STRUCTURAL COMPARISON OF NATIVE AND DEOXYCHOLATE-TREATED PURPLE MEMBRANE

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ABSTRACT Lipid-depleted purple membrane prepared by extraction with deoxycholate has been compared with the native structure. X-ray and electron diffraction photographs show a reduction in cell dimension from 62.4 to 57.3 Å, and a substantial change in the distribution of diffraction intensity compared with the native specimens. Low-dose electron microscopy has been used to obtain a projected density map of lipid-depleted membranes. The projected structure shows that the deoxycholate treatment removes a boundary layer of lipid, which in the native form separates adjacent trimers of bacteriorhodopsin. The map also provides an improved estimate of the molecular envelope of the protein. A plausible arrangement for the lipid molecules in both the native and the lipid-depleted membranes is proposed, but the precise positions of individual molecules cannot yet be specified.

INTRODUCTION

The purple membrane fraction of the cell membrane of *Halobacterium halobium* consists of a single protein, bacteriorhodopsin (bR), together with a variety of lipids, in a 3:1 mass ratio. Much has been learned about the structure of the protein by x-ray diffraction (Blaurock, 1975; Henderson, 1975), neutron diffraction (Trewhella et al., 1983), and electron microscopy and electron diffraction (Henderson and Unwin, 1975; Leifer and Henderson, 1983). These diffraction methods have considerable power in studying the molecular structure of the purple membrane because it is a two-dimensional crystal (space group p3) containing trimers of the protein. Relatively little has been learned about the structural arrangement of lipid molecules within this crystal, however. The most likely positions for lipid molecules can, of course, be inferred from the projected structural map of the protein (Unwin and Henderson, 1975), but neither the precise positions of individual lipid molecules nor the degree of order nor the packing arrangement is known. The packing of protein trimers may involve only protein-lipid-protein contacts, or it may be determined partially by direct protein-protein contacts between adjacent trimers.

It has been reported by Hwang and Stoeckenius (1977) that 80% of the lipid phosphorus is removed from purple membrane by extraction with sodium deoxycholate. Hwang and Stoeckenius also stated that the crystallinity of the membrane was retained after treatment with this detergent, but that small intensity changes occurred in the x-ray diffraction rings. These changes in intensity might be caused by (*a*) shrinkage of the lattice parameter (caused by the removal of mass from the unit cell) with the result that the molecular transform is sampled at different places than for the native membrane, (*b*) changes in the contribution made by the lipids themselves to the measured diffraction intensities, (*c*) rotation of protein trimers within the plane of the membrane, or (*d*) tilting and translational movements of individual bR molecules relative to one another.

Two-dimensional Fourier analysis of the deoxycholate-extracted membranes would distinguish between these possibilities. In view of the potential that the lipid-depleted form of purple membrane might have for an understanding of the structural role of the lipids, we carried out combined electron diffraction and electron microscopy on the deoxycholate-treated membranes. The resulting map shows that there is no observable modification of the molecular bonding between monomers within the bR trimers. Furthermore, the apparent rotation of trimers within the plane of the membrane is very small. The cell dimension is reduced by ~5 Å, and this reduction appears to be accounted for entirely by the removal of a boundary layer of lipid molecules that completely surrounds the protein trimers in the native membrane. The two-dimensional map of the lipid-depleted membrane therefore provides a more accurate estimate of the molecular boundary for the protein, which in turn provides a clearer outline of the lipid positions within the native membrane. The two-dimensional map of lipid-depleted membrane also shows two possible positions that together can accommodate up to four lipid molecules per molecule of bR. A direct measurement of the lipid content is broadly consistent with this stoichiometry.

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METHODS

Purple membrane was isolated from *H. halobium* S-9 according to standard procedures (Oesterhelt and Stoeckenius, 1974). Initial electron diffraction experiments with membranes that had been extracted with sodium deoxycholate (DOC) by the method of Hwang and Stoeckenius (1977) showed that individual membrane patches tended to contain multiple crystalline domains. A modified procedure was therefore developed, which produced membrane patches composed of single crystalline domains.

