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Mechanisms of Membrane Protein Insertion into Liposomes during Reconstitution Procedures Involving the Use of Detergents. 2. Incorporation of the Light-Driven Proton Pump Bacteriorhodopsin†

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ABSTRACT: A method has been developed for identifying the step in a detergent-mediated reconstitution procedure at which an integral membrane protein can be associated with phospholipids to give functional proteoliposomes. Large liposomes prepared by reverse-phase evaporation were treated with various amounts of the detergents Triton X-100, octyl glucoside, or sodium cholate as described in the preceding paper [Paternostre, M.-T., Roux, M., & Rigaud, J. L. (1988) *Biochemistry* (preceding paper in this issue)]. At each step of the solubilization process, we added bacteriorhodopsin, the light-driven proton pump from *Halobacterium halobium*. The protein–phospholipid detergent mixtures were then subjected to SM_2 Bio-Beads treatments to remove the detergent, and the resulting vesicles were analyzed with respect to protein insertion and orientation in the membrane by freeze-fracture electron microscopy, sucrose density gradients, and proton pumping measurements. The nature of the detergent used for reconstitution proved to be important for determining the mechanism of protein insertion. With sodium cholate, proteoliposomes were formed only from ternary phospholipid–protein–detergent micelles. With octyl glucoside, besides proteoliposome formation from ternary mixed micelles, direct incorporation of bacteriorhodopsin into preformed liposomes destabilized by saturating levels of this detergent was observed and gave proteoliposomes with optimal proton pumping activity. With Triton X-100, protein insertion into destabilized liposomes was also observed but involved a transfer of the protein initially present in phospholipid–Triton X-100–protein micelles into Triton X-100 saturated liposomes. Our results further demonstrated that protein orientation in the resulting proteoliposomes was critically dependent upon the mechanism by which the protein was incorporated.

The data presented in the preceding paper (Paternostre et al., 1988) define the conditions for a stepwise solubilization of large liposomes by different detergents. The lipid–detergent interactions can be analyzed in terms of a three-stage model (Lichtenberg, 1985). Accordingly, as increasing amounts of detergents are added to a suspension of liposomes, the detergent is first incorporated into the bilayers, leading to alterations in membrane permeability without solubilization. At a critical detergent concentration, when the bilayers are saturated with detergent, gradual disintegration of the membrane starts and lipid–detergent mixed micelles begin to form. The end point of solubilization is reached when all the membrane material is converted into mixed micelles. It is generally assumed that this solubilization process represents the reverse of detergent-mediated reconstitution of pure liposomes or proteoliposomes (Helenius & Simons, 1975; Eytan, 1982). One possible sequence of events during detergent-mediated reconstitution is the following: Initially, lipid–detergent and lipid–protein–detergent micelles are formed and the detergent is then removed. At a certain point, the micelles are no longer soluble, and structures containing lipids and proteins are formed that, upon reorganization, are transformed into vesicles. The last phase of detergent removal corresponds to the removal of residual detergent from detergent-saturated proteoliposomes.

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With regard to protein–lipid association during detergent removal two kinds of events may occur: (1) Pure liposomes are first formed and the protein incorporates into the preformed liposomes, and/or (2) the protein participates in the gradual formation of proteoliposomes. Since formation of lipid–protein complexes from a micellar solution depends on the changes in local composition within the particles formed upon detergent removal, the aqueous solubility of each constituent and the composition of the initial mixed micelles can be key factors in determining the products of reconstitution (Mimms et al., 1981; Jackson & Litman, 1982, 1985). On the other hand, Eytan and collaborators (Eytan, 1982; Eytan & Broza, 1978), who compared cytochrome oxidase incorporation into proteoliposomes by different reconstitution procedures, have suggested that the rate of detergent removal may be crucial in proteoliposome formation. They proposed that upon slow detergent removal vesicle formation may precede incorporation of protein into the lipid bilayer and that upon rapid detergent removal the proteins are incorporated during the liposome formation which corresponds to the micellar–lamellar transition. Furthermore, Helenius et al. (1981) demonstrated that a critical factor in determining the mechanism of protein reconstitution is the state of aggregation of the proteins when membranes begin to form from the solubilized lipids. Other factors involved include vesicle size, lipid composition, and ionic conditions (Eytan & Broza, 1978; Eytan et al., 1975, 1976). Despite all this information, the molecular mechanisms by which membrane proteins are inserted into the liposomes during detergent removal are still unclear. The purpose of this work is therefore to obtain more precise information about the main parameters involved in working out a general rationale for the reconstitution of membrane proteins into liposomes.

We describe in this paper easily reproducible conditions under which protein insertion into liposomes can be analyzed at each step of the solubilization–reconstitution process. The procedure adopted was to add a protein to large liposome solutions containing the appropriate amount of detergent for the desired step in the lamellar to micellar transition. Next, detergent was removed and the vesicles formed were characterized with respect to protein incorporation, protein orientation in the membrane, and biological activity. This procedure was carried out with bacteriorhodopsin (BR)[1] as a prototypic integral membrane protein. Bacteriorhodopsin, the chromoprotein of the *Halobacterium halobium* membrane, is one of the best characterized electrogenic proton pumps [for a recent review see Dencher (1985)]. Furthermore, various methods for incorporating this protein into liposomes have been published, including detergent-mediated reconstitutions (Van Dijck & Van Dam, 1982). However, the mechanisms that trigger BR incorporation into the lipid bilayer are still far from clear.

The results presented in this paper indicate that the mechanism of detergent-mediated BR incorporation into proteoliposomes is critically dependent upon the nature of the detergent used. Although with sodium cholate proteoliposome formation only arose from mixed phospholipid–protein–detergent micelles, with octyl glucoside and Triton X-100 optimal reconstitutions were not achieved by complete solubilization in the form of ternary mixed micelles but by integration of the protein into destabilized liposomes. In agreement with many data [see, for example, Eytan (1982) and Helenius et al. (1981)] protein was found more asymmetrically oriented in the reconstituted liposomes when integrated into preformed liposomes than when integrated during proteoliposome formation by detergent depletion of ternary mixed micelles.

Materials and Methods

Chemicals. Purified egg-yolk phosphatidylcholine and derived phosphatidic acid were isolated according to the methods of Singleton et al. (1965) and Allgyer and Wells (1979), respectively.

The chemicals used in this study and their sources were as follows: pyranine (Eastman Kodak); [^{14}C]phosphatidylcholine (Amersham); dansylphosphatidylethanolamine (Molecular Probes); Sephadex PD-10 columns (Pharmacia); SM_2 Bio-Beads (Bio-Rad); Triton X-100 (Merck); *n*-octyl β-D-glucopyranoside (Sigma). Cholic acid (Prolabo) was recrystallized as described by Kagawa and Racker (1971). All other chemicals were of analytical grade.

Preparation of Liposomes. Large unilamellar liposomes were prepared by reverse-phase evaporation as described in the preceding paper. Buffers used were 20 mM Pipes–KOH or 20 mM KH_2PO_4, pH 7.2, supplemented with 110 mM K_2SO_4, and contained 200 μM pyranine when stated. Liposomes (about 20 mM phospholipid) were sized through 0.4 and 0.2 μM polycarbonate membranes before use.

Preparation of Bacteriorhodopsin Monomers. Purple membrane was isolated from *Halobacterium halobium* (strain S9) according to the method of Oesterhelt and Stoeckenius (1974). Preparations of bacteriorhodopsin monomers in Triton X-100 and octyl glucoside were carried out as described by Dencher and Heyn (1982). Solubilization of purple membrane by sodium cholate was performed as described by Bakker and Caplan (1978). The treated BR was centrifuged 1 h at 200000*g* to remove any unsolubilized material. The final concentrations of BR monomers were 1 mg of BR/mL in 0.5% TX 100, 1 mg of BR/mL in 100 mM octyl glucoside, and 0.2 mg of BR/mL in 0.5% cholate.

Detergent Removal. To remove detergent, Bio-Beads [extensively washed before use as described by Holloway (1973)] were added directly to the protein–lipid–detergent mixtures at a concentration of 80 mg of wet beads/mL. The mixtures were gently stirred at room temperature. After 3 h of incubation, a second portion of 80 mg of wet beads was added for an additional incubation period of 2 h. The turbid suspensions were transferred to dialysis bags and dialyzed overnight at 4 °C against 500 mL of buffer containing Bio-Beads outside the bags (Philippot et al., 1983). This dialysis step was chosen for cholate-mediated reconstitutions to avoid a too long contact with Bio-Beads for removal of high detergent concentration.

Due to the detergent-induced changes in permeability of the liposomes, removal of nonencapsulated material (e.g., pyranine) or changes of the external buffer (e.g., for pH-meter measurements) were performed only after complete detergent removal. For TX 100 and OG this was done after the Bio-Beads treatment by dialysis or passage through PD 10 Sephadex G-25 columns. For cholate-mediated reconstitutions, this was done only after the overnight dialysis by passage through PD 10 Sephadex columns.

Vesicle Characterization. After reconstitution, proteoliposomes were submitted to discontinuous flotation gradients. The vesicle–protein mixtures (1 mL) were thoroughly mixed with 1 mL of sucrose (60% w/w) in 20 mM Pipes and 100 mM K_2SO_4, pH 7.2, supplemented with 0.05% TX 100 (due to the impermeability of the proteoliposomes to sucrose) in

[1] Abbreviations: EPC, egg phosphatidylcholine; EPA, egg phosphatidic acid; OG, octyl β-D-glucoside; TX 100, Triton X-100; Pipes, 1,4-piperazinediethanesulfonic acid; pyranine, 8-hydroxy-1,3,6-pyrenetrisulfonate; BR, bacteriorhodopsin; ΔpH, transmembrane pH gradient; R_{eff}, effective molar ratio of detergent to phospholipid; PM, purple membrane sheets.

