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The effect of temperature and protein content on the dispersive properties of bacteriorhodopsin from *H.halobium* in reconstituted DMPC complexes free of endogenous purple membrane lipids: A freeze-fracture electron microscopy study

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Ten reconstituted bilayer complexes of bacteriorhodopsin from *Halobacterium halobium* and 1,2-dimyristoyl-*sn*-glycerol-3-phosphocholine with protein/lipid mole ratios of between 1:1440 and 1:67, have been produced entirely free (less than 0.02%) of endogenous purple membrane phospholipid as judged by ^{31}P-NMR methods using Triton X-100 and cholate as detergents for solubilization of the protein and reconstitution by detergent dialysis. The nitroxide spin-label Tempo has been employed to determine the bilayer gel to liquid-crystalline phase transition temperature of the reconstituted complexes which was shown to broaden with increasing protein content, but remain centered at 23–24°C. Freeze-fracture electron micrographs of the recombinants showed that the arrangement of the protein particles depended upon the temperature from which the complexes were quenched for study, the protein content of the complexes as well as the rate of freezing. In recombinants quenched from below the bilayer phase transition temperature, protein particles were restricted to areas in the bilayers of low structural order at dilute protein content but randomly dispersed at high protein content, regardless of the rate of sample freezing. When quenched from above the bilayer phase transition temperature, complexes quenched at a faster rate of freezing produced randomly dispersed particles whilst a slower rate of freezing produced areas devoid of protein particles. No indication of the characteristic hexagonal packing of bacteriorhodopsin molecules, as reported for the purple membrane (Blaurock, A.E. and Stoeckenius, W. (1971) Nature, 233, 152–155) or reconstituted complexes still containing endogenous purple membrane phospholipids (Cherry, R.J. et al. (1978) J. Mol. Biol. 121, 282–298), were observed. The results are interpreted in terms of a lipid-mediated promotion for bacteriorhodopsin association into the hexagonal lattice, possibly through association with the negatively charged lipids of the purple membrane at the bilayer surface.

Introduction

Mechanisms which control the association of integral membrane proteins are not well understood but they most probably involve, to some degree at least, specific molecular interactions which take place at the membrane bilayer surface [1,2]. Such associations between these kinds of proteins, which in the case of one particular protein, bacteriorhodopsin from *Halobacterium halobium*, leads to a highly organized arrangement of the protein into a characteristic lattice [3], may occur either as a result of lipid–lipid, lipid–protein or protein–protein interactions. Although in the case of bacteriorhodopsin the aggregation seems not to have any functional significance since the protein still acts as a proton pump as the monomer [8], other proteins do associate, for example within electron transport complexes or in cell capping and patching. Studies of how integral proteins do interact with each other within the

Abbreviations: bR, bacteriorhodopsin; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; T_c, phospholipid bilayer gel to liquid-crystalline transition temperature; T_q, temperature from which a protein-lipid complex was quenched for freeze-fracture electron microscopy; DPhPGP, 2,3-di-*O*-phytanyl-*sn*-glycero-1-phosphoryl-3′-*sn*-glycerol-1′-phosphate; MDP, methylene diphosphonate; Tempo, 2,2,6,6-tetramethylpiperidine-*N*-oxyl.

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plane of the membrane are therefore instructive, especially when these can lead to suggestions about how the cell can trigger or manipulate such association through metabolic or biosynthetic means.

The three-dimensional structure of the integral membrane protein bacteriorhodopsin (bR) from the purple membrane of *H. halobium* is known in some detail [5]. Each monomer contains even closely packed α-helical segments which extend roughly perpendicular to the plane of the membrane and is closely associated with two other identical monomers to form a regular trimeric unit in the plane of the membrane. Bacteriorhodopsin is thus an extreme example of a protein that self-associates within the plane of the bilayer to form specialized paracrystalline patches of trimers arranged with hexagonal symmetry in the purple membrane [3]. Whilst bacteriorhodopsin is exceptional, there are several examples of integral membrane proteins that form domains by lateral segregation within the plane of the membrane. Gap junction complexes are formed by association of a particular group of integral membrane protein units (connexons) with the exclusion of other proteins [6]. Similarly, at the post-synaptic junction, individual acetylcholine receptor molecules are self-associated [7]. How such proteins overcome the randomising effect of lateral diffusion in the fluid mosaic membrane and form domains depends on a variety of mechanisms. Some interact with peripheral membrane proteins, whilst others can interact with cytoskeletal components [4].

Evidence has been presented to show that the only components required to form hexagonal arrays of bacteriorhodopsin in the purple membrane are the endogenous purple membrane lipids and the protein molecules [8]. Here, Triton X-100 solubilized purple membrane, that is, micelles containing monomeric bacteriorhodopsin and endogenous lipids, reformed the hexagonal two-dimensional structures after removal of the detergent by dialysis to form protein-lipid bilayer complexes. Various factors may be responsible for the association properties of bR. For example, the negatively charged headgroups of the bacterial membrane may order the monomers into hexagonal crystalline aggregates through electrostatic interactions. Alternatively, bacteriorhodopsin may be forced into aggregates because it is relatively insoluble in the negatively charged lipids of the purple membrane, especially at the very high protein/lipid mole ratio of about 10:1 of the purple membrane [9]. One factor which could be responsible for the insolubility of the protein in a bilayer could be imperfect matching of the hydrophobic surface of the protein to the bilayer core provided by the lipid acyl chains. In order to minimize exposure of the protein surface to the lipid, the protein particles may be forced to aggregate in the plane of the membrane [10]. Alternatively, both factors (or even others) may influence the association of integral proteins in general in bilayer membranes under different circumstances.

Freeze-fracture electron microscopy has been used to study such aggregation behaviour for bR in exogenous phospholipid bilayers produced by a variety of methods [8,11–13]. Protein aggregation appears to depend upon the lipid composition of bR-lipid bilayers in which either up to 90% of the endogenous purple membrane lipids were removed or all endogenous lipids were retained [11]. The exogenous lipids used for vesicle formation have included azolectin and soybean phosphatidylcholine, which have heterogeneous acyl chains. Bacteriorhodopsin, reconstituted into bilayers of the well defined homogeneous lipid, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), but with all the endogenous purple membrane phospholipids still retained, has been studied by electron microscopy, X-ray diffraction and circular dichroism [8].

