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Lipid-Induced Modulation of the Protein Packing in Two-Dimensional Crystals of Bacteriorhodopsin

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The mechanism by which bacteriorhodopsin (BR), the light-driven proton pump from the purple membrane (PM) of *Halobacterium halobium*, arranges in a 2D hexagonal array has been studied by reconstitution of BR in complexes of two types of bilayer made either with PM-derived lipids or with PM lipids and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC). The unit cell dimensions of the 2D protein crystals, determined by correlation averaging analysis of freeze-fracture electron micrographs, were compared with the lattice constant of the PM. In complexes made with delipidated BR and with the polar lipids extracted from *H. halobium* cells (HHPL), BR trimers are arranged in a hexagonal lattice with the same lattice constant of 5.9 ± 0.2 nm as found in the PM. In BR-containing complexes made with PM-derived lipids and DMPC at several protein:lipid mole ratios, BR trimers are also arranged in a hexagonal lattice, but with a unit cell dimension of 9.2 ± 0.2 nm, which is about one-third larger compared to that measured in PM (Michel *et al.*, 1980). In a subclass of this type of complexes, orthogonal BR arrays were observed with a lattice constant of 5.9 × 9.9 ± 0.2 nm. It appears that insertion of DMPC into the BR/PM-derived lipid complexes increases the center-to-center distances in both array types by a discrete amount.

INTRODUCTION

Bacteriorhodopsin (M_r ~26 000), the light-driven proton pump and only protein species of the purple membrane of *Halobacterium halobium*, packs in a two-dimensional array with hexagonal symmetry within the plane of the membrane (Blaurock and Stoeckenius, 1971). In this arrangement, fewer than 10 lipid molecules per BR molecule are associated with the protein (Kates *et al.*, 1982).

The reasons why BR and other integral membrane proteins arrange into 2D arrays in the bilayer are unclear. Although the BR arrangement into hexagonal arrays is not essential for its function (Cherry *et al.*, 1978), it is of considerable interest to define the principles and factors necessary for 2D crystallization. It is this fortuitous 2D arrangement which has enabled electron microscopic and diffraction methods to be applied and permits the resolution of structural details of BR to 2.7 Å (Henderson *et al.*, 1990).

The hexagonal 2D array formation could be promoted through protein density (protein–protein interactions) or more specific lipid–protein interactions. However, it has been shown that BR, purified entirely free of PM lipids and reconstituted at a wide range of protein densities into DMPC bilayers, does not arrange into 2D arrays, suggesting that protein density is not important for array formation (Sternberg *et al.*, 1989).

It has been shown that when the PM-derived lipids are partially removed from the PM by nonsolubilizing amounts of detergent, a loss of hexagonal protein packing and formation of the orthorhombic BR pattern occurs (Michel *et al.*, 1980). This observation suggests that lipids may control the arrays formed in the PM.

The lipids of the purple membrane (PML) contain mainly 2,3-di-*O*-phytanyl-*sn*-glycero-1-phosphoryl-3′-*sn*-glycerol-1′-phosphomethyl (DPhPGP), 2,3-di-*O*-phytanyl-1-*O*-β-D-Gal-*p*-3-sulfate-(1 → 6)-α-D-Glc-*p*-*sn*-glycerol (DPhGLS), 2,3-di-*O*-phytanyl-*sn*-glycero-1-phosphoryl-3′-*sn*-glycerol (DPhPG), and traces of 2,3-di-*O*-phytanyl-*sn*-glycero-1-phosphoryl-3′-*sn*-glycerol-1′-sulfate (DPhPGS) with saturated phytanyl groups ether linked to the glycerol backbone. *H. halobium* polar lipids (HHPL), extracted from the whole bacterial cell, are similar in composition to the PML, although the red membrane of *H. halobium* does not contain the sulfate derivative, which is exclusive to the PM (Kates *et al.*, 1982; Smith, 1988).

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It has been demonstrated that well-defined 2D arrays of BR can be reconstituted in DMPC bilayers by adding back one or more components of the PM-derived phospholipids, either DPhPGP or DPhPGS, both having highly charged polar head groups, and 4 *M* NaCl (Sternberg *et al.*, 1992).

Here we describe the structure of 2D arrays of BR reconstituted in bilayers of PM-derived lipids with and without DMPC. The unit cell dimensions of these protein crystals, determined by correlation averaging analysis of freeze-fracture electron micrographs, are compared with the unit cell dimensions of the native and orthorhombic PM.

MATERIALS AND METHODS

Bacterial Cells, PM, and BR Isolation

H. halobium cells were grown and harvested, and the PM was isolated and purified as described previously (Oesterhelt and Stoeckenius, 1974). Delipidation of the BR was performed as detailed previously (Huang *et al.*, 1980; Sternberg *et al.*, 1989).

Lipids

DMPC, which was pure by thin-layer chromatography analysis, was obtained from Sigma Chemical Co. and used without any purification. *H. halobium* polar lipids (HHPL) were extracted from the whole bacterial cells as described previously (Kates *et al.*, 1982, Gale and Watts, 1991). The individual lipid types were separated by two-dimensional thin-layer chromatography (Schwietz, 1982) adapted from earlier methods (Kates *et al.*, 1965).

