Reconstitution of Membrane Proteins: Sequential Incorporation of Integral Membrane Proteins into Preformed Lipid Bilayers†

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ABSTRACT: Several integral membrane proteins can be inserted sequentially into preformed unilamellar vesicles (ULV's) composed of dimyristoylphosphatidylcholine (DMPC) and cholesterol in a gel phase. Thus, proteoliposomes of DMPC, cholesterol, and bacteriorhodopsin from *Halobacterium halobium* rapidly incorporate UDPglucuronosyltransferase (EC 2.4.1.17) from pig liver microsomes, cytochrome oxidase from beef heart mitochondria, and additional bacteriorhodopsin, added sequentially. This process of spontaneous incorporation can be regulated to produce complex artificial membranes that contain phospholipids and proteins at ratios (mol/mol) equivalent to what is found in biological membranes. The ability of the lipid–protein bilayers to incorporate additional integral membrane proteins is not affected by annealing of the proteoliposomes at 37 °C nor by the order of addition of the proteins. Bacteriorhodopsin-containing vesicles formed by the sequential addition of integral membrane proteins demonstrate light-driven proton pumping. Therefore, they have retained a vesicular structure. Vesicles containing one or two different proteins will fuse with each other at 21 °C or with ULV's devoid of proteins. Incorporation of bacteriorhodopsin or UDPglucuronosyltransferase into proteoliposomes containing DMPC, with or without cholesterol as impurity, also occurs above the phase transition for DMPC. The presence of a protein in a liquid-crystalline bilayer provides the necessary condition for promoting the spontaneous incorporation of other membrane proteins into preformed bilayers.

We have described recently a simple method for inserting (reconstituting) pure integral membrane proteins into preformed unilamellar lipid vesicles (ULV's)[1] of DMPC, containing either myristate (Scotto & Zakim, 1985) or cholesterol (Scotto & Zakim, 1986) as impurities. We have shown that bacteriorhodopsin, microsomal UDPglucuronosyltransferase, or mitochondrial cytochrome oxidase can be incorporated spontaneously into ULV's of DMPC plus the above impurities when the vesicles are in a gel state. The orientation of the incorporated proteins appeared to be nonrandom. The significance of myristate or cholesterol for promoting these spontaneous insertions of membrane proteins into preformed ULV's of DMPC appeared to be that they caused defects in packing, e.g., lateral phase separation (Jain, 1983; Scotto & Zakim, 1985, 1986; Jain & Zakim, 1987). The simplicity of the spontaneous insertion of membrane proteins into preformed bilayers as described by Scotto and Zakim (1985, 1986), and the mildness of the conditions required, suggests that these methods might be suitable for reconstituting membranes of considerable heterogeneity with regard to the protein components. Moreover, because ULV's containing impurities promote spontaneous insertion of integral membrane proteins, the presence of a membrane protein in ULV's also is likely to promote the spontaneous insertion of other membrane proteins into the same preformed bilayer. There is precedence, in fact, for this idea (Enoch et al., 1979; Eytan & Racker, 1977). The purposes of the experiments reported in this paper were to investigate whether the reconstitution methods described previously (Scotto & Zakim, 1985; 1986) can be used to incorporate sequentially several integral membrane proteins into the same ULV's and whether prior insertion of a membrane protein can promote subsequent spontaneous insertion of other membrane proteins into bilayers.

Materials and Methods

Materials. DMPC was purchased from Avanti, Birmingham, AL. [^{14}C]DMPC was obtained from Amersham, Arlington Heights, IL. All other chemicals used were the best available commercial grades.

Purification of DMPC. Several lots of DMPC were used without further purification. When necessary, due to impurities in a gram lot of lipid or arising from degradation of phospholipids (caused by the presence of residual water), DMPC was repurified, essentially according to Colacicco (1972). This was done because residual impurities in DMPC alter the properties that are important for spontaneous insertion of proteins into bilayers. The silicic acid used for the purification of DMPC was washed extensively prior to use. The absorbent was washed first with a gradient of chloroform and methanol, from 100% chloroform to 100% methanol, and then with methanol/water (1:1) followed by reactivation.

Preparation of Vesicles of DMPC. DMPC (70 mg) in chloroform was added to a stainless steel tube and solvent removed under a stream of dry nitrogen. The lipids were dispersed in 10 mL of 10 mM KCl and 1 mM ascorbate. The mixture then was sonicated in a stainless steel tube suspended in a water bath at 30 °C. Sonication was carried out for 30 min, with a Heat Systems W225 sonicator, under a stream of dry argon. The standard tip was used at 30% of maximum power; the output was pulsed so that power was delivered for 60% of the total time of sonication. These precautions were taken to prevent degradation of the phospholipid during sonication (Hauser, 1971). Samples appeared clear at the end

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[1] Abbreviations: DEAE, diethylaminoethyl; DMPC, dimyristoylphosphatidylcholine; Tris, tris(hydroxymethyl)aminomethane; TCA, trichloroacetic acid; ULV, unilamellar lipid vesicles.

