Fung, B. K., & Stryer, L. (1978) *Biochemistry 17*, 5241–5248.
Gibson, S. M., & Strauss, G. (1984) *Biochim. Biophys. Acta 769*, 531–542.
Hoekstra, D. (1982) *Biochemistry 21*, 2833–2840.
Johnson, M. E., Simon, S., Kauffmann, J. W., & MacDonald, R. C. (1973) *Biochim. Biophys. Acta 291*, 587–591.
Kendall, D. A., & MacDonald, R. C. (1982) *J. Biol. Chem. 257*, 13892–13895.
Lagerwerff, J. V., Ogata, G., & Eagle, H. E. (1961) *Science (Washington, D.C.) 133*, 1486–1487.
LeNeveu, D. M., Rand, R. P., Parsegian, V. A., & Gingell, D. (1977) *Biophys. J. 18*, 209–230.
MacDonald, R. I., & MacDonald, R. C. (1983) *Biochim. Biophys. Acta 735*, 243–251.
Maggio, B., Ahkong, Q. F., & Lucy, J. A. (1976) *Biochem. J. 158*, 647–650.
Michel, B. E., & Kaufmann, M. R. (1973) *Plant Physiol. 51*, 914–916.
Miller, C., Arvan, P., Telford, J. N., & Racker, E. (1976) *J. Membr. Biol. 30*, 271–282.
Minetti, M., Aducci, P., & Viti, V. (1979) *Biochemistry 18*, 2541–2548.
Morgan, C. G., Thomas, E. W., & Yianni, Y. P. (1983) *Biochim. Biophys. Acta 728*, 356–362.
Nichols, J. W., & Pagano, R. E. (1981) *Biochemistry 20*, 2783–2789.
Ohno, H., *Maeda*, Y., & Tsuchida, E. (1981) *Biochim. Biophys. Acta 642*, 27–36.
Oku, N., & MacDonald, R. C. (1983) *Biochemistry 22*, 855–863.
Pagano, R. E., Martin, O. C., Schroit, A. J., & Struck, D. K. (1981) *Biochemistry 20*, 4920–4927.
Parsegian, V. A., & Rand, R. P. (1983) *Ann. N.Y. Acad. Sci. 416*, 1–11.
Portis, A., Newton, C., Pangborn, W., & Papahadjopoulos, D. (1979) *Biochemistry 18*, 780–790.
Saez, R., Alonso, A. V., & Goni, F. M. (1982) *FEBS Lett. 137*, 323–326.
Schwartz, M. A., & McConnell, H. M. (1978) *Biochemistry 17*, 837–840.
Smith, C. L., Ahkong, Q. F., Fisher, D., & Lucy, J. A. (1982) *Biochim. Biophys. Acta 692*, 109–114.
Szoka, F., Magnusson, K.-E., Wojcieszyn, J., Hou, Y., Derzko, Z., & Jacobson, K. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1685–1689.
Tilcock, C. P. S., & Fisher, D. (1979) *Biochim. Biophys. Acta 557*, 53–61.
Tilcock, C. P. S., & Fisher, D. (1982) *Biochim. Biophys. Acta 688*, 645–652.
Waggoner, A. S., & Stryer, L. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 579–589.
Walter, H. (1977) in *Methods in Cell Separation* (Catsimpoolas, N., Ed.) Vol. 1, pp 307–354, Plenum Press, New York.
Wojcieszyn, J. W., Schlegel, R. A., Lumley-Sapanski, K., & Jacobson, K. A. (1983) *J. Cell Biol. 96*, 151–159.
Yi, P. N., & MacDonald, R. C. (1973) *Chem. Phys. Lipids 11*, 114–134.
Zingsheim, H. P., & Plattner, H. (1976) *Methods Membr. Biol. 7*, 1–146.

Reconstitution of Membrane Proteins. Spontaneous Association of Integral Membrane Proteins with Preformed Unilamellar Lipid Bilayers†

Anthony W. Scotto and David Zakim*
Division of Digestive Diseases, Department of Medicine, Cornell University Medical College, New York, New York 10021
Received January 7, 1985

ABSTRACT: We have developed a simple method for reconstituting pure, integral membrane proteins into phospholipid–protein vesicles. The method does not depend on use of detergents or sonication. It has been used successfully with three different types of integral membrane proteins: UDPglucuronosyltransferase (EC 2.4.1.17) from pig liver microsomes, cytochrome oxidase (EC 1.9.3.1) from pig heart, and bacteriorhodopsin from *Halobacterium halobium*. The method depends on preparing unilamellar vesicles of dimyristoylphosphatidylcholine (DMPC) that contain a small amount of myristate as fusogen. Under conditions that the vesicles of DMPC have the property of fusing, all of the above proteins incorporated into the bilayers. Two events appear to be involved in forming the phospholipid–protein complexes. The first is a rapid insertion of all proteins into a small percentage of total vesicles. The second is slower but continued fusion of the remaining phospholipid–protein vesicles, or proteoliposomes, with small unilamellar vesicles of DMPC. This latter process was inhibited by conditions under which vesicles of DMPC themselves would not fuse. On the basis of proton pumping by bacteriorhodopsin and negative staining, the vesicles were unilamellar and large. The data suggest that insertion of the above integral membrane proteins into vesicles occurred independently of fusion between vesicles.

Careful studies of the properties of proteins that are integral components of membranes depend on dissolution of the membrane with detergents, on separation of the protein of interest from other membrane components, and finally on reintroduction of the protein into a matrix of phospholipid. The latter may have a variety of physical structures, depending on the complex of protein and phospholipid being studied. The complex of most interest, because of its resemblance to naturally occurring biological membranes, is reconstitution of integral membrane proteins into unilamellar bilayers of

† This work was supported by a grant from the National Science Foundation (PCM-8311967). The method of reconstitution described in this paper has been assigned to the Cornell Research Foundation, Inc.

0006-2960/85/0424-4066$01.50/0

phospholipid. Protein–phospholipid structures of this form can be obtained in several different ways. As far as is known, the method for preparing these structures does not affect the properties of the protein or the interactions in the vesicle between protein and phospholipid, given that the function of the protein is stable under the conditions used to reconstitute the protein–phospholipid vesicle. The most popular techniques for achieving this end include cosonication of protein and phospholipid (Racker, 1973; Carroll & Racker, 1977; Banerjee et al., 1977) and slow dialysis of mixtures of integral membrane protein, phospholipid, and detergent (Kagawa & Racker, 1971; Hinkle et al., 1972; Racker, 1972; Kagawa et al., 1973). There is apparently direct insertion of selected small proteins, such as mellitin (Vogel, 1981; Jahnig, 1983; Vogel et al., 1983) or microsomal cytochrome b_5 (Enoch et al., 1977), into preformed phospholipid vesicles. In a limited number of instances, the use of specific mixtures of phospholipids in the bilayer has led to spontaneous incorporation into the bilayers of large integral membrane proteins, for example, cytochrome oxidase (Eytan et al., 1976; Eytan & Broza, 1978). Small proteins that insert spontaneously into vesicles, as mellitin, appear to attach to the membrane by a single region of α-helix, and the driving force for insertion is believed to be a transition from a random coil in water to an α-helix in the bilayer (Jahnig, 1983). The mechanism by which a protein like cytochrome oxidase incorporates spontaneously into bilayers with limited specific compositions is unclear.