Formation of the BR/Lipid Complexes

Most of BR–lipid complexes were prepared by detergent dialysis as described previously (Sternberg *et al.*, 1992). In short, HHPL or the individual lipid types (DPhPGP, DPhPGS) and DMPC were first dissolved individually in chloroform. The HHPL alone or the lipid mixtures of DMPC and required lipid fraction of HHPL, DPhPGP, or DPhPGS were dried under a stream of nitrogen and the remaining traces of solvent were removed under high vacuum (10^{-2} Torr) for several hours. A small volume (<1 ml) of cholate elution buffer (see below) was then added to the lipid mixtures for solubilization by agitation at room temperature until optically clear.

BR, entirely free of endogenous PM lipids, was solubilized in cholate and the BR–detergent micelles concentrated to 5–10 ml by ultrafiltration. The required amount of protein was mixed with the solubilized lipids and the solution was dialyzed at room temperature in the dark against 1 liter of buffer (10 m*M* Tris, 2 m*M* EDTA, 150 m*M* KCl, 0.02% NaN_3, pH 8.0) containing 1.2 g of sodium cholate. The buffer was renewed once or twice a day for about 5 days until a slight cloudiness could be observed. Once vesicles were formed, the samples were dialyzed against the same buffer, but without cholate, and washed. SM2 Biobeads (10 ml slurry) were added in order to complete the detergent removal for about 5 more days. Cholate was successively removed to a level of 1 cholate per 700 lipid molecules determined by measuring the residual detergent doped with [^{14}C]cholate (Sternberg *et al.*, 1992).

Complexes, made of BR, DMPC, and all the lipids of the PM (PML), were produced by inserting DMPC into PM fragments mediated by a nonspecific phospholipid-transfer protein (nsTP) from bovine liver as previously described (Gale and Watts, 1991). All the BR–lipid complexes produced were purified on a sucrose density gradient (10–45%, w/v) by centrifugation (100 000*g*; 4°C, 17 hr). In each case only the single major band, after washing three times, was used to determine the phospholipid:protein mole ratio, using a phosphate assay (Rouser *et al.*, 1970) and a modified Lowry method (Markwell *et al.*, 1981) and for freeze-fracture electron microscopy.

The absorption spectrum of BR, with λ_{max} at 500 nm, in all the complexes was very similar to that for BR in the PM.

Freeze-Fracture Electron Microscopy

The BR/PM-derived lipid complexes were studied by freeze-fracture electron microscopy after quenching at either 55 or 4°C and in the presence or absence of 4 *M* NaCl, glucose (1 or 10% w/v), or trehalose (1 or 10% w/v) as described previously (Sternberg *et al.*, 1992). Both *H. halobium* cells and isolated PM patches were also studied by the same method but quenched at room temperature, for comparison with the reconstituted complexes.

Samples were frozen with liquid propane using the sandwich technique to give a cooling rate of $>10^4$ K s^{-1}, which has been shown to be fast enough to prevent reorganization of protein particles in the bilayers during quenching (Sternberg, 1992). The samples were fractured and shadowed in a Balzers BAF-400D freeze-fracture device at −120°C. The cleaned replicas were examined in a Jeol JEM 100B or a Tesla BS 500 electron microscope.

Image Processing

Optical diffractograms of the electron micrographs were used to judge the quality of the protein arrays in bilayers. Image areas showing reasonable crystalline order (between one and three diffraction orders) were digitized using an Eikonix CCD camera 1412. The digitized areas were either 1024 by 1024 or 512 by 512 pixel arrays and the step size was 15 μm, which corresponds to a pixel size at the specimen level of 0.69 and 0.46 nm on micrographs taken on the Tesla microscope at a magnification of 21 600-fold and 32 600-fold, respectively, and 0.48 nm on micrographs taken on the Jeol microscope at a magnification of 31 200-fold.

The digitized image areas were processed using standard correlation averaging techniques (Saxton and Baumeister, 1982). Surface relief reconstructions were performed according to Guckenberger (1985). The programs used are implemented in the SEMPER (Saxton *et al.*, 1979) and EM (Hegerl and Altbauer, 1982) program systems.

RESULTS

All BR-containing complexes made from PM-derived lipids and formed with or without DMPC and, for comparison, PM of *H. halobium* cells and isolated PM patches were examined by freeze-fracture electron microscopy and image processing.