The modified DOC-extraction procedure began with the addition of 1.0 ml of 0.1% DOC to a wet pellet containing 20 mg of bR, centrifuged from distilled water, to produce a starting solution containing ~0.1% DOC and 20 mg/ml of protein. The concentration of DOC was then doubled, at intervals of >8h, by the addition of small aliquots of concentrated stock solutions, to produce a final solution containing 6% DOC and 7 mg/ml protein. After at least 8 h, the membranes were washed by centrifugation on a sucrose gradient, using 7% DOC to make up all solutions. The DOC was then removed by a slow dialysis procedure in which the initial dialyzing solution was 7% DOC. At intervals of >8 h, half of the dialysate was removed and replaced by distilled water. This operation was repeated at least 16 times. The specimen was protected from exposure to light throughout this procedure by wrapping all containers in aluminum foil.

Samples used for measuring the lipid content of DOC-extracted membranes were prepared by the same method, with tracer quantities of ^{3}H-labeled DOC (I.C.N. Radioisotopes and Amersham, Radiochemicals) added. After thorough washing in distilled water, the amount of DOC present in the membranes was measured to be 0.33 (± 0.1) mol per mol of bR (three determinations).

Lipids were extracted from membranes with chloroform-methanol according to the procedure described by Kushwaha et al. (1975). The amount of bR in the sample was estimated before lipid extraction from the peak optical density in the visible absorption spectrum, assuming a molar extinction coefficient (light-adapted) of 55,000, or by measurement of dry weight.

Three types of measurement of lipid content were performed. First, the total lipids extracted from native and DOC-treated membranes were dried by evaporating the chloroform phase first under N_2 and then in vacuo, and simply weighed. Second, lipid hexose was measured using the phenol-sulfuric acid method of Dubois et al. (1956) with glucose as standard. Third, lipid phosphate was measured by the method of McClare (1971).

Electron diffraction patterns and low-dose images were obtained from glucose-embedded membranes prepared on hydrophobic carbon films by the procedure of Unwin and Henderson (1975). Diffraction patterns were recorded on a Philips EM400 equipped with a cold stage, at a specimen temperature of −120°C to minimize radiation damage, but images were recorded at room temperature with the specimen mounted on a high-resolution specimen stage in a Philips EM301. Accurate measurement of the cell dimension from both the native and DOC-extracted membranes was carried out by allowing a thin layer of ice to condense on the membrane specimen at −150°C, after which the stage temperature was raised to −120°C to allow the condensed, vitreous ice to crystallize into the cubic form (Dubochet et al., 1982). Such specimens exhibit a sharp diffraction ring at a Bragg spacing of 3.66 Å, which can be used to calibrate the camera length precisely. The lattice parameter of native purple membrane was found to be 62.4 Å, measured relative to the ring from cubic ice, while that of DOC-extracted membranes was found to be 57.3 Å, a shrinkage of 5.1 Å.