10-mL centrifuge tubes; 1–2 mL of 20%, 10%, 5%, and 2.5% sucrose (w/w) was successively layered, followed by 0.5 mL of buffer. These density gradients were centrifuged at 30000 rpm for 1 h in a Beckman SW 41 rotor. The fractions were collected and analyzed for lipid and protein content. Lipid content was determined by using [^{14}C]phosphatidylcholine. Bacteriorhodopsin was assayed by absorption spectroscopy (λ = 560 nm) in the presence of TX 100 in order to avoid light scattering by liposomes.

Freeze-fracture electron microscopy was done as detailed in the preceding paper.

Light-Induced Proton Movements. Changes in internal pH were measured as changes in the fluorescence intensity of the pH-sensitive fluorescent probe pyranine trapped within the vesicle as detailed elsewhere (Seigneuret & Rigaud, 1985, 1986a,b). Proton uptake into the BR liposomes was assayed by the pH-meter essentially as described earlier (Rigaud et al., 1983). The transmembrane pH difference was determined from [^{14}C]methylamine uptake by using the flow dialysis apparatus (Colowick & Womack, 1969) with the interpretation method described earlier (Hellingwerf et al., 1979; Rigaud et al., 1983).

RESULTS

Reconstitution Procedure. The standard procedure for studying the incorporation of BR into liposomes was the following: Liposomes prepared by reverse-phase evaporation were resuspended at the desired concentrations in the buffer used for their preparation (reconstitution experiments have been performed between 2.5 and 10 mM phospholipid without significant changes). Then TX 100, octyl glucoside, or cholate was added under vortex mixing at the desired detergent to lipid ratio. After 5–10 min of incubation (our preceding study showed that the time of detergent equilibration was complete within a few minutes), bacteriorhodopsin was added to give the desired final lipid to protein ratio. Unless otherwise stated, BR was generally added as a solution of detergent-solubilized monomeric BR. No significant changes in protein incorporation were observed when the detergent and the protein were first mixed and then added to the liposome suspensions. The detergent–protein–phospholipid mixtures were kept at room temperature for 5 min to 2 h under gentle stirring, and the detergent was then removed.

Besides the importance of the nature of the detergent and of the detergent to phospholipid ratio during protein insertion (see below), one of the parameters critically affecting the results of this reconstitution procedure was the method of detergent removal.

Maximum light-induced proton accumulation by BR liposomes requires a sealed vesicle. In view of the drastic effects of the detergents upon the liposome basic permeability (Paternostre et al., 1988), it was imperative that residual detergent could be minimized. We mainly focused our attention on the removal of Triton X-100. Due to its low critical micelle concentration, this detergent cannot be easily removed by dialysis (Allen et al., 1980), and therefore a different method was required, namely, adsorption by direct contact with hydrophobic resin beads (Holloway, 1973).

Figure 1 shows the time course of Triton X-100 removal from various detergent–phospholipid mixtures in the presence of SM_2 Bio-Beads. For this purpose [^{3}H]Triton X-100 has been added externally to preformed liposomes at different detergent to phospholipid ratios. After equilibration, the different solutions were mixed with a fixed amount of Bio-Beads (80 mg of wet beads/mL), and the adsorption of TX 100 was monitored as a function of time. Starting from effective detergent to phospholipid molar ratios of 0.64 and less, most of the detergent was removed from the medium by processes that gave rise to essentially monoexponential decay curves (data not shown), with half-times of about 10 min. It is noteworthy that less than 1% of the initial TX 100 was detected after 3 h of incubation. According to the results presented in the preceding paper, for R_{eff}'s below 0.64 all the detergent present in the solution is monomeric. Hence, the present results indicate that nonmicellar detergent can be efficiently adsorbed into Bio-Beads and, consequently, that in Triton X-100 mediated reconstitution experiments residual detergent can be completely removed. With higher detergent concentrations (R_{eff} > 0.64) the process of Triton X-100 removal was no longer monoexponential, and its half-time increased drastically with the initial detergent concentrations. Since the maximum capacity of Bio-Beads is not reached in our experimental conditions,[2] it has to be assumed that the access of TX 100 to the pores of the beads is hampered by the prior binding of other detergent molecules. Consequently, raising the bead concentration may accelerate TX 100 removal. The inset in Figure 1 demonstrates that, indeed, the rate of

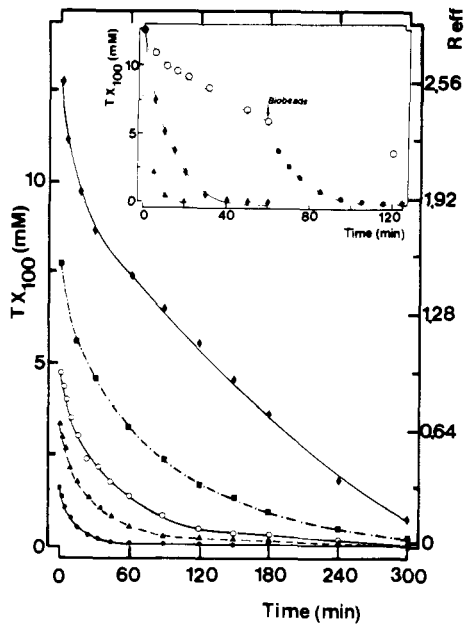

FIGURE 1: Time course of Triton X-100 removal by SM_2 Bio-Beads. Liposomes prepared by reverse-phase evaporation were resuspended at a concentration of 5 mM phospholipid and treated by different amounts of [^{3}H]TX 100 (millimolar as indicated in the figure). Then 160 mg of Bio-Beads were added to 2 mL of the lipid–detergent mixtures and maintained in suspension by gentle agitation. Aliquots from the supernatant (25 μL) were collected as a function of time and analyzed for their radioactivity. R_{eff} = ([TX 100]$_{total}$ − 0.18 mM)/[phospholipid] is the effective molar detergent to phospholipid ratio. (Inset) Influence of the SM_2 Bio-Beads concentration. Liposomes (5 mM phospholipid) were solubilized in the presence of 12.5 mM TX 100 and then mixed with different proportions of Bio-Beads: 80 (○), 240 (◆), and 460 (▲) mg/mL. The arrow indicates when additional fresh Bio-Beads (80 mg/mL) were added to the phospholipid–detergent mixtures.

[2] Titration of SM_2 Bio-Beads with ^{3}H-labeled TX 100 yielded an adsorptive capacity of 185 mg of TX 100/g of wet beads [these results together with a detailed analysis of TX 100 adsorption onto Bio-Beads will be published separately (Rigaud et al. unpublished results)]. On the other hand, titration of SM_2 beads with [^{14}C]dipalmitoyllecithin yielded an adsorptive capacity of 1 mg of phospholipid/g of wet beads for pure liposomes and 2 mg of phospholipid/g of wet beads for phospholipid–detergent micelles: This indicated that in our experimental conditions, starting from 4 mg phospholipid/mL, lipid loss in the presence of 80 mg of Biobeads is negligible.

Table I: Effect of the Rate of Triton X-100 Removal on the Proton Pumping Activities of Reconstituted BR Proteoliposomes[a]

Bio-Beads (mg/mL)	total H^+ uptake (nequiv of H^+)	initial rate of H^+ uptake (nequiv of H^+/min)
80	500	300
160	450	250
320	350	150
480	300	135

[a] Liposomes were treated by solubilizing Triton X-100 concentrations (R_{eff} = 2.5) and incubated for 1 h in the presence of BR previously solubilized in Triton X-100 [final phospholipid concentrations 5 mM; final lipid/BR ratio 60 (w/w)]. Then the detergent was removed by different concentrations of Bio-Beads. The H^+ pumping activities of the resulting proteoliposomes were measured by the pH-meter technique as described under Materials and Methods.

detergent removal was critically dependent upon the amount of Bio-Beads present in the solution. Starting from a solubilized sample containing 12.5 mM TX 100 and 5 mM phospholipid, the removal of the detergent was essentially complete after a 10-h incubation with 80 mg of Bio-Beads/mL. If after 3 h of incubation in the presence of this amount of Bio-Beads additional fresh beads were added, the detergent removal was greatly accelerated and completed after only an additional 2 h of incubation. Comparatively, in the presence of 460 mg of Bio-Beads/mL, the detergent could be eliminated in only 15 min. However, at this point it is important to note that the rate of detergent removal is important for optimal reconstitution results. Table I indicates that both initial rates and total extents of H^+ pumping diminish when the amount of Bio-Beads and thus the rate of detergent removal increase. This may be due on the one hand to the formation of multilamellar structures and to the scramble orientation of BR in the membrane upon rapid detergent removal on the other. To overcome these difficulties and minimize lipid loss,[2] we first removed the detergent with only a few beads (80 mg of Bio-Beads/mL), and after 3 h (the micellar to lamellar transition being complete), we added the same amount of fresh beads for fast removal of the residual detergent. The vesicles obtained under these conditions consist of a fairly homogeneous unilamellar vesicle population with mean diameters of about 200 nm (data not shown).