To date, only one freeze-fracture electron microscopic study of bR in phosphatidylcholine bilayers, free of endogenous purple membrane lipids, has been reported [13]. In that study, it was shown that the protein remains dispersed above the bilayer lipid phase transition temperature (T_c) in phosphatidylcholine bilayers of a variety of acyl chain lengths including dilauroyl (C_{12}), dimyristoyl (C_{14}), dipalmitoyl (C_{16}) and distearoyl (C_{18}). Only in didecanoyl (C_{10}) phosphatidylcholine bilayers was the bacteriorhodopsin found to be extensively aggregated. However, only one lipid/protein ratio and one temperature (above the phase transition for each lipid) was examined for each chain-length lipid. For the bR-DMPC complex, the ratio studied was 278:1 (mol/mol) and the complex was quenched from 34°C.

In this present study, bR-DMPC complexes with a wide variety of protein-lipid ratios have been studied by freeze-fracture electron microscopy, ^{31}P-NMR and spin-label methods. ^{31}P-NMR has been used to show that the solubilized complexes were totally free (< 0.02%) of endogenous purple membrane phospholipids and the nitroxide spin-label Tempo employed to determine the bilayer gel to liquid-crystalline phase transition temperature (T_c). Using freeze-fracture electron microscopy, the effect of quenching dispersions from both above and below T_c of the complex is reported, as well as the solubility of bR in the complete absence of endogenous purple membrane lipids. In addition, the rate of freezing of the complexes is found to have significant effects on the protein distribution as visualized in the micrographs of complexes quenched from above T_c, with significant reordering of the protein into patches taking place when the complexes were quenched at a slower (approx. 10^4 K·s^{-1}) rate rather than more quickly (approx. 10^5 K·s^{-1}). These results are compared with the solubility of bR in bilayers of DMPC in the presence of endogenous purple membrane lipids

[4,8]. The results reported here suggest that the solubility of bacteriorhodopsin molecules in DMPC is more complicated than described earlier [8,13]. Protein solubility appears to depend upon lipid/protein ratio, the presence or absence of endogenous lipids, as well as the physical state of the bilayer acyl chains which implies an involvement of the highly charged phospholipids characteristic of the purple membrane, in determining the aggregation behaviour of bacteriorhodopsin.

Materials and Methods

Purification of purple membrane. Purple membrane was isolated from cultures of *Halobaterium halobium* [14]. Each five litre culture yielded approximately 100 mg of bacteriorhodopsin as determined by absorbance at 560 nm and protein determination. Purple membrane was separated from any remaining red membrane on a 30–50% sucrose density gradient (100000 × *g*; 4°C; 17 h). The spectral ratio at 280 and 560 nm, A_{280}/A_{560}, of purified purple membrane was typically 2.

Reconstitution methods. Bacteriorhodopsin/DMPC complexes in which all the endogenous purple membrane lipids have been removed, were produced by detergent solubilization and reconstitution [15] with some modifications, in particular to facilitate the more efficient removal of detergent during dialysis. A purple membrane pellet, containing between 20 and 60 mg of bR as judged from the A_{560}, was solubilized to give micelles of bacteriorhodopsin with endogenous purple membrane lipids by stirring overnight in the dark at 25°C with 5 ml of 5% (v/v) Triton X-100, 100 mM Tris-HCl (pH 7.0) at a detergent/bacteriorhodopsin ratio 4.5–13.5 : 1 (w/w). After 15 h, the solubilized purple membrane was centrifuged (100000 × *g*; 4°C; 30 min) to pellet any unsolubilized purple membrane or aggregated bR. The supernatant was loaded onto a Pharmacia Sephadex G-75 (fine) column (70 × 2.6 cm) which had been pre-equilibrated with 1% cholate (w/v), 150 mM NaCl, 10 mM Tris-HCl (pH 8.0) at 25°C. The Sephadex slurry and cholate buffers were previously degassed. Bacteriorhodopsin/cholate micelles were eluted with the above buffer (flow rate, 30 $cm^3 \cdot h^{-1}$), collected in 10 cm^3 fractions and analyzed at 538 nm for bR and at 280 nm for Triton X-100 micelles. Bacteriorhodopsin/cholate fractions (usually about 6) were pooled and concentrated to 6 cm^3 using an Amicon pm10 ultrafiltration membrane.

The lipid, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), was synthesized according to establish methods [16], dried from chloroform/methanol (2 : 1, v/v) in a glass vial under vacuum (10^{-2} torr; 8 h), resuspended in 1% cholate buffer and tip sonicated until the bilayer milkiness disappeared. The appropriate amount of bacteriorhodopsin/cholate suspension was added, stirred in the dark for 15 min and then dialysed initially against 0.1% cholate, 150 mM NaCl, 10 mM Tris-HCl (pH 8.0), 0.025% azide at 25°C in the dark. Once vesicles had formed, after about 4–5 days, the same buffer was used for dialysis but without the cholate, together with Amberlite XAD-2 beads (BDH, Poole, U.K.), which had previously been washed three times in acetone, then boiled in distilled water with frequent changes. Finally, the dialysis buffer was changed to 10 mM Tris-HCl (pH 7.5). The dialysate (usually 10–12 cm^3) was loaded onto a linear sucrose gradient (5–35% for low to intermediate and 15–45% for high protein containing complexes) (250000 × *g*; 4°C; 4 h). The major band (if more than one was observed) was washed free of sucrose in 10 mM Tris-HCl (pH 7.5) three times.

One bR-DMPC complex was also produced in which the endogenous purple membrane phospholipids were not removed [8] for comparitive ^{31}P-NMR experiments (see below).

