An improved freeze-fracture electron microscope procedure has been developed and applied to the study of the association of bacteriorhodopsin in large proteoliposomes reconstituted by reverse-phase evaporation with egg lecithin. Due to the improved accuracy and resolution of this procedure, intramembrane particles, the diameter of which (4.5 nm) closely matched that of bacteriorhodopsin monomer, could be observed at high lipid to protein ratios (≥40 w/w). At lower lipid to protein ratios, larger particles (diameter 7.5 nm) progressively appeared, resulting in bimodal particle size distributions up to a lipid to protein ratio of 1, where the large particles were the sole species present. These large particles were interpreted as corresponding to bacteriorhodopsin oligomers.

Because of the large size and homogeneity of proteoliposomes, accurate particle density measurements could be performed. These confirmed the occurrence of a lipid to protein ratio-dependent bacteriorhodopsin monomer-oligomer equilibrium and further allowed us to identify the oligomer as a trimer or a tetramer.

In complementary experiments, it was found that the bacteriorhodopsin monomer and oligomer had identical visible lipid to protein ratios (240 w/w). At lower lipid to protein ratios, larger particles (diameter 7.5 nm) progressively appeared, resulting in bimodal particle size distributions up to a lipid to protein ratio of 1, where the large particles were the sole species present. These large particles were interpreted as corresponding to bacteriorhodopsin oligomers.

In particular, the state of aggregation of bacteriorhodopsin (BR), the light-activated proton pump of Halobacterium halobium, has been investigated. In its native purple membrane, BR is organized in a two-dimensional hexagonal lattice of protein trimers (4, 16). Such organization gives rise to a characteristic excitonic CD signal due to interactions between protein chromophores. A similar protein arrangement and CD spectrum can be recovered in reconstituted bacteriorhodopsin-lipid vesicles below the order-disorder transition temperature of lipid hydrocarbon chains (12, 17, 18).

Above this temperature, no excitonic CD is usually observed (12, 17, 18), and conventional freeze-fracture electron microscopy shows the presence of isolated intramembrane particles the mean diameter of which is 2-3 times larger than that determined for BR monomer by electron microscopy of glucose-embedded purple membrane (4). This size difference led some authors to the conclusions that BR may be oligomeric in such proteoliposomes (12). Such a conclusion may, however, be incorrect if one does not take into account the possible enlargement of intramembrane particles by plastic deformation of membrane proteins during fracturing and by variable metal deposition during shadowing.

Although such deformation and enlargement of particles is endemic to the freeze-fracture procedure, they can be minimized by using appropriate fracturing and shadowing procedures. We describe below such an improved procedure which we have used to study the aggregation state of bacteriorhodopsin in reconstituted bacteriorhodopsin-lipid vesicles as a function of the protein to lipid ratio. For this, we make use of BR proteoliposomes reconstituted by reverse-phase evaporation (19), the large size and homogeneity of which appear particularly well suited for such studies. Our results show quite unambiguously that bacteriorhodopsin is in a monomeric form at high lipid to protein ratios and starts to

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The abbreviations used are: BR, bacteriorhodopsin; Pipes, 1,4-piperazinediethanesulfonic acid.
self-associate to form well defined oligomers (trimers or tetramers) for lower lipid to protein ratios. This conclusion is based, first of all, on analysis of the sizes of intramembrane particles and is also confirmed by analysis of intramembrane particle densities.

We finally conclude that the freeze-fracture procedure described here may be useful for the study of other membrane proteins.

MATERIALS AND METHODS

BR Reconstitution—BR proteoliposomes were reconstituted using the reverse-phase evaporation technique described previously (19) with a 9:1 molar mixture of egg phosphatidylcholine and phosphatidic acid as lipids and 20 mM Pipes, pH 7.1, 110 mM K₂SO₄, as buffer. Lipid to protein ratios ranging from 80:1 to 1:1 (w/w) were used. After reconstitution, proteoliposomes were sized through 400 nm and 200 nm polycarbonate membranes.