BR/HHPL Complexes, the Native PM, and Isolated PM Patches

Freeze-fracture electron micrographs of PM either on *H. halobium* cells (Fig. 1a) or isolated in patches (Fig. 1b) show a closely similar BR lattice as seen in complexes made from totally delipidated BR and the polar lipids extracted from *H. halobium* cells (BR/HHPL (1:7 mol:mol)) (Figs. 1c and 1d). The arrays formed did not depend upon the sample quenching temperature (22°C in Figs. 1a and 1b, 4°C in Fig. 1c, and 55°C in Fig. 1d) or whether the complexes were quenched in the presence (Fig. 1c) or absence (Fig. 1d) of 4 *M* NaCl. In all cases the BR trimers are arranged in a well-defined hexagonal lattice as

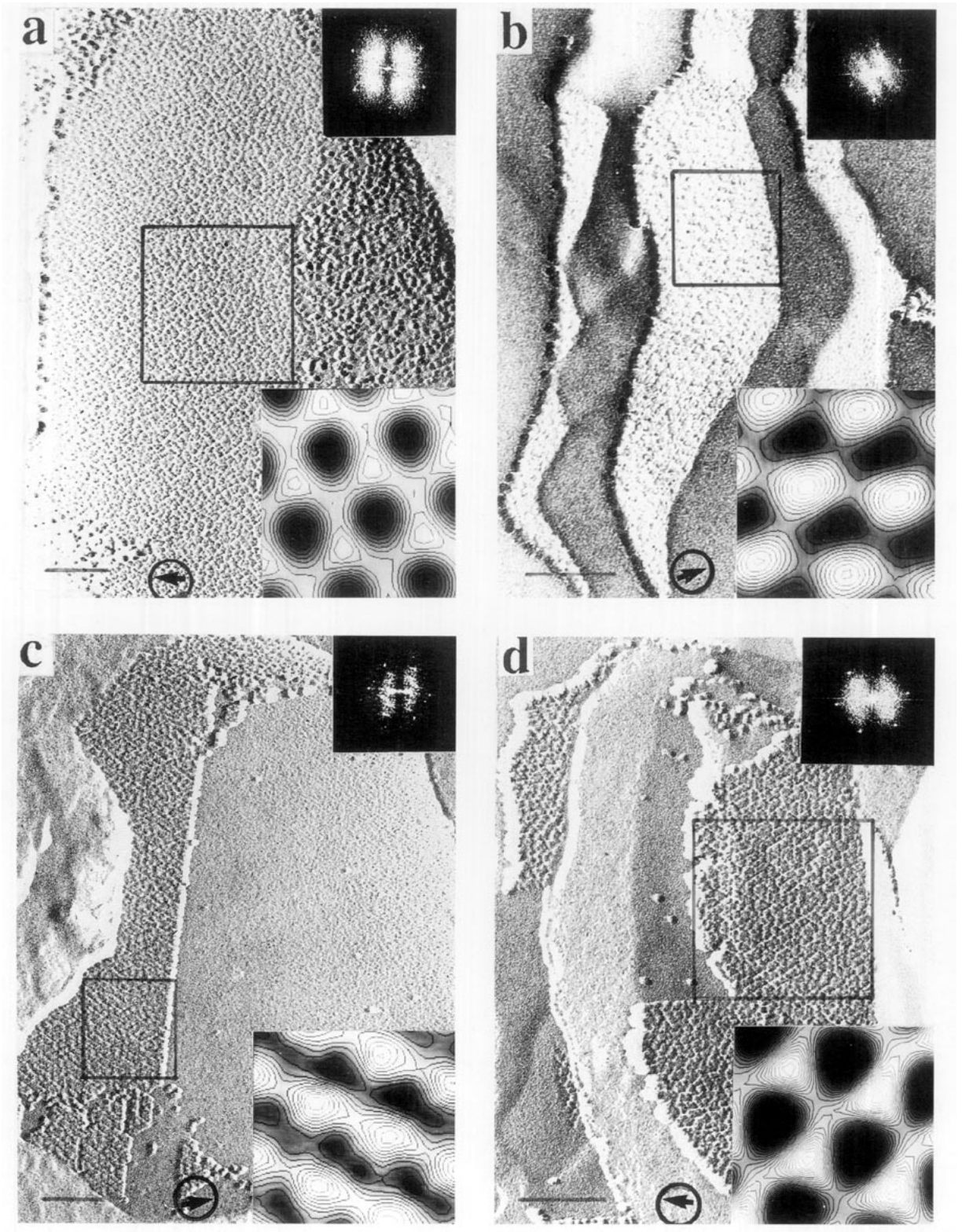

FIG. 1. Electron micrographs of a freeze-fractured *Halobacterium halobium* cell (a), isolated PM (b), and complexes made of BR/HHPL (1:7 mol/mol) with (c) and without (d) added 4 *M* NaCl. Temperatures before quenching were 22°C (a,b), 4°C (c), and 55°C (d). In all cases the BR trimers are arranged in a hexagonal lattice as shown by the calculated diffraction pattern (smaller insets in a–d) and the averages displayed as contour maps (larger insets in a–d), all with similar lattice dimensions of 5.9 ± 0.2 nm. Shadows are white in the contour maps. Bars represent 100 nm, the shadowing direction is marked by a circled arrow, and the processed areas are marked by black squares.

shown by both the calculated diffraction pattern (small insets in Figs. 1a–1d) and the correlation averages displayed as contour maps (large insets in Figs. 1a–1d). Here, in the limit of error, the same lattice dimension of 5.9 ± 0.2 nm is determined (Table I) in both naturally occurring membranes and reconstituted complexes.