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of sonication. When vesicles containing cholesterol were prepared, cholesterol dissolved in chloroform (10 mg/mL) was added to a glass tube. Trace amounts of [^{14}C]DMPC were added in order to simplify quantitation of the ratio of phospholipid to protein in the final lipid–protein complex. DMPC in chloroform then was added to effect a comixing of the two lipids. Solvent was removed under a stream of dry nitrogen, leaving a film of cholesterol and DMPC on the vessel wall. The dried residue was hydrated for 1 h in 10 mM Tris, pH 7.5 (30 °C), 100 mM KCl, and 1 mM ascorbate. Vesicles were prepared by sonication, as described above. Sonicated vesicles were centrifuged for 30 min at 39000 rpm in a Beckman 40 rotor to remove titanium particles and any multilamellar vesicles that may have remained at the end of sonication. Temperature during centrifugation was 37 °C. The concentration of DMPC was determined after centrifugation by assay of inorganic phosphorus. The specific activity of the radiolabel was determined in separate aliquots by counting in a liquid scintillation counter.

Density Gradient Centrifugation. Lipid vesicles, lipid–protein complexes (proteoliposomes), and proteins were separated from each other by centrifugation on glycerol gradients containing 10 mM Tris, pH 8.0. The compositions of gradients are given in the text and legends to the figures and tables. The gradients contained 0.5 mL of fluroinert FC-40 at the bottom of the tubes. Equilibrium conditions were achieved unless noted otherwise. Gradients were fractionated from the top with an ISCO Model 185 density gradient fractionator.

Purification of Bacteriorhodopsin. Halobacterium halobium strain E1 was a generous gift of Drs. W. Stoeckenius and R. Bogomolni. The bacterium was grown and membrane purified essentially according to Oesterhelt and Stoeckenius (1982).

Proton Pumping. Proton pumping by bacteriorhodopsin was measured at 30 °C in a water-jacketed, glass chamber. The preparation was illuminated by a 250-W quartz–halogen bulb with a Corning 3-69 filter. The light-dependent change in pH was measured as described by Racker and Stoeckenius (1974) in the presence of 150 mM KCl, pH 6.2. Vesicles for these experiments were prepared in 150 mM KCl, pH 6.2.

Purification of UDPglucuronosyltransferase. The enzyme was purified from pig liver and assayed as described in Hochman and Zakim (1983). All experiments were carried out with the type of pure enzyme designated GT_{2P} (Hochman & Zakim, 1983).

Purification of Cytochrome Oxidase. A stabilized, freeze-dried preparation of beef heart cytochrome oxidase was obtained from Biozyme Laboratories Ltd., Great Britain. The preparation was resuspended in 20 mM sodium phosphate, pH 7.0, and 1% octyl glucoside and purified by DEAE chromatography according to Mason et al. (1973). The pooled fractions containing cytochrome oxidase were concentrated to 1.0 mL by negative pressure in a dialysis concentrator from Bio-Molecular Dynamics, Beaverton, OR. Excess detergent was removed from the protein by extended centrifugation (250000*g* for 60 h) at 4 °C on a 10–60% glycerol gradient containing 10 mM Tris-HCl, pH 7.4. A band of soluble cytochrome oxidase was recovered from the gradient and dialyzed overnight at 4 °C against buffer containing a final concentration of 0.1% octyl glucoside.

Analytical Methods. Proteins were measured by TCA precipitation followed by analysis with the Lowry method (Peterson, 1977) or by coprecipitation with S-RNA (Polacheck & Cabib, 1981) followed by protein determination with bicinchoninic acid (Smith et al., 1985). Phospholipids were measured by a modification (Scotto & Zakim, 1985) of the malachite green method of Chalvardjian and Rudniski (1970).

Results

Bacteriorhodopsin Is Incorporated Spontaneously into Proteoliposomes. ULV's composed of DMPC and cholesterol aggregate below the transition temperature. These vesicles also fuse rapidly at 21 °C. The rate of vesicle fusion decreases, however, at lower temperatures, and there is no fusion of vesicles at 5 °C (Scotto & Zakim, 1986). The integral membrane proteins used in these experiments will insert spontaneously into ULV's of DMPC and cholesterol at all temperatures below the phase transition (Scotto & Zakim, 1986). Therefore, ULV's comprising DMPC plus 12 mol % cholesterol were equilibrated at 5 °C prior to mixing with bacteriorhodopsin, which also was equilibrated at 5 °C. When the ULV's and bacteriorhodopsin were mixed at this temperature, all the bacteriorhodopsin became incorporated into ULV's. Thus, after bacteriorhodopsin was mixed with ULV's and the mixture was subjected to density gradient centrifugation, all the bacteriorhodopsin was recovered as lipid–protein complexes. Some residual protein-free DMPC remained at the top of such gradients. Lipid–protein complexes of DMPC, cholesterol, and bacteriorhodopsin separated from protein-free ULV's of DMPC plus cholesterol by density gradient centrifugation were reisolated by a second density gradient centrifugation. This was done to ensure that the lipid–protein complexes were stable, that is, that the complexes did not break down to yield protein-free vesicles. The data in Figure 1A show that this did not happen. All the phospholipid associated with bacteriorhodopsin after fractionation of a density gradient remained associated with the protein during a second density gradient fractionation. No phospholipid was found at the top of the gradient in Figure 1A. In addition, the vesicular structure of the proteoliposomes was maintained, as demonstrated by proton pumping (data not shown).

The vesicles containing bacteriorhodopsin and lipids formed at 5 °C fused with each other when warmed to 21 °C. These vesicles also fused with protein-free ULV's of DMPC plus cholesterol at 21 °C. Treatment at 21 °C of proteoliposomes formed at 5 °C led to an increase in their size but not to a change in the lipid to protein ratio of the proteoliposomes (data not shown). In order to increase the lipid to protein ratio of proteoliposomes formed by mixing sonicated bacteriorhodopsin and ULV's at 5 °C, it was necessary to add additional ULV's and then to treat the mixture at 21 °C.