Although several methods and, in particular, dialysis are available for efficient removal of octyl glucoside and sodium cholate (Philippot et al., 1981; Allen et al., 1980), it was of interest to test the capacity of Bio-Beads to bind these two detergents by using the batch procedure described above. For this purpose, liposomes containing bacteriorhodopsin were prepared by reverse-phase evaporation (Rigaud et al., 1983). When illuminated, these proteoliposomes displayed a light-induced proton movement resulting in a large alkalinization of the external medium (inset Figure 2). Upon addition of detergents at concentrations saturating the bilayers (R_{eff} = 0.6, 1.25, or 0.3 for TX 100, OG, and cholate, respectively) this light-induced proton movement was abolished by the permeabilization of the membrane. We followed the recovery of the initial steady-state proton uptake as a function of the time of incubation in the presence of Bio-Beads: It is clear from the data reported in Figure 2 that SM_2 Bio-Beads adsorb not only Triton X-100 and OG in accordance with previous reports (Holloway, 1979; Philippot et al., 1981) but also the cationic detergent cholate. Although cholate removal was slow, it was nevertheless complete.

Triton X-100 Mediated Reconstitution. (*a*) *Transport Activities.* Figure 3A shows the effect of the initial TX 100/phospholipid ratio on the transport activity of the reconstituted BR proteoliposomes. Liposomes prepared by reverse-phase evaporation were treated with different amounts of TX 100 in the presence of BR previously solubilized in the same detergent. The detergent–phospholipid–BR mixtures were then incubated for various times at room temperature followed by SM_2 Bio-Beads treatment. After detergent removal, the light-induced H^+ movements in the resulting proteoliposomes were measured by the pH-meter technique and/or using the pH-sensitive fluorescent probe pyranine trapped inside the liposomes (Seigneuret & Rigaud, 1985, 1986b). Net inwardly directed proton movements were always observed. This resulted in light-induced reversible alkalinization of the external medium when measured by the pH-meter technique or light-induced fluorescence quenching when measured with entrapped pyranine.[3]

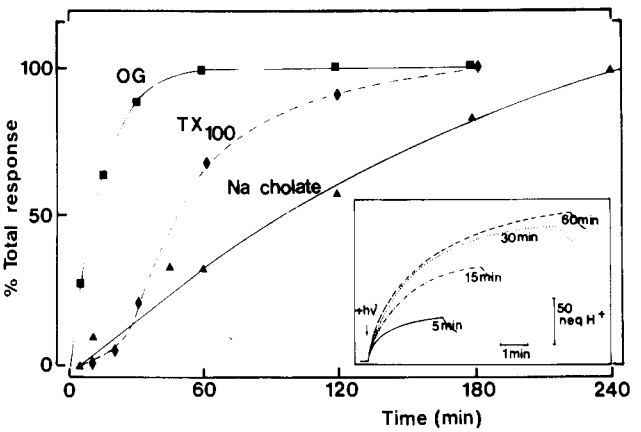

FIGURE 2: Time course of Triton X-100, octyl glucoside, and sodium cholate removal by SM_2 Bio-Beads. Liposomes containing bacteriorhodopsin were prepared by reverse-phase evaporation, resuspended at a concentration of 5 mM phospholipid, and treated with saturating amounts of TX 100 (3.4 mM corresponding to an R_{eff} of 0.64), of octyl glucoside (23.5 mM corresponding to an R_{eff} of 1.3), or sodium cholate (4.3 mM corresponding to an R_{eff} of 0.3). Then Bio-Beads (80 mg/mL) were added, and the light-induced proton uptake by BR proteoliposomes was measured as a function of time. Recovery of the light-induced pH changes is expressed as percent of the initial response before detergent treatment. (Inset) Changes in external pH generated upon illumination of BR proteoliposomes pretreated by a saturating amount of OG and incubated for different times with 80 mg/mL Bio-Beads.

From parts A and B of Figure 3 it is obvious that the proton pumping efficiency after reconstitution is critically dependent not only upon the initial TX 100/phospholipid ratio but also upon the time of incubation of BR with detergent–phospholipid mixtures before detergent removal. The turbidities of the phospholipid–detergent–BR mixtures before detergent removal are plotted in Figure 3A (dashed curve): As described in the preceding paper, the maximum turbidity observed at R_{eff} = 0.64 corresponded to the onset of the lamellar to micellar transition (R^{Sat}), whereas at R_{eff} = 2.5 all the liposomes initially present have been transformed into micelles (R^{Sol}). When BR was incubated in the presence of liposomes treated with subsolubilizing TX 100 concentrations (R_{eff} < 0.64), no

[3] It should be stressed that in the absence of valinomycin the initial H^+ pumping rates as well as the steady-state total H^+ extent pumpings were much slower in all the samples analyzed. The low proton pumping activity observed in the absence of valinomycin is due to the well-known retroinhibitory effect of the transmembrane electrical potential, $\Delta\psi$, which develops across proteoliposomes when all the ions present in the medium are relatively impermeant (Seigneuret & Rigaud, 1986b). In the presence of the antibiotic valinocmyin the $\Delta\psi$ is overcome by compensatory K^+ movements and a large ΔpH (acidic inside) develops. These observations also made on our reconstituted proteoliposomes confirm that TX 100 removal was very efficient and led to BR liposomes with a very low basic ionic permeability.

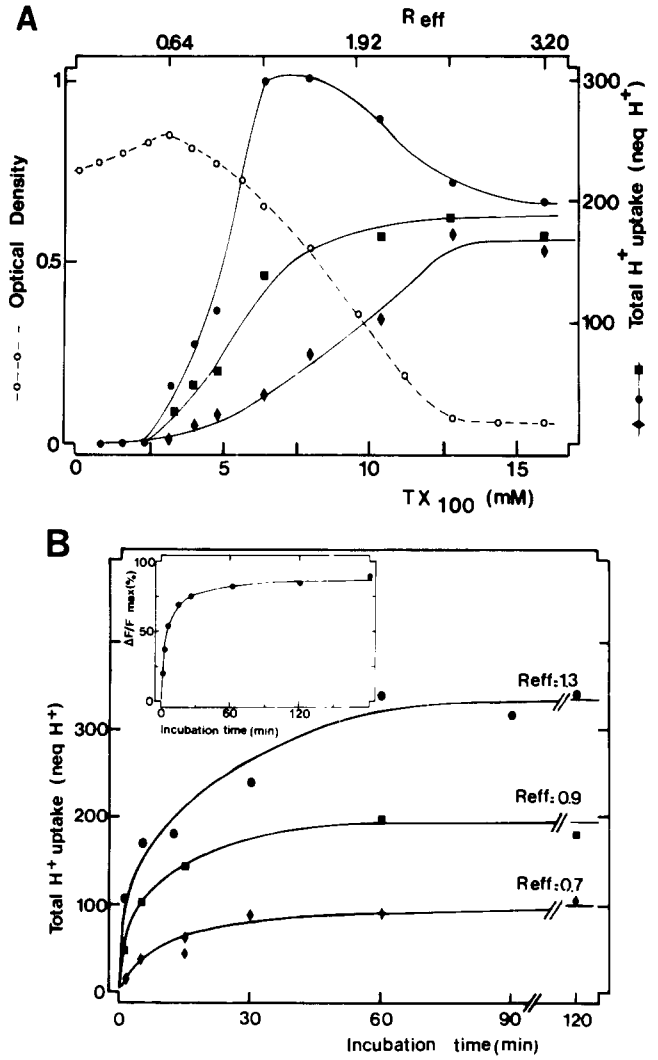

FIGURE 3: Triton X-100 mediated reconstitution of bacteriorhodopsin. (A) Liposomes prepared by reverse-phase evaporation were treated with variable amounts of TX 100 in a final volume of 2.250 mL. Then 250 μL of BR previously solubilized in 0.5% TX 100 was added under vortex mixing. The final TX 100 concentrations, which take into account the small amount of detergent added with the protein, are indicated in the figure. Final phospholipid concentration 5 mM, i.e., 4 mg/mL; final BR concentration 100 μg/mL. Incubation medium: 10 mM Pipes, 120 mM K_2SO_4, pH 7.2, supplemented with 200 μM pyranine. The different phospholipid–BR–TX 100 mixtures were incubated at room temperature for 2 min (◆), 5 min (■), or 1 h (●) followed by Bio-Beads treatment and overnight dialysis against a buffer containing 2 mM Pipes, 130 mM K_2SO_4, pH 7.2. Two milliliters of the reconstituted proteoliposomes were transferred to pH-meter cuvettes, and light-induced pH changes were measured as described under Materials and Methods. Dashed curve (–O–) represents turbidity of the phospholipid–BR–TX 100 mixtures before detergent removal. (B) Time course of BR incorporation as measured by the pH-meter technique. Same experimental conditions as in (A). Proteoliposomes were reconstituted from phospholipid–TX 100–BR mixtures (R_{eff}'s of 1.3 (●), 0.9 (■), or 0.7 (◆). After various periods (as indicated in the figure), detergent was removed and H^+ pumping activities of the resulting proteoliposomes measured by the pH-meter technique. (Inset) Time course of BR incorporation as measured by light-induced changes in pyranine fluorescence intensity. Proteoliposomes were reconstituted from phospholipid–TX 100–BR mixtures at an R_{eff} of 1.3 and incubated for various periods before detergent removal. Maximal changes in fluorescence of pyranine trapped inside liposomes ($\Delta F/F\%$) were measured as described under Materials and Methods.

proton pumping activity could be detected after reconstitition, whatever the time of incubation before detergent removal. The results in our preceding paper have shown that up to this ratio no liposome solubilization occurs, the free TX 100 concentration in the aqueous phase being below the critical micelle concentration. These observations indicate that, in the absence of micelles, BR cannot be directly incorporated into preformed large liposomes, even if the latter are destabilized by a saturating level of TX 100.