Freeze-Fracture Procedure—The samples were cryoprotected with glycerol (30% w/w) and rapidly frozen in liquid propane, either using conventional Balzer's gold planchets or an ultrarrapid "sandwich" freezing procedure (20). In the latter case, a small drop of the sample solution (about 0.1 μl) was compressed between two thin copper plates and rapidly plunged into liquid propane. The fracturing was performed at −150 °C with a nitrogen-cooled knife for the conventionally frozen samples, and by opening a "sandwich" immediately before shadowing, for the ultrarrapidly frozen samples. The replication of the fractured surfaces was performed in the direction of fractures using tungsten-tantalum (W-Ta) alloys in four to six steps, each lasting a few seconds and separated by about 10-s periods during which the partly shadowed, fractured surfaces were allowed to cool. In order to avoid contamination of the fractured surfaces, the samples were protected, between each shadowing step, by a liquid nitrogen-cooled blade. The replicas were cleaned in chromic acid, washed with distilled water, and observed in a Philips 301 electron microscope. Low magnification calibration was performed with the grating replica and intermediate magnifications with glutaraldehyde-fixed, negatively stained catalase crystals (21). The sizes of particles appearing on fracture surfaces were measured in a direction perpendicular to the direction of shadowing using a finely graduated ruler placed under a large field stand magnifier (×3). The precision of individual measurements was estimated to be about 0.1 mm, which corresponds to an error in particle size measurement of about ±0.8 nm. For the comparison of the data obtained using the above described procedure with other procedures, some samples were shadowed with Pt-C and others rotary-shadowed with W-Ta in conventional ways (20).

Spectroscopic Methods—CD spectra were recorded with a Mark V Jobin Yvon CD spectrometer using 1-mm quartz sample cells. Measurements of light-induced proton transport in proteoliposomes using pyranine fluorescence were performed on a Perkin-Elmer MPF 44A spectrofluorimeter equipped with a magnetic stirrer and a temperature control system. Excitation was performed at 460 nm and emission was recorded at 510 nm. Proteoliposomes were diluted to a final concentration of 0.8 mM phospholipids in 4 mM Pipes, pH 7.1, 130 mM K₂SO₄, containing 1 μM pyranine as an external pH probe, and supplemented with 0.1 μM valinomycin. A 150-watt xenon lamp (Osram) equipped with heat and yellow filters were used as the actinic light source.

RESULTS

Freeze-Fracture Morphology of BR Reconstituted Proteoliposomes—A typical low magnification freeze-fracture electron micrograph of BR reconstituted proteoliposomes prepared by reverse-phase evaporation is shown in Fig. 1A. Most of the material appears as large spherical, unilamellar vesicles. The size distribution of vesicles, shown in Fig. 1B for two different lipid to protein ratios, is relatively broad and centered around an average diameter of about 150 nm.

This value is in good agreement with dynamic light scattering measurements (19, 22) and with estimation of vesicle diameter from the entrapped aqueous volume (19, 22). Most important for the present study, the size distribution of proteoliposomes is independent of the lipid to protein ratio, a feature which can be related to the fact that all reconstituted preparations are sized through 200-nm nucleopore membranes (see "Materials and Methods"). Higher magnification views of BR-proteoliposomes reconstituted at high lipid to protein ratios are shown in Fig. 2, together with views of pure lipid vesicles and of Triton X-100-solubilized BR. As opposed to pure lipid material (Fig. 2A), the BR-proteoliposomes clearly show the presence of intramembrane particles (Fig. 2B). These appear to be randomly dispersed on both concave and convex fracture surfaces (for all preparations tested, the ratio of particle densities on convex and concave fractures was always close to 1). On the other hand, no particles are visible outside of the vesicles, in the surrounding ice, such as those clearly seen on the pictures of detergent-solubilized BR (Fig. 2E), indicating that all protein is incorporated into proteoliposomes during the reconstitution. This was observed for all lipid to protein ratios tested.

A quantitative analysis of the intramembrane particle size distribution (Fig. 2D) indicates that the average diameter of the particles is about 4.5 nm, which is very close to the dimensions of a monomeric BR (2.5 × 3.5 × 4.5 nm) as

![Fig. 1](attachment:image.png)
Fig. 2. A, preparation of pure liposomes. B, preparation of bacteriorhodopsin proteoliposomes at a lipid to protein ratio of 40 (w/w). C, the same preparation as in B, but freeze-fractured in a conventional way and shadowed with Pt. D, size distribution of intramembrane particles measured on bacteriorhodopsin proteoliposomes such as those shown in B and C, for a lipid to protein ratio of 40 (w/w); closed circles, improved freeze-fracture procedure; open circles, conventional freeze-fracture procedure. E, freeze-fracture view of bacteriorhodopsin-Triton X-100 micelles. F, particle size distribution for bacteriorhodopsin-Triton X-100 micelles as measured from micrographs such as that shown in E. Magnification is the same for all micrographs. The bar in E represents 200 nm.