Lipid and protein analysis. For all bacteriorhodopsin/DMPC complexes produced, the final lipid/protein ratio was determined. The amount of DMPC in the complex was determined by perchloric acid digestion and inorganic phosphate analysis [17]. The amount of bacteriorhodopsin (26 kDa) was determined by the modified Lowry method and the protein content adjusted accordingly [18].

NMR. High resolution ^{31}P-NMR spectra were recorded on a Nicolet (360 MHz for ^{1}H) spectrometer at 145.9 MHz, shimmed on ^2H_2O which was also used as a lock. A sweep width of 6 kHz was used and spectra were collected into 4K points with a relaxation delay of 2 s. Methylene diphosphonate (MDP) in 5 mM Tris-HCl (pH 8.0, ^2H_2O) in an insert tube provided a standard against which chemical shifts could be measured. A line broadening of 2 Hz was used after typically 5000 scans. Purple membrane or bacteriorhodopsin-DMPC complexes were pelleted (100000 × *g*; 25°C; 20 min) and stirred with 4% SDS 100 mM Tris-HCl (pH 7.0) in ^2H_2O for 1 h. Any unsolubilized membrane (generally none) was pelleted by centrifugation (100000 × *g*; 25°C, 15 min) and the clear yellow/orange supernatant used for NMR measurements.

Tempo partitioning. The water and fluid-lipid soluble nitroxide spin-label Tempo (2,2,6,6-tetramethylpiperidine-*N*-oxyl) (50 µl of 10^{-3} M in 10 mM Tris-HCl (pH 7.5)), was added to a bacteriorhodopsin complex (approx. 5 mg lipid in 0.5 cm^3 buffer) and the complex pelleted (100000 × *g*; 25°C; 15 min). After decanting the supernatant, the pellet was loaded into a sealed 100 ml micropipette using a drawn out pasteur pipette. The sealed sample tube was then placed in a 4 mm ESR tube containing silicon oil to stabilize the temperature. The exact temperature was determined by a thermocouple placed in the oil immediately above the ESR cavity.

ESR spectra were recorded on a Bruker ESP 300 spectrometer using a conversion time of 164 ms and a

time constant of 82 ms over 1K points. A sweep width of 0.01 T with a modulation amplitude of 0.1 mT was used. Temperature was controlled by a Bruker liquid nitrogen VT-unit. Spectra were recorded every 3 deg and 5 min were allowed between measurements for temperature equilibration. The apparent partition coefficient (f) was calculated from the line heights for the spin-label partitioned between liquid-crystalline bilayers and in the aqueous phase [19].

Freeze-fracture electron microscopy. The different bR-DMPC complexes were studied by freeze-fracture electron microscopy after quenching from a range of temperatures from 6 to 55°C. Freeze-fracture electron micrographs were also produced from *H. halobium* cells and also from the isolated purple membrane patches of those cells. Samples were quenched using liquid propane and the sandwich technique, in which the complexes were mounted between two plain-faced copper holders to give a quenching rate of approx. 10^5 K·s^{-1}. To effect a slower cooling rate for the complexes when required, one of the copper holders used has a depression onto which the sample was positioned; in this situation the sample layer is thicker and the cooling rate on quenching about an order of magnitude slower (approx. 10^4 K·s^{-1}) than when using the plain-faced holders [20].

The samples were fractured and shadowed in a Balzers BAF-400D freeze-fracture device at −150°C. The cleaned replicas were examined in a Tesla BS-500 or Jeol JEM-100B electron microscope.

Results

Ten bR-DMPC reconstituted complexes, with mole ratios of between 1:1440 and 1:67 were prepared, as well as one complex with the endogenous lipids still remaining. The experimentally determined absorption spectra for bR in both the purple membrane and when reconstituted into DMPC bilayers was the same with an absorption maximum at 560 nm as shown in Fig. 1 for the 1:67, bR-DMPC complex. In these complexes, bR does pump protons (unpublished observations) and this is also taken as evidence for bR integrity.

Phospholipid composition of reconstituted complexes

The high resolution ^{31}P-NMR spectrum of SDS solubilized purple membrane, pH 7.8 (Fig. 2a) reveals two spectral lines of similar intensity (height ratio 0.96:1) that are 1.26 ppm apart, at 15.63 ppm and 16.89 ppm upfield from MDP; the MDP peak is not shown. The major phospholipids of the purple membrane are diether analogues of phosphatidylglycerol phosphate [9] and the (monoester)phosphate is assigned the resonance at 15.63 ppm upfield from the MDP at pH 7.8 (Gale and Watts, to be published). Fig. 2b shows the spectrum of the 1:68 bR-DMPC complex solubilized in 4% SDS. As with all other similarly produced bR-DMPC complexes, no evidence for purple membrane phospholipids was obtained, with only a single

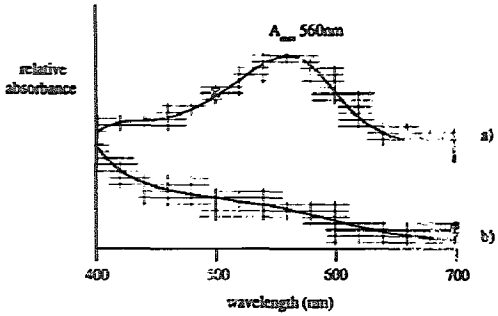

Fig. 1. Experimentally determined absorption spectra of bR in a DMPC reconstituted bilayer complex (bR/DMPC mole ratio of 1:67) free of endogenous purple membrane phospholipid (a) and the same complex bleached in the presence of hydroxylamine as a blank (b) in deionized water at room temperature.