The most generally useful method for reconstituting large integral membrane proteins, based on its popularity, is the formation of protein–lipid vesicles by slow dialysis of a mixture of the protein, phospholipid, and detergent. This and the method of cosonication have been used almost exclusively as a means for studying the influence of the lipid milieu on the properties of the protein. These techniques have proved extremely valuable in this context. On the other hand, these commonly used techniques for reconstituting complexes of protein and lipid can provide little information on the mechanism(s) by which integral membrane proteins in cells might enter biological membranes. Thus, although many proteins insert into intracellular membranes as they are translated on ribosomes attached to these membranes (Blobel & Dobberstein, 1975), it is clear that this is not so for all such proteins (Ross & Shatz, 1977, Maccecchini et al., 1979; Date et al., 1980). We describe in this paper easily controlled conditions under which three different integral membrane proteins insert rapidly into preformed unilamellar vesicles of dimyristoylphosphatidylcholine (DMPC).[1] These data indicate that at least some large, membrane-bound proteins contain information that facilitates their insertion into preformed phospholipid vesicles and that this aspect of the biology of membranes is susceptible to analysis in relatively simple, in vitro systems.

Experimental Procedures

Materials. DMPC, bacteriorhodopsin, hemoglobin, and cytochrome *c* were purchased from Sigma. Aldolase and lactic acid dehydrogenase were obtained from Boehringer Mannheim. All other chemicals used were the best available commercial grades.

Preparation of Vesicles of DMPC. Lots of DMPC were used without further purification. DMPC (70 mg) was dispersed in 10 mM Tris, pH 8.0, 30 °C, 100 mM KCl, and 1 mM ascorbate. The mixture was sonicated at 30 °C in a glass tube suspended in a water bath. Vesicles produced this way (above the temperature for the phase transition) are annealed (Lawaczeck et al., 1976). 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. Samples appeared clear at the end of sonication. When vesicles containing myristate were prepared, myristate, dissolved in ethanol (3 mg/mL), was added to a glass tube. Solvent was removed under a stream of dry nitrogen, leaving a film of myristate on the vessel wall. DMPC in buffer was then added, and vesicles were prepared by sonication, as described above. Samples of vesicles were centrifuged at 140000*g* for 30 min at 37 °C to remove titanium particles and any multilamellar vesicles, which may have remained at the end of sonication. The concentration of DMPC was determined after centrifugation by assay of inorganic phosphorus.

Density Gradient Centrifugation. Lipid vesicles, 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 in the legends to figures and tables. The gradients contained 0.25 mL of fluoroinert FC-40 at the bottom of the tubes. The gradients were centrifuged at 34000 rpm for 17 h at 30 °C in a Spinco SW41 rotor. Equilibrium conditions were achieved unless noted otherwise. Gradients were fractionated from the top with an ISCO Model 185 density gradient fractionator.

Proton Pumping. Proton pumping of bacteriorhodopsin was measured in a water-jacketed glass chamber at 30 °C. 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 essentially as described by Racker & Stoeckenius (1974) in the presence of 150 mM KCl.

Monomer of Bacteriorhodopsin. Purple membrane sheets were comminuted by treating with 5% Triton X-100 in 100 mM sodium acetate, pH 5.0, for 48 h in the dark. The treated bacteriorhodopsin was centrifuged at 8000*g* for 1 min to remove any remaining sheets and then freed of excess Triton X-100 by centrifugation on a 10–60% glycerol gradient, which was centrifuged at 275000*g* for 60 h. After 60 h of centrifugation, two distinct bands of bacteriorhodopsin were present. The sedimentation rates of these two populations of bacteriorhodopsin were consistent with the conclusion that the slow sedimenting band contained monomers of bacteriorhodopsin and that the rapidly sedimenting band contained trimers and larger structures (Dencher & Heyn, 1980). Both bands of bacteriorhodopsin were dialyzed overnight against 1 L of sodium acetate, pH 5.0, to reduce the glycerol content.

Purification of UDPglucuronosyltransferase. Enzyme was purified from pig liver and assayed as described in Hochman & Zakim (1983). All experiments were carried out with the type of pure enzyme designated as 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% Triton X-100 and purified by DEAE chromatography according to Mason et al. (1973). The pooled fractions containing the cytochrome oxidase were concentrated to 1 mL by negative pressure in a dialysis concentrator from

[1] Abbreviations: DMPC, dimyristoylphosphatidylcholine; ULV, unilamellar lipid vesicle(s); Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; Cl_3CCOOH, trichloroacetic acid.

Bio-Molecular Dynamics, Beaverton, OR. Excess detergent was removed from the protein by extended (250000*g* for 60 h) centrifugation at 4 °C on a 10–60% glycerol gradient containing 10 mM Tris–HCl, pH 7.4. The isolated band of soluble cytochrome oxidase was recovered from the gradient and dialyzed overnight at 4 °C against buffer before reconstitution experiments were performed.

Analytical Methods. Proteins were measured by Cl_3CCO-OH precipitation followed by analysis with the Lowry method (Peterson, 1977). Phospholipids were measured by a modification of the malachite green method of Duck-Chong (1979). Samples for analysis from glycerol gradients were digested with concentrated HNO_3 at an initial temperature of 95 °C. The temperature was raised gradually to 140 °C over the course of 3 h. The mixture then was taken to dryness by heating to 195 °C. Phosphorus was measured as in Duck-Chong (1979).

Results

Conditions for Incorporation of UDPglucuronosyltransferase into Preformed Vesicles of DMPC. The integral membrane protein of primary interest in this laboratory is hepatic microsomal UDPglucuronosyltransferase. We observed previously that two different pure, delipidated types of this enzyme inserted rapidly into preformed unilamellar vesicles of phosphatidylcholine (Hochman & Zakim, 1983). The pure delipidated preparations of this enzyme contained small amounts of residual cholate. This residual cholate was not removed routinely by us because removal by extensive dialysis did not appear to affect results from kinetic experiments carried out after reconstitution into micelles of lysophosphatidylcholines. We found, however, that extensive dialysis of the type of UDPglucuronosyltransferase designated GT_{2P}, to reduce levels of cholate to below those normally present, destroyed the ability of GT_{2P} to insert rapidly into preformed bilayers of DMPC. Typical data are shown in Figure 1A for isolation of lipid, protein, and lipid–protein complexes after density gradient centrifugation of a mixture of DMPC and a preparation of GT_{2P} that had been dialyzed extensively to remove small amounts of residual cholate. The data in Figure 1A show that this preparation of GT_{2P} did not form lipid–protein complexes after addition to ULV of DMPC. Instead, the GT_{2P} sedimented completely to the bottom of the gradient, and all the phospholipid floated on the top of the gradient. By contrast, addition of a small amount of cholate to the preparation of GT_{2P} used in Figure 1A led to formation of lipid–protein complexes on mixing with ULV of DMPC (Figure 1B). The data in Figure 1 show, therefore, that some small but critical concentration of residual cholate was essential for incorporation of GT_{2P} into the preformed vesicles of DMPC.