Complexes Containing BR/DMPC and the Individual Types of the PM-Derived Lipids

Freeze-fracture electron micrographs of reconstituted complexes of BR with PM-derived lipids and DMPC do show a hexagonal BR arrangement when DPhPGP or DPhPGS is present in the complex, both as individual lipid fractions (Fig. 5a and Fig. 5b) and in several combinations (as in the PM lipids (PML, Figs. 2a and 2b), as well as in the polar lipids of the *H. halobium* cell membrane (HHPL, Figs. 4a–4c). As confirmation of this observation, the calculated diffractograms (small insets in Figs. 2a and 2b, 4a–4c, and 5a and 5b) are shown in which the unit cell dimension for the BR arrays was determined to be 9.2 ± 0.2 nm (Fig. 3a), which is about one-third larger than the lattice constant of the PM (Table I), for all types of complexes.

BR/DMPC Complexes Formed from PM Fragments

Freeze-fracture electron micrographs of liposomes made from BR/DMPC/PML (1:46:9, mole ratios) prepared by inserting DMPC into PM with the lipid-transfer protein, show two types of BR arrays, as seen in Figs. 2a and 2b, namely hexagonal arrays (Figs. 2a and 2b) and orthogonal arrays (Fig. 2b; one of them marked by an arrow). Calculated diffractograms (small insets in Figs. 2a and 2b) and relief reconstructions, displayed as 3D shaded representations (upper large insets in Figs. 2a and 2b), show the hexagonal and orthogonal arrangement of the corresponding arrays.

A center-to-center distance of 9.2 ± 0.2 nm is found in the hexagonal array (Fig. 3a) which is close to the larger dimension of the center-to-center protein unit distance of 9.9 nm in the orthogonal array (Fig. 3b), whereas the shorter dimension (5.9 nm) is again similar to that for the native PM (Fig. 3b; Table I).

BR/DMPC Complexes Containing H. halobium Polar Lipids

The temperature at which the complexes were quenched appears unimportant in determining crystal formation. Quenching at 55°C, which is well above the temperature of the gel to liquid phase transition (T_m) of DMPC (T_m ~23°C), the bulk lipid in the complex, gave micrographs (Fig. 4a) with features similar to those obtained by quenching at 4°C (Figs. 4b and 4c). Indeed, the array is seen to be even better ordered in the complex (here BR/DMPC/HHPL, 1:44:8 mole ratios shown as an example) when quenched at 55°C rather than 4°C. Higher order reflections are visible in the calculated diffractogram of the micrograph of a sample quenched at 55°C (inset Fig. 4a), whereas in complexes quenched at 4°C, broad reflections are seen and these sometimes coalesce to Debye–Scherer rings (Figs. 4b and 4c).

BR/DMPC Complexes Containing Individual H. halobium Polar Lipids

Freeze-fracture electron micrographs of liposomal complexes containing BR reconstituted with the individual lipid fractions DPhPGP (BR/DMPC/DPhPGP, 1:15:5, mole ratios) or DPhPGS (BR/DMPC/DPhPGS, 1:16:9, mole ratios) in addition to the synthetic DMPC show hexagonal BR crystals (Figs. 5a and 5b) (Sternberg *et al.*, 1992). To confirm this crystallinity power spectra from selected areas of the negatives are mounted in the right upper corner of the micrographs.

Electron micrographs of complexes of BR with DMPC and PM-derived lipids such as PML, HHPL, DPhPGP, or DPhPGS, which showed hexagonal protein arrays in the presence of 4 *M* NaCl, as seen in Figs. 2a and 2b, 4a–4c, and 5a–5b, showed no such arrays when the NaCl was not added or subsequently removed by dialysis below a level of 2 *M* NaCl or substituted by glucose (1 or 10% w/v) or trehalose (1 or 10% w/v) (electron micrographs not shown).

TABLE I

Unit Cell Dimensions and Space Groups Calculated from the Diffraction Pattern from Micrographs of Various BR-Containing Membranes

Type of Specimen	Lattice type	Space group	Lattice dimensions (nm)
Purple membrane in the *H. halobium* cell	Hexagonal	$p3$	5.9 ± 0.1
Purple membrane patch	Hexagonal	$p3$	5.8 ± 0.1
BR/HHPL vesicle[a] 1:7 mol:mol	Hexagonal	$p3$	5.9 ± 0.2[b]
BR/DMPC/PML vesicle[c]	Hexagonal	$p3$	9.2 ± 0.2[d]
1:46:9, mole ratios	Orthogonal	$p22_12_1$	5.9 × 9.9 ± 0.2
BR/DMPC/HHPL vesicle[a] 1:44:8, mole ratios	Hexagonal	$p3$	9.4 ± 0.2[b]
BR/DMPC/PhPGS vesicle[a] 1:16:9, mole ratios	Hexagonal	$p3$	9.5 ± 0.2

[a] Made by detergent dialysis (see text for details).
[b] Averaged from three calculations.
[c] Made by inserting DMPC into PM patches (see text for details).
[d] Averaged from six calculations.