Sonicated preparations of bacteriorhodopsin could be incorporated spontaneously into proteoliposomes already containing a mixture of bacteriorhodopsin, DMPC, and cholesterol. For example, if variable amounts of bacteriorhodopsin were added at 5 °C to proteoliposomes containing complexes with 127 mol of DMPC/mol of protein, the ratio of DMPC to protein (mol/mol) in complexes isolated subsequently by density gradient centrifugation decreased to 93 or 73, which was the change appropriate for complete insertion into the proteoliposomes of the second increment of bacteriorhodopsin. We did not attempt to define whether or at what final lipid/protein the proteoliposomes would become saturated with protein in these particular experiments.

The ability of proteoliposomes containing DMPC, cholesterol, and bacteriorhodopsin to fuse with each other at 21 °C or with sonicated preparations of bacteriorhodopsin at 5 °C was not affected by annealing the proteoliposomes for several hours at 37 °C. These data indicate that lipid vesicles with the property of promoting spontaneous incorporation of membrane proteins retain this property and the ability to fuse

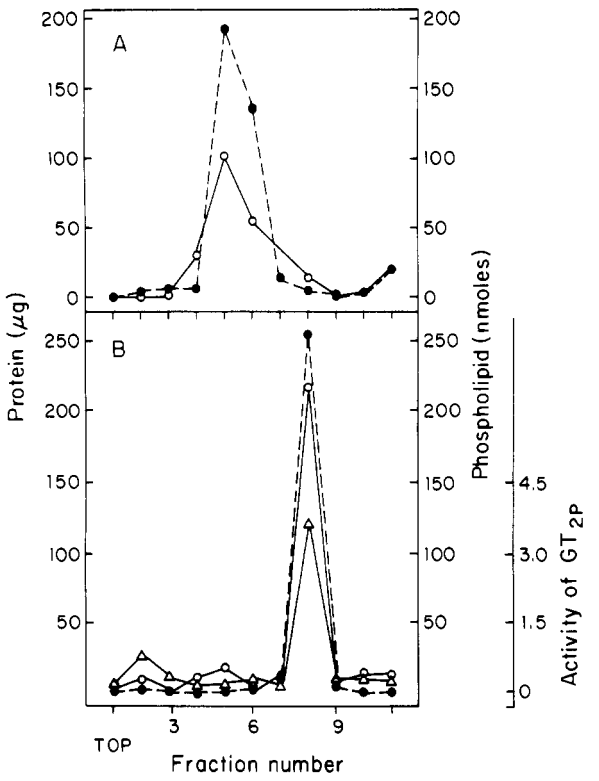

FIGURE 1: Sequential incorporation of two integral membrane proteins into a single population of ULV's. A total of 3 mL of sonicated bacteriorhodopsin (2.5 mg/mL) and 3 mL of preformed ULV's comprising DMPC and 12 mol % cholesterol (7.1 mg of phospholipid/mL) were chilled separately to 5 °C and mixed. Spontaneous incorporation of protein into ULV's was allowed to proceed for 10 min. Vesicles then were warmed to 21 °C to initiate vesicle fusion. Thirty minutes later the mixture was chilled to 5 °C and layered on a 5–60% glycerol gradient containing 10 mM Tris-HCl, pH 8.0, and 100 mM KCl. The gradient was centrifuged for 16 h at 23 000 rpm in an SW-27 rotor at 5 °C. The purple band of proteoliposomes was removed by side puncture of the tube. This fraction was dialyzed against buffer for 2 h at 5 °C. The bacteriorhodopsin-containing proteoliposomes, isolated as above, were layered on a 10–75% glycerol gradient and centrifuged for 2 h at 39 000 rpm in an SW-41 rotor. The distribution of protein and phospholipid in this gradient is shown in (A). (B) Proteoliposomes isolated as above, comprising 62 μg of bacteriorhodopsin at a phospholipid to protein ratio of 145 (mol/mol), were mixed with 100 μg of GT_{2P} and treated at 5 °C for 45 min. The mixture was layered on a 10–75% glycerol gradient and centrifuged for 2 h at 39 000 rpm in an SW-41 rotor at 5 °C. The gradients were fractionated from the top. Phospholipid (●), protein (○), and GT_{2P} activity (△) were determined as described under Materials and Methods.

with other vesicles even after incorporation of proteins. The data suggest, therefore, that the methods described previously (Scotto & Zakim, 1985, 1986) for forming proteoliposomes spontaneously from integral membrane proteins and small ULV's will be useful for sequential assembly of synthetic membranes of increasing complexity.

Sequential Incorporation of Bacteriorhodopsin and Microsomal UDPglucuronosyltransferase into a Single Vesicle. The demonstration above of the sequential incorporation of two separate aliquots of bacteriorhodopsin into the same lipid vesicle reflects the persistence in proteoliposomes of the mechanism promoting the spontaneous insertion of bacteriorhodopsin into ULV's. The finding, nevertheless, leaves open to question whether the mechanism of sequential insertion is limited to the addition of a single type of protein or whether different integral membrane proteins could be added serially to the same membrane. To examine the possibility that different integral membrane proteins could be incorporated sequentially into the same vesicle, proteoliposomes containing bacteriorhodopsin were "constructed" at 5 °C and isolated by density gradient centrifugation (Figure 1A).