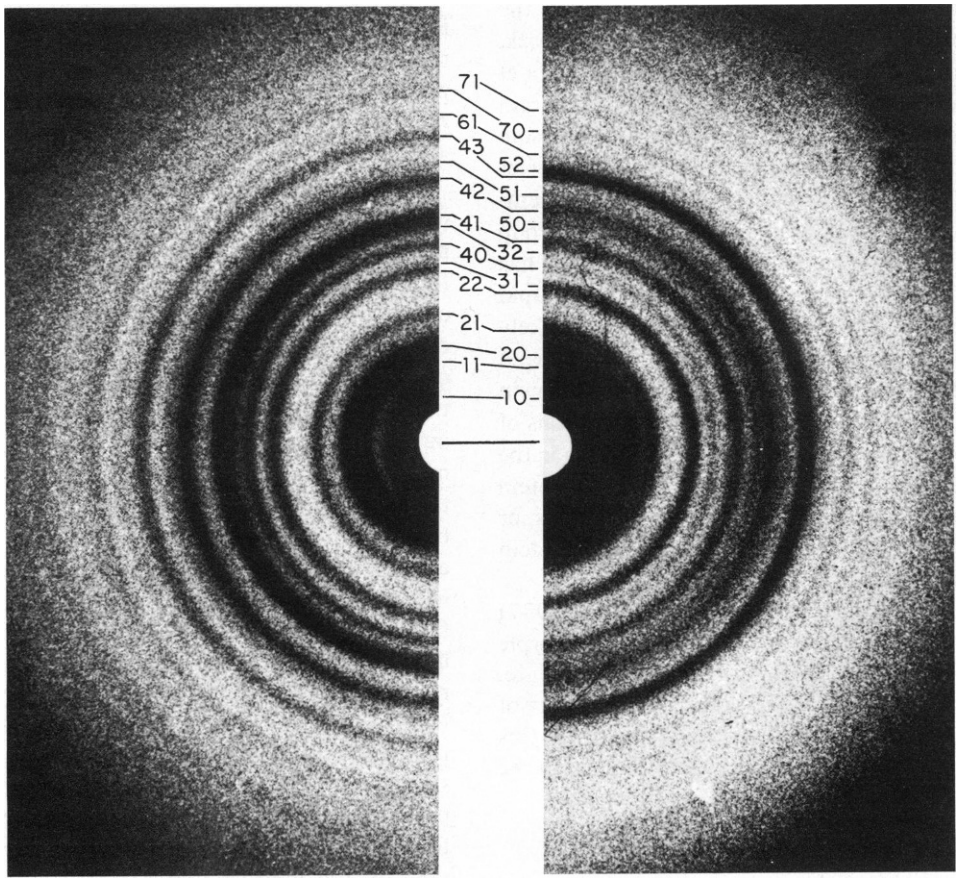

FIGURE 1 Comparison of x-ray diffraction patterns recorded from pellets of native purple membrane (*left*) and DOC-treated purple membrane (*right*). A similar sample-to-film distance was used in both cases.

Measurement of electron diffraction intensities was carried out as described by Baldwin and Henderson (1984). Image analysis was carried out on five electron micrographs as described by Unwin and Henderson (1975). However, two more micrographs were treated by a real-space method based on the procedures of Saxton et al. (1984), Frank (1982), and Crepeau and Fram (1981) before Fourier analysis. This procedure gave significant improvements in resolution and will be described separately (Henderson, R., manuscript in preparation).

RESULTS

The crystallinity of specimens obtained after treatment with DOC was first confirmed by recording x-ray diffraction patterns of pelleted membranes. Fig. 1 shows a comparison of x-ray patterns recorded for native membranes and for DOC-extracted membranes, respectively. The x-ray diffraction patterns were recorded at the same specimen-to-film distance, and confirmed that the cell dimension of the DOC-treated membranes was smaller than that of the native membranes. Rather large differences in the distribution of diffraction intensity are also evident in this figure. As expected, the electron diffraction intensities of DOC-extracted membranes (shown in Table I) are also very different from those of native purple membranes.

TABLE I
STRUCTURE FACTORS FOR THE DOC-TREATED PURPLE MEMBRANE

h	k	A	ϕ
1	0	14	−122
1	1	76	170
2	0	30	−168
2	1	14	−172
1	2	28	−16
3	0	20	99
3	1	28	−147
2	2	47	110
1	3	19	127
4	0	52	−52
4	1	45	−41
3	2	46	−11
2	3	41	−47
1	4	59	−87
5	0	35	−1
5	1	27	10
4	2	50	122
3	3	42	132
2	4	45	−157
1	5	26	−82
6	0	28	−50
6	1	27	−75
4	3	45	143
3	4	19	−145
2	5	26	−75
1	6	24	162
7	0	27	−154
6	2	11	−69
5	3	10	77
4	4	10	5
3	5	31	−44
2	6	19	169
1	7	19	98
6	3	17	3
4	5	15	95

Structure factors were obtained to a resolution of 6.2 Å by combining the amplitudes from four electron diffraction patterns and phases from seven electron micrographs, the latter being recorded at somewhat different values of defocus. The values of the structure factors obtained are listed in Table I. The phase residual in the merged data set was 25°. The values of the amplitudes, $|F|$, are, in general, accurate to between ±5 and ±10%, except for the (2, 5) and the (4, 3) reflections, which seem to be more sensitive than others to small tilt angles, and may therefore be accurate to only about ±25%. The intensity of the (0, 3) reflection is also relatively weak and very sensitive to the tilt angle.

The Fourier map obtained with these structure factors is shown in the top right-hand half of Fig. 2. Superimposed on this map is an estimate of the molecular boundary obtained by drawing a perimeter located at a distance of 5 Å from the center of the points of maximum density in the Fourier map.