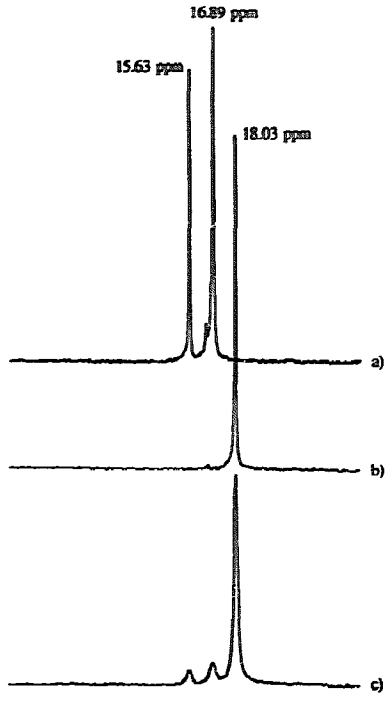

Fig. 2. High-resolution ^{31}P-NMR (145.9 MHz) spectra of SDS solubilized membranes (4% SDS; 100 mM Tris-HCl (pH 7.4)) containing bacteriorhodopsin (bR) in purified purple membrane (a), a bR-DMPC complex (1:68; bR/DMPC mole ratio) with all endogenous purple membrane phospholipids removed by detergent exchange (b), and a bR-DMPC complex (1:72; bR/DMPC mole ratio) in which no attempt was made to remove endogenous purple membrane lipids (c). Chemical shifts (ppm) are relative to MDP (not shown). Bar = 5 ppm.

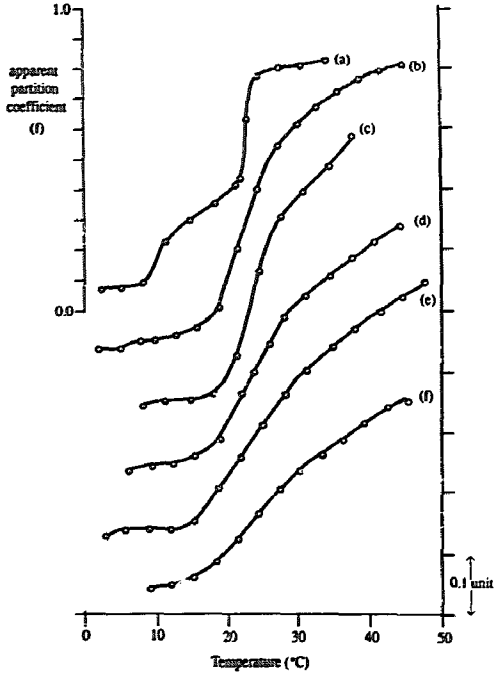

Fig. 3. Phase transition curves for DMPC bilayers (a) and bR-DMPC complexes free of endogenous purple membrane lipids. (b–f) determined by ESR from the partitioning parameter, (f), for the spin-label Tempo with increasing temperature. The mole ratios of the bR-DMPC complexes were 1:218 (b); 1:187 (c); 1:131 (d), 1:95 (e) and 1:67 (f). The apparent partition coefficient, f, determined as described in the text, is given for bR-free DMPC bilayers in the left hand scale, with each transition curve being displaced vertically by 0.1 unit of (f) in the lowest temperature value for clarity since all the curves overlap.

resonance being recorded 18.03 ppm upfield from the MDP peak at the same chemical shift as solubilized DMPC.

As a control, complex with bR-DMPC mole ratio of 1:72 was produced using octyl glucoside, in which no attempt was made to remove endogenous purple membrane lipids [8]. This solubilized complex showed three ^{31}P-NMR resonances corresponding to the major purple membrane lipid (DPhPGP) and DMPC (Fig. 2c).

This evidence confirms that even in these relatively high protein/lipid ratio complexes, there is no bacterial DPhPGP and the only phospholipid present in these complexes is DMPC as judged by ^{31}P-NMR and within the limits (approx. 0.02%) of the method.

Tempo partitioning

Fig. 3 shows the apparent partition coefficients (f), determined from the ESR spectral heights as an indication of the concentration of the label in each phase [19], for Tempo partitioning into the bilayer with increasing temperature for the 1:67; 1:95, 1:131; 1:187, 1:218 bR-DMPC complexes and protein-free DMPC bilayers. Increasing the protein concentration in each case broadens the temperature range over which the DMPC gel to liquid-crystalline transition occurs. However, the midpoint for the transition is maintained at 23–24°C, as shown earlier for complexes in which purple membrane lipids were not fully removed [21,22].

Freeze-fracture electron microscopy

Representative freeze-fracture electron micrographs, from eighty five produced, for the bR-DMPC complexes entirely free of endogenous purple membrane lipids and quenched from below T_c are shown in Fig. 4 and quenched from a temperature above T_c in Fig. 5. The packing density of bR in the DMPC bilayers observed in the electron micrographs, appears to depend upon the temperature (T_q) from which the complex was quenched, the bR/DMPC ratio in the complex, as well as the rate of freezing of the complexes when quenched from above T_c.

Electron micrographs of bR-DMPC complexes quenched from temperatures (6–15.5°C) below T_c at all protein/lipid ratios, reveal similar features whether they are quenched slowly (approx. 10^4 K·s^{-1}) or more quickly (approx. 10^5 K·s^{-1}). Complexes with a low protein content display two kinds of ridges termed $\lambda/2$ (zig-zag) and λ (wave-like) as shown in Figs. 4a, b, respectively, and similar to those observed in protein-free DMPC [23] and 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol [24] bilayers when quenched from temperatures between the pre- and main bilayers phase transition temperature. The protein particles can be seen to decorate these DMPC-ridges (Figs. 4a, c–f) or are localized in structural defects of these lipid-ridges (Fig. 4b) forming areas rich in protein particles. With increasing protein content the repeat distance of both ripple types are found to increase (Figs. 4a–e) until at higher protein content (1:222; bR/DMPC mole ratio) initially the λ ripples are no longer observed (Fig. 4c) and then at very high protein content (1:68; bR/DMPC mole ratio), the $\lambda/2$ ripples also disappear and only very few ripples are decorated by protein particles (Fig. 4f). These features were essentially similar in all micrographs regardless of whether the samples were quenched from 6, 13.5 or 15.5°C, except that no zig-zags were seen in 6°C quenched samples.