The purified GT_{2P} type of UDPglucuronosyltransferase was added to the proteoliposomes isolated as in Figure 1A. Mixing was at 5 °C for 45 min. The mixture then was layered on top of a 10–75% glycerol gradient and centrifuged for 2 h at 39 000 rpm, in an SW41 rotor at 5 °C. Shown in Figure 1B are the distributions of protein, and phospholipid, and activity of GT_{2P} in fractions collected from this gradient. GT_{2P} under these conditions (2 h of centrifugation) will remain near the top of the gradient (Scotto & Zakim, 1985). Longer periods of centrifugation are required to sediment this enzyme on glycerol gradients. The data show, however, that almost all the activity of the GT_{2P} comigrated with the proteoliposomes, which sedimented into the gradient. In addition, comparison of the lipid to protein ratios in Figure 1 (parts A and B) shows a decrease in the lipid to protein ratio of the proteoliposomes isolated in Figure 1 (part B vs. A) from a phospholipid to protein ratio of 2.04 nmol/μg of protein for the proteoliposomes in Figure 1A to 1.18 nmol/μg of protein for the proteoliposomes in Figure 1B. The symmetrical profile for the distribution of the components in Figure 1B suggests too that the GT_{2P} incorporated uniformly into the bacteriorhodopsin-containing "parent" proteoliposomes and not preferentially into a subpopulation of these proteoliposomes. It was determined separately that the preparation of GT_{2P} used in Figure 1B did not incorporate into vesicles due to residual cholate (Scotto & Zakim, 1985).

A small amount of GT_{2P} was found at the top of the gradient in Figure 1B. We do not have a certain explanation for this result. As pointed out by data in following sections, however, the absence of the complete incorporation of GT_{2P} into proteoliposomes in Figure 1B is unlikely to reflect saturation of the vesicles with protein. More likely, the unincorporated GT_{2P} represents highly aggregated GT_{2P} that will not associate with the bilayer. Or the portion of GT_{2P} at the top of the gradient could represent partially denatured enzyme that will not reconstitute into bilayers but that nevertheless retains some enzymatic activity.

Electron microscopy of the proteoliposomes isolated in Figure 1B showed no loss of the vesicular structure after the incorporation of GT_{2P}. And in separation experiments, the integrity of the vesicles containing bacteriorhodopsin or bacteriorhodopsin plus GT_{2P} was confirmed by measurement of proton pumping. The magnitude of proton pumping was the same for vesicles in Figure 1 (data not shown).

Sequential Incorporation of Bacteriorhodopsin and Cytochrome Oxidase into a Single Vesicle. Another pair of proteins, bacteriorhodopsin and cytochrome oxidase, were also used to form a proteoliposome containing two proteins. Proteoliposomes containing bacteriorhodopsin and cytochrome oxidase were formed by the same procedure used in Figure 1, except that 200 μg of cytochrome oxidase was added to the "parent" bacteriorhodopsin-containing proteoliposomes. Addition of cytochrome oxidase to vesicles isolated as in Figure 1A shifted the ratio of lipid to protein from 1.89 nmol/μg of protein in proteoliposomes containing bacteriorhodopsin, DMPC, and 12 mol % cholesterol to 1.15 nmol of DMPC/μg of protein in vesicles isolated after addition of cytochrome oxidase.

Sequential Incorporation of Three or More Integral Membrane Proteins into ULV's of DMPC and Cholesterol. We attempted to extend the sequential addition of integral membrane proteins beyond the stage of proteoliposomes containing two different proteins. The sequential incorporation of GT_{2P}

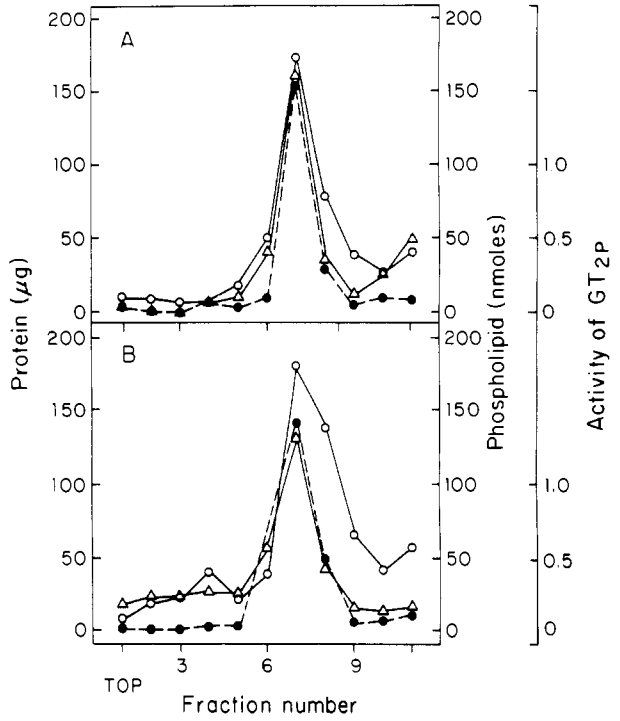

FIGURE 2: Sequential incorporation of four integral membrane proteins into preformed lipid bilayers. Proteoliposomes containing bacteriorhodopsin, DMPC, and 12 mol % cholesterol were prepared and isolated as described in Figure 1. The lipid to protein ratio of these proteoliposomes was 49 (mol/mol). The proteoliposomes (0.7 mL) were placed at 5 °C. The following proteins were added sequentially at 30-min intervals: GT_{2P} (100 μg), cytochrome oxidase (100 μg), and sonicated bacteriorhodopsin [200 μg (A) or 400 μg (B)]. After an additional 30 min, the mixtures in (A) and (B) were layered on 10–75% glycerol gradients and centrifuged at 39 000 rpm for 20 h in an SW-41 rotor at 5 °C. The gradients were fractionated from the top and analyzed as in Figure 1: phospholipid (●), protein (○), and GT_{2P} activity (△).