Small amounts of detergents are known to facilitate the incorporation of some pure, integral membrane-bound proteins into preformed bilayers (Eytan et al., 1976). The importance of small amounts of cholate for the incorporation of GT_{2P} into vesicles of DMPC hence was not completely surprising. We noted, however, that simple visual inspection of a mixture of GT_{2P} and ULV of DMPC allowed us to predict accurately whether a given preparation of GT_{2P} became incorporated into the vesicles. Thus, density gradient centrifugation of mixtures of GT_{2P} and ULV of DMPC confirmed that protein–lipid complexes always were formed when addition of GT_{2P} to a clear suspension of vesicles of DMPC was followed by development of turbidity (Figure 1B). Complexes of GT_{2P} and DMPC were not formed, on the basis of density gradient centrifugation of mixtures of GT_{2P} and DMPC, when addition of enzyme to lipid had no affect on the apparent turbidity of

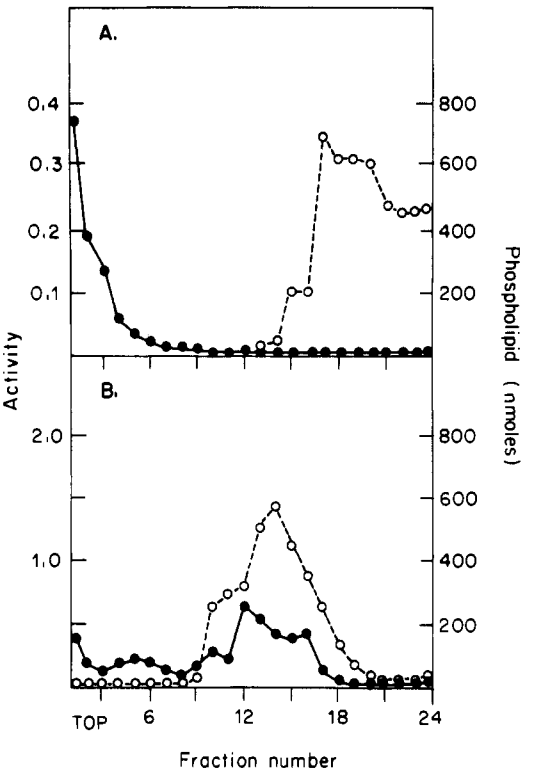

FIGURE 1: Distribution of DMPC, GT_{2P}, and GT_{2P}–lipid complexes and lipid after centrifugation into glycerol gradients. ULV of DMPC were prepared as described under Experimental Procedures. Mixtures of DMPC vesicles and purified GT_{2P} were separated by centrifugation for 17 h at 34 000 rpm in an SW41 rotor using a 10–60% glycerol gradient containing 10 mM Tris-HCl, pH 7.5 at 30 °C. The gradient was fractionated from the top. (A) A mixture of purified GT_{2P} (278 μg) and DMPC vesicles (2.78 mg) was treated for 2 h at 30 °C prior to centrifugation. (B) An aliquot of purified GT_{2P} (278 μg) was enriched with 50 μg of cholate, then mixed with DMPC vesicles (2.70 mg), and treated for 2 h at 30 °C prior to centrifugation. Phospholipids (●) were determined as phospholipid phosphorus. Activity of GT_{2P} (○) in the gradient was assayed as described under Experimental Procedures.

the previously clear suspension of vesicles. The turbidity, when it occurred, appeared instantly on addition of GT_{2P} plus cholate to vesicles of DMPC. This immediate change in turbidity suggested that addition of GT_{2P} plus cholate not only facilitated insertion of GT_{2P} into vesicles of DMPC but also triggered a rapid growth by fusion in the size of the phospholipid vesicles. This seemed a reasonable explanation for the rapid change in light scattering by the ULV. Moreover, it is known that detergents can induce fusion between small ULV (Enoch & Strittmatter, 1979; Alonso et al., 1982). We considered the possibility, therefore, that the importance of contamination of GT_{2P} with cholate for incorporation of GT_{2P} into the ULV of DMPC in Figure 1B was cholate-induced fusion of the vesicles. We decided, therefore, to test the following two ideas: (1) that the influence of cholate on formation of lipid–protein complexes between GT_{2P} and DMPC was due to effects of cholate on the ULV of DMPC and not due to effects of cholate on the GT_{2P} and (2) that the property that promotes fusion of vesicles of phosphatidylcholine might be the property of the vesicle that was important for incorporation of GT_{2P} into vesicles. The validity of these ideas was examined by determining whether or not GT_{2P} would be incorporated into ULV of DMPC containing a fusogen other than cholate.

Small ULV of DMPC fuse slowly when kept at 18 °C, which is slightly below the temperature for the gel to liquid-crystalline phase transition for such vesicles (Prestegard & Fellmeth, 1974). The rate of fusion of these vesicles at 18 °C

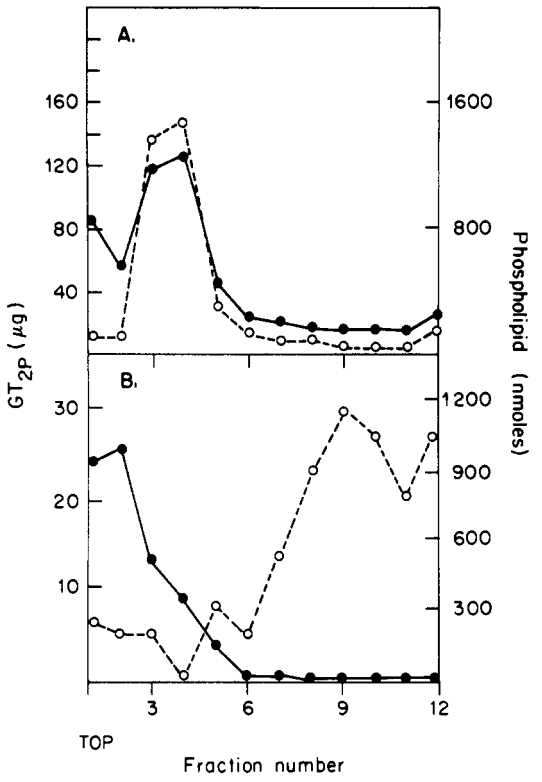

FIGURE 2: Distribution of lipid, GT_{2P}, and GT_{2P}–lipid complexes after centrifugation into glycerol gradients. Centrifugation conditions and analysis of the gradient were as described in Figure 1. (A) Purified GT_{2P} (250 μg) was mixed at 30 °C with ULV of DMPC in a ratio of 1:10 (w/w). The DMPC contained 11.3 mol % myristate. The mixture was treated at 18 °C for 10 min and then warmed to 30 °C for 10 min prior to centrifugation. (B) Purified GT_{2P} (165 μg) was mixed at 30 °C with ULV of pure DMPC in a ratio of 1:10 (w/w). The mixture was treated at 18 °C for 120 min and then warmed to 30 °C prior to centrifugation. Phospholipid (●) was determined as phospholipid phosphorus. Activity of GT_{2P} in the gradient (○) was assayed as described under Experimental Procedures.

is reported to be accelerated greatly by incorporating small amounts of myristate into the vesicles (Kantor & Prestegard, 1975). Therefore, we determined whether GT_{2P} would form lipid–protein complexes when added to ULV of DMPC containing added myristate.