DISCUSSION

In the present study the unit cell dimensions of two types of reconstituted BR/PM-derived lipid com-

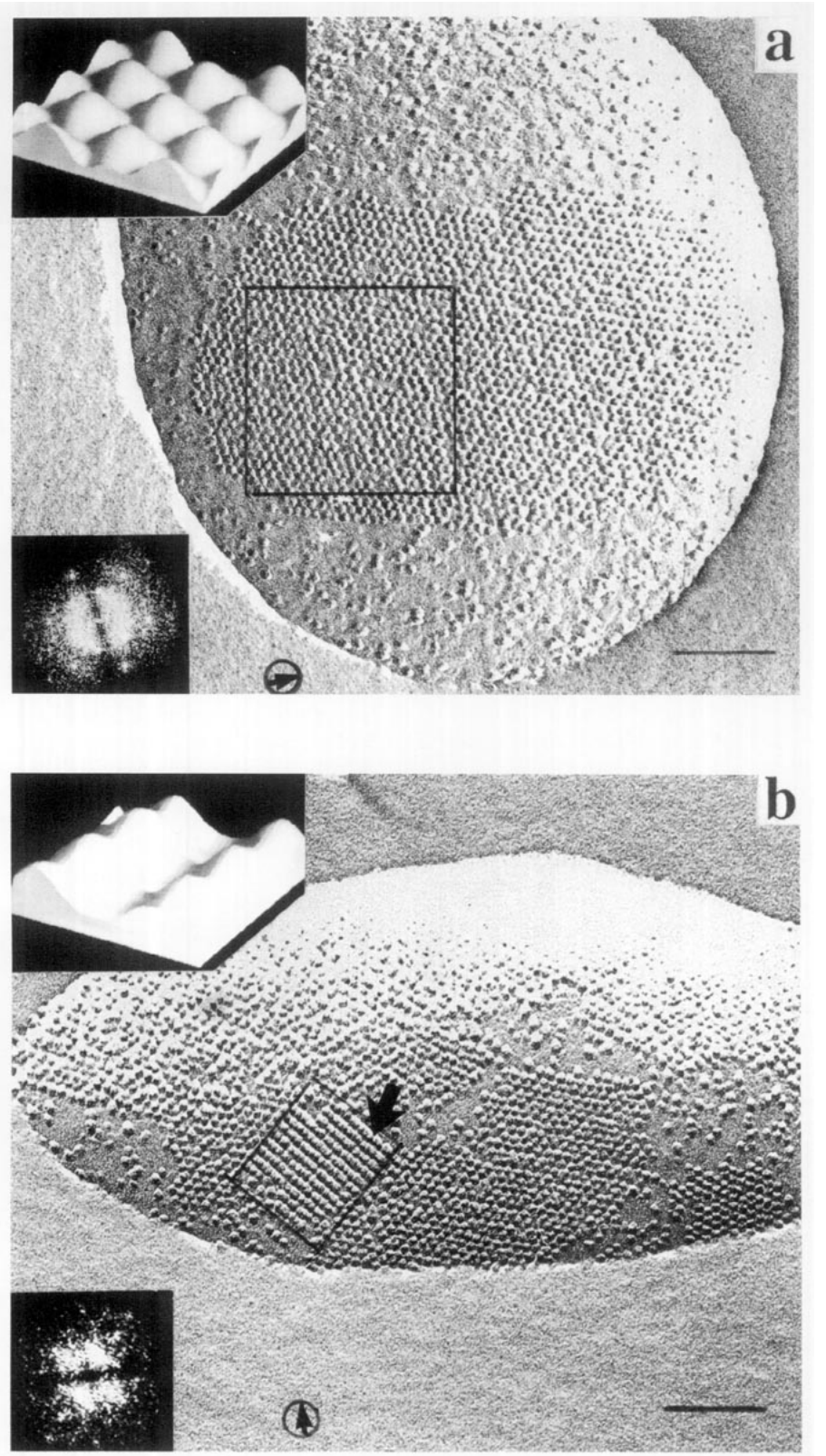

FIG. 2. Freeze-fracture electron micrographs of a complex made from BR/DMPC/PML (1:46:9; mole ratios) in 4 *M* NaCl, prepared with a nsTP (see text for details), and quenched at 55°C, displaying two types of BR arrays: hexagonal arrays in (a) and (b) and an orthogonal array, marked by an arrow, in (b). Bars represent 100 nm, shadowing direction is marked by a circled arrow, and processed areas are marked by black squares. Calculated diffraction pattern (smaller insets in (a) and (b)) and relief reconstructions, displayed as 3D shaded representations (larger insets in (a) and (b)), show the hexagonal and orthogonal arrangement of the corresponding arrays.