and then cytochrome oxidase into isolated proteoliposomes containing bacteriorhodopsin was performed at 5 °C, essentially as described in Figure 1. Proteoliposomes containing DMPC, 12 mol % cholesterol, and bacteriorhodopsin were prepared at 5 °C. The ratio of DMPC to protein in these vesicles was 1.88 nmol/μg of protein. A total of 100 μg of GT_{2P} was added to vesicles containing a total of 132 μg of bacteriorhodopsin and the mixture held at 5 °C for 30 min. We did not determine the exact rate of incorporation of GT_{2P} into bacteriorhodopsin-containing proteoliposomes in Figure 1A, but the data in Figure 1B show that GT_{2P} becomes nearly fully incorporated into proteoliposomes containing bacteriorhodopsin within a minimum of 30 min. Therefore, 30 min after GT_{2P} was added to proteoliposomes prepared as in Figure 1A, 200 μg of cytochrome oxidase was added. Thirty minutes later, an additional 200 μg of sonicated bacteriorhodopsin was added. The mixture was kept at 5 °C for an additional 30 min and then layered on top of a 10–75% glycerol gradient and centrifuged as in Figure 1A for 20 h.

The data in Figure 2A show the distribution of protein, phospholipid, and activity of GT_{2P} in fractions after gradient centrifugation of the mixture. There is a fairly uniform distribution of the components in tubes 6–9. As expected from the experiments examining sequential insertion of bacteriorhodopsin and GT_{2P} or bacteriorhodopsin and cytochrome oxidase, a relatively small amount of protein did not seem to incorporate into the proteoliposomes. Some protein was found in the bottom two fractions of the gradient (tubes 10 and 11), but there was little phospholipid associated with protein in these tubes. On the basis of visual inspection of the gradient, the protein at the bottom was not purple membrane. Most important, however, is that four sequential additions of protein to a single population of vesicles was associated with four independent events of spontaneous insertion of membrane proteins into preformed ULV's or vesicular lipid–protein complexes.

Shown in Figure 2B are distributions of protein, phospholipid, and activity of GT_{2P} after density gradient centrifugation of proteoliposomes prepared as in Figure 2A except that 400 μg of sonicated bacteriorhodopsin was the last component added to the mixture. As in Figure 2A, the data in Figure 2B show that essentially all the protein added to the proteoliposomes became incorporated into lipid–protein complexes. A small amount of unincorporated bacteriorhodopsin was seen in the gradient in Figure 2B just above the cushion of fluorinert. It is likely, therefore, that the proteoliposomes in Figure 2B reached a limit in terms of spontaneous incorporation of proteins into the membranes of the proteoliposomes. An interesting aspect of the data in Figure 2 is that there was not a uniform increase in the concentration of protein in vesicles with increasing addition of protein. Moreover, whereas addition of GT_{2P} to bacteriorhodopsin-containing liposomes, as in Figure 1, increased the density of the proteoliposomes, the distribution of densities of proteoliposomes in Figure 2, parts A and B, is the same. What is different between parts A and B of Figure 2 is the proportion of vesicles with different densities. It seems, therefore, that some but not all the vesicles became selectively more concentrated in protein in part B vs. A of Figure 2. There is a shift of the percent of complex with a concentration of protein of 0.36 nmol of DMPC/μg of protein. As the total protein increases, more complex is formed with this concentration of protein, which corresponds to a concentration of approximately 1 mol of protein/18 mol of DMPC. Had there been a uniform and random distribution of proteins in all vesicles, i.e., random spontaneous insertion, one would have expected the maximum density of vesicles in Figure 2B to be greater vs. those in Figure 2A. Since this was not so, the data suggest that the insertion of the final protein component (the bacteriorhodopsin) was not a completely random process. We do not have a certain explanation for this finding, but the distribution of protein and phospholipid in Figure 2B suggests that 0.36 nmol of DMPC/μg of protein represents the maximum concentration of protein attainable in the proteoliposomes.

Spontaneous Insertion of Membrane Proteins into Proteoliposomes in Liquid-Crystalline State. The presence of cholesterol or myristate as impurities in ULV's of DMPC promotes the spontaneous insertion of membrane proteins into these bilayers but only for phospholipids in a gel state. We found, however, that bacteriorhodopsin inserted spontaneously into proteoliposomes containing DMPC, 12 mol % cholesterol, and bacteriorhodopsin when the lipids were in a fluid phase as well as a gel phase. Proteoliposomes containing bacteriorhodopsin were prepared and isolated by density gradient centrifugation as in Figure 1A. These proteoliposomes were annealed at 37 °C for more than 2 h. The temperature was lowered to 30 °C, and additional bacteriorhodpsin was added to the proteoliposomes. This mixture was centrifuged on a density gradient as in Figure 1A. On the basis of prior studies of the effects of cholesterol on spontaneous incorporation of bacteriorhodopsin into ULV's of DMPC, we expected to find bacteriorhodopsin at the bottom of the gradient and bacteriorhodopsin-containing proteoliposomes in the middle of the gradient. Instead, we found all the bacteriorhodopsin present