Myristate is not the only fusogen for ULV of DMPC. Impurities present in DMPC in amounts too low to detect by chromatography have been reported to promote fusion of these ULV (Kantor & Prestegard, 1975). We have also found this to be the case for some lots of DMPC. All experiments reported were carried out with lots of DMPC for which added myristate was essential for promoting spontaneous fusion of ULV.

Myristate-induced fusion of ULV of DMPC is reported to occur only over a small range of temperatures. Myristate-induced fusion does not occur at 30 °C, for example (Kantor & Prestegard, 1975). For this reason, GT_{2P} was added to small ULV of DMPC plus myristate that were at 30 °C. The mixture then was cooled to 18 °C and kept at 18 °C for 10 min. It then was rewarmed to 30 °C and placed on top of a 10–60% glycerol gradient, which was centrifuged for 15 h at 30 °C. The results of this experiment are shown in Figure 2A. All the GT_{2P} in this experiment was found as a protein–lipid complex. Shown in Figure 2B is the distribution of GT_{2P} and phospholipid after density gradient centrifugation of a mixture of GT_{2P} and vesicles of DMPC incubated at 18 °C but in the absence of myristate. GT_{2P} did not incorporate into the vesicles of DMPC under this set of conditions. It was determined separately that the preparation of GT_{2P} used in Figure 2 did not incorporate into vesicles of DMPC when a mixture of enzyme and phospholipid was allowed to stand at 30 °C (data not shown). This result indicated that contamination with residual cholate of the specific preparation of GT_{2P} used in Figure 2 was less than that required for facilitating incorporation of GT_{2P} into the liposomes, as shown in Figure 1B.

Formation of Complexes between Bacteriorhodopsin and DMPC and between Cytochrome Oxidase and DMPC. The data in Figures 1 and 2 reveal a potentially interesting and simple mechanism for reconstituting pure, delipidated GT_{2P} into phospholipid–protein complexes. Review of the literature on the general problem of reconstituting pure, integral membrane proteins into phospholipid–protein complexes reveals a few instances in which spontaneous reconstitution of large, integral membrane proteins, i.e., reconstitution in the absence of detergent dialysis or cosonication of protein and lipid, has occurred (Eytan et al., 1976; Klausner et al., 1980). It was important to consider, therefore, whether the data in Figures 1 and 2 were applicable only to GT_{2P} or whether they might have more general significance for reconstitution of integral membrane proteins. Hence, we determined whether pure cytochrome oxidase and bacteriorhodopsin could be reconstituted to form lipid–protein complexes under conditions identical with those employed in Figure 2. We selected these two integral membrane proteins because reconstitution of them usually has utilized the detergent dialysis method or cosonication. Moreover, it is relatively simple to obtain these two membrane-bound proteins in pure form.

The data in Figure 3 demonstrate that bacteriorhodopsin forms protein–lipid complexes spontaneously when added to ULV of DMPC at 18 °C if the vesicles have the property of being able to fuse. The experiment in Figure 3A is identical with that in Figure 2A except that bacteriorhodopsin was substituted for GT_{2P}. By contrast, bacteriorhodopsin did not form a lipid–protein complex when added to ULV of DMPC that contained no myristate (Figure 3B). Moreover, bacteriorhodopsin did not incorporate into liposomes of DMPC containing myristate when the mixture of protein and lipid was treated at 30 °C (data not shown). A small amount of phospholipid (12 mol of phospholipid per mole of bacteriorhodopsin) was associated with the bacteriorhodopsin that sedimented to the bottom of the density gradient in Figure 3B. The lack of functional significance for DMPC that associates with bacteriorhodopsin under conditions used in Figure 3B is discussed below.

Interactions between DMPC and pure cytochrome oxidase were studied as in Figures 2 and 3. As for GT_{2P} and bacteriorhodopsin, cytochrome oxidase also spontaneously formed a lipid–protein complex at 18 °C when added to ULV of DMPC containing myristate, but not when added to ULV of DMPC that do not contain myristate. At 30 °C, a temperature at which ULV of DMPC plus myristate do not fuse, there was no significant association of cytochrome oxidase with DMPC (data not shown).

Interactions between ULV of DMPC and Lactic Acid Dehydrogenase, Aldolase, Cytochrome c, or Hemoglobin. The interaction of several soluble proteins with DMPC vesicles containing myristate was studied in order to determine whether the data in Figures 2 and 3 reflected properties limited to integral membrane proteins or whether any protein, membrane or soluble, would incorporate into vesicles under the conditions that the vesicles were able to fuse. Lactic acid dehydrogenase, aldolase, cytochrome *c*, and hemoglobin (1 mg) were mixed

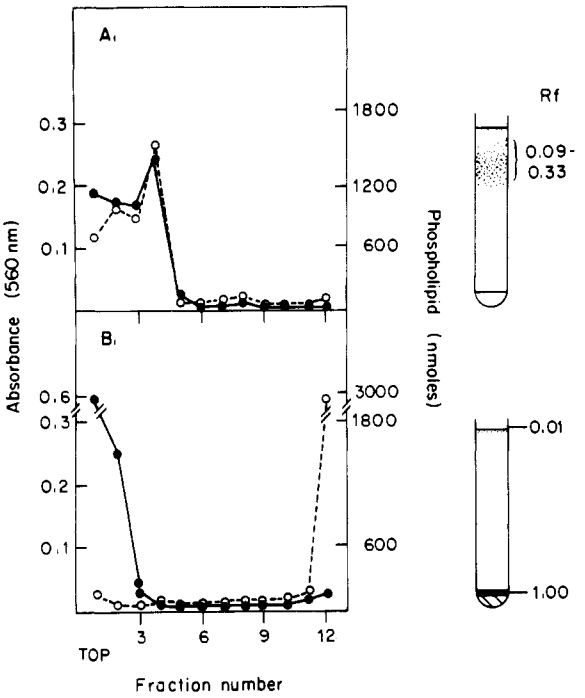

FIGURE 3: Distribution of lipid, bacteriorhodopsin, and bacteriorhodopsin–lipid complexes after centrifugation into glycerol gradients. Conditions for centrifugation and analysis of the mixtures were as described in Figure 1. (A) Bacteriorhodopsin (250 μg) was mixed at 30 °C with 2.5 mg of ULV of DMPC containing 11.3 mol % myristate. The mixture was treated at 18 °C for 10 min and then warmed to 30 °C prior to centrifugation. (B) Bacteriorhodopsin (250 μg) was mixed at 30 °C with 2.5 mg of ULV of DMPC. The mixture was treated at 18 °C for 10 min and then warmed to 30 °C prior to centrifugation. The amount of bacteriorhodopsin in different fractions is reported as the absorbance at 560 nm (○). Phospholipid (●) was determined as phospholipid phosphorus. The representation of test tubes show that the protein–lipid complexes were easy to visualize. R_f is the distance a complex migrates from the top of the gradient vs. the distance for free bacteriorhodopsin. The material in (B) with R_f 0.01 is for retinal.

at 30 °C with DMPC vesicles containing myristate, and the temperature was reduced to 18 °C for 2 h. The temperature then was restored to 30 °C, and the samples were centrifuged for 24 h on 10–40% glycerol gradients. Lactic acid dehydrogenase, aldolase, and hemoglobin sedimented to the bottom of the gradient in these experiments. There was no measurable phospholipid associated with these proteins. Cytochrome *c* sedimented only about halfway into the gradient and was associated with a small amount of lipid: 3.9 mol of phospholipid per mole of protein. The basis for this last result was not examined in greater detail because the amount of phospholipid associated with the protein was quite small (see below). Moreover, the most important conclusion to be derived from the data with soluble, intracellular proteins is they do not form lipid–protein complexes with ULV of DMPC under conditions identical for forming such complexes between pure integral membrane proteins and ULV of DMPC. It seems reasonable to conclude, therefore, that at least some integral membrane proteins but not soluble proteins have a property that specifically allows them to become associated with ULV of DMPC under the condition that the vesicles are able to fuse.