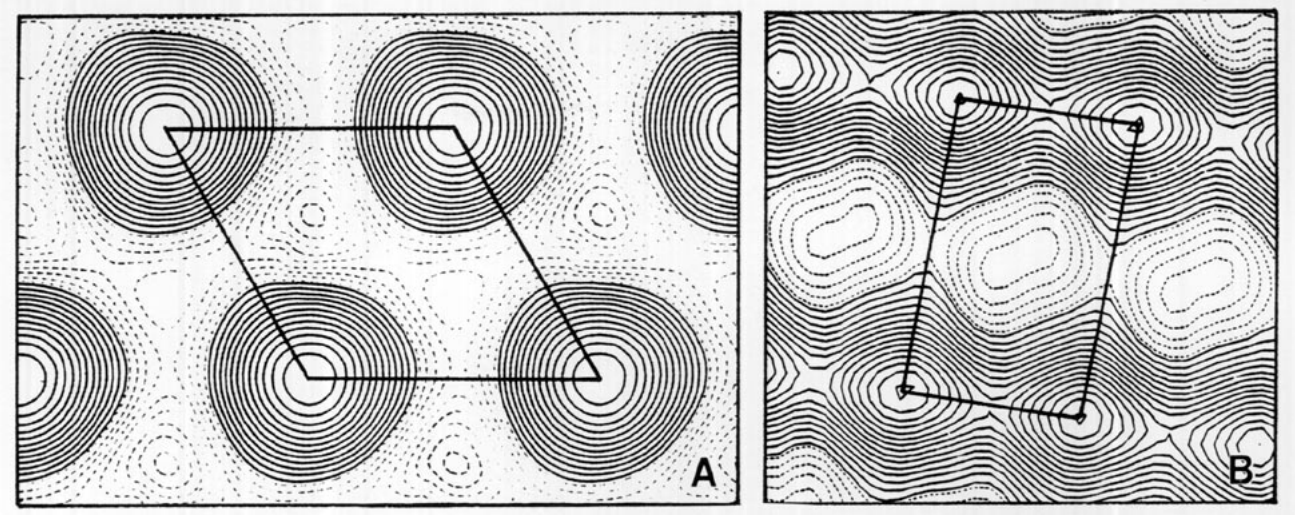

FIG. 3. Relief reconstructions of two-dimensional arrays of BR from the electron micrographs shown in Fig. 2. The hexagonal array has a center-to-center distance of 9.7 nm (A) and the orthogonal array has center-to-center distances of 5.9 by 9.9 nm (B).

plexes, with and without the addition of the synthetic lipid DMPC, were determined by correlation averaging analysis of freeze-fracture electron micrographs and compared with the unit cell dimensions of the native and the orthorhombic PM.

In complexes made from delipidated BR and the polar lipids extracted from *H. halobium* cells (but without added synthetic lipid) BR trimers are arranged in a hexagonal lattice as seen in Figs. 1c and 1d. The lattice dimension in these complexes of 5.9 ± 0.2 nm is close to that found in the native PM and is similar to that measured in isolated PM patches (6.18 nm; Michel *et al.*, 1980). The unit cell dimensions are independent of the temperature at which the complexes were quenched or of the presence or absence of 4 *M* NaCl (Table I).

In reconstituted complexes made from BR, PM-derived lipids, and additional DMPC as a synthetic lipid component, BR trimers are arranged in a hexagonal lattice when DPhPGP or DPhPGS is present in the complex, both as individual lipid fractions (Figs. 5a and 5b) or in several combinations, namely as PML (Figs. 2a and 2b) and HHPL (Figs. 4a, 4b, and 4c). That is true at all temperatures at which the complexes were quenched, with a protein-to-total lipid molar ratio of less than 1:100, but only when 4 *M* NaCl is present (Sternberg *et al.*, 1992). The lattice constant of the hexagonal assemblies found in all complexes of this type is about the same (Table I), with a value of 9.2 nm, which is about one-third larger than the lattice constant for the protein crystals in the native PM.

In addition to the hexagonal BR assemblies, orthogonal BR arrays are visible in complexes made by inserting DMPC into PM fragments mediated by a nonspecific transfer protein (Fig. 2b). In the orthogonal arrays the center-to-center distances are found to be 5.9 by 9.9 ± 0.2 nm (Fig. 3b; Table I). The dimension of the orthogonal array is slightly larger, whereas the shorter dimension is close to that found in orthorhombic arrays in the PM after detergent treatment (5.76 × 7.35 nm; Michel *et al.*, 1980).

The center-to-center distances became greater by about one-third in the hexagonal as well as in the orthogonal arrays by insertion of a synthetic lipid into the BR/PM-derived complexes. The dimensions of the protruding mass of the protein trimers in the hexagonal as well as in the orthogonal arrays seem to be the same as those in the native and the orthorhombic PM. However, the space between the BR oligomers in the hexagonal as well as in the orthogonal arrays appears to be filled with lipid (Figs. 3a and 3b).

The formation of the orthorhombic two-dimensional crystal form of BR in PM and in complexes formed from PM fragments and containing DMPC may be similar in origin and caused by delipidation. In both cases the PM had been treated with detergents, either directly (Michel *et al.*, 1980) or followed by reconstitution into DMPC.