Above this critical ratio of 0.64, BR is incorporated into liposomes as indicated by the light-induced H^+ movements measured (Figure 3A). However, one striking feature of our results is the critical dependence of the resulting H^+ pumping efficiency upon the time of BR incubation with detergent–phospholipid mixtures. Increasing the time of incubation of the protein with the detergent–phospholipid suspension gradually increased the proton pumping efficiency of the resulting proteoliposomes, except in samples reconstituted at $R_{eff} \geq 2.5$. Maximal H^+ pumping activities were obtained after about 1 h of incubation, as shown in Figure 3B. Interestingly, after 1 h of incubation, H^+ pumping efficiency was optimal for samples incubated at detergent to phospholipid ratios much lower than those necessary for complete solubilization of the initial phospholipid suspensions. Samples reconstituted from an R_{eff} of 1.3 mol of TX 100/mol of phospholipid displayed a H^+ pumping extent about twice as great as samples reconstituted from an R_{eff} of 2.5.

(*b*) *Sucrose Density Gradients.* To understand H^+ pumping activities as a function of both the initial detergent to phospholipid ratios and the length of incubation, reconstituted vesicles were investigated by means of flotation on discontinuous sucrose density gradients.

When the recombinants resulting from a 2-min incubation at an R_{eff} of 1.3 were analyzed, two liposome populations emerged from the sucrose density gradient patterns shown in Figure 4Aa. Just below the top of the gradient at the interface between 2.5 and 5% (w/w) sucrose, 60–70% of the liposomes were found to contain little or no protein. At the interface between 10 and 20% (w/w) sucrose, most of the BR was found associated with the remaining phospholipids. The pattern of the sucrose density gradient of proteoliposomes resulting from a 5-min incubation (Figure 4Ab) showed an additional band at the interface between 5 and 10% (w/w) sucrose that contained about half of the protein and phospholipids initially present. Concomitantly, protein-free liposomes now only constituted 30% of the total phospholipids. Finally, after an incubation period of 1 h (Figure 4Ac), proteoliposomes banded into one population containing all the protein and phospholipid located at the interface between 5 and 10% (w/w) sucrose. In the absence of phospholipid all of the protein was found at the bottom of the gradient, while in the absence of protein all phospholipid was found at the 5–2.5% (w/w) sucrose interface (data not shown).

The most likely explanation accounting for the sucrose gradients is that when BR is added to the phospholipid–detergent mixtures, it is first rapidly incorporated in the mixed phospholipid–detergent micelles already present in an equilibration time of less than 2 min. If detergent is removed at this step, proteoliposomes will be formed only from these micelles. It can be noted that there is good agreement between the percentage of solubilized phospholipid deduced from the turbidity measurements (30% of the phospholipids are solubilized at an R_{eff} of 1.3) and the percentage of reconstituted proteoliposomes after 2 min of incubation deduced from the sucrose density gradient. Increasing the time of incubation before detergent removal would allow a transfer of BR from ternary mixed micelles to detergent-saturated liposomes, thus reducing the proportion of protein-free liposomes. After 1 h of incubation, the process is complete and no protein-free liposomes can be detected. On the other hand, the consequence

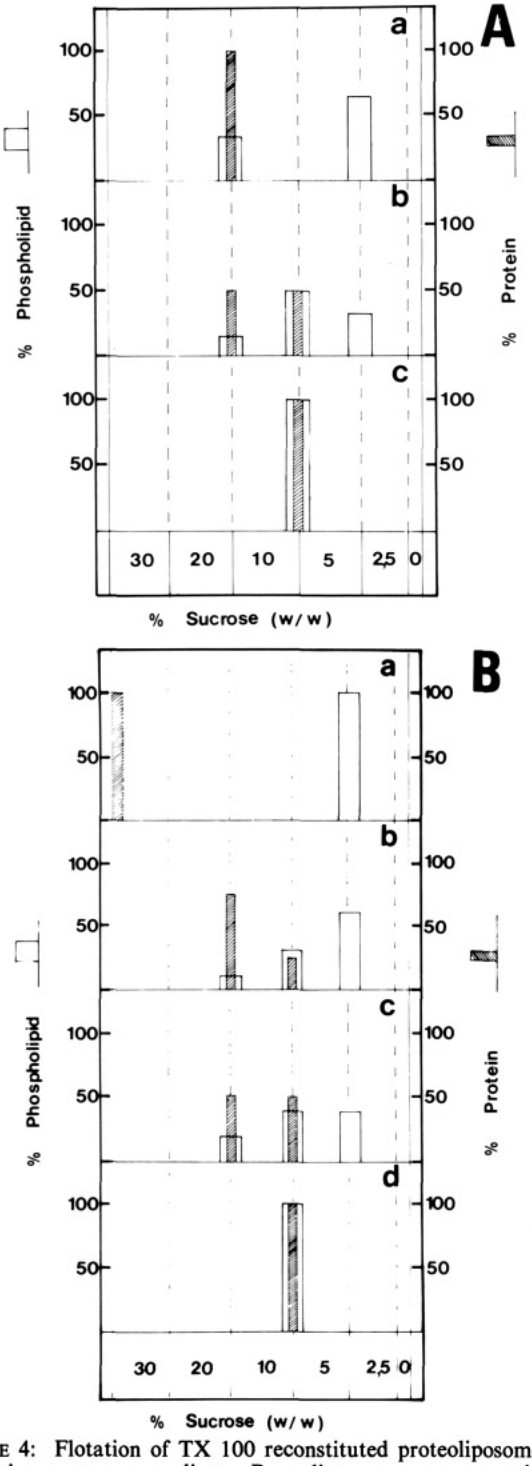

FIGURE 4: Flotation of TX 100 reconstituted proteoliposomes in discontinuous sucrose gradients. Proteoliposomes were reconstituted as outlined in the legend of Figure 3A except that liposomes were prepared in the presence of [^{14}C]phosphatidylcholine. Samples were submitted to flotation in sucrose gradients as described under Materials and Methods. Fractions were collected from the bottom, dissolved with 1% TX 100, and analyzed for BR (absorption at 550 nm) and [^{14}C]phosphatidylcholine [100%, total BR (hatched bars) or phospholipid (open bars) concentrations present in each sucrose gradient]. (A) Sucrose gradient patterns of protein–lipid recombinants formed from phospholipid–BR–TX 100 mixtures at an initial R_{eff} of 1.3 incubated 2 min (a), 5 min (b), or 1 h (c) before detergent removal. (B) Sucrose gradient patterns of protein–lipid recombinants formed from phospholipid–BR–TX 100 mixtures at R_{eff}'s of 0.6 (a), 0.7 (b), 0.9 (c), and 1.3 (d) and incubated 1 h before detergent removal.

of this transfer process is to increase the number of liposomes containing BR, i.e., the internal volume in which H^+ can be pumped upon illumination. Accordingly, the H^+ pumping activities were found to increase with the length of incubation (Figure 3A,B).

A similar interpretation can be advanced in order to understand the results of protein insertion into liposomes starting from different detergent to phospholipid ratios. Results from flotation in sucrose gradients of vesicles reconstituted from different initial TX 100–phospholipid ratios are summarized in Figure 4B. When reconstitutions were performed at $R_{eff} < 0.64$ (Figure 4Ba), all the protein was found at the bottom of the gradient, indicating the nonincorporation of BR into liposomes treated with subsolubilizing TX 100 concentrations. Above an R_{eff} of 0.64, all the protein was recovered associated with phospholipids. However, the heterogeneity of the resulting proteoliposomes was clearly dependent upon the starting detergent to phospholipid ratio. Liposomes reconstituted from an R_{eff} of 0.7 consisted of three populations. In the first, about 75% of the protein migrated with about 10% of the phospholipid and constituted the densest population. The remaining 25% of the protein, which comprised the second population, occurred as a complex with about 20% of the phospholipids at the interface between 5 and 10% (w/w) sucrose. In the third population, most of the lipids floated at the top of the sucrose gradient, which was devoid of protein. Increasing the initial Triton X-100 concentrations above an R_{eff} of 0.7 reduced the percentage of protein-free liposomes while shifting the BR from the densest population band to the intermediary band at the interface between 5 and 10% (w/w) sucrose. At R_{eff}'s of 1.3 (Figure 4Bd) and above (data not shown), only one band was observed in the flotation sucrose gradient containing all lipid and protein.