The equivalent Fourier map of native purple membrane is shown for comparison in the bottom left-hand half of Fig. 2. Structure factors for this map were obtained from work published previously (Henderson and Unwin, 1975; Leifer and Henderson, 1983), and phases have been partially refined by application of the molecular replacement method (Baldwin, J., private communication) to data from the native p3 form and the reconstituted $p22_12_1$ form of purple membrane (Michel et al., 1980).

The quaternary structure of the bR trimer in the two cases is indistinguishable at this resolution. There is probably a small counterclockwise rotation of the trimer within the plane of the membrane by 1.5–2°, but at this resolution it is only just detectable. The distance of nearest contact between trimers, measured as the nearest distance between the centers of maximum density on the outer rims of adjacent trimers, is reduced from 16.5 Å in the native membrane to 11.5 Å in the DOC-extracted membrane. The reduction in cell dimension from 62.4 to 57.3 Å therefore seems to be accounted for entirely by the removal of a boundary layer of lipid, one molecule wide, from the space between protein trimers. Enough space probably remains to accommodate three pairs of lipid molecules at one of the threefold axes between trimers in the DOC-treated membrane, and in both membranes there is a space of similar size and shape at the center of the protein trimer.

The density in the map of the DOC-treated membrane looks a little rounder and less peaky than in the native map, even though the resolution in the DOC map is nominally higher (6.2 compared with 6.6 Å). This is probably because the structure factor amplitudes in the DOC structure are falling off faster as a function of resolution. This may be caused by an intrinsically higher disorder or, more

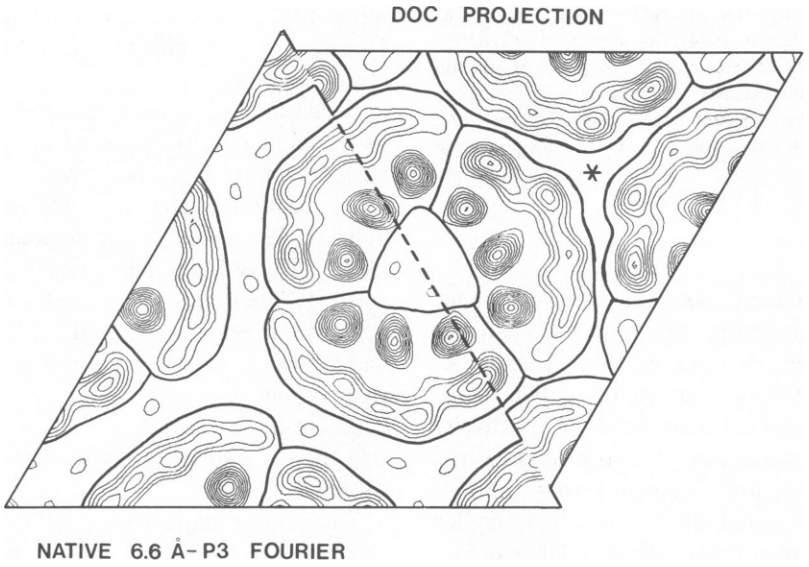

FIGURE 2 Comparison of the projected density maps of native and DOC-extracted purple membrane obtained by direct imaging in the electron microscope. The probable molecular outline of single protein molecules is shown.

likely, by the effect of radiation damage on the DOC electron diffraction patterns that were obtained from much smaller crystals than the native diffraction patterns.

Finally, the results (Table II) of the lipid analysis of the DOC-treated membranes show that the lipid-depleted membrane contains ~30% of the normal lipid content, this being composed of more glycolipid (45%) and less phospholipid (20%), with a small quantity of residual DOC remaining.

DISCUSSION

The Fourier map obtained for the two-dimensional projection of DOC-extracted purple membrane rules out the possibility of movement of protein monomers within the trimer, or, to a first approximation, of rotation of trimers within the lattice. On the other hand, the lattice parameter is reduced by >8% after DOC treatment, and the molecular transform is therefore sampled quite differently in the two cases. This accounts for the observed intensity changes out to a resolution of 6 Å.

Ordered lipid molecules may also make a significant contribution to the diffraction intensity for the native membrane at higher resolutions. Both the removal and the repositioning of such lipid could occur in the DOC form. As discussed below, a comparison of the projected structures of native and DOC-treated membranes indicates that ordered lipids are removed by the detergent and that at least some of the remaining lipids occupy positions in the DOC-extracted membrane that are different from those adopted in the native membrane.