Electron micrographs from complexes at all bR-DMPC ratios quenched from a temperature above T_c using a faster cooling rate (approx. 10^5 K·s^{-1}), show well dispersed and irregularly distributed protein particles with representative micrographs shown in Figs. 5a, b and c. However, electron micrographs of bR-DMPC complexes quenched also from $T_q > T_c$, but using a slower cooling rate as described in Materials and Methods [20], show distinct and often regular pat-

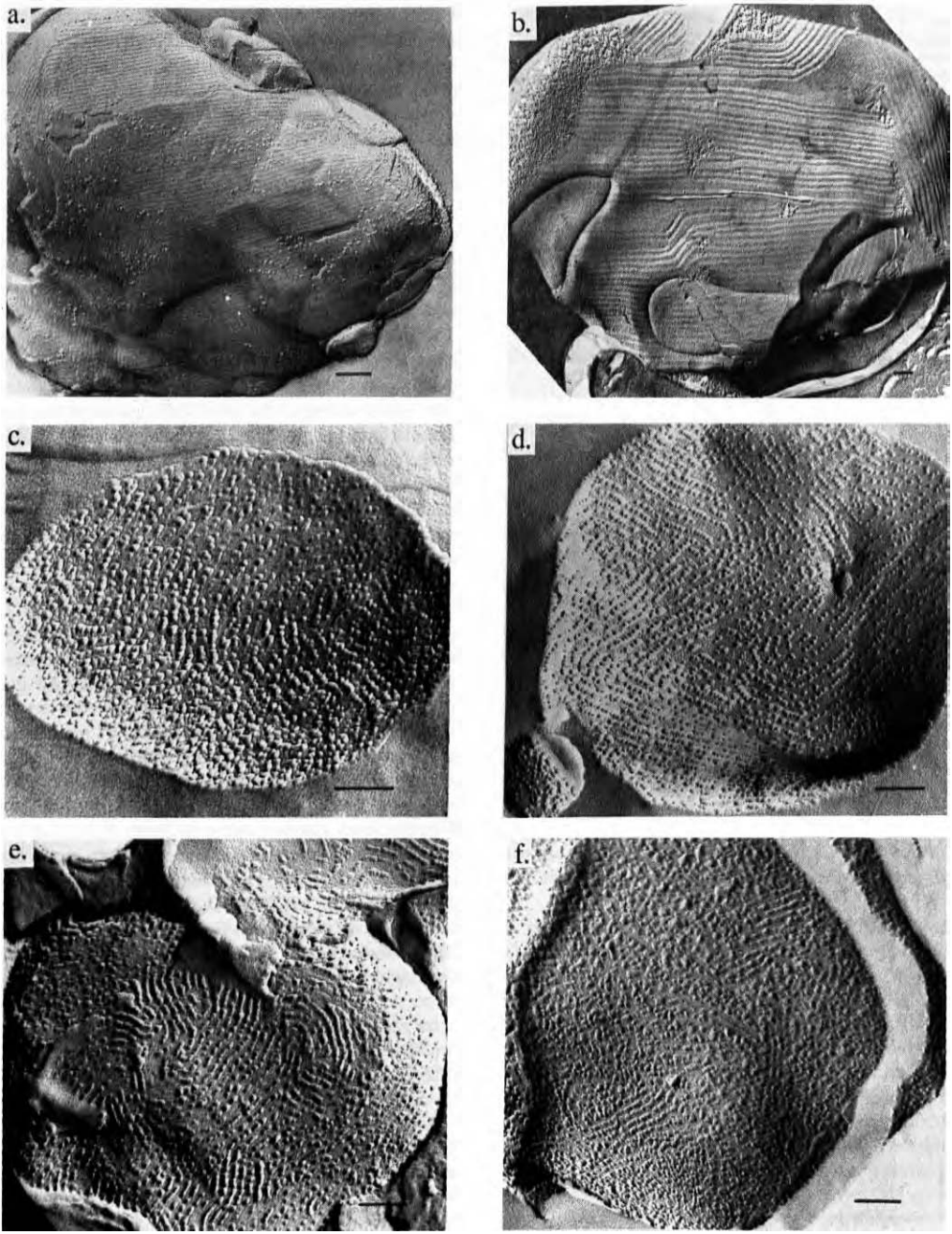

Fig. 4. Freeze-fracture electron micrographs of bR-DMPC complexes totally free of endogenous lipid, quenched from 13.5 °C (a); 15 °C (b + c) and 15.5 °C (d, e and f), temperatures which are below the bilayer phase transition temperature (T_c). The bR/DMPC mole ratios are 1 : 1440 (a), 1 : 379 (b), 1 : 222 (c), 1 : 168 (d), 1 : 141 (e) and 1 : 68 (f). Similar results were obtained for complexes quenched both more quickly and more slowly (see text). Bars represent 100 nm; shadowing direction is from bottom to top of micrographs.