These results clearly indicate that BR insertion into proteoliposomes cannot simply be related to the percentage of initial phospholipid solubilization but that a mechanism other than the simple formation of proteoliposomes by detergent removal from phospholipid–TX 100–BR micelles is involved. Transfer of the protein from micelles to the detergent-saturated liposomes clearly occurs but is dependent upon the amount of phospholipids solubilized. At an R_{eff} of 0.7, only 25% of the protein is transferred to about 30% of the phospholipid initially present. The protein-rich liposome population that contains 75% of the protein and 10% of the total phospholipid is probably generated by detergent depletion of the ternary BR–TX 100–phospholipid micelles initially present. Increasing initial R_{eff} increases the number of initial mixed micelles, and consequently the transfer of the protein to detergent-saturated bilayers appears more efficient. Thus, at an R_{eff} of 0.9, corresponding to about 20% of initial phospholipid solubilization, 50% of the protein is transferred, and the amount of protein-free liposomes represents now only 30% of the total phospholipids. Finally, our results indicate that transfer is complete at an R_{eff} of 1.3, corresponding to initial preparations where about 30–40% of the liposomes are solubilized. At this point, homogeneous populations of proteoliposomes, as seen by sucrose density gradients, are obtained.[4]

According to these observations, i.e., increases in the amount of BR-containing liposomes with initial R_{eff}'s, H^+ pumping activities were found to increase drastically between R_{eff}'s of 0.6 and 1.3 (Figure 3). Surprisingly, although reconstitutions from detergent to phospholipid ratios higher than 1.3 yielded

[4] Freeze-fracture electron micrographs of BR liposomes reconstituted from a R_{eff} of 1.3 indicate the presence of vesicles with particles randomly dispersed on all the fracture faces (less than 5% of the faces are devoid of particles). For comparison, proteoliposomes reconstituted from a lower R_{eff} indicate a large proportion of fracture faces devoid of particles (data not shown).

suspensions comparable with respect to flotation on sucrose gradients, H^+ activities were found to consistently decrease. Results presented in the following will show that at higher TX 100 concentrations the protein is more randomly oriented in the reconstituted vesicles.

Octyl Glucoside Mediated Reconstitutions. (*a*) *Transport Activities.* Figure 5A shows the resulting transport activities of proteoliposomes reconstituted from pure liposomes treated with different amounts of octyl glucoside and incubated for 10 min with BR previously solubilized in the same detergent. In accordance with the results of the preceding paper (Paternostre et al., 1988) and as depicted by the changes in turbidity reported in Figure 5A (dashed curve), OG concentrations of 23.5 and 37 mM (i.e., R_{eff}'s of 1.3 and 4) corresponded, respectively, to the onset and total solubilization of the initial liposome suspensions. In contrast to what was observed in TX 100 mediated reconstitutions, H^+ pumping activities in the case of OG-mediated reconstitutions were detected in vesicles reconstituted from initial OG concentrations close to but significantly below those necessary for saturating the initial preformed liposomes. Thus, low H^+ pumping activity was measured at about 17 mM octyl glucoside. At slightly higher concentrations, H^+ pumping activities rose drastically and were maximal in proteoliposomes reconstituted from initial liposome suspensions containing 23–25 mM OG, which corresponded to initial R_{eff}'s of 1.3–1.5. For reconstitutions performed above this critical range of ratios, the total H^+ extents and maximal fluorescence intensity changes of entrapped pyranine slightly decreased to an R_{eff} of 4. At this level, which corresponded to reconstitutions from isotropic micellar solutions, the transport activities of the reconstituted liposomes were independent of increasing OG concentrations. Thus, the most striking feature of the data presented in Figure 5A is that optimal reconstitution of BR occurred approximately at the onset of the solubilization of the preformed pure liposomes present in the incubation medium.

It was further observed that optimal incorporation of BR into vesicles occurred very rapidly, since after 5 min of incubation of solubilized BR with OG-treated liposomes the resulting initial rates and total proton pumpings were already maximal. What is more, lengthening the time of incubation before detergent removal reduced the initial H^+ pumping rates of the resulting proteoliposomes [after 90 min of incubation, the initial rates dropped by 25% (data not shown)]. This last observation may be accounted for by an inhibition of the BR's activity by OG in accordance with the progressive chromophore loss reported to occur in the presence of this detergent (Dencher & Heyn, 1983).

Furthermore, when two reconstitutions were performed, one such that solubilized BR was added to OG-treated liposomes (experiments reported in Figure 5A) and the other such that the appropriate amount of OG was first added to the protein and next added to preformed liposomes, identical results were obtained after only 5 min of incubation. Therefore, there must have been rapid equilibration, i.e., movement of BR and/or phospholipid between micelle populations on the one hand and micelles and detergent-saturated liposomes on the other hand.

(*b*) *Sucrose Density Gradients.* To quantitate the extent of protein reconstitution, proteoliposomes were analyzed on discontinuous sucrose density gradients. Results are summarized in Figure 5B and are in complete agreement with the corresponding H^+ pumping activities in Figure 5A. When reconstitutions were performed below 17 mM OG, all the protein was found at the bottom of the gradient and most of the lipid banding at the 2.5–5% (w/w) sucrose interface as protein-free liposomes (Figure 5Ba). This demonstrated again that BR was not incorporated into liposomes treated with low

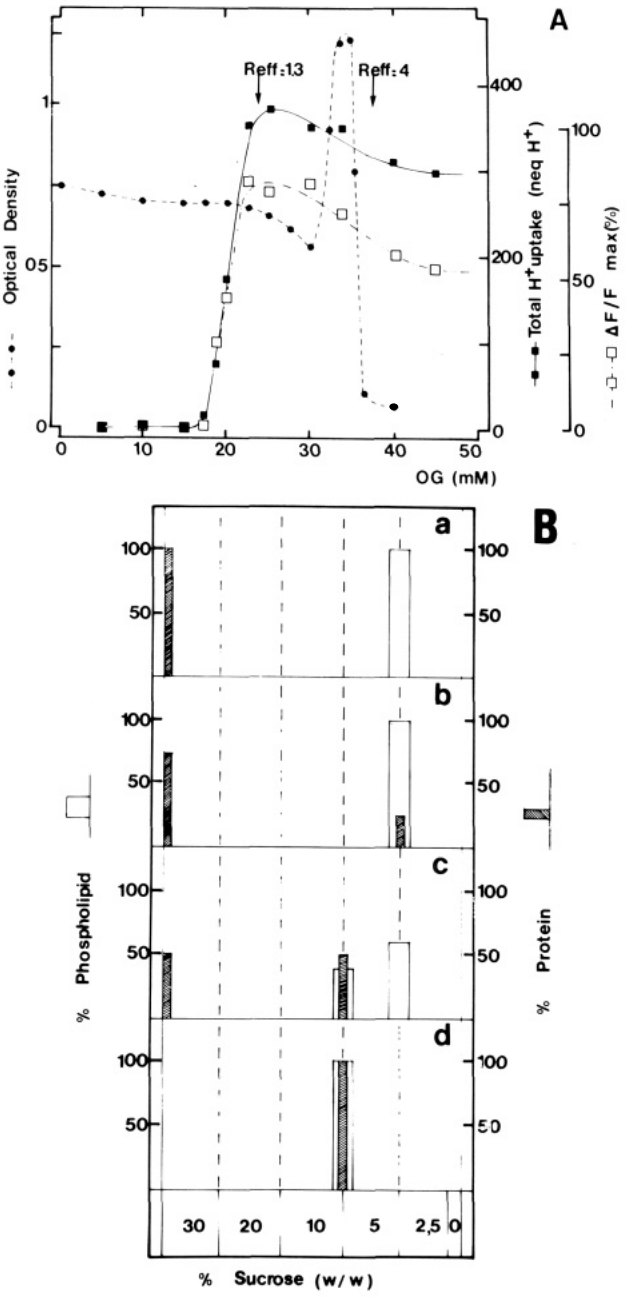

FIGURE 5: Octyl glucoside mediated reconstitution of bacteriorhodopsin. Liposomes prepared by reverse-phase evaporation were first treated with variable amounts of OG in a final volume of 2.375 mL. Then 125 μL of BR previously solubilized in 100 mM OG was added under vortex mixing. Final concentrations: phospholipids, 5 mM; BR, 50 μg/mL. Incubation medium: 20 mM Pipes, 110 mM K_2SO_4, pH 7.2, supplemented with 200 μM pyranine. After a 10-min incubation, OG was removed from the phospholipid–OG–BR mixtures by Bio-Bead treatment followed by overnight dialysis against buffer containing 2 mM Pipes, 130 mM K_2SO_4, pH 7.2, to remove external pyranine and change the external buffer. (A) Light-induced H^+ movements by reconstituted proteoliposomes as measured by the pH-meter (■) or changes in pyranine fluorescence intensity (□). Dashed curve (-●-) represents turbidity of phospholipid–OG–BR mixtures before detergent removal. R_{eff}'s of 1.3 and 4 indicate, respectively, the onset and total solubilization of the liposomes initially present (R_{eff} = $[OG]_{total}$ – 17 mM/[phospholipid]). (B) Flotation of OG-reconstituted proteoliposomes in discontinuous sucrose gradients. Proteoliposomes reconstituted from initial OG concentrations of 10 (a), 17 (b), 20 (c), and 25 mM (d) [100%, total BR (hatched bars) or total phospholipid (open bars) amounts in each sucrose gradient].

subsolubilizing detergent concentrations. For reconstitutions at OG concentrations above about 17 mM, the amount of nonincorporated BR decreased, and concomitantly the protein was found associated with phospholipids (parts b and c of Figure 5B). In samples reconstituted from an initial detergent concentration of 24 mM (R_{eff} = 1.3), the reconstituted material was collected at the interface between 5 and 10% (w/w) sucrose in one broad purple band containing all the phospholipid and protein (Figure 5Bd). For reconstitutions performed at R_{eff}'s above 1.3, similar band patterns were observed in sucrose density gradients, with BR and phospholipid floating as a single band at the 5–10% (w/w) sucrose interface. Thus, density gradient centrifugation not only confirmed the previous observation that optimal H^+ pumping activities occurred in samples reconstituted from liposome–OG suspensions at the onset of solubilization but also revealed that protein incorporation was complete and relatively homogeneous among the liposomes.