determined by electron microscopy of the purple membrane (4). Consistently, the data shown in Fig. 2D (closed circles) and 2F indicate that similar average particle diameter is observed with Triton X-100-solubilized BR, which is known to be monomeric (23). Fig. 2, C and D (open circles), shows, respectively, a high magnification view and the corresponding size distribution of the same BR-proteoliposome preparation after conventional freeze-fracturing. As can be seen, much larger particles are observed, the average diameter of which is about 10 nm.

Influence of the Lipid to Protein Ratio on Intramembrane Particle Size—Fig. 3 shows a series of electron micrographs of BR-proteoliposomes of different protein to lipid weight ratios, from 80:1 to 1:1 (w/w), i.e. from 0.012 to 0.5 weight% BR. As can be expected, the surface density of intramembrane particles increases with decreasing lipid to protein ratio. But the striking observation is that the size of the intramembrane particles also increases with increasing protein content. This is best seen on the histograms of particle size distributions, such as those shown in Fig. 4. At high (<40 (w/w)) lipid to protein ratios, this distribution is symmetric and centered at about 4.5 nm. At intermediate ratios (20:1 to 3:1 (w/w)), the distribution is bimodal, probably indicating the appearance of rather well defined, larger particles. At the lowest lipid to protein ratio tested (1(w/w)), the particle size distribution is again almost symmetric, but is now centered at about 7.5 nm. Lower lipid to protein ratios were not tested since these resulted in incomplete incorporation and partial bleaching of BR. The simplest explanation of these data is to assume that at high lipid to protein ratios BR is monomeric. When the protein content increases above a given lipid to protein ratio, the BR monomer starts to self-associate into a rather well defined oligomer, as can be deduced from the presence of a bimodal particle size distribution. This self-association process seems to be completed at a lipid to protein ratio of about 1 (w/w) (i.e. 31 molecules of lipid per BR). It is difficult to give an exact number of BR molecules present in these oligomers, but if one takes into account the value of the
FIG. 3. Freeze-fracture views of bacteriorhodopsin proteoliposomes as a function of increasing protein content. A, B, C, D, E, and F correspond, respectively, to lipid to protein ratios of 80, 40, 20, 10, 5, and 1 (w/w), i.e., to BR weight percentages of 0.012, 0.024, 0.048, 0.091, 0.167, and 0.5. Note the increasing density and size of intramembrane particles with increasing bacteriorhodopsin concentration. Magnification is the same for all micrographs. The bar in F represents 200 nm.
Particle Density-Independent information on the aggregate a priori. The first correction should take into account available for particle counting. For this reason, we have used greater than 8. It was first necessary to check that the particle distribution from measurement of the surface density of intramembrane protein ratio tested to protein ratio (1 (w/w)). The average diameter of particles present at the lowest lipid to protein ratio tested (7.5 nm), their number can hardly be greater than 4. We have also found that when the freezing rate is increased by the use of our ultrarapid “sandwich” freezing procedure (20), no change in the particle size distribution is observed (Fig. 4, lowest dotted line).

**Influence of Lipid to Protein Ratio upon Intramembrane Particle Density—**Independent information on the aggregation properties of BR within proteoliposomes can be obtained from measurement of the surface density of intramembrane particles as a function of the lipid to protein ratio. In principle, the exact particle density can be measured over the whole of each sample, then the comparison of such a value with the theoretical density of BR polypeptides (calculated from the known composition of the samples) can be used to estimate the composition of each class of particles. However, in order to obtain the exact number of particles per proteoliposome from particle counting over the observed fracture surfaces, several corrections must be made, which are difficult to evaluate a priori. The first correction should take into account the partition of particles between convex and concave fracture faces. Other corrections are related to the heterogeneity of vesicle sizes and the heterogeneity of fracture diameters for each size. Additionally, when unidirectional shadowing is used, some portions of the fractured vesicle surfaces (depending on the shadowing angle and on the vesicle size) are not available for particle counting. For this reason, we have used two different approaches: one approximate and the second one more quantitative. Both approaches rely on particle counting on particular proteoliposome size classes, in order to obviate the problem of proteoliposome size heterogeneity. It was first necessary to check that the particle distribution was not a function of proteoliposome size. The number of particles counted per fractured proteoliposome was plotted as a function of the squared diameter of the fractured proteoliposomes for each lipid to protein ratio investigated (not shown). In each case, a linear relation was found, indicating that the particle surface density was independent of proteoliposome size. This confirms the homogeneity of the reconstituted system with regard to protein distribution.