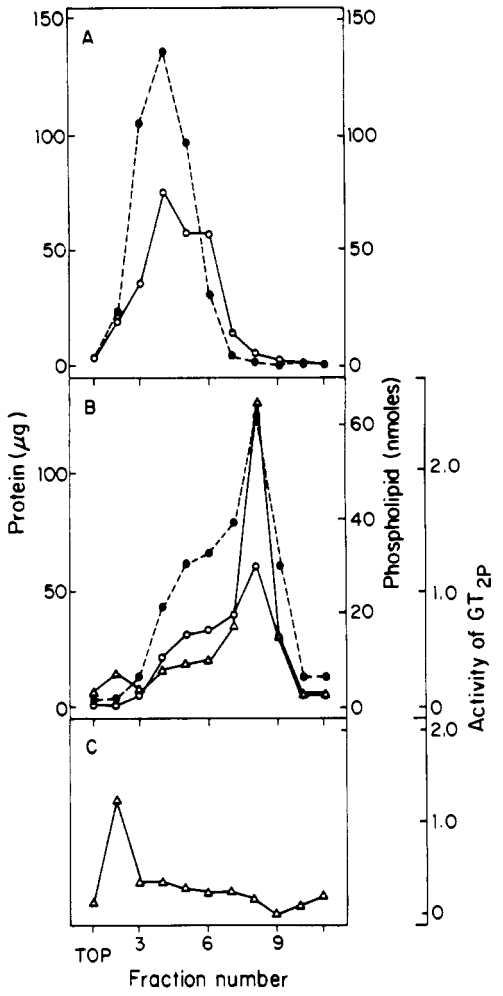

FIGURE 3: Spontaneous incorporation of GT_{2P} into fluid-phase proteoliposomes of bacteriorhodopsin and pure DMPC. Proteoliposomes of bacteriorhodopsin and DMPC were formed by cosonication of these components. The proteoliposomes were isolated by density gradient centrifugation as described in Figure 1. (A) Vesicles containing DMPC and bacteriorhodopsin were reisolated on a 10–75% glycerol gradient as described below. (B) An aliquot of proteoliposomes identical with those shown in panel A was treated for 30 min at 30 °C with 200 μg of GT_{2P} and then was layered on a 10–75% glycerol gradient. (C) A total of 100 μg of purified GT_{2P} was diluted in buffer and layered on a 10–75% glycerol gradient. Gradients were centrifuged at 30 °C for 1 h at 39 000 rpm in an SW-41 rotor. The gradients were fractionated from the top: protein (○), phospholipid (●), and GT_{2P} activity (△).

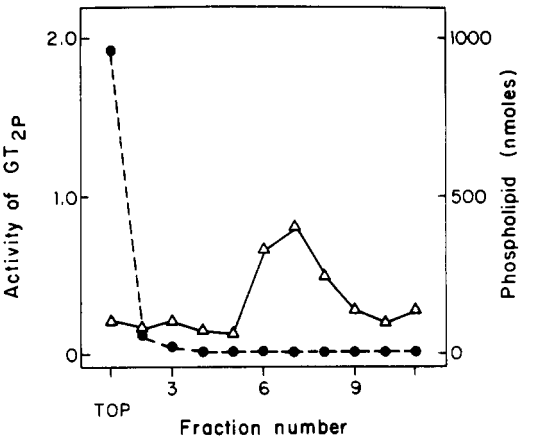

FIGURE 4: Distribution of DMPC and GT_{2P} after equilibrium centrifugation. ULV's of DMPC were prepared as described under Materials and Methods. DMPC vesicles (3 mg) and purified GT_{2P} (100 μg) were mixed at 30 °C and incubated at this temperature for 30 min. The mixture of DMPC vesicles and GT_{2P} was separated by centrifugation for 17 h at 39 000 rpm in an SW-41 rotor on a 10–75% gradient containing 10 mM Tris-HCl, pH 7.5, at 30 °C. The gradient was fractionated from the top. Phospholipids (●) were determined as phospholipid phosphorus. Activity of GT_{2P} (△) in the gradient was assayed as described under Materials and Methods.

