopsin into seven relatively hydrophobic transmembrane segments has been determined (Engelman *et al.*, 1981). The two shortest hydrophobic stretches are each 20 residues long; for alpha helices, the rise per residue is 1·5 Å, giving a transmembrane dimension of 30 Å. X-ray experiments reported by Blaurock (1975) showed that the extracted lipids from *Halobacterium halobium* membranes form bilayers with a polar group separation of about 40 Å, again implying a hydrophobic thickness of about 30 Å. By varying the length of the phosphatidylcholine acyl chains, the equilibrium thickness of the hydrophobic region of the phosphatidylcholine bilayer was varied from 15·5 to 37·5 Å, considerably thinner and thicker, respectively, than the bacteriorhodopsin hydrophobic surface. For present purposes, the bilayer hydrophobic region is defined to begin at C-2 of the phosphatidylcholine acyl chains; since the two chains are staggered (Hauser *et al.*, 1981), we use the average of the two C-2 positions.

H. halobium, strain S9, was grown on a defined medium (Engelman & Zaccai, 1980) and purple membranes were isolated from washed cells by the methods of Oesterhelt & Stoeckenius (1971). Bacteriorhodopsin was separated from its native lipid by the method of Huang *et al.* (1980). The protein concentration of the bacteriorhodopsin fractions was estimated by the Lowry method, modified by the addition of sodium deoxycholate (Dunn & Maddy, 1976). The long-chain phosphatidylcholines di-erucic (di-22 : 1, *cis*-13) and di-nervonic (di-24 : 1, *cis*-15) were generous gifts from Drs Martin Caffrey and Gerald Feigenson (Caffrey & Feigenson, 1981). All other phosphatidylcholines were from Calbiochem. Unsaturated lipids were kept in an inert gas atmosphere until the dialysis step.

The phosphatidylcholines were lyophilized and, for all except di-24 : 1, 2% (w/v) sodium cholate in either buffer K (10 mM-Tris·HCl (pH 8), 150 mM-NaCl, 0·025% (w/v) NaN_3 or in 150 mM-KCl (pH 8) was added. The mixture was sonicated in a bath sonicator for a few seconds at a temperature above the lipid t_m value. Since di-24 : 1 phosphatidylcholine was apparently insoluble in 2% (w/v) sodium cholate, this lipid was hydrated in buffer K and sonicated at 30 to 35°C with a Branson probe sonicator to produce small vesicles. Under dim red light, the lipid and bacteriorhodopsin solutions were mixed and then dialyzed in a foil-covered flask at 4°C for at least three days, with two buffer changes per day. After three days, less than 0·2 to 1% of the original deoxycholate (measured using ^{14}C-labeled deoxycholate) remained; this represents 0·4 to 1·6 deoxycholate molecules per 100 phosphatidylcholine molecules.

The vesicles were centrifuged through continuous, 10% to 45% (w/w) sucrose gradients. Most preparations yielded a single band, except those made with di-10 : 0 or di-24 : 1 phosphatidylcholine, which gave several sharp bands or one very broad band. The vesicle bands were washed several times in 0·1 M-sodium acetate (pH 5) and were then ready for use, except for the samples made with di-10 : 0 phosphatidylcholine. These samples were sonicated at room temperature for one

to three minutes, to break up large multilamellar vesicles, and were then washed or dialyzed further.

The vesicles were spray-frozen by the method of Bachman & Schmitt (1971) using a Balzers Spray-Freeze Apparatus. The spray-gun was adapted for thermal control by cementing copper tubing around the outside, through which water was circulated. Fracturing and replication were performed in a Balzers freeze-etch unit at temperatures of -115 to -135°C and pressures of 10^{-6} to 8×10^{-6} kPa. Deposition of platinum and carbon from electron beam electrodes was controlled by a quartz crystal monitor to 25 and 200 Å, respectively.

The reconstituted vesicles had diameters ranging up to several micrometres. Most consisted of one or a few layers, except for those made with di-10 : 0 phosphatidylcholine, which consisted of many tightly packed layers. To obtain more clearly interpretable fracture faces, the di-10 : 0 phosphatidylcholine samples were sonicated to break up the tightly layered structures. The resulting vesicles were heterogeneous in size and tended to clump. Both convex and concave fracture faces in all preparations exhibited similar densities of intramembranous particles, indicating random, symmetric orientation of bacteriorhodopsin across the bilayer. In the buffer used for freeze-fracture, 0·1 M-sodium acetate (pH 5), all samples were purple. In buffer K (pH 8), a variation in color among the preparations was observed, showing a decreasing absorption maximum with decreasing lipid chain length (Table 1).