Time Course for Incorporation of Bacteriorhodopsin into Preformed Vesicles of DMPC. The remainder of the experimental work reported in this paper deals with incorporation of bacteriodhodopsin into vesicles of DMPC.

The data in Figure 3A suggest that complete incorporation of bacteriorhodopsin into vesicles of DMPC could occur relatively rapidly, but provide no specific information in this regard. We were interested in determining more accurately the rate of incorporation of bacteriorhodopsin into vesicles of DMPC. Bacteriorhodopsin was added to vesicles of DMPC that contained 11 mol % myristate. The ratio of DMPC to bacteriorhodopsin in the initial mixture was 10:1 (w/w). All components of the system were kept at 30 °C prior to mixing. After being mixed, the system was placed at 18 °C. Temperature equilibrium was reached in 10 s. Aliquots of the mixture were removed at various times after the mixture reached 18 °C and were warmed by placing them in a water bath at 30 °C. The individual samples then were centrifuged at 30 °C on glycerol gradients. A minor problem with the design of this experiment was that fusion of ULV of DMPC containing myristate will begin to occur at about 22 °C (see below). Incorporation of bacteriorhodopsin into vesicles of DMPC begins to occur, therefore, while the system is being cooled to 18 °C. In fact, the lowest temperature reached is 22 °C during 5 s of cooling from 30 °C. Thus, conditions under which the ULV containing myristate would fuse were achieved only transiently for the sample cooled for 5 s.

Table I: Time Dependence of Myristate-Induced Incorporation of Bacteriorhodopsin into ULV of DMPC[a]

time (min:s)	R_f, glycerol gradient 10–60%	R_f, glycerol gradient 40–100%	phospholipid:protein ratio w/w	phospholipid:protein ratio mol/mol
0:0	1.00	0.51–0.58	0.46–0.54	12–14
0:05		0.49–0.55	0.61	16
0:15		0.42–0.48	0.88	23
0:30		0.12–0.16	1.23	32
1:00		0.12–0.17	2.96	77
15:0	0.33–0.48		3.14	117
30:0	0.24–0.49		4.30	162
60:0	0.24–0.44		4.70	175
120:0	0.24–0.29		7.35	277

[a] Bacteriorhodopsin was added to preformed ULV of DMPC containing 11.3 mol % myristate. Samples were mixed at 30 °C, cooled to 18 °C for the time shown, and then returned to 30 °C. The mixtures were centrifuged at 30 °C for 17 h at 34000 rpm in an SW41 rotor using either 10–60% or 40–100% glycerol gradients containing 10 mM Tris-HCl, pH 7.5. The gradients were fractionated from the top. Times in the table are from the onset of the cooling cycle. The meaning of R_f is the same as in Figure 1.

The preparation of bacteriorhodopsin used in these experiments contained a residual phospholipid of 4.5 mol per mole of protein. This value was increased to about 12 mol of phospholipid per mole of protein by a brief period of mixing of ULV of DMPC and bacteriorhodopsin at 30 °C. The significance of this lipid in association with bacteriorhodopsin in the absence of conditions for fusion of ULV is uncertain. The associated phospholipids did not appear to be important for function in that the bacteriorhodopsin–phospholipid complex formed at 30 °C did not pump protons (see below). Also, the amount of phospholipid associated with bacteriorhodopsin under conditions in which fusion of ULV did not occur did not increase with time. Within 5 s of onset of the cooling cycle, the ratio of phospholipid to bacteriorhodopsin in isolated lipid–protein complexes was increased vs. the control (Table I). Although the change at 5 s was small, it was reproducible. Moreover, as shown below, the lipid associated with bacteriorhodopsin after 5 s of cooling was important for the function of this protein. For samples removed from the 18 °C water bath at times longer than 5 s, there was a progressive growth in the ratio of phospholipid to protein in the isolated lipid–protein complexes. Interestingly, however, whereas all the bacteriorhodopsin occurred as a complex with DMPC, only a minor portion of the DMPC was associated with bacteriorhodopsin. Most of the DMPC floated at the top of the

gradient, which was devoid of protein. The data in Table I show, therefore, that there is an almost instantaneous complete incorporation of bacteriorhodopsin into a small population of vesicles of DMPC. This event appears to be completed about 5 s after onset of the cooling cycle. Subsequently, more lipid becomes incorporated into the complex of bacteriorhodopsin and DMPC, presumably by fusing with these complexes. This process of fusion between vesicles of DMPC and complexes of DMPC plus bacteriorhodopsin can be stopped by warming the system to 30 °C. We conclude that the formation of the complexes of lipid and bacteriorhodopsin isolated as in Figure 3 is due to two events. First, there is a rapid incorporation of bacteriorhodopsin into a small proportion of ULV of DMPC. Second, the protein–lipid complexes become increasingly richer in DMPC. This secondary process of growth seems to be mediated by fusion of protein-free vesicles with protein-rich complexes. The secondary phase of vesicle formation is sensitive to temperature in the same way that initial fusion of ULV of DMPC is sensitive to temperature.

Functional Status and Morphology of Bacteriorhodopsin in Lipid–Protein Complexes. The bacteriorhodopsin used for the experiments in Figure 3A,B was in crystalline arrays. These could interact with DMPC by inserting into vesicles or possibly by extracting DMPC from the vesicles. In the former instance, but not the latter, the protein–lipid complexes would be vesicular in structure. The simplest and most significant way for establishing whether the protein–lipid complexes of bacteriorhodopsin and DMPC were vesicular was to determine whether the complexes were active in proton pumping. Bacteriorhodopsin was incorporated into ULV of DMPC as in Figure 3A, and the ability of the resulting lipid–protein vesicles to sustain a pH gradient was measured. The entire mixture of bacteriorhodopsin and DMPC was used in these experiments. The data in Figure 4A show a typical rapid increase in pH (proton pumping from outside to inside of the vesicle) when light was shined on the bacteriorhodopsin–lipid complexes, followed by a rapid decline of the pH gradient across the liposomal membranes when illumination was turned off. The data in Figure 4B show the pH gradient sustained by bacteriorhodopsin reconstituted with DMPC by cosonication as in Hwang & Stoeckenius (1977). The duration of cosonication in Figure 4B was 6 min, which yielded the preparation that sustained a maximum pH gradient vs. other times for cosonication. The data in Figure 4 establish several important points. Thus, whereas bacteriorhodopsin mixed with DMPC at 30 °C did not pump protons, the complexes produced by mixing bacteriorhodopsin and DMPC at 30 °C followed by 5 s of cooling in an 18 °C water bath did pump protons. The extent of the proton gradient sustained by this preparation was smaller, however, than the gradient maintained by a mixture of bacteriorhodopsin and ULV of DMPC plus myristate cooled from 30 °C for 10 s. A reasonable interpretation of the difference in pH gradients for samples cooled for 5 vs. 10 s is that the sample cooled in an 18 °C water bath for 5 s contained a mixture of free bacteriorhodopsin as well as complexes of bacteriorhodopsin with DMPC. The extent of the proton gradient maintained by the protein–lipid complexes cooled at 18 °C for 30 s was less than that for the sample cooled for 10 s. This last result can be accounted for by growth in size of vesicles in the interval between 15 and 30 s, which would make the vesicles more leaky to protons. These results emphasize the conclusion reached above that incorporation of bacteriorhodopsin into ULV of DMPC containing myristate occurs rapidly and is complete under conditions used in Figure 4 within a few seconds or less.