in one population of lipid complexes with a lipid to protein ratio of 93 and an R_f value of 0.35–0.39 vs. 123 and 0.32–0.36, respectively, for the parent vesicles. It appeared, therefore, that the presence of bacteriorhodopsin in the bilayer of the parent vesicles promoted spontaneous inserted of additional amounts of bacteriorhodopsin into membranes in a fluid state. In order to examine further the spontaneous incorporation of an integral membrane protein into proteoliposomes in the fluid state, we prepared preoteoliposomes by cosonication of bacteriorhodopsin and pure DMPC. The bacteriorhodopsin was sonicated for 6 min prior to cosonication with DMPC. The sedimentation behavior of these vesicles in a glycerol gradient is shown in Figure 3A. As compared with proteoliposomes generated spontaneously from bacteriorhodopsin and preformed ULV's of DMPC plus cholesterol (Figure 1A), those in Figure 3A are less uniform. There appears to be a bimodal distribution of densities of the proteoliposomes in Figure 3A. We did not determine whether this was a characteristic property of vesicles prepared by cosonication. The proteoliposomes isolated from the gradient in Figure 3A were annealed for several hours at 37 °C. The temperature then was lowered to 30 °C, and 100 μg of GT_{2P} was added. This mixture was layered on a glycerol gradient and centrifuged for 1 h at 37 °C. The relevant distributions of protein, phospholipid, and activity of GT_{2P} are shown in Figure 3B. The GT_{2P} activity comigrated with the leading edge of the proteoliposomes. As noted already, and as shown in Figure 3C, lipid-free GT_{2P} will be at the top of a 10% glycerol gradient after centrifugation under the conditions in Figure 3. Thus, GT_{2P} was "dragged" into the gradient in Figure 3 by virtue of its association with the bacteriorhodopsin-containing proteoliposomes. By comparison with the data shown for the "parent" proteoliposomes in Figure 3A, there was a downward shift in the density of the proteoliposomes in the gradient in Figure 3B. These observations together indicate that the GT_{2P} incorporated into the bacteriorhodopsin-containing proteoliposomes in the liquid-crystalline phase. Moreover, as shown in Figure 4, GT_{2P} added to ULV's of pure DMPC at 30 °C did not incorporate into vesicles. The data in Figure 4 are for a gradient centrifuged for 17 h that shows a clear separation of ULV's from GT_{2P}. In addition to this interesting finding, it is clear that GT_{2P} did not incorporate uniformly into all the proteoliposomes in Figure 3B but incorporated preferentially into a subpopulation with a relatively high concentration of bacteriorhodopsin. We examined next whether GT_{2P} in proteoliposomes would promote spontaneous insertion of bacteriorhodopsin into lipids in the liquid-crystalline phase. Proteoliposomes containing GT_{2P} were prepared by adding enzyme to ULV's of DMPC and 12 mol % cholesterol at 5 °C (Scotto & Zakim, 1986). This protocol was used because GT_{2P} is unstable to treatment with sonic energy. Also, we wanted to avoid the use of detergents. GT_{2P} was treated at 5 °C with ULV's comprising DMPC and 12 mol % cholesterol. The vesicles then were allowed to fuse for 30 min at 21 °C prior to annealing at 37 °C for 2 h. The proteoliposomes prepared in this way did not sediment beyond an R_f value of 0.60 in a glycerol density gradient of 10–60% (Scotto & Zakim, 1986). Sonicated bacteriorhodopsin was added either to vesicles of DMPC, 12 mol % cholesterol, and GT_{2P} or to ULV's of DMPC and 12 mol % cholesterol, but no GT_{2P}, which had been treated sim-

Table I: Spontaneous Incorporation of Bacteriorhodopsin into GT_{2P}-Containing Proteoliposomes in a Fluid State[a]

sample	R_f	GT_{2P} activity	phospholipid/protein (w/w)
ULV + GT_{2P}	0.3–0.6	753	9.87
ULV + GT_{2P} + BR	0.75–0.92	753	5.20
ULV + BR	0.79–1.00		0.53

[a] GT_{2P} (240 μg) was incorporated into preformed ULV's (3 mg) comprising DMPC and 12 mol % cholesterol (ULV + GT_{2P}) at 5 °C (Scotto & Zakim, 1986). The vesicles were warmed to 21 °C and allowed to fuse for 30 min, prior to annealing at 37 °C for 2 h. ULV's of DMPC and 12 mol % cholesterol but no GT_{2P} were treated in an identical manner (ULV) to provide a control population of fused vesicles. Lipid–protein vesicles containing GT_{2P} (ULV–GT_{2P}) and control vesicles (ULV) were mixed with sonicated bacteriorhodopsin (200 μg) for 30 min at 30 °C. The mixtures were layered on top of 10–75% glycerol gradients and centrifuged for 17 h at 39 000 rpm in an SW41 rotor. The R_f's of the purple or white regions were recorded, and the colored bands were removed by side puncture of the gradient tubes. GT_{2P} activity as well as phospholipid and protein content were measured as described under Materials and Methods.

ilarly to the vesicles containing GT_{2P} at 30 °C. These additions were at 30 °C. Thirty minutes after the additions of bacteriorhodopsin, the mixtures were layered on top of glycerol gradients and centrifuged for 17 h at 39 000 rpm. The purple regions of these gradients were removed by side puncture and analyzed as described under Materials and Methods. The data in Table I show that spontaneous insertion of bacteriorhodopsin into vesicles in a liquid-crystal state was promoted by the presence of GT_{2P} in the vesicles and that bacteriorhodopsin did not insert into ULV's of DMPC plus cholesterol at 30 °C under the conditions used in Table I (Scotto & Zakim, 1985). Although membrane proteins, as components of bilayers, promoted the spontaneous insertion of other membrane proteins into a bilayer in a liquid-crystalline state, there was no evidence in any experiment that the proteins promoted fusion between vesicles in a liquid-crystalline state.

The problem of whether bacteriorhodopsin or GT_{2P} promoted insertion of each other into ULV's in a gel phase could not be resolved in a clear-cut fashion. This was so because the residual lipids that can be extracted from purple membrane, which include retinal, are fusogenic for ULV's of DMPC in a gel phase (data not shown).