For the first, semiquantitative estimation of the evolution of the particle density with the lipid to protein ratio, particles were counted for a particular size class of fractured proteoliposome surfaces, namely those of diameter 200 ± 10 nm and compared with the theoretical number of particles which should have occurred in the absence of oligomerization (Table I). Above a lipid to protein ratio of 20 (w/w), the ratio R of these two quantities remains constant and is equal to about 8. This represents the correction factor which should be applied to take into account the fact that only a portion of the proteoliposome surface is actually observable. At a lipid to protein ratio of 20 (w/w) and below, the value of R increases with decreasing lipid to protein ratio, due to BR oligomerization which reduces the number of particles. At a lipid to protein ratio of 1, where oligomerization is completed according to particle size measurement, this ratio is close to 32. Taking into account the correction factor of 8, the value for the degree of BR oligomerization is about 4.

The second more quantitative approach for the estimation of particle densities takes advantage of the presence in each preparation of a certain number of large vesicles displaying almost flat central regions. An example of such vesicles is shown in Fig. 5.

Counting the particles on such large surfaces gives the most accurate particle density values. These values are compared in Table I with the expected densities of BR molecules, calculated for corresponding lipid to protein ratios. At high lipid to protein ratios, the two densities are equal within experimental error (after dividing the calculated density by 2 to take into account the fact that particles distribute equally between concave and convex fracture faces). This reinforces the conclusion that the particles observed at high lipid to protein ratios correspond to BR monomers.

For lipid to protein ratios of 20 (w/w) and below, the measured values of the particle densities deviate progressively from the expected ones, confirming that BR aggregation is taking place. The degree of BR oligomerization obtained from the ratio of the experimental to the expected particle density values for the lowest lipid to protein ratio tested here is 3.4.

**Influence of Cholesterol upon Intramembrane Particle Size and Distribution—**It has been shown recently that cholesterol can induce an important aggregation of intramembrane particles in BR-proteoliposomes (24). We repeated this experiment, using the above described freeze-fracture procedure in order to compare the aggregation properties of BR in the presence and absence of cholesterol. The main difference between cholesterol-containing samples and those described above occurs at low lipid to protein ratios, where one observes intramembrane particles heavily clustered, leaving large, smooth areas completely devoid of particles. An example of such behavior is shown in Fig. 6 for a cholesterol to phospholipid molar ratio of 2:1 and a protein to lipid ratio of 3 (w/w). Small portions of the hexagonally packed BR, similar to those observed in the native purple membrane, are also occasionally observed (see arrow in Fig. 6).

**Effect of BR Aggregation on Circular Dichroism Spectra—**The freeze-fracture data described above indicate that the lipid to protein ratio and/or lipid composition have a strong influence on the oligomerization-aggregation behavior of BR. It was thus interesting to investigate how these parameters affect the circular dichroism (CD) spectrum of BR. Indeed, it
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Evolution of intramembrane particle density of bacteriorhodopsin proteoliposomes as a function of lipid to protein ratio