The intramembranous particle of the thin-bilayer sample, di-10 : 0 phosphatidylcholine, were extensively aggregated (Fig. 1(a)), and only fracture faces with very low or very high particle densities were observed. All vesicles made with di-12 : 0, di-14 : 0, di-16 : 0, di-18 : 0 and di-22 : 1 phosphatidylcholine had dispersed intramembranous particles, as shown in representative electron micrographs in Figure 1(b) to (e). The thickest bilayer sample, di-24 : 1, yielded fracture faces with a variety of intramembranous particle distributions ranging from apparently dispersed, through slightly aggregated, to occasional ordered

TABLE 1

Absorption maxima of bacteriorhodopsin-containing vesicles

	pH5		pH8	
Acyl chain	Below t_m	Above t_m	Below t_m	Above t_m
10 : 0	n.d.†	550‡	n.d.	495
12 : 0	n.d.	545	n.d.	495
14 : 0	555	555 (35)	490	495 (35)
16 : 0	555	550 (50)	550	505 (50)
18 : 0	555	540 (60)	555	525 (60)
22 : 1	n.d.	550	n.d.	n.d.
24 : 1	550	n.d.	n.d.	n.d.

All values are ±10 nm. Samples were kept in room light immediately before measurements were made.

† Not determined.

‡ All measurements were performed at 15 to 20°C, except where noted in parentheses.