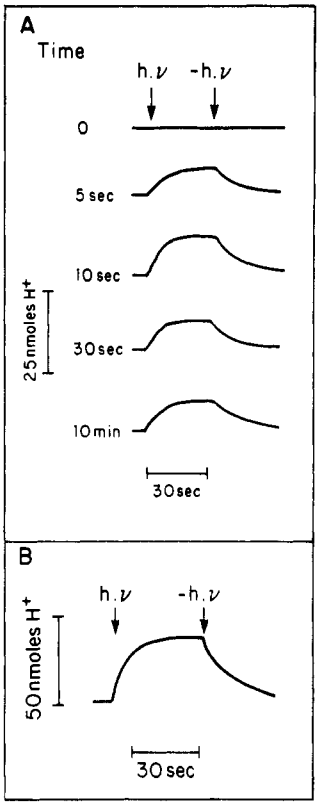

FIGURE 4: Proton pumping by reconstituted bacteriorhodopsin and DMPC at 30 °C. The concentration of bacteriorhodopsin in panel A was 220 μg/mL. In panel B, it was 600 μg/mL. Illumination of the vesicles was carried out with a 250-W quartz–halogen bulb fitted with a Corning 3-69 filter. Vesicles were prepared as described in Figure 1 except the buffer was replaced with 150 mM KCl. (A) Proton pumping of bacteriorhodopsin reconstituted into ULV of DMPC containing 11.3 mol % myristate at 18 °C for the indicated time from onset of cooling from 30 °C to warming. (B) Proton pumping of bacteriorhodopsin reconstituted into preformed DMPC vesicles by cosonication for 6 min. Assays were carried out at 30 °C.

It is important to point out that the efficiency of proton pumping depends on the technique used for reconstituting bacteriorhodopsin. Reconstitution by cholate dialysis yields vesicles that sustain a larger pH gradient across the membrane vs. reconstitution by cosonication (Hwang & Stoeckenius, 1977). The difference is believed to reflect greater leakiness of proteoliposomes prepared by cosonication as compared with cholate dialysis (Hwang & Stoeckenius, 1977). The proteoliposomes in Figure 4A would be expected to be extremely leaky because the lipid portion was composed of DMPC and the vesicles were quite large (Figure 5). Large size and variation in size with a changing ratio of lipid to bacteriorhodopsin is the most likely explanation for greater pumping by complexes made by cooling at 18 °C for 10 s vs. those made by cooling at 18 °C for 30 s and longer. It is significant, in this regard, that the size of the pH gradient maintained by complexes made by cooling for 10 s was as large as that maintained by complexes made by cosonication (Figure 4B).

The data in Figure 5A indicate the size of vesicles of DMPC and bacteriorhodopsin prepared by cooling a mixture of protein and ULV of DMPC containing myristate, as in Figure 3A. The vesicles were isolated by density gradient centrifugation after treatment of the mixture of lipid and bacteriorhodopsin at 18 °C for only 1 min. Shown in Figure 5B for comparison are vesicles of bacteriorhodopsin and DMPC prepared by cosonication.

Incorporation of Bacteriorhodopsin into ULV of DMPC as a Function of the Concentration of Myristate. An important

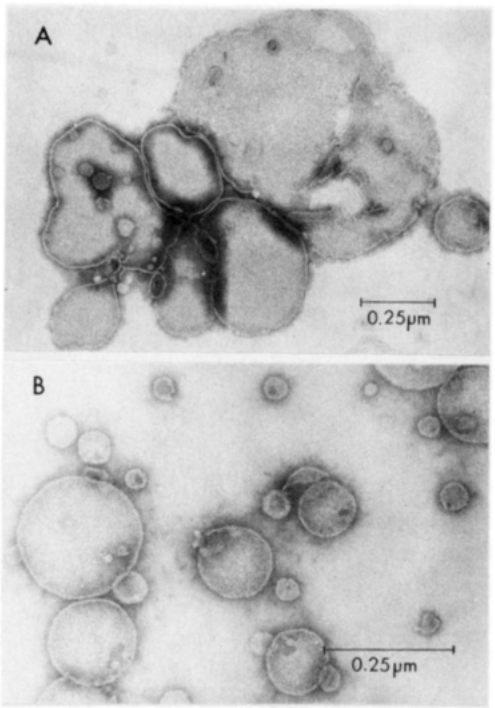

FIGURE 5: Electron microscopy of proteoliposomes prepared from bacteriorhodopsin and ULV of DMPC. The bacteriorhodopsin–DMPC complexes were isolated on a 10–40% glycerol gradient as in Figure 1. The ULV of DMPC contained 11.3 mol % myristate. The fraction containing the proteoliposomes was prepared for electron microscopy by negative staining, with 5% uranyl acetate, of samples adhered to glow-discharged carbon film grids. The mixtures of bacteriorhodopsin and ULV of DMPC were treated at 18 °C for 1 min (A) prior to centrifugation. (B) Vesicles of bacteriorhodopsin and DMPC prepared by sonication for 6 min.

Table II: Influence of Concentration of Myristate in ULV of DMPC on Reconstitution of Bacteriorhodopsin[a]

mol of myristate	R_f of bacteriorhodopsin after centrifugation	
	1 min[b]	120 min[b]
3.0	0.92–0.99	0.42–0.63
1.5	1.00	0.57–0.71
0.75	1.00	0.64–0.76
0.375	1.00	0.68–0.79
0.18	1.00	0.71–0.85
0	1.00	1.00

[a] Bacteriorhodopsin was mixed at 30 °C with preformed ULV of DMPC containing the designated amount of myristate. Samples were cooled to 18 °C for the specified time period and then returned to 30 °C prior to centrifugation at 30 °C on 10–60% glycerol gradients as in Figure 1. The initial ratio of bacteriorhodopsin to DMPC was 1:10 (w/w). R_f values are the relative positions of the purple band from the top of the gradient after centrifugation, as in Table II. Analyses for the phospholipid and protein were carried out as in Table I. [b] Interval of treatment at 18 °C.