Cholate-Mediated Reconstitutions. (*a*) *Transport Activities.* Because of the low extent of purple membrane solubilization by cholate (see Materials and Methods), reconstitutions were performed by adding to cholate-pretreated liposomes a solution of BR monomers solubilized in Triton X-100. It was checked in control experiments that the small amount of TX 100 added together with the protein did not affect the results of cholate-mediated reconstitutions (for reconstitutions at a lipid to protein ratio of 40, the TX 100 to phospholipid ratio was 4 times lower than the critical effective ratio for the onset of liposome solubilization by this detergent). The reconstitution mechanism was indeed not affected for three reasons: First, the turbidity changes of the phospholipid suspensions with increasing cholate concentrations were not affected by the presence of this amount of TX 100 (dashed curve in Figure 6A). In this connection, the onset of phospholipid solubilization and total solubilization were obtained at R_{eff}'s of 0.3 and 0.9, respectively, similar to those previously reported in the absence of TX 100 (Paternostre et al., 1988). Second, identical H^+ pumping activities were measured whether BR was initially added as solubilized monomers in TX 100 or in octyl glucoside. Third, in reconstitutions at high lipid to protein ratios (≥160) in which BR solubilized in cholate was used, similar H^+ pumping activities were recovered after reconstitution, whether TX 100, OG, or cholate was used to solubilize BR (data not shown).

Figure 6A shows the influence of the cholate concentration on the transport activities of the resulting proteoliposomes. No proton pumping activities were detected in samples reconstituted from initial cholate concentrations below those corresponding to the onset of the phospholipid solubilization (R_{eff} = 0.3). Only for reconstitutions performed above this critical ratio were H^+ pumping activities measured whose amplitudes increased progressively with increasing initial detergent concentrations up to an R_{eff} of 0.9. Consequently, for cholate-mediated reconstitutions, the efficiency of BR reconstitution was directly related to the initial percentage of phospholipid solubilization, and optimal H^+ pumping was obtained in samples reconstituted from an isotropic BR–cholate–phospholipid solution. Lastly, it is noted that the length of BR incubation with phospholipid–cholate suspensions before detergent removal did not affect the resulting H^+ pumping activities; similar activities were measured after both 5 min and 2 h of incubation (data not shown).

(*b*) *Sucrose Density Gradients.* Some representative sucrose density gradient patterns of vesicle preparations reconstituted from different cholate/phospholipid ratios are shown in Figure 6B. When reconstitutions were performed at detergent to phospholipid ratios below the critical ratio corresponding to the lamellar to micellar transition, all the protein was found at the bottom of the gradient. This indicated that BR was not incorporated into liposomes treated by subsolubilizing

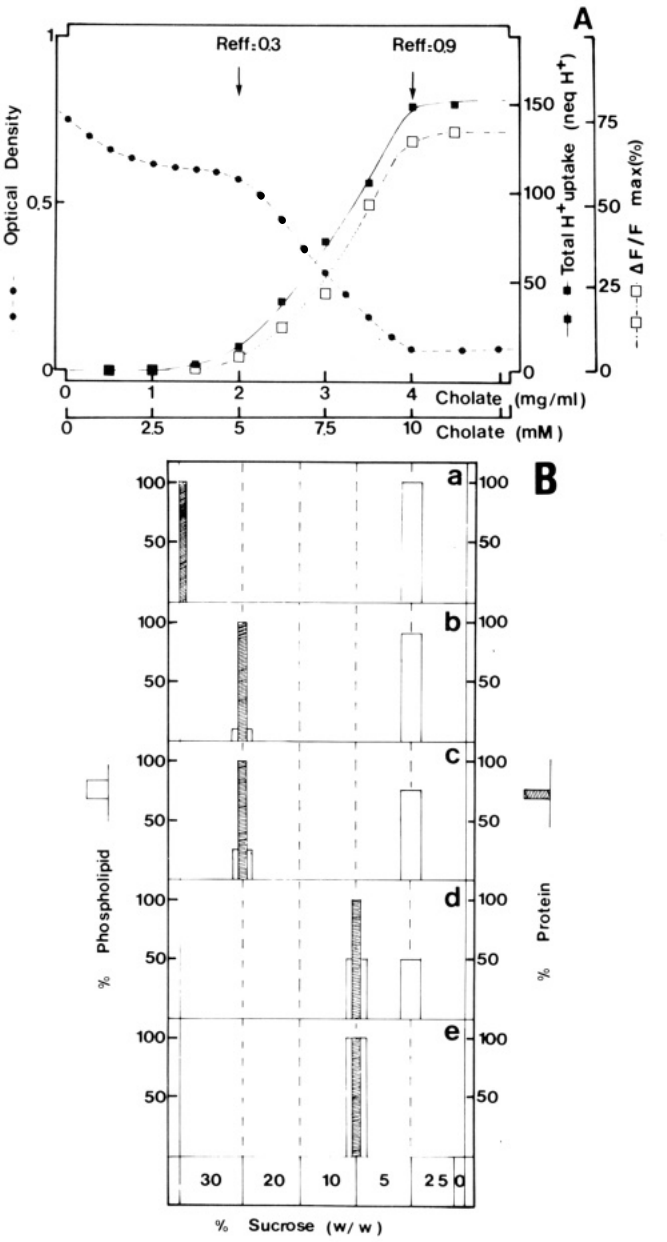

FIGURE 6: Sodium cholate mediated reconstitution of bacteriorhodopsin. Liposomes prepared by reverse-phase evaporation were first treated with variable amounts of cholate in a final volume of 2.150 mL. Then 350 μL of BR previously solubilized in TX 100 was added. Final concentrations: phospholipids, 7 mM; BR, 140 μg/mL. Incubation medium: 20 mM Pipes, 110 mM K_2SO_4, pH 7.2, supplemented with 200 μM pyranine. After a 10-min incubation, cholate was removed first by Bio-Beads treatment, followed by overnight dialysis against the same buffer as during incubation. Removal of external pyranine and change of the external buffer was achieved by passage through Sephadex G-25 column after dialysis. (A) Light-induced H^+ movements by reconstituted proteoliposomes as measured by the pH-meter (■) or changes in pyranine fluorescence (□). Dashed curve (–●–) represents turbidity of phospholipid–BR–cholate (+TX 100) mixtures before detergent removal. R_{eff}'s of 0.3 (R_{eff} = [cholate]$_{total}$ − 2.8mM/[phospholipid]) and 0.9 (R_{eff} = [cholate]$_{total}$ − 3.7mM/[phospholipid]) denote, respectively, the onset and the total solubilization of the initial liposome suspension. (B) Flotation of cholate-reconstituted proteoliposomes in discontinuous sucrose gradients. Proteoliposomes reconstituted from initial cholate concentrations of 2.5 (a), 5.5 (b), 6.5 (c), 7.5 (d), and 10 mM (e).

Table II: Detergent-Mediated Reconstitutions of Solubilized BR or Purple Membrane Sheets

(A) Triton X-100 and Cholate Mediated Reconstitutions[a]

detergent	detergent (mM)	total H^+ uptake (nequiv of H^+) BR	total H^+ uptake (nequiv of H^+) PM	initial rates (nequiv of H^+/min) BR	initial rates (nequiv of H^+/min) PM
Triton X-100	4 (R_{eff} = 0.8)	200	0	185	0
	6.5 (R_{eff} = 1.3)	520	0	200	0
	12.5 (R_{eff} = 2.5)	400	180	110	60
cholate	6	40	0	150	0
	7.5	80	0	175	0
	10 (R_{eff} = 1)	150	20–40	185	

(B) Octyl Glucoside Mediated Reconstitutions[b]

lipid/protein (w/w)	total H^+ uptake (nequiv of H^+) BR	total H^+ uptake (nequiv of H^+) PM	initial rates (nequiv of H^+/min) BR	initial rates (nequiv of H^+/min) PM
160	340	300	130	120
80	400	380	220	220
40	380	350	500	210
20	350	310	950	230

[a] Phospholipid, 5 mM; lipid/protein ratio, 80 (w/w); buffer, 20 mM Pipes, 10 mM K_2SO_4, pH 7.2. [b] Phospholipid, 5 mM; final OG concentration, 23.5 mM; buffer, 20 mM Pipes, 110 mM K_2SO_4, pH 7.2.

cholate concentrations (Figure 6Ba). For samples reconstituted from detergent to phospholipid ratios slightly above this critical ratio, all the protein initially present was recovered associated with phospholipids. The resulting recombinants from cholate–BR–phospholipid suspensions at an R_{eff} of 0.35 indicated that all the BR formed a complex with only 10% of the total phospholipids and that most of the lipids floated as protein–free liposomes Figure 6Bb). When the cholate concentration in the initial suspension was further increased, more lipids became associated with the protein at the expense of the protein–free liposome population. Only vesicles reconstituted from pure micellar phospholipid–BR–cholate suspensions were collected in flotation sucrose gradient as a single band containing all the protein and phospholipid (Figure 6Be). It is noted that there is good agreement between the percentage of initial phospholipid solubilization deduced from the turbidity measurements in Figure 6A (10, 25, 50, and 100% for initial cholate concentrations of 5.5, 6.5, 7.5 and 10 mM, respectively) and the percentage of phospholipids associated with the protein deduced from density gradient analysis (Figure 6B). Therefore, the most likely mechanism for cholate-mediated reconstitutions is that when BR is added to phospholipid–detergent mixtures above the onset of solubilization, it is incorporated into the mixed phospholipid–detergent micelles already present. On cholate removal proteoliposomes are only formed from these micelles. Raising the initial cholate concentration increases the number of ternary mixed micelles and, consequently, the number of BR-containing liposomes. Optimal BR reconstitution is observed when the starting solution consisted only of micelles.