<table>
<thead>
<tr>
<th>Lipid/BR (w/w)</th>
<th>BR weight %</th>
<th>Lipid/BR (mol/mol)</th>
<th>Calculated BR surface density (BR/μm²)⁴</th>
<th>Measured particle density (BR/μm²)⁴</th>
<th>Calculated number of BR per liposome of 200-nm diameter</th>
<th>Measured number of particles per fractured 200-nm proteoliposome</th>
<th>R'</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>0.012</td>
<td>2,500</td>
<td>1,150</td>
<td>500</td>
<td>140</td>
<td>18</td>
<td>8</td>
</tr>
<tr>
<td>40</td>
<td>0.024</td>
<td>1,250</td>
<td>2,240</td>
<td>1,200</td>
<td>280</td>
<td>34</td>
<td>8.2</td>
</tr>
<tr>
<td>20</td>
<td>0.048</td>
<td>625</td>
<td>4,400</td>
<td>2,000</td>
<td>550</td>
<td>52</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>0.091</td>
<td>312</td>
<td>8,500</td>
<td>3,500</td>
<td>1,000</td>
<td>65</td>
<td>15</td>
</tr>
<tr>
<td>5</td>
<td>0.167</td>
<td>155</td>
<td>15,800</td>
<td>3,500</td>
<td>2,000</td>
<td>75</td>
<td>27</td>
</tr>
<tr>
<td>3</td>
<td>0.25</td>
<td>93</td>
<td>24,400</td>
<td>3,600</td>
<td>3,100</td>
<td>95</td>
<td>32</td>
</tr>
<tr>
<td>1</td>
<td>0.5</td>
<td>31</td>
<td>51,000</td>
<td>7,500</td>
<td>6,400</td>
<td>200</td>
<td>32</td>
</tr>
</tbody>
</table>

* The surface density of BR was calculated by assuming a surface area of 875 Å² for a BR molecule and of 70 Å² for a phospholipid.
* Particle density measured on the flat central regions of large fracture faces (see Fig. 5). Taking into account that particles distribute equally between concave and convex fracture faces, values of surface density of particles can be deduced by multiplying by 2 the measured particle density.
* Ratio of the calculated number of BR per liposome (diameter 200 nm) and the measured number of particles per fracture face (diameter 200 nm).

is well known that when organized in a two-dimensional hexagonal lattice, such as in the purple membrane, BR gives rise to an intense CD doublet in the visible region which is replaced by a less strong single positive band when this protein is monomeric (12, 17, 18).

Fig. 7 shows the CD spectra of BR proteoliposomes at low lipid to protein ratios in the absence and presence of cholesterol, as well as spectra of purple membrane- and Triton X-100-solubilized BR, for comparison. As can be seen, the low lipid to protein ratio cholesterol-containing samples show an excitonic doublet. In the absence of cholesterol, the spectrum displays a characteristic monomer CD signal. It thus appears that the oligomeric BR species observed at low lipid to protein ratios does not display an excitonic interaction between adjacent chromophores. Only when BR is extensively aggregated, in the presence of cholesterol, is this interaction observed on CD spectra.

**Effect of Oligomerization on Proton Pumping and Leakage of BR Proteoliposomes—**Finally, we have tested whether the oligomerization of BR affects its function as a light-induced proton pump. For this, we have measured the proton accumulation properties of BR proteoliposomes reconstituted at different lipid to protein ratios, using the fluorescence probe pyranine, added outside the proteoliposome to measure external pH variations.² The pyranine response to actinic illumination of BR-proteoliposomes is shown in Fig. 8A for different lipid to protein ratios. In all cases, light induces a time-dependent increase of pyranine fluorescence, indicative of external alkalization which reversed in the dark. This confirms the predominantly inside-out orientation of BR (i.e., inward proton pumping) in the reconstituted system (26). In Fig. 8B, the initial rates of light-induced proton pumping (V_ON) and of dark proton back leakage (V_OFF) calculated from the initial slopes of fluorescence responses in the light and the dark are plotted as a function of the inverse of the lipid to protein ratio. For V_ON, a linear dependence is obtained, indicating that the specific activity of BR is constant within this range of lipid to protein ratios.

Thus, the BR oligomerization process appears to have no detectable influence on its proton pumping function. On the other hand, we had previously used pyranine as an entrapped probe inside BR-proteoliposomes to measure internal pH variations associated with proton pumping (25, 26). However, we have recently found that its use as an external probe allows external pH variation to be measured with more accuracy than with potentiometric techniques (unpublished observations).

![FIG. 5. An example of large bacteriorhodopsin proteoliposomes which served for measurements of intramembrane particle density on their almost flat central parts (lipid to protein ratio of 10 (w/w)). The bar represents 200 nm.](image-url)
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FIG. 6. Freeze-fracture view of bacteriorhodopsin proteoliposomes containing cholesterol. Cholesterol to lipid molar ratio is 2:1 and lipid to protein ratio is 1 (w/w). The bar represents 200 nm.