question to consider is how much myristate is needed to catalyze the incorporation of bacteriorhodopsin, or other integral membrane proteins, into ULV of DMPC. The data presented so far were for vesicles containing approximately 11 mol % myristate. The data in Table II show that quite small amounts of myristate in DMPC are sufficient for catalyzing the formation of bacteriorhodopsin–DMPC complexes. The data presented are the relative densities of different lipid–protein complexes expressed in terms of a ratio of the distance migrated from the top to the bottom of the gradient (Figure 1B). We know from independent measurements of the ratio of phospholipid to protein, as a function of the distance from the top of the gradient, that the distance migrated

Table III: Influence of Vesicle Size on Incorporation of Bacteriorhodopsin into Preformed ULV of DMPC[a]

expt	treatment of vesicles at 18 °C prior to adding bacteriorhodopsin	R_f of bacteriorhodopsin after centrifugation
1	0	0.41–0.62
2	1	0.39–0.62
3	5	0.47–0.72
4	20	0.59–0.79
5	60	0.64–0.79
6	120	0.69–0.84
7	120	1.00

[a] ULV of DMPC containing 5.6 mol % myristate were allowed to fuse by treatment at 18 °C for the duration of time indicated in column 2. Bacteriorhodopsin in a ratio of 1 part per 10 parts DMPC (w/w) was added and treatment at 18 °C continued for an additional 120 min. Mixtures were warmed to 30 °C and centrifuged at 30 °C on glycerol gradients as in Figure 1. Vesicles that floated at the top of the gradient after centrifugation of experiment 6 were collected. Bacteriorhodopsin was added to these vesicles in a ratio of 1:10 (w/w) and the mixture treated for 120 min at 18 °C. This mixture then was centrifuged as above on a glycerol gradient. The results from this gradient are shown as experiment 7. The meaning of R_f is the same as in Figure 1 and Table II.

is an accurate reflection of the phospholipid:protein ratio of the complexes (Table I). One exception is that complexes with a low ratio of phospholipid to protein and free protein migrate completely to the bottom of the gradient, under conditions used in Table II. That is, the gradient has poor resolving power for complexes with a low phospholipid:bacteriorhodopsin ratio. Nevertheless, the data in Table II show that bacteriorhodopsin is incorporated into ULV at extremely low levels of myristate in the ULV. The data do not allow for detailed conclusions about the influence of the concentration of myristate on the rate of incorporation of bacteriorhodopsin into the ULV, which it seems from Table I and Figure 4 can be addressed only by techniques for measuring rates of rapid reactions. On the other hand, the data in Table II do show that the secondary phase of formation of the protein–DMPC complex, the "growth phase", becomes slower as the concentration of myristate declines.

Influence of the Size of Vesicles on the Incorporation of Bacteriorhodopsin into DMPC. Small ULV of DMPC fuse to form larger vesicles, but there appears to be a maximum size of about 950 Å that limits the process of fusion (Wong et al., 1982). Whether this limit reflects a size-dependent loss of the property catalyzing fusion of small ULV or that vesicles larger than 950 Å are unstable is uncertain. If the former were so, then the size of vesicles might be a determinant of whether bacteriorhodopsin could incorporate into ULV of DMPC plus myristate. It seems, on the other hand, from the data in Table I and Figure 5A, that vesicles of bacteriorhodopsin and DMPC that are larger than 950 Å continue to grow in size.

Vesicles of DMPC containing 5.5 mol % myristate were allowed to fuse at 18 °C for varying periods before addition of bacteriorhodopsin. The vesicles then were warmed to 30 °C and centrifuged on density gradients of glycerol. The data in Table I show that bacteriorhodopsin–lipid complexes will have an R_f value of 0.24–0.29 after mixing of bacteriorhodopsin and ULV of DMPC at 30 °C followed by treatment of the mixture for 2 h at 18 °C. The data in Table III show, however, that none of the bacteriorhodopsin–lipid complexes had this level of enrichment of lipid vs. protein. This result indicates that the pretreatment of the ULV of DMPC at 18 °C prior to mixing with bacteriorhodopsin prevented maximum incorporation of DMPC into protein–lipid complexes. Further, as the duration of pretreatment of DMPC at 18 °C was increased, protein–lipid complexes formed after addition of

Table IV: Temperature Dependence of Incorporation of Bacteriorhodopsin into ULV of DMPC[a]

	lipid/protein ratio (mol/mol) at time (min)				
T (°C)	1	10	30	60	120
37					17
30					15
25	17	17	15	13	17
22	14	19	46	82	159
18	68		162	175	277

[a] Bacteriorhodopsin was mixed at 37 °C with ULV of DMPC containing 5.5 mol % myristate. Samples were cooled to the temperatures shown for the time periods indicated and then returned to 37 °C. The mixtures were centrifuged at 37 °C on 10–60% glycerol gradients as described in Figure 1.

bacteriorhodopsin were progressively less enriched in lipid. Since pretreatment of ULV of DMPC containing myristate at 18 °C promotes fusion of small vesicles, these data are compatible with the idea that large vesicles will not fuse with bacteriorhodopsin–DMPC complexes. To determine whether large ULV interacted with bacteriorhodopsin, the vesicles floating on top of the gradient for experiment 6 of Table III were collected. These were the vesicles "left over" after fusion for 2 h followed by addition of bacteriorhodopsin at 18 °C and thus would be expected to be large ULV. Bacteriorhodopsin was added to these vesicles and the mixture held at 18 °C for 2 more hours. Density gradient centrifugation of this mixture revealed that the bacteriorhodopsin did not become associated with a significant amount of phospholipid (experiment 7, Table III). The absence of incorporation of bacteriorhodopsin into the "remnant" vesicles from this experiment supports the conclusion that bacteriorhodopsin does not associate with phospholipids when the ULV of DMPC plus myristate are large. These data explain too why all the DMPC does not become associated with lipid–protein complexes, that is, why all the DMPC does not become associated with bacteriorhodopsin under the conditions used in Table I. In separate experiments, we determined that complexes of bacteriorhodopsin and DMPC that have appeared to reach a maximum size (ratio of phospholipid to protein) will increase further in size on addition of freshly prepared ULV of DMPC plus myristate (data not shown).

Effect of Temperature on Incorporation of Bacteriorhodopsin into ULV of DMPC. Fusion of DMPC vesicles containing myristate is reported to occur only over a limited range of temperature, which is a few degrees centigrade below the midpoint temperature for the gel to liquid-crystalline phase change (Kantor & Prestegard, 1975). In view of the relationship between conditions for fusion of ULV and insertion of bacteriorhodopsin into vesicles, the insertion of bacteriorhodopsin was examined as a function of temperature in the range of the phase transition for vesicles of DMPC containing added myristate. Bacteriorhodopsin at 37 °C and ULV of DMPC at 37 °C were mixed and then cooled to the temperatures indicated in Table IV. Bacteriorhodopsin inserted into the ULV over a limited range of temperatures. Insertion occurred at 18 and 22 °C, but not at higher temperatures. Although the bacteriorhodopsin mixed with DMPC at 25, 30, and 37 °C had more associated phospholipid as compared with protein that was not mixed with DMPC, none of the bacteriorhodopsin–phospholipid complexes prepared at temperatures above 22 °C had activity in proton pumping. The secondary rate of growth of phospholipid–bacteriorhodopsin complexes was faster at 18 vs. 22 °C.