State of Protein Aggregation. In the absence of detergents, bacteriorhodopsin occurs as crystalline sheets of purple membrane with the BR molecules arranged in a two-dimensional hexagonal lattice of protein trimers (Dencher, 1985). Since all the experiments reported here so far were conducted with the detergent-solubilized monomers of BR, we also attempted to establish whether the reconstitution described above could be performed with large aggregates of BR. The insertion characteristics of the soluble monomeric and membranous proteins were compared by incubating each one in the presence of liposomes treated with different detergents. Some of the results are reported in Table II. For Triton X-100 or cholate mediated reconstitution of the oligomeric protein, only the samples reconstituted from totally solubilized liposomes displayed light-induced H^+ movements. Proton activities, however, were lower than those observed when reconstitutions were performed with the same amount of monomeric BR. Furthermore, when purple membrane sheets were incubated 4 h with phospholipid–TX 100 micelles, H^+ pumping activities rose to a level comparable to that obtained with BR monomers (data not shown). A time-dependent increase in H^+ pumping activity was also observed with cholate but appeared to be less efficient since after 4 h of purple membrane sheets incubation with phospholipid–cholate micelles, H^+ pumping values were still half those found after reconstitution from initially solubilized protein. These results tend to support the interpretation that to obtain efficient Triton X-100 or cholate mediated reconstitutions, BR must be solubilized. The present high detergent concentrations and long incubations of purple membrane sheets with phospholipid–detergent micelles required to obtain proteoliposomes may reflect the difficulty of solubilizing these membranes with TX 100 or cholate (Heyn & Dencher, 1982). In contrast, in the presence of OG, purple membrane sheets insertion occurred in preformed liposomes destabilized by saturating levels of this detergent. The results reported in Table IIB indicated that although purple membrane sheets insertion indeed occurred, the amount incorporated was limited to a lipid to protein ratio of 80. Above this initial lipid/purple membrane ratio, identical initial rates of H^+ pumping were measured, in accordance with the constant amount of incorporated protein determined from sucrose density gradient analysis (data not shown). By comparison, efficient and total protein insertion, starting from solubilized BR, was obtained up to a lipid to protein ratio of 20. In this case, the initial rates increased proportionally with the amount of BR initially present.

Protein Orientation and Comparison of the Different Reconstituted Proteoliposomes. In a previous study (Seigneuret & Rigaud, 1985), we showed that the use of the pH-sensitive probe pyranine entrapped in the intraliposomal aqueous space, combined with the effects of one-sided inhibition by lanthanide ions, allowed us to detect the relative orientation of BR in proteoliposomes. Indeed, lanthanide ions are nonpermeant BR inhibitors that act from the carboxyl-terminal side only and therefore block inside-out BR molecules in reconstituted systems.

Actinic illumination of reconstituted BR proteoliposomes containing entrapped pyranine induced a time-dependent decrease in fluorescence intensity. This indicated internal acidification (Figure 7), and therefore that most of the BR molecules in the sample had an inside-out orientation. In the presence of 5 mM Gd^{3+}, inhibition of inside-out BR allowed detection of a light-induced rise in fluorescence. This clearly indicates that proteoliposomes contain a significant fraction of BR which pumps protons outward and is thus oriented right-side out. Gd^{3+} concentrations above 5 mM yielded a similar positive response, suggesting that this concentration completely inhibits inside-out BR (data not shown).

If it is assumed that the orientation of BR in the vesicle does not affect significantly the rate of H^+ pumping, the percentage of BR in each orientation can be calculated from the initial rates of the changes in the fluorescence intensity of pyranine, in the presence and absence of Gd^{3+}.

The orientations of BR in proteoliposomes reconstituted at different detergent to phospholipid ratios are reported in Table III, together with the corresponding initial rates and total

Table III: Comparison of the H^+ Pumping Efficiencies of Bacteriorhodopsin Proteoliposomes Reconstituted from Triton X-100, Octyl Glucoside, or Sodium Cholate[a]

sample	detergents	pyranine $\Delta F/F$ (%)	[^{14}C]methylamine distribution ΔpH	pH meter: total H^+ (nequiv of H^+)	pH meter: initial rate (nequiv of H^+/min)	inside-out (right-side-out) orientation (%)	proteoliposome size (nm)
1	Triton X-100 (6.5 mM) (R_{eff} = 1.3)	85	2.2	520	430	80–85 (15–20)	160
2	Triton X-100 (12.5 mM) (R_{eff} = 2.5)	40	1.65	345	220	65–70 (35–30)	200
3	OG (24 mM) (R_{eff} = 1.3)	80	2.45	400	530	95 (5)	100
4	OG (40 mM) (R_{eff} = 4.5)	60	1.9	300	370	70–75 (25–30)	200
5	cholate (9 mM) (R_{eff} = 1)	75	2.2	150	360	70–75 (25–30)	70

[a] Liposomes were incubated in the presence of BR previously solubilized in Triton X-100 and the indicated amounts of Triton X-100, OG, or cholate. For all samples: final phospholipid concentration, 5 mM; final lipid/BR ratio, 40 (w/w); buffer, 20 mM Pipes, 110 mM K_2SO_4, pH 7.2. After detergent elimination, H^+ pumping activities were measured by the pH-meter technique, by fluorescence quenching intensity of entrapped pyranine, and by distribution of [^{14}C]methylamine by flow dialysis. The percentage of inside-out orientation of BR was determined from the effects of Gd^{3+} on the light-induced fluorescence changes of pyranine. Proteoliposome sizes are expressed as the most frequent diameters observed on size distribution histograms derived from freeze-fracture electron microscopy studies.

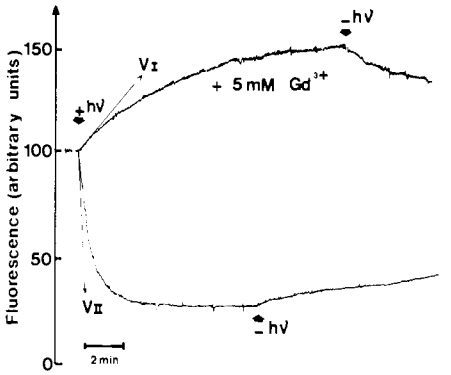

FIGURE 7: Bacteriorhodopsin orientation in reconstituted proteoliposomes. BR proteoliposomes were reconstituted as depicted in the legend of Figure 5A from an effective OG to phospholipid ratio of 1.3 in the presence of pyranine. Proteoliposomes pretreated with 0.2 μM valinomycin were illuminated in the absence or presence of 5 mM $Gd(NO_3)_3$. First arrow, light on; second arrow, light off. V_I and V_{II} denote the initial rates of fluorescence changes in the presence or absence of Gd^{3+}, respectively.

extents of H^+ pumping determined by the pH-meter technique, by steady-state pyranine fluorescence quenchings, and by the amplitudes of the pH gradients across proteoliposomes assessed by [^{14}C]methylamine distribution. Since reconstitutions with the three detergents were performed under exactly the same experimental conditions (buffer composition, lipid concentration, lipid/protein ratio, and prior BR solubilization in TX 100), Table III allows direct comparison of the efficiency of reconstitutions with the different detergents.

It should be recalled that the initial rates of H^+ pumping determined by the pH-meter technique are related to protein insertion into the membranes of the reconstituted proteoliposomes. Since we know from sucrose density gradient experiments that protein incorporation was complete in all the reconstitutions reported in Table III, any variation in the amplitude of the initial H^+ pumping rates should be related to the orientation of the protein in the membranes: Thus, for a constant amount of protein incorporated, the initial rates will decrease with the random orientation of BR in the bilayer. The data in Table III indicate that there is a fairly good agreement between the amplitudes of the initial pumping rates and the protein orientation determined by the one-sided lanthanide ions. For example, in reconstituted proteoliposomes in which 95% of the BR is oriented inside out, the initial rates should be 2.25 times higher than in reconstituted proteoliposomes in which only 70% of the protein is oriented inside out. This is indeed what was observed when OG-mediated reconstitutions at an R_{eff} of 1.3 (sample 3) were compared with TX 100 mediated reconstitutions at an R_{eff} of 2.5 (sample 2).

Whatever the detergent and initial detergent to phospholipid ratio, inwardly directed proton activities were always observed. This was confirmed by the preferential inside-out orientation detected in all the samples analyzed. However, the degree of BR orientation was critically dependent upon the nature of the detergent used for reconstitution, and for each detergent, upon the initial detergent to phospholipid ratio. Interestingly, for TX 100 (samples 1 and 2) and OG (samples 3 and 4) better inside-out orientations and consequently higher initial rates and total extent of proton pumping were observed for the samples reconstituted from starting detergent to phospholipid ratios below those necessary for complete solubilization of the initial material. These results support the idea that the insertion of a protein into preformed liposomes leads to proteoliposomes with better asymmetric protein insertion than when proteoliposomes are formed by detergent removal from ternary phospholipid–detergent–protein micelles (Eytan, 1982; Helenius et al., 1981). It should, in particular, be stressed that the best orientation of BR (95% inside out) was observed with OG-mediated reconstitutions from an R_{eff} of 1.3, i.e., before any liposome solubilization had occurred.