Discussion

The formation of proteoliposomes with more than one membrane protein or complex of proteins usually is not achieved with standard procedures for reconstitution (Eytan, 1982), unless the proteins are coinserted during sonication (Shertzer et al., 1977), cholate dilution, or detergent dialysis (Racker & Stoeckenius, 1974; Serrano et al., 1976; Yamaguchi et al., 1981). A sequential incorporation of integral membrane proteins into the same bilayer has been accomplished in several instances by sequential sonication (Winget et al., 1977), by combining different methods [as in Takabe and Hammes (1981), Dewey and Hammes (1981), and van der Bend et al. (1984)], or by fusing two populations of proteoliposomes each containing a different protein. There are reports too of spontaneous insertion of cytochrome b_5 into liposomes containing stearoyl-CoA desaturase or into microsomes (Enoch et al., 1979), of spontaneous insertion of mitochondrial proteins into preformed proteoliposomes (Eytan & Racker, 1977), and of spontaneous insertion of P-450 (Yang, 1977). In addition, the activity of pure UDPglucuronosyltransferase has been reported to be reconstituted by liver microsomal membranes (Jansen & Arias, 1975). Despite these observations, it is somewhat surprising that there have been no reported attempts to assemble complex synthetic membrane systems by sequential additions of integral membrane proteins into preformed vesicles. The data presented above suggest that this is not difficult to do, that it can be done under mild, easily controlled conditions, and that it can be done in such a way that the events of membrane assembly per se can be studied. Moreover, we believe our data, when considered in the context of work reported previously (Scotto & Zakim, 1985, 1986), indicate the general applicability of the experimental protocols we have elaborated. It seems, in fact, that synthetic membranes of considerable complexity, with regard to the content of both lipids and proteins, can be assembled quickly and easily. For example, all membrane proteins tested to date have inserted into properly prepared ULV's in a gel phase (Scotto & Zakim, 1985, 1986; Jain & Zakim, 1987; Scotto et al., 1986). Moreover, the data presented above show that vesicles containing an integral membrane protein promote spontaneous insertion of other integral membrane proteins into proteoliposomes in a liquid-crystalline phase and that with proper selection of conditions proteoliposomes will fuse with other proteoliposomes or with ULV's devoid of proteins. In each case, the event of insertion/fusion is susceptible to study, which is a major advantage of protocols for spontaneous assembly of membranes from proteins and preformed lipid bilayer membranes as compared with formation of lipid–protein complexes by cosonication of lipids and proteins, or detergent dialysis. Another advantage of the experimental approach described above is that multiprotein proteoliposomes can be assembled under conditions such that the ratios of protein to lipid can be controlled. Current popular procedures for reconstitution do not regulate the final ratio of lipid to protein of the reconstituted membranes. In addition, these methods rarely result in reconstituted vesicles that approach physiological levels of the ratio of protein to lipids (Eytan, 1982; Etemadi, 1985). Our method is successful in achieving physiologically low lipid to protein ratios while forming either membranes with a single protein or membranes containing many proteins.

Another limiting feature of most reconstitution methods is the relatively small size of reconstituted vesicles. Since we can regulate the fusion of proteoliposomes with each other or with protein-free ULV's, the size and radius of curvature of bilayers can be increased by controlled periods of fusion. Although this may not result in a uniform increase in vesicle size, the formation of complex proteoliposomes ranging from small vesicles to those the size of cells (0.1–10 μm) allows for the study of the effects of membrane curvature on a variety of membrane properties. It is possible, too, to examine the effect of lipid to protein ratios on protein structure and function in a continuous experiment. Although we have presented data only for reconstitution in bilayers of DMPC, we know from preliminary experiments that the method is general and that the sequential incorporation of integral membrane proteins as reported above should also be possible with vesicles comprising lipids other than DMPC (A. W. Scotto and D. Zakim, unpublished data).

We have proposed previously that the mechanism for spontaneous insertion of integral membrane proteins into preformed bilayers is the presence of relatively long-lived structural or packing defects in bilayers in a gel phase (Scotto & Zakim, 1986; Jain & Zakim, 1987). The experimental basis for this idea was that the condition required in a bilayer for promoting spontaneous insertion of integral membrane proteins is the presence of impurities, which cause or stabilize defects in packing (Eytan, 1982; Eytan et al., 1976; Eytan & Racker,

1977; Jain, 1983; Jain & Zakim, 1987; Scotto & Zakim, 1985, 1986). When viewed in the context of this idea, it is not surprising that the presence of proteins embedded in a bilayer also creates conditions for spontaneous insertions of other integral membrane proteins. Thus, the effect of the reconstitution of integral membrane proteins into artificial bilayers often has been discussed with respect to the closeness of fit between the protein and lipid, i.e., the length of the hydrophobic region of protein spanning the bilayer vs. the length of the apolar region of the lipids and the "sealing" of the bilayer along the boundary of the lipid–protein interface. For example, the incorporation of Ca^{2+} ATPase into a bilayer of pure phospholipids increases leakage of ions, suggesting that the reconstituted membrane has several defects in the packing at the lipid–protein interface (Warren et al., 1974).

Defects in packing promote fusion between vesicles (Jain & Zakim, 1987; Jain et al., 1984; Young & Young, 1984) as well as spontaneous insertion of membrane proteins into vesicles containing packing defects. The events associated with packing defects can be classified as (1) small order fusion events, i.e., spontaneous insertions of integral membrane proteins into ULV's that do not fuse with each other; (2) moderate order fusion events, i.e., fusion between small ULV's fusion to other ULV or the incorporation of purple membrane patches; and (3) large order fusion events, i.e., fusion between large vesicles. The magnitude of the packing defect appears to determine the order of the fusion event in the context of this classification. Small concentrations of myristate (0.1 mol %) in ULV's of DMPC promote spontaneous insertion of integral membrane proteins but not fusion between ULV's. Large concentrations of myristate (10 mol %) in ULV's of DMPC promote both of these events (Scotto & Zakim, 1985). The presence of myristate and bacteriorhodopsin in vesicles of DMPC in the gel state promotes fusion between large vesicles, which is not observed for ULV's of DMPC containing myristate as the only impurity. The defects due to the presence of proteins embedded in ULV's of DMPC in a liquid-crystal phase seem to be small order, therefore, since they promote only the insertion of protein and not the fusion of vesicles.

Registry No. DMPC, 13699-48-4; EC 2.4.1.17, 9030-08-4; cytochrome oxidase, 9001-16-5; cholesterol, 57-88-5.

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