FIG. 7. Circular dichroic spectra of purple membrane and bacteriorhodopsin proteoliposomes (BR concentration, 38 μM). Trace 1, purple membrane. Trace 2, bacteriorhodopsin in proteoliposomes (lipid/BR = 1 (w/w)) in the presence of cholesterol (egg phosphatidylcholine/phosphatidic acid/cholesterol, 9:1:5). Trace 3, bacteriorhodopsin in proteoliposomes (lipid/BR = 1 (w/w)) in the absence of cholesterol. Inset, Trace a, purple membrane; Trace b, Triton X-100-solubilized purple membrane.

The first result of this study is that our improved freeze-fracture-shadowing procedure can provide a relatively good estimation of the size of intrinsic membrane proteins. The choice of BR for testing this procedure was particularly appropriate.

FIG. 8. Light-induced proton movements in bacteriorhodopsin proteoliposomes reconstituted at different lipid to protein ratios. A, proteoliposomes (lipid/protein ratios ranging from 5 to 160 (w/w)) were resuspended in a medium containing 150 mM K$_2$SO$_4$, 4 mM Pipes (pH 7.1) and 1 μM pyranine and supplemented with 0.1 μM valinomycin. Light-induced changes in the fluorescence intensity of pyranine were recorded as described under "Materials and Methods." B, initial rates of light-induced pyranine fluorescence response ($V_{an}$, closed circles) and of dark reversal response ($V_{od}$, open circles) calculated from Part A as a function of the lipid to protein ratios.

other hand, the lipid to protein ratio dependence of $V_{off}$ is more complex. Above lipid to protein ratios of 20, i.e. 0.048 weight % BR, $V_{off}$ was nearly constant, indicating very little influence of the protein content on the proton passive permeability of the membrane. However, below this lipid to protein ratio, a dramatic increase of $V_{off}$ occurred and at a lipid to protein ratio of 1, $V_{off}$ was 20-fold higher than at a lipid to protein ratio of 80. It is worth remarking that the onset of this increase of proton passive permeability corresponds to that of the BR oligomerization process described above.

DISCUSSION

The first result of this study is that our improved freeze-fracture-shadowing procedure can provide a relatively good estimation of the size of intrinsic membrane proteins. The choice of BR for testing this procedure was particularly appropriate.
proteins, since it is one of the smallest intrinsic membrane proteins ($M_r = 27,000$). With such a small protein, the relative enlargement due to metal deposition may be expected to lead to an important overestimation of its size. The results shown here demonstrate, however, that the average diameter of the intramembrane particles corresponding to the BR monomers is only some 1–1.5 nm larger than the average molecular size of BR. It is very likely that for larger intrinsic membrane proteins, this relative enlargement will be even smaller. It is also shown here that the same procedure can be used to detect discrete association phenomena of intrinsic membrane proteins, even those involving limited oligomerization.

This situation contrasts with the conventional freeze-fracture procedure, which when used to study bacteriorhodopsin proteoliposomes under conditions where BR is monomeric leads to a mean size of intramembrane particles of the order of 10 nm, i.e. an enlargement of about 6–7 nm in comparison with the true molecular diameter. Such large values of intramembrane particle diameters have led Casadio and Stoeckenius (12) to suggest that BR may existing reconstituted proteoliposomes as nonamers or dodecamers. This appears, however, highly speculative in view of the important enlargement associated with conventional freeze-fracture procedures.

Although, in principle, intramembrane particle diameters could be related to molecular diameters through an appropriate correction procedure, this is difficult to perform in practice. In particular, it appears to us quite misleading to use a single multiplicative correction factor associated with a particular shadowing procedure (7, 14, 15). Indeed, the metal thickness has no reason to be proportional to the molecular diameter. It would be more reasonable to subtract a constant value from the measured mean particle size for each procedure. In this regard, we have found (data not shown) that when the conventional freeze-fracture procedure is used, the BR oligomer shows a mean particle diameter of about 12 nm as compared to 10 nm for a monomer. If we assume that the enlargement of both species is the same, 1.5 nm for our procedure and about 6 nm for conventional freeze-fracture, then corrected values for monomer and oligomer will be about the same (respectively, 3.5 nm and 6 nm). In our opinion, an acceptable procedure for accurate size determination of membrane proteins is, however, only realized when the absolute enlargement is significantly smaller than the molecular diameter. This appears to be the case in our study (1–1.5 nm as compared to about 3.5 nm). Analysis of the dimensions of the intramembrane particles indicate that BR is monomeric down to lipid to protein ratios of about 40, which correspond to 1250 lipids per BR molecule. At lower lipid to protein ratios, the BR molecules start to self-assemble into a rather well defined oligomer. This self-association seems to be completed at the lipid to protein ratio of about 1 w/w, i.e. for about 30 lipids per BR. We have also performed measurements of the density of the intramembrane particles in reconstituted proteoliposomes. In order to obtain the most accurate quantitative values for this density we have taken advantage of the presence in our preparations of a few very large proteoliposomes displaying almost flat central portions. The results clearly showed that BR undergoes a limited self-association process which begins at a lipid to protein ratio of 40 and is completed at a lipid to protein ratio of 1. At this lowest lipid to protein ratio, evaluation of particle densities indicates that the BR oligomer is composed of 3–4 BR polypeptides.