Surprisingly, although the inside-out orientation of bacteriorhodopsin in OG-mediated reconstitutions samples reconstituted at an R_{eff} of 1.3 was better than in TX 100 mediated samples reconstituted at an R_{eff} of 1.3, the level of total H^+ pumping was observed to be lower, while the steady-state pH gradients measured by [^{14}C]methylamine distribution were similar. To interpret these results, it has to be recalled that the level of H^+ pumping depends not only on the initial pumping rates but also on the internal volume in which the protons are pumped: Thus, for a constant internal buffering capacity, more protons will need to be pumped in larger internal volumes in order to elicit a given decrease in the internal pH. Accordingly, electron microscopy data indicate that the proteoliposomes in sample 3 have a mean diameter of about 100 nm, compared to about 160 nm for those in sample 1. The same explanation applies to understand the low H^+ pumping level measured in the proteoliposomes reconstituted with cholate, whose mean diameter was about 70 nm.

CONCLUSION

The studies described in this and the preceding paper (Paternostre et al., 1988) represent an effort to understand the different mechanisms by which an integral membrane protein, bacteriorhodopsin, can associate with phospholipid in detergent-mediated reconstitutions. To this end, stepwise solubilization of preformed liposomes by different detergents was

employed as a means of varying and controlling the composition of the starting phospholipid–detergent mixtures in which the protein was incubated.

The data reported in this paper allow us to identify, depending upon the kind of the detergent, three mechanisms by which BR can associate with phospholipids to give functional proteoliposomes. The results from the reconstitution studies with sodium cholate demonstrated that proteoliposome formation arose only from detergent depletion of BR–phospholipid–cholate micelles. No protein incorporation into preformed liposomes, even destabilized by saturating levels of cholate, could be detected. Maximal H^+ pumping activities and homogeneous protein distribution were measured in samples reconstituted from micellar solutions. In the case of Triton X-100 mediated reconstitutions, although no protein was found associated with phospholipids until the starting material contained mixed micelles, the efficiency of the reconstitution was not related to the amount of mixed micelles initially present in the incubation medium. Optimal reconstitutions were detected in samples reconstituted from TX 100–phospholipid–BR suspensions where about 60–70% of the phospholipid was still present as TX 100 saturated liposomes. A time-dependent transfer from micelles to these detergent-saturated liposomes was observed, allowing formation of homogeneous proteoliposomes with a final lipid to protein ratio similar to the initial ratio. At this time we have no definite explanation for the specific mechanism by which transfer of BR occurs: Clearly transfer is time-dependent but also depends upon the number and/or composition of the mixed micelles present in the incubation medium. Furthermore, we already know from preliminary experiments that such mechanism does not occur when dodecyl octa(oxyethylene) ether ($C_{12}E_8$) is substituted for TX 100 or during TX 100 mediated reconstitution of the Ca^{2+}-ATPase of sarcoplasmic reticulum. Finally, the results from octyl glucoside mediated reconstitutions indicate that reconstitution is optimal when starting from a suspension at a detergent to phospholipid ratio around the critical ratio for the onset of liposome solubilization. Thus, proteoliposomes can be formed by direct incorporation of BR into preformed liposomes, provided the liposomes are destabilized by saturating levels of OG. It is interesting to compare the present findings with other examples of the insertion of integral membrane proteins into preformed liposomes. In all those instances where spontaneous incorporations of integral membrane proteins have been analyzed, the data show that phospholipid bilayers must have the property that is required for spontaneous fusion. Indeed, insertions of membrane proteins are achieved in the presence of impurities such as fatty acids, cholesterol, lysophospholipids, or detergents and have been reported to depend upon the state of the bilayer, the size of the liposomes, and phospholipid composition (Scotto & Zakim, 1985, 1986; Eytan et al., 1975, 1976; Eytan & Broza, 1978; Greenhut & Rosenman, 1985). However, in a recent review, Jain and Zakim (1987) have proposed that the putative effect of these impurities is the formation of organizational defects in the bilayers that may act as sites for fusion of vesicles with other vesicles but importantly also for fusion of vesicles with aggregates of proteins. It is thus possible to envisage direct incorporation as fusion of a lipid envelope of adhering proteins with the liposomes in order to explain the striking similarities between direct protein incorporation and membrane fusion. In this respect, the experiments reported in this paper demonstrate that (i) the interaction of BR with OG-saturated liposomes is a rapid and random process; (ii) the association of BR with preformed liposomes occurs independently of fusion between large unilamellar liposomes; and (iii) spontaneous insertion of crystalline arrays of BR, although limited, occurs into OG-saturated liposomes. In conclusion, our findings on the different ways by which BR associates with phospholipids in the presence of detergents may have some bearing on the observations of other authors. In many instances, octyl glucoside was proved to be useful in facilitating the direct incorporation of membrane proteins in lipid bilayer membranes (Curman et al., 1980; Bullock & Cohen, 1986; Helenius et al., 1981). Furthermore, detergent-mediated reconstitutions of various membrane proteins have been observed to be more efficient when starting from non totally solubilized material as well as in the presence of OG (Racker et al., 1979; Van Dijck & Van Dam, 1982) than in the presence of TX 100 (Kramer & Heberger, 1986) or sodium cholate (Pick, 1986).

Another important aspect of our study is that the mechanism by which BR can be associated with phospholipids to give proteoliposomes is shown to critically affect the final orientation of the protein into the bilayer. When BR was incorporated into preformed liposomes, it oriented unidirectionally, whereas proteoliposomes with more random orientation were obtained upon detergent depletion of mixed BR–detergent–phospholipid micelles, i.e., when incorporation and vesicle formation occurred simultaneously (Table III). A possible mechanism explaining unidirectional orientation of BR when it is incorporated into preformed liposomes is that the proteins are inserted through the hydrophobic domain of the membrane, always by their more hydrophobic moiety first (Eytan, 1982). The carboxylic tail of BR is the most hydrophilic, containing at least five COOH, while the NH_2-terminal region is more hydrophobic. Thus, the latter will be the first to penetrate the membrane, leading to almost inside-out orientation of BR into the resulting proteoliposomes.

Besides providing information concerning the way by which proteins may associate to phospholipids during detergent-mediated reconstitutions, we believe that an important benefit of our study is the finding that the reconstitution method described in this paper is a method of choice for protein reconstitution, more suitable than the usual methods using detergents. The almost standard procedure for detergent-mediated reconstitution consists in cosolubilization of membrane proteins and lipids with detergents to form a suspension of mixed micelles, followed by detergent removal. According to Mimms et al. (1981) the composition of the resulting preparation will be determined by the relative initial concentrations of lipid, protein, and detergent and by the aqueous solubilities of each constituent, which determine the rate at which a given constituent can be exchanged between particles as a result of detergent removal. Since phospholipid and protein solubilities are generally small, it is therefore expected that starting from lipid and protein in the same micelle, detergent removal will lead to the formation of proteoliposomes with a lipid to protein ratio similar to that in the initial ternary micelle. The consequence is that, even if a protein can be inserted into a preformed liposome, this step can be missed during detergent removal from micellar solutions since proteoliposomes can be readily and preferentially formed from the ternary phospholipid–detergent–protein micelles initially present. This is clearly what is observed in OG and TX 100 mediated reconstitution of BR. Apparently, when the detergent is removed from a micellar solution, disruption of most of the micelles initially present is simultaneous, and the differences in solubilities of the different constituents do not interfere in the composition of the resulting proteoliposomes. In this respect, the rate of detergent removal has been shown to drastically

modify the composition of the final proteoliposomes [Table I; see also Eytan (1982) and Jackson and Litman (1985)].

An added advantage of the reconstitution procedure described in this paper relies on the batch procedure using SM_2 Bio-Beads as detergent-removing agent: It provides a reproducible and easy way to achieve unilamellar, relatively large, and impermeable liposomes. Except for cholate-mediated reconstitutions, the liposomes obtained have diameters ranging from 100 to 200 nm, depending on the nature and the initial amount of detergent, and thus are well suited for transport measurements. On the other hand, the efficient removal of traces of detergent allow formation of proteoliposomes with a low passive permeability, and light-induced pH gradients as large as 2 pH units can be generated across the membranes.

In summary, the advantage of the method described in this paper, i.e., incubating a protein in detergent-treated liposome suspensions at each step of the solubilization processes, is to allow a rapid and easy determination of the experimental conditions for optimal detergent-mediated reconstitution of bacteriorhodopsin. The relative ease and reproducibility of this method should make it a useful assay tool in the optimal reconstitution of other membrane proteins. We already know from preliminary experiments that the method is useful for reconstitution of the Ca^{2+}-ATPase of the sarcoplasmic reticulum and the H^+-ATPase of the chloroplasts. However, it has to be stressed from these preliminary experiments that the mechanisms of reconstitution of other membrane proteins may be different from those described in this paper for bacteriorhodopsin. The underlying process that leads to the formation of specific reconstituted form is not well understood. Future detailed studies of the reconstitution processes with other membrane proteins and other detergents are likely to result in the formulation of a general set of principles that would serve as a guide in the formulation of reconstitution experiments.

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