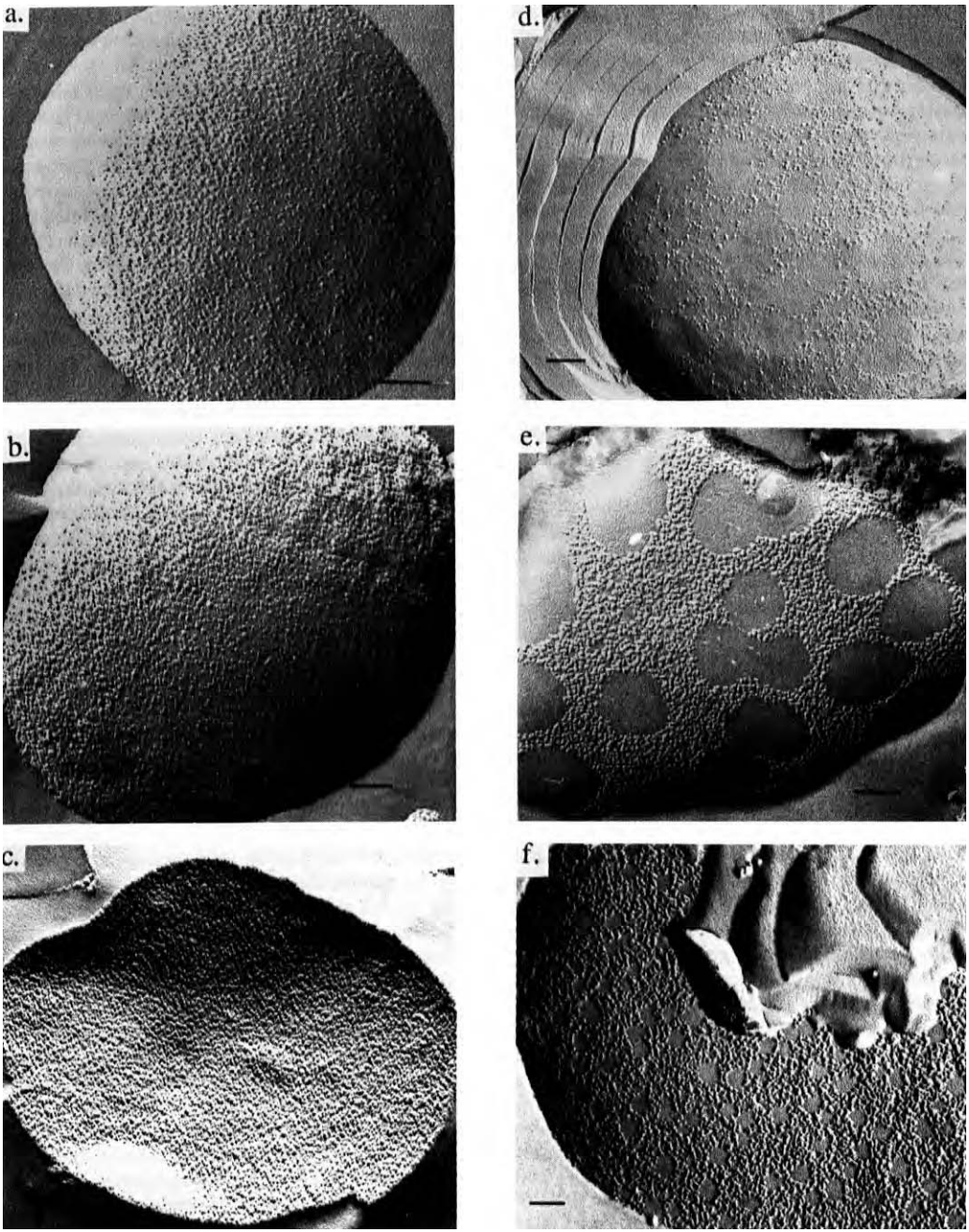

Fig. 5. Freeze-fracture electron micrographs of bR-DMPC complexes totally free of endogenous lipid, quenched from 55°C in all cases, a temperature which is above the bilayer phase transition temperature, quenched more quickly (approx. 10^5 K·s^{-1}) (a–c) and more slowly (approx. 10^4 K·s^{-1}) (d–f) as described in the text. The protein/lipid mole ratios were 1:222 (a), 1:182 (b), 1:95 (c), 1:1440 (d), 1:187 (e) and 1:68 (f). Bars represent 100 nm; shadowing direction is from bottom to top of micrographs.

terns of particle-free areas (Figs. 5d, e and f). The protein particle-free islands are almost circular and of varying diameters (100–130 nm, 130–200 nm and 50–60 nm in Figs. 5d, e and f, respectively) and more clearly pronounced at intermediate and high protein content in the bilayers (Figs. 5e and f). Such islands are less distinct in micrographs from complexes with lower (Fig. 5d) protein content.

In electron micrographs of all the bR-DMPC complexes produced totally free (< 0.02%) of endogenous purple membrane phospholipid, regardless of protein content or whether quenched from a temperature either below or above T_c, bR did not display a hexagonal packing similar to that observed in the purple patches of *H. halobium* cell membranes, in isolated purple membranes (micrographs not shown) and as reported earlier [3,5], or DMPC-endogenous lipid complexes containing bR [4]. In marked contrast to these observations, bR aggregates do display a hexagonal-like packing in bR-DMPC complexes which contain endogenous purple membrane lipids [8].

Discussion

In the present study, Triton X-100 and subsequently cholate, has been used to produce a wide range of bR-DMPC complexes that are free of endogenous purple membrane lipids. The visible absorption spectrum for bR in such complexes (Fig. 1) was very similar to that for bR in purple membranes indicating that the protein is structurally intact after the reconstitution procedure. The ^{31}P-NMR spectra of all complexes when solubilized in detergent were similar and confirmed that DMPC was the only phospholipid present as the representative spectrum in Fig. 2b demonstrates. When observed by ^{31}P-NMR, the resonances of different types of phospholipids with fully isotropic motion in detergent micelle have different chemical shifts [25,26] due to the different intra-molecular hydrogen bonding [27] and chemical environment, and hence chemical shielding of the phosphoester headgroup moieties. Thus phospholipids with different headgroups can readily be assigned and their relative amounts determined from the integrated intensity of the resonance lines [28] under suitable instrumental conditions. High resolution ^{31}P-NMR therefore appears to be a good technique for identifying the phospholipid headgroup types present in bR-DMPC complexes and also possibly in other reconstituted systems, especially those containing mixed phospholipids.

The bilayer pre-transition observed at 10–12°C for pure DMPC bilayer (Fig. 3) is not evident for DMPC bilayers containing bacteriorhodopsin for any of the complexes produced. Earlier DSC results for bR-DMPC complexes with the endogenous purple membrane lipids retained, also reveal no pre-transition for the lipid [22]. It therefore appears that this perturbation of the lipid bilayer is an effect of the protein itself and not any residual purple membrane lipids, as shown with other integral proteins in reconstituted saturated phospholipid bilayers [29].