The long-range order of the hexagonal, as well as of the orthogonal arrays of particles, is relatively imperfect. The highest order reflections in the diffractograms (Figs. 4a and 5a) were always found in BR/DMPC complexes containing PM-derived lipids quenched at temperatures above T_m, with crystalline arrangement maintained over 15–20 particles. In the same complexes but quenched at temperatures below T_m the coherent lattice domains are small (4–6 particle distances; a small crystal is marked by an arrow in Fig. 4c) and sometimes at an angle to each other due to the competition between gel-phase lipid long-range ordering and protein ordering. Gaps are seen in the lattice domains and

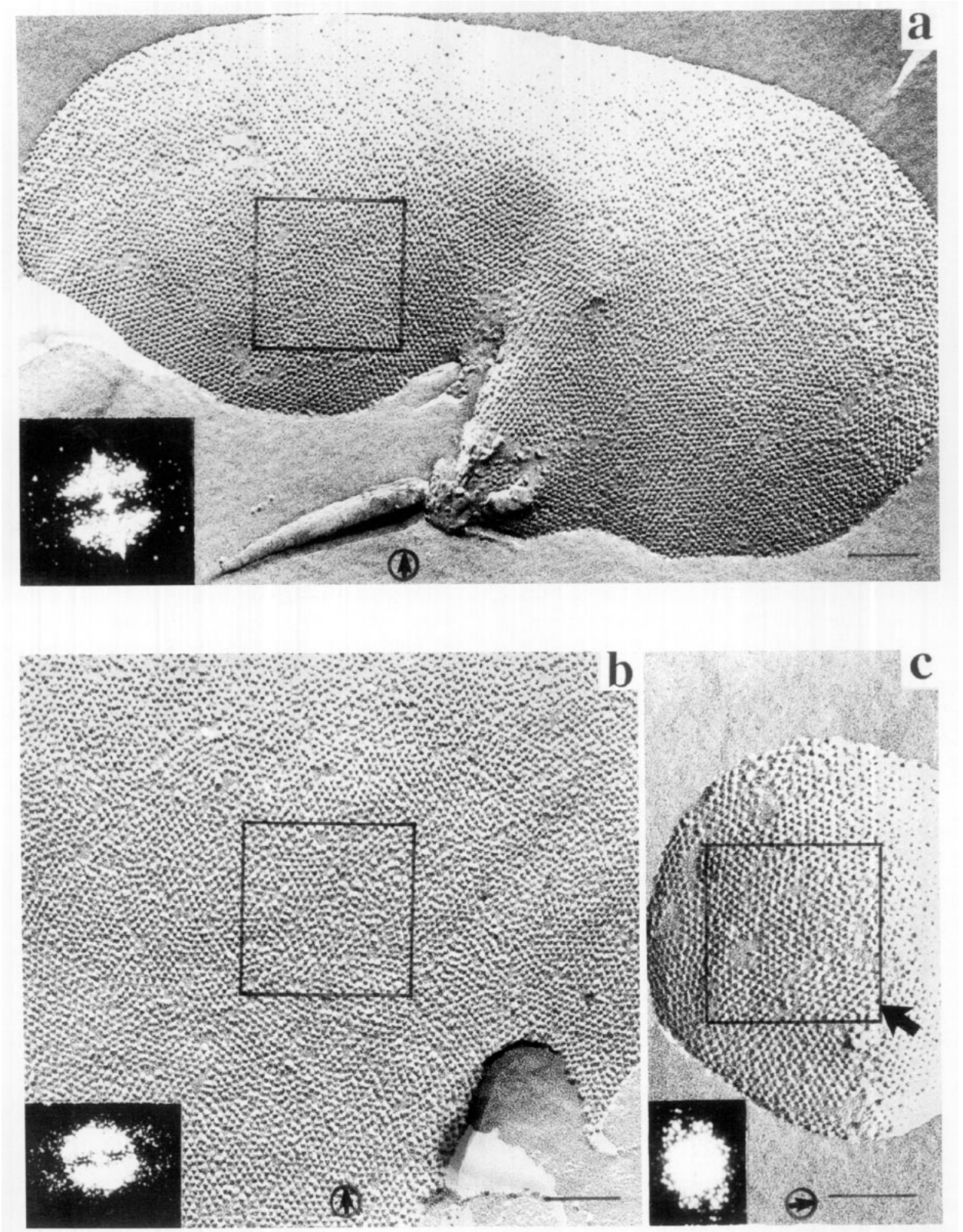

FIG. 4. Freeze-fracture electron micrographs of a complex containing membranes made (see text for details) from BR/DMPC/HHPL (1:44:8, mole ratios) in 4 *M* NaCl quenched at 55°C (a) and 4°C (b,c). Bar represents 100 nm and the shadowing is marked by a circled arrow. The insets are calculated diffractograms from selected regions of the micrographs (marked by black squares) showing first- and *second-order reflections when quenched at 55°C (inset in (a)) and Debye–Scherer rings when quenched at 4°C (insets in (b) and (c)).* A small crystalis is marked by an arrow in (c).