It is evident from the two maps shown in Fig. 2 that the crystallographic packing of trimers in the native purple membrane involves only protein-lipid-protein contacts, and that there are no direct protein-protein contacts between trimers. At the same time, it is known that the bR trimers are packed with extremely good long-range and short-range order, from the fact that sharp electron diffraction spots can be observed to a resolution of 2.6 Å or better (Hayward and Glaeser, 1980). It is unlikely that such a good crystallographic order of the trimers could be achieved if all of the lipid molecules lying between the trimers were crystallographically disordered. Thus, we conclude that some of the lipid molecules must be crystallographically ordered between protein trimers.

The molecular envelope shown in Fig. 2 assigns ~72% of the area of the native purple membrane to protein and ~28% of the area to lipid. The envelope should probably be enlarged slightly in order to bring the protein molecules in

TABLE II
LIPID ANALYSIS OF NATIVE AND DOC-TREATED PURPLE MEMBRANE

	Native	DOC-treated	Reference
Total weight of lipid extractable (g/g)	22%	6.6%	This work
Hexose content measured as glucose (g/g)	2.86%	1.23%	This work
Phosphorus atomic content (g/g)	1.0%	0.2%	Hwang and Stoeckenius (77)
		0.1% after repeated extraction	Kates et al. (82)
Residual DOC by ^{14}C-label (mol/mol bR)	0.0	0.33	This work

the lipid-depleted membrane into tight Van der Waals contact at the threefold symmetry axis, which already shows too small an area to accommodate a lipid molecule, and also along the full length of the interface where trimers are in obvious protein-protein contact. The measured ratio of areas of protein and lipid is, however, already slightly larger than that expected from the estimated mass ratio of 75% protein and 25% lipid. Perhaps the molecular packing in the lipid-depleted membrane leaves some small gaps between protein trimers. This could account for the fact that contrast in negatively stained membranes after the DOC treatment seems to be somewhat higher than for native membranes, where it is very weak (Hwang and Stoeckenius, 1977).

The measured decrease in lattice constant after DOC extraction suggests that <40% of the original lipid remains in the DOC-treated membranes. This figure is calculated on the assumption that lipid occupies an area of ~940 Å^2 on each side of the native membrane (28% of the area of the unit cell) and from the fact that the area of the unit cell in extracted membranes is reduced by ~530 Å^2. The molecular area of diether dihydrophytoyl phosphatidyl glycerol in monolayer films is quoted to be ~60 Å^2 (Blaurock, 1975). All of the above data are therefore finally consistent with the idea that there are ~10 mol of lipid per mole of protein in the native purple membrane and 4 mol (or less) of lipid per mole of protein in DOC-extracted membranes. This figure for the total lipid in DOC-extracted membranes is higher than published estimates of the amount of lipid (estimated from lipid phosphorus) that remains in the membrane after DOC treatment (Hwang and Stoeckenius, 1977; Kates et al., 1982). However, as shown in Table II, the amount of glycolipid is not reduced as much as the phospholipid, while the total lipid measurement is in reasonable agreement with the area estimates.

The general arrangement of the lipids in the native unit cell can now be deduced, as illustrated by the schematic model shown in Fig. 3. The two adjacent dihydrophytoyl chains of the lipid molecules are modeled as an oval with a minor axis of 5.0 Å and a major axis of 10 Å. These ovals are then fitted into the space available to the lipids in the native membrane, based upon the molecular envelope of bR seen in the DOC-extracted membrane. The precise positions and orientations of lipid molecules, and whether lipid molecules on opposite sides of the membrane "bilayer" are in register or are offset from each other, cannot be determined from the existing data. However, the kind of model-building illustrated by Fig. 3 suggests that there are three pairs of lipid molecules at each of the crystallographic threefold axes of the native membrane, and that there are two pairs of lipid molecules located roughly midway between the two nontrimer threefold axes. A shortcoming of the model shown in Fig. 3 is the fact that squalene molecules, which are thought to be present at the level of 1–2 mol per mole of bR (Kates et al., 1982), are not treated explicitly. Unfortunately, there is not yet enough known about (*a*) whether there is an integer stoichiometry of squalene within the purple membrane unit cell, and (*b*) whether squalene molecules adopt an extended conformation (spanning the lipid bilayer) or a hairpin conformation (confined to one half of the bilayer). As a result, it is not yet possible to construct a structural model that reflects the unique chemical structure of this neutral lipid.