For all complexes, a well defined main gel to liquid crystalline phase transition is observed (Fig. 3). For pure DMPC bilayers, the mid-point occurs sharply at 23.5°C. In the absence of endogenous purple membrane lipids, bacteriorhodopsin maintains the midpoint of the main transition for the supporting DMPC bilayer at 22–24°C for all the complexes produced. However, the Tempo partitioning experiments (Fig. 3) reveal a broadening of the bilayer phase transition with increasing bacteriorhodopsin content. Earlier fluorescence depolarization studies show a similar qualitative effect for bR-DMPC complexes still containing endogenous purple membrane phospholipids [22]. The broadening of the temperature range over which the gel-liquid crystalline transition occurs arises presumably through a decrease in the size of the lipid pool able to undergo the transition as the protein/lipid ratio of the complex decreases. Since the complexes are cooled and heated rather slowly during the determination of the transition temperatures using the ESR Tempo method (slower than 1.5 $C^\circ \cdot min^{-1}$) it may be that the lipid-rich areas between protein aggregates and seen in the electron micrographs, undergo the phase transition essentially unperturbed by the protein. If this is the case, the measured lipid transition temperature should be similar to that measured for protein-free bilayers but of reduced cooperativity, as is observed in Fig. 3, and this cooperativity will be determined by the size of the lipid pool able to undergo the transition. Micrographs of complexes quenched from below T_c reveal that the distances between the lipid ripples become wider with random protein particle arrangements interspersed between areas containing ripples (Figs. 4a–e) until finally the ripples almost disappear (Fig. 4f) confirming that the lipid pool decreases with increasing protein content. Electron micrographs from samples quenched from above T_c at a slower cooling rate, show distinct and regular patterns of particle-free areas (Figs, 5d, e and f) until at high protein content (1:68; bR/DMPC mole ratio), nearly all the lipid is protein affected with the protein-free areas being very small or almost completely disappeared (Fig. 5f).

A number of integral proteins change only the breadth of the lipid phase transition without altering the temperature of the transition, whereas others change both the breadth and the temperature of the transition for bilayers of saturated phospholipids. The effect of an integral protein upon these characteristics of the phase transition behaviour of a bilayer of a saturated acyl chain phospholipid, may therefore reflect the propensity for that protein to either aggregate or not self associate

with decreasing temperature. Such aggregation will therefore determine whether lipid pools, essentially free of protein, are created in the bilayer. A similar phenomenon was also observed for the $(Ca^{2+} + Mg^{2+})$-stimulated ATPase from sarcoplasmic reticulum [30]. The degree of lipid co-operativity has been shown to depend upon the size of lipid pool undergoing the gel to liquid-crystalline bilayer transition [19]. Thus, proteins which are well dispersed (such as rhodopsin from mammalian retinal discs membranes) alter both the width and temperature of the bilayer transition [29,31]. The situation is clearly complicated if proteins change their aggregation state with either protein content or temperature, although the general principle still applies to any one situation.

Freeze-fracture electron microscopy is a good technique for investigating the interior of a membrane and the arrangement and distribution of particles (presumed to be proteins) within the plane of the bilayer [32,33]. It has been assumed that the cytoplasmic membranes of *H. halobium* fracture along the interior plane [3]. In the purple membrane patches the outer lamellar shows a smooth fracture face which lacks proteins, while the inner lamellar reveals a hexagonal array of particles [3]. Apparently the bR remains embedded in the cytoplasmic monolayer of the bacterial membrane after fracturing. A similar hexagonal pattern of bR particles was also observed in the present study using the freeze-fracture technique in purple membrane patches isolated from the bacterial membranes (micrograph not shown), in agreement with others [3]. The electron micrographs presented here for bR-DMPC complexes without endogenous purple membrane phospholipids (Figs. 4 and 5) also clearly reveal the presence of such particles presumed to be bR. Bacteriorhodopsin monomers are 3.5 nm by 4.5 nm in the plane of the bilayer [5] and thus the particles observed in Figs. 4 and 5 are, from their size (8–10 nm in diameter), most probably small aggregates of bR symmetrically oriented in the DMPC bilayers. Freeze-fracture electron microscopy and diffraction techniques have been used to define the polypeptide content of a 11.9 nm wide single membrane particle in purple membranes and it was concluded that such a particle contains 9–12 bacteriorhodopsin monomers, that is, 63–84 transmembrane α-helices [34].

A considerable amount of freeze-fracture electron microscopic data has been published on bacteriorhodopsin containing vesicles. For example, vesicles have been produced in which 80–90% of the endogenous purple membrane lipids were removed using deoxycholate treatment followed by sucrose density gradient centrifugation [11]. Electron microscopy, together with X-ray diffraction and circular dichroism, revealed that the protein-associated particles did not form the regular hexagonal lattice obtained in isolated purple membrane or whole cells. When partially delipidated bacteriorhodopsin was reconstituted with total *H. halobium* lipid extract, patches of protein formed which had a highly ordered planar hexagonal lattice structure, although no preferential orientation was obtained [11]. Such indications imply a rôle for the endogenous lipids in forming the ordered hexagonal two-dimensional patches of bR. A link between protein orientation and particle size has been suggested although in that study no temperature studies were performed and lipids of heterogeneous fatty acyl chain composition (azolectin and soybean PC) were used.

Freeze-fracture results presented here of bR in DMPC vesicles with no endogenous purple membrane lipids present, reveal in no case the regular hexagonal lattice observed in isolated purple membrane patches [5] or in the purple membrane patches of the bacterial cells [34] whether quenched from below (Fig. 4) or above (Fig. 5) the phospholipid bilayer phase transition temperature (Fig. 3). Even at the higher protein/lipid ratios, the bR particles are randomly distributed (Figs. 4f and 5c) and in the protein-rich areas observed in electron micrographs made from complexes quenched more slowly from temperatures above T_c, show no large ordered aggregates (Figs. 5e, f). The protein particles are close packed but they are not ordered into the hexagonal two dimensional crystalline array as in the purple membrane patches [3] or in DMPC complexes (quenched from a temperature below T_c) in which the endogenous purple membrane lipids are still present [8]. These results add further support to a role of the highly negatively charged headgroups of the purple membrane phospholipids such as DPhPGP [9] in ordering the protein particles into a hexagonal lattice through association at the membrane bilayer surface.