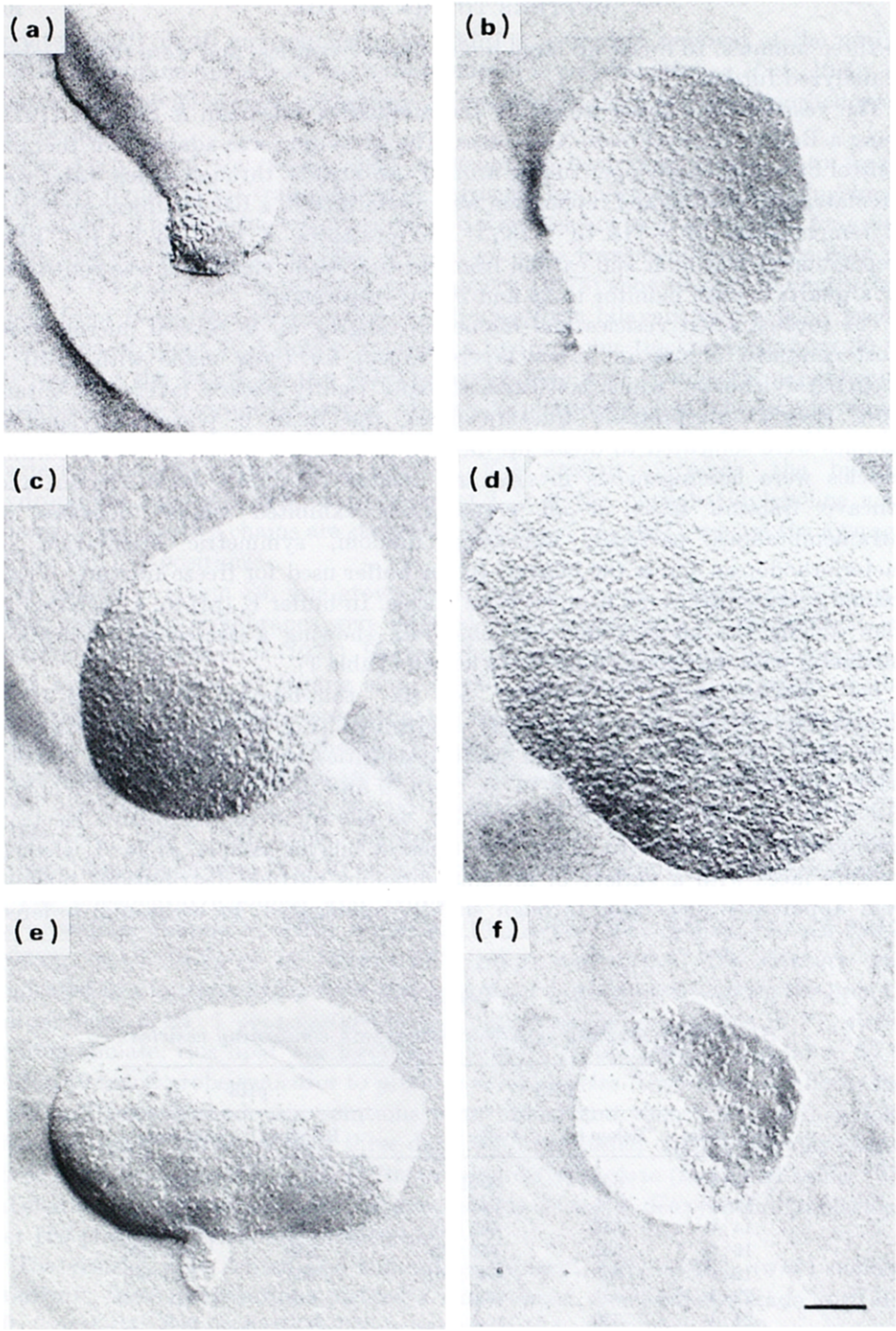

FIG. 1. Bacteriorhodopsin in phosphatidylcholine vesicles above the lipid t_m value, in 0·1 M-sodium acetate (pH 5). BR, bacteriorhodopsin; PC, phosphatidylcholine: (a) Br-di-10 : 0 PC, 15°C; (b) BR-di-12 : 0 PC, 22°C; (c) BR-di-14 : 0 PC, 34°C; (d) BR-di-18 : 0 PC, 66°C; (e) BR-di-22 : 1 PC, 35°C; (f) Br-di-24 : 1 PC, 45°C; kept at 45°C for 5 h before spray-freezing. The bar represents 100 nm.

arrays. The example shown in Figure 1(f) was obtained after five hours of incubation above t_m, which should certainly be adequate for thermal equilibration and protein diffusion in a fluid bilayer, and a similar range of distributions was obtained after 20 minutes of equilibration time. These results are summarized in Table 2. For all samples examined, no differences in intramembranous particle organization were observed at pH 8 (buffer K) or in buffer K containing 1·0 M-NaCl.

Thus, the basic result of this work is that bacteriorhodopsin remains dispersed in phosphatidylcholine bilayers with hydrophobic thicknesses ranging from 19·5 to 34·5 Å, a range from 10 Å thinner to 4 Å thicker than the protein hydrophobic surface. Since the free energy of exposure of hydrophobic surface to water is substantial (Kauzmann, 1959), this lack of protein aggregation suggests that adaptive lipid or protein conformational changes occur with relative ease within this range of mismatch. These results are consistent with the ability of organisms such as *Acholeplasma laidlawii* to grow even when large variations in the acyl chain composition of their membrane lipids are made (McElhaney & Tourtelotte, 1969; Silvius & McElhaney, 1978). Furthermore, the observation that bacteriorhodopsin does aggregate at bilayer thicknesses of 15·5 and 37·5 Å provides information on the limits of this adaptability and stimulates discussion of possible molecular mechanisms of adaptation to mismatch.

A protein structural change large enough to alter its hydrophobic thickness by several ångström units seems unlikely, since it would require stretching or compression of the transmembrane helices, while deformation of the lipids at the protein boundary may occur more readily. For clarity, the two directions of bilayer deformation, thickening and thinning, will be considered separately. For the first case, Parsegian *et al.* (1979) and Lis *et al.* (1982) have estimated the work of lateral compression of phosphatidylcholine bilayers to be less than 2 kT per

TABLE 2

Intramembranous particle distribution above lipid phase transition temperature

Acyl chain	Lipid t_m (°C)	Temperature (°C)	HC thickness (Å)†	Lipid : protein		Distribution
				Weight	Molar	
10 : 0	0	15	15·5	6·0	267	Aggregated
12 : 0	−1·8‡	22	19·5	5·1	207	Dispersed
14 : 0	23‡	34	23	7·5	278	Dispersed
16 : 0	41·4§	50	26	5·8	200	Dispersed
18 : 0	54·9§	60	29·5	6·0	200	Dispersed
22 : 1	11‖	35	34	14·1	400	Dispersed
24 : 1	24‖	45	37·5	10–20	300–500	Dispersed, aggregated and ordered arrays

† Data on the thickness of bilayer hydrocarbon regions is from Lewis & Engelman (1983), accompanying letter.

‡ From Van Dijck *et al.* (1976).

§ From Mabrey & Sturtevant (1976).