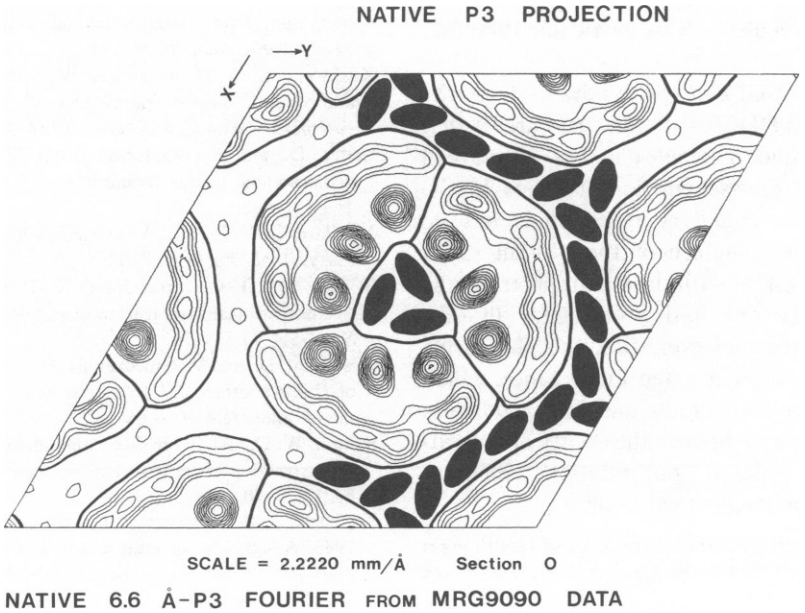

FIGURE 3 Schematic model of the possible distribution of lipid molecules in native purple membrane. The molecular envelope shown in Fig. 2 has been drawn to outline the spaces available to lipid molecules. The lipid molecules are assumed to contain parallel dihydrophytoyl chains, modeled here as ovals with a minor axis of 5 Å and a major axis of 10 Å.

A similar model of lipid distribution has been discussed previously by Jost et al. (1978), but the model shown in Fig. 3 differs from the earlier one in a number of significant points. The model shown in Fig. 3 accommodates 20 lipid chains per bR molecule, whereas the model of Jost et al. included only 18. The difference is due in part to the more accurate value for the molecular envelope afforded by the present studies of lipid-depleted membranes. It should be noted that the model of Jost et al. places only four lipid chains on each side of the bilayer at the threefold symmetry axis within the protein trimer, whereas we believe that the molecular envelope of the protein will accommodate six chains at that position. Other differences also exist in the detailed distribution of lipid chains, but both models agree on the key point that the protein trimers are surrounded completely by a boundary layer of lipid that is one molecule wide.

The residual lipid molecules in DOC-treated membranes must be confined solely to the threefold axis at the center of the trimer and just one of the threefold axes between trimers, shown with an asterisk in Fig. 2. The packing of lipid molecules and their degree of structural order within the center of the trimer are unlikely to be affected by the detergent treatment. However, there seems to be only one possibility for the packing of oval-shaped lipid molecules at the external threefold axis: the narrow, elongated shape of the open space between the envelope of adjacent trimers requires that the long axis of the oval should point out radially from the threefold symmetry axis. It is interesting that the same position and orientation of three lipid molecules does not provide a satisfactory space-filling arrangement when it is translated to the Fourier map of the native membrane. It is thus quite probable that there is a major structural difference in the positions and orientations of lipid molecules at this particular threefold axis.

The major conclusion that can now be drawn from the comparison of native and DOC-extracted structures is that at least some of the lipid molecules in the native cell membrane must adopt precise positions relative to the protein. Removal of the boundary layer lipids has no observable effect on the structure of the protein itself, however, and this is consistent with the small spectroscopic changes observed by Hwang and Stoeckenius (1977). These authors have noted, however, that the photocycle kinetics are altered in DOC-extracted membranes, which suggests that the molecular contacts and lattice-bonding forces between trimers may influence the ability of individual bR monomers to undergo conformational changes required in the normal photochemical cycle.

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