Circular dichroism and rotational diffusion measurements of bR in DMPC bilayers [21] have been used to suggest that for complexes with more than approx. 40 lipid molecules per bacteriorhodopsin, the protein molecules exist in a monomeric state, while in gel state bilayers below this temperature, the bacteriorhodopsin molecules self-associated into the same two-dimensional hexagonal crystal observed in the purple membrane. Additionally, calorimetric studies show the existence of a second thermally induced exothermic transition (in addition to the main transition of DMPC at 23–24°C) at 17°C, i.e. 6–7 C° below the main phase transition of the pure lipid [22]. This second transition was attributed to disaggregation of the purple membrane lattice. However, in those studies, all the endogenous purple membrane lipids were still present in the complexes. Band 3, the erythrocyte anion transporter, has also been shown to self-associate reversibly with temperature in both the physiological environment of the red cell membrane [35] as well as in reconstituted complexes with DMPC [16]. However, this self-association of band 3 appears to be protein-mediated, because of the similarity in aggrega-

tion in two very different lipid environments, and not lipid-mediated as suggested by the present study for bacteriorhodopsin although such inter-protein interactions may still take place at the bilayer surface.

Studies of integral membrane protein aggregation phenomena in model systems may help in the understanding of the mechanisms and triggering events which can lead to protein associations and signalling between proteins in the physiological situation. Such interactions could be under cellular control if lipid head group modification, for example phosphorylation, is determined metabolically or in response to second messengers.

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References

1 Watts, A. (1987) J. Biomembr. Bioenerg. 19, 625–653.
2 Watts, A. (1987) in Membrane Receptors, Dynamics and Energetics, (Wirtz, K.A.W., ed.) pp. 329–340 Plenum Press, New York.
3 Blaurock, A.E. and Stoeckenius, W. (1971) Nature New Biol., 233, 152–155.
4 Cherry, R.J. (1979) Biochim. Biophys. Acta 559, 289–327.
5 Henderson, R. and Unwin, N. (1975) Nature 257, 28–32.
6 Unwin, P.N.T. and Zampighi, G. (1980) Nature 283, 544–549.
7 Kistler, J., Strand, R.M., Klymkowsky, M.W., Labancette, R. and Fairclough, R.H. (1982) Biophys. J. 37, 371–383.
8 Cherry, R.J., Müller, U., Henderson, R. and Heyn, M.P. (1978) J. Mol. Biol. 121, 282–298.
9 Kates, M. (1978) Prog. Chem. Fats Lipids 15, 304–342.
10 Israelachivili, J.N., Marcelja, S. and Horn, R.G. (1980) Q. Rev. Biophys. 13, 121–200.
11 Hwang, S.B. and Stoeckenius, W. (1977) J. Membr. Biol. 33, 325–350.
12 Van Dijck, W.M., Nicolay, K., Levinssen-Bijvelt, J., Van Dam, K. and Kaptein, R. (1981) Eur. J. Biochem. 117, 639–645.
13 Lewis, B.A. and Engelman, D.M. (1983) J. Mol. Biol. 166, 203–210.
14 Oesterhelt, D. and Stoeckenius, W. (1974) Meth. Enzymol. 31, 667–678.
15 Huang, K.S., Bayley, H. and Khorana, H.G. (1980) Proc. Natl. Acad. Sci. USA 77, 323–327.
16 Dempsey, C.E., Ryba, N.J.P. and Watts, A. (1986) Biochemistry 26, 2180–2187.
17 Rouser, G., Fleischer, S. and Yamamoto, A. (1970) Lipids 5, 494–496.
18 Rehorvek, M. and Heyn, M.P. (1979) Biochemistry 18, 4977–4983.
19 Marsh, D. and Watts, A. (1982) in Liposomes, from Physical Structure to Therapeutic Action (Knight, G.G., ed.) Ch. 5, Elsevier, Amsterdam.
20 Costello, M.J., Fetter, R. and Höchli, M. (1982) J. Microsc. 125, 125–136.
21 Heyn, M.P., Cherry, R.J. and Dencher, N.A. (1981) Biochemistry 20, 840–849.
22 Heyn, M.P., Blume, A., Rehorek, M. and Dencher, N.A. (1981) Biochemistry 20, 7131–7115.
23 Luna, E.J. and McConnell, H.M. (1977) Biochim. Biophys. Acta 466, 381–392.
24 Watts, A., Harlos, K., Maschke, W. and Marsh, D. (1978) Biochim. Biophys. Acta 510, 63–74.
25 London, E. and Feigenson, G.W. (1979) J. Lipid Res. 20, 408–412.
26 Seelig, A. and Seelig, J. (1985) Biochim. Biophys. Acta 815, 153–158.
27 Henderson, T.O., Glonek, T. and Myers, T.C. (1974) Biochemistry 13, 623–628.
28 Sotirhos, N., Herslof, B. and Kenne, L. (1986) J. Lipid Res. 27, 386–390.
29 McElhaney, R.N. (1986) Biochim. Biophys. Acta 864, 361–421.
30 Lentz, B.R., Clubb, K.W., Alford, D.R., Höchli, M. and Meissner, G. (1985) Biochemistry 24, 433–442.
31 Silvius, J. (1982) in Lipid-Protein Interactions, Vol. 2 (Jost, P.C. and Griffith, O.H., eds.), Wiley Interscience, New York.
32 Branton, D. (1966) Proc. Natl. Acad. Sci. USA 55, 1048–1067.
33 Meyer, H.W. and Winkelmann, H. (1969) Protoplasma 68, 253–270.
34 Fisher, K.A. and Stoeckenius, W. (1977) Science 197, 72–74.
35 Mühlebach, T. and Cherry, R.J. (1985) Biochemistry 26, 975–983.