Interestingly, we have found a direct correlation between the amount of BR oligomers (as deduced from the freeze-fracture results) and the rate of proton passive permeability of the proteoliposomes. The possibility that membrane protein oligomers could provide pathways of passive permeability in membranes has already been suggested (27). Another problem raised the present study is the relation between the BR oligomer found in proteoliposomes and the BR trimer of the purple membrane hexagonal lattice. The isolated oligomer does not appear to display any excitonic CD signal (note that this cannot be due to some chromophore rearrangement during reconstitution since an excitonic CD was observed in cholesterol-containing proteoliposomes). If, as suggested (28), the excitonic doublet is due to chromophore interactions within the trimer, then it must be concluded that the isolated BR oligomer is structurally different from the trimer. However, the hypothesis that the CD excitonic doublet is an intrinsic property of the trimer has never received direct experimental support. In this regard, according to recent retinal localization data by neutron diffraction of the purple membrane (29), it appears that the interchromophore distance between BR in the trimer is similar to that between neighboring BRs in adjacent trimers. Excitonic interactions outside trimers may be important in determining the CD spectrum. Thus, despite the absence of a CD doublet, the oligomer we observe may be structurally similar to the hexagonal lattice trimer. In any case, in view of our data, as well as those of Hiraki et al. (30), who found that at low pH the excitonic CD signal of regenerated brown membrane is removed although the lattice structure is retained, it seems that CD cannot be used as an unequivocal probe of the BR aggregation state.

Several intrinsic membrane proteins reconstituted with lipids above the order-disorder transition temperature have been shown to aggregate at low lipid to protein ratios (31–33). Usually such effects occur at lipid to protein ratios of the order of 100–200 (mol/mol) and result in the segregation of protein patches. These are interpreted as resulting from perturbation of the lipid phase by the protein, which is minimized by squeezing the protein out of the bulk lipids (34).

The BR oligomerization process described here appears to be of a different nature. Indeed, it begins at a very high lipid to protein ratio, 40 (w/w) (i.e. about 1250 lipids/protein). Furthermore, it is limited to the formation of BR trimers or tetramers and does not lead to protein segregation even when the ratio is very low (1 w/w, i.e. about 30 lipids/protein). Thus, it appears likely that the BR oligomerization is due to an intrinsic affinity of BR for itself rather than to an effect of lipid-protein interactions. On the other hand, the formation of the purple membrane-like patches observed when cholesterol is present in the liposomes may be attributed to a lipid-mediated effect. It is likely that the decrease of lipid mobility introduced by cholesterol promotes the aggregation of BR trimers into patches as is found with phospholipids below the order-disorder transition (12, 17, 18). Such properties may be related to the formation of the BR hexagonal lattice during the biogenesis of the purple membrane in Halobacterium, or to properties which occur when lipids are brought below the phase transition temperature, in reconstituted systems. Indeed, the first step may be the formation of such oligomers which afterward segregate when a very high local protein concentration is reached. The hexagonal lattice would form when these oligomers are separated by about one layer of lipid (see Ref. 35). Thus, formation of BR patches would occur in a sequential and progressive way rather than by a rapid all or nothing phase separation. This could explain why a two-dimensional crystalline arrangement is formed when BR segregates, unlike what is observed with other membrane proteins.

**Acknowledgments**—We would like to thank J. C. Dedieu for excellent technical assistance, V. Luzzati and A. Tardieu for useful discus-
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