‖ From Caffrey & Feigenson (1981).

molecule for a decrease in molecular surface area of 25%, which increases the bilayer thickness by 33%. Thickening of a bilayer requires extension of the acyl chains, with loss of conformational entropy and, to accommodate the decrease in surface area, the head groups may tilt. Such tilting occurs in the $L\beta'$ phase and in phospholipid crystals (Hauser *et al.*, 1981), and the energy required for these conformational changes should be quite small until the limit of full extension of the acyl chains is reached. It is remarkably consistent with these suggestions that bacteriorhodopsin aggregation was observed in di-10 : 0 phosphatidylcholine bilayers, for which the maximum hydrophobic thickness with all-*trans* chains is about 26 Å (Lewis, 1981), 4 Å shorter than the bacteriorhodopsin hydrophobic surface, while aggregation did not occur in the di-12 : 0 phosphatidylcholine vesicles, with a maximum thickness of 31 Å.

In the second case, bilayer thinning increases the molecular surface area, which may not be accommodated so easily by conformational changes of the head group. The fact that stretching planar lipid bilayers along the bilayer plane results in membrane breakage with a 2 to 3% area increase (Kwok & Evans, 1981) indicates that, in this direction, bilayer adaptation may not occur as readily. Again, the present observations are consistent with this suggestion: bacteriorhodopsin aggregation in thick bilayers occurred at a much smaller degree of mismatch, 7 Å, than in thin bilayers, 14 Å. Further experiments are necessary both to measure the extent of lipid (and/or protein) deformation in these mismatched bilayers and to test the suggestion that bilayer thickening occurs with a lower free energy cost than does bilayer thinning.

Two further points merit brief discussion. First, it should be noted that the present investigation is structural rather than functional; the lack of bacteriorhodopsin aggregation over a wide range of bilayer thicknesses does not indicate whether the protein remains active within the same range. In fact, the blue shift with decreasing chain length at pH 8, where bacteriorhodopsin should have a significant net charge, may indicate some protein perturbation in thin bilayers. For the calcium ATPase from sarcoplasmic reticulum, optimal activity occurs over a fairly narrow range of bilayer thicknesses (Caffrey & Feigenson, 1981; Johannsson *et al.*, 1981; Moore *et al.*, 1981). In the case of gramicidin in solvent-containing black lipid membranes, the activity was found to decrease as the bilayer thickness was increased (Haydon & Hladky, 1972).

Finally, it is useful to compare the present results with those obtained for another transmembrane protein, rhodopsin. Chen & Hubbell (1973) found by freeze-fracture that in its native dark-adapted state, rhodopsin remained dispersed in vesicles made with di-10 : 0 phosphatidylcholine, while it aggregated in di-18 : 1-*trans* phosphatidylcholine vesicles, both above the lipid t_m value. Assuming that the thickness of di-18 : 1-*trans* phosphatidylcholine bilayers is similar to that of di-18 : 0 phosphatidylcholine, these results suggest that the hydrophobic surface of dark-adapted rhodopsin is at least 5 Å thinner than that of bacteriorhodopsin. In addition, Kusumi & Hyde (1982) have used saturation transfer electron spin resonance to study rotational diffusion of rhodopsin in phosphatidylcholine bilayers. Their results, which suggest that the hydrophobic thickness of rhodopsin is best matched by di-14 : 0 and di-16 : 0 phosphatidyl-

choline above t_m, are also consistent with a hydrophobic thickness of about 25 Å for rhodopsin. Since the native rod outer segment membrane has a high content of long-chain lipids (Stone *et al.*, 1979), protein aggregation in a thick bilayer may well be physiologically important in this case. However, these two studies differ in the degree of rhodopsin aggregation observed in thinner and thicker bilayers, and further studies on both rhodopsin and bacteriorhodopsin will be necessary for a complete comparison.

In summary, since variations in lipid bilayer thickness over a range of 15 Å have no detectable effect on the planar organization of bacteriorhodopsin, it is likely that hydrophobic mismatch is not a significant factor in the planar organization of many biological membranes. However, there may be specific proteins, such as rhodopsin, for which thickness effects are, in fact, important, and no generalizations can be made until data are available for more proteins. From the bilayer thicknesses at which aggregation does occur for bacteriorhodopsin and for rhodopsin, we would predict that thickness effects are more likely to be significant for thin proteins in thick lipid bilayers than for thick proteins in thin bilayers.

We thank Drs Martin Caffrey and Gerald Feigenson for generous gifts of di-22 : 1 and di-24 : 1 phosphatidylcholine. We are also grateful to Drs James Wade and George Palade for use of their Balzers Freeze-Etch devices, to Dr John Flory for use of the electron microscope, and to Dr Richard Henderson for helpful discussions concerning the manuscript. This research was supported by the National Institute of General Medical Sciences (grants GM22778 and GM29243 to D.M.E.).

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Received 27 January 1982, and in revised form 16 December 1982

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Edited by A. Klug