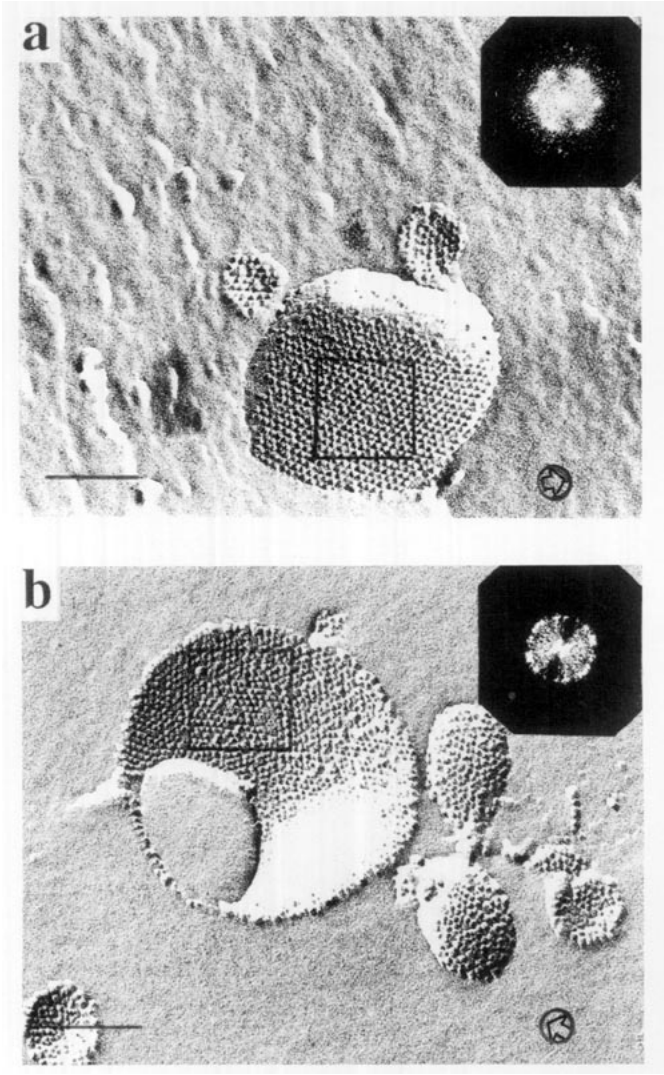

FIG. 5. Freeze-fracture electron micrographs of complexes containing individual lipid fractions of the *H. halobium* cell membranes in a combination of BR/DMPC/DPhPGP (1:15:5, mole ratios) (a) and of BR/DMPC/DPhPGS (1:16:9, mole ratios) (b). All complexes were in 4 *M* NaCl and quenched at 55°C. Bar represents 100 nm, the shadowing is marked by a circled arrow, and the processed areas are marked by black squares. In both cases the BR trimers are arranged in hexagonal arrays as shown from the calculated diffractograms from selected areas of the negative (insets in (a) and (b)).

thus second-order reflections are repressed. Broad reflections in the calculated diffractograms may even coalesce to a Debye–Scherer ring, thereby reducing their intensity (Figs. 4b and 4c).

Second-order reflections are found in complexes made of BR/DMPC/HHPL (Fig. 4a) as well as of BR/DMPC/DPhPGP (Fig. 5a) when quenched at temperatures above T_m. This is higher indeed than that we obtained using the same procedure for the native (Fig. 1a) and isolated PM (Fig. 1b). Therefore these relatively well-ordered protein arrays in complexes of different lipid composition are potentially good specimens for electron diffraction studies (Downing, 1991). Structural information may be obtained from such complexes about the nature of the intermolecular interactions between BR molecules, and between BR and the highly charged individual lipids, DPhPGP and DPhPGS, of the PM. However, high salt (4 *M* NaCl), the presence of which precludes the use of conventional negative staining electron microscopy methods, is necessary to promote well-ordered two-dimensional BR array formation in complexes made from DMPC and the PM-derived lipids, independent of whether these lipids are the HHPL or PML mixtures or the individual PM lipids such as DPhPGP and DPhPGS (Sternberg *et al.*, 1992).

The well-ordered BR pattern generated within bilayers of these complexes under the influence of high salt disappeared when the salt was dialyzed below a level of 2 *M* (micrographs not shown). Addition of glucose (1 and 10% by weight) or trehalose (1 and 10% by weight) to the samples instead of high salt does not reveal any hexagonal BR arrangement in the complexes containing DMPC and the bacterial lipids investigated (micrographs not shown). Interaction of BR with the charged head groups of two of the PM-derived phospholipids (DPhPGP and DPhPGS), limited dehydration, and relatively high protein density (BR:lipid <1:100, mol:mol), but not so much an acyl chain effect, may therefore be partial requirements for good ordering of BR in 2D arrays (Sternberg *et al.*, 1992).

Although BR trimers in reconstituted complexes are not as densely packed, and the unit cell dimension is extended by about one-third compared with the PM, well-ordered two-dimensional BR arrays can be formed in DMPC bilayers by salt and the presence of one or more components of the PM-derived phospholipids, either DPhPGP or DPhPGS.

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