It was not possible via the experimental plan in Table IV to test the effect of temperature on insertion of bacteriorhodopsin into ULV at temperatures below 18 °C. This was so because cooling through the range 22–18 °C causes rapid insertion and secondary growth of phospholipid–bacteriorhodopsin vesicles. To obviate this problem, ULV of DMPC prepared by sonication at 30 °C and containing 5.5 mol % myristate were cooled quickly to 5 °C. Bacteriorhodopsin at 5 °C was added to the ULV at 5 °C. The mixture of ULV and protein was allowed to stand at 5 °C for 2 more hours and then centrifuged at 5 °C on a glycerol gradient. The complex of phospholipid–bacteriorhodopsin isolated from this gradient had a phospholipid to protein ratio of 299:1 (mol/mol). This ratio was essentially the same as the ratio for complexes isolated after mixing ULV of DMPC and bacteriorhodopsin at 18 °C for 2 h. In fact, this ratio seems to be the largest ratio achievable after mixing 10 parts DMPC with 1 part protein. Insertion of bacteriorhodopsin into ULV of DMPC occurred, therefore, at temperatures that have been reported to inhibit fusion of the ULV per se. Moreover, the phase of secondary growth of the protein–lipid complexes appeared to occur as rapidly at 5 °C as at 18 °C. Monitoring the time-dependent change in light scattering at 5 °C of ULV of DMPC containing myristate showed that there was fusion of the vesicles at this temperature but that the rate of fusion was much slower at 5 °C as compared with 18 °C (data not shown).

Incorporation of Noncrystalline Arrays of Bacteriorhodopsin into ULV of DMPC. Bacteriorhodopsin occurs as crystalline sheets, in the absence of detergents. These sheets can be comminuted by treatment with detergents to arrays believed to be as small as monomers (Dencher & Heyn, 1980). Since all experiments reported above were conducted with sheets of bacteriorhodopsin, we also determined whether the reconstitutions described above could be due to the physical form of the bacteriorhodopsin. The crystalline sheets of protein were treated with Triton X-100, as described by Dencher & Heyn (1980) for preparing monomers. We considered it necessary, however, to reduce concentrations of Triton X-100 in this preparation. Thus, as shown in Figure 1B, detergent can catalyze incorporation of integral membrane proteins into preformed ULV. Triton X-100 was removed, therefore, by density gradient centrifugation of Triton-treated bacteriorhodopsin, as described under Experimental Procedures. Two bands of bacteriorhodopsin were obtained. Each of these failed to sediment at 200000*g* for 45 min, indicating that the bacteriorhodopsin in these bands was extensively disaggregated as compared with the starting material and suggesting, in fact, that the relatively slower sedimenting of the two bands consisted of monomers of bacteriorhodopsin (Dencher & Heyn, 1980). Each of the bands of bacteriorhodopsin isolated as above inserted into ULV of DMPC containing 11 mol % myristate when mixed at 18 °C with DMPC. Neither inserted when the DMPC contained no added myristate. These results show that large aggregated bacteriorhodopsin is not essential for insertion of this protein into ULV of DMPC.

Discussion

The data presented above represent a new method for reconstituting pure, integral membrane proteins into lipid bilayers. The general utility of this method remains to be established through testing with a greater number of integral membrane proteins. It is notable, nevertheless, that the method is successful with three different integral membrane proteins, each from a different type of biologic membrane. Moreover, we already know from preliminary experiments that the method is generally useful with regard to the phospholipid com-

position of ULV (A. W. Scotto and D. Zakim, unpublished data). It seems likely, therefore, that the conditions for incorporating UDPglucuronosyltransferase, cytochrome oxidase, and bacteriorhodopsin into bilayers of DMPC will be useful with other membrane proteins and other species of phospholipid. Cytochrome oxidase, as mentioned already, has been reported to incorporate spontaneously into preformed unilamellar vesicles of phospholipids (Eytan et al., 1976; Eytan & Broza, 1978). The requirements for incorporation were the presence of specific mixtures of phospholipids, such as phosphatidylcholine and phosphatidylethanolamine, or the presence of stearylamine in bilayers of phosphatidylcholine. The influence of temperature on these systems was not reported. It also has been shown that phospholipase A_2 from pig pancreas associates with ULV under conditions that the ULV will fuse spontaneously (Jain et al., 1982, 1984; Jain & deHaas, 1983). We believe that the data reported in these papers are based on the same events as described in this paper and that the previous reports of spontaneous incorporation of cytochrome oxidase into preformed liposomes are evidence of the general utility of the phenomenon we are reporting.

As compared with the usual methods for reconstituting integral membrane proteins, the method described in this paper is more gentle, more rapid, and less costly. In addition, although reconstitution by our method depends on adding myristate to DMPC, the amount of myristate required is quite low. In fact, if care is not taken to avoid warming during preparation of ULV by sonication of DMPC, amounts of myristate sufficient to catalyze reconstitution probably could be produced during preparation of the ULV.

Potential shortcomings of the method for reconstitution that is described above are that the vesicles of phospholipid and protein have a more heterogeneous distribution of sizes as compared with vesicles prepared by detergent dialysis or cosonication and that a mixture of ULV plus membrane protein contains lipid–protein vesicles and ULV devoid of protein. It appears too that inhibition of the secondary growth phase of vesicle formation, at least for the case that the lipids are DMPC, cannot be accomplished at low temperatures because the secondary phase of growth of the protein–lipid vesicles proceeds relatively rapidly under this condition. This could be a problem if inhibition of the secondary growth phase requires raising the temperature above 23 °C (for ULV of DMPC) and the reconstituted protein has a high degree of thermal lability.

More careful control of the rates of the two events associated with the present method of reconstitution, that is, what we are calling the rate of insertion of protein into bilayers and the subsequent rate of growth of the lipid–protein complexes via accretion of additional ULV, should allow for controlling the size of the lipid–protein vesicles and should allow for production of lipid–protein vesicles with a variety of defined sizes, which cannot be accomplished easily by detergent dialysis or cosonication. Separating populations of vesicles containing proteins from those not containing protein may be a problem when proteins are unstable at 30 °C. On the other hand, even the potential instability of the activity of membrane-bound enzymes at 30 °C does not detract from using the method described above to study the process of incorporation of integral membrane proteins into preformed phospholipid membranes, which it seems to us is the most significant advantage of this method vs. detergent dialysis and cosonication.

Mechanism of Insertion. The data show that insertion of large integral membrane proteins into preformed phospholipid bilayers depends on two factors. The protein must have an as yet undefined characteristic, and the phospholipid bilayer must have the property that is required for spontaneous fusion. Although it is clear that several different conditions catalyze fusion of ULV, for example, detergents above the temperature for the main phase transition or the presence of fatty acids in bilayers at a temperature below that for the main transition (Kantor & Prestegard, 1975), it is not clear what characteristic of bilayers actually accounts for fusion. Nevertheless, this property appears to be essential for incorporating integral membrane proteins into the bilayers. The question arises, therefore, as to the significance of this property for the process of reconstitution of protein–lipid complexes. That is, does reconstitution occur simultaneously with and as a consequence of fusion of ULV, or does it occur independently of fusion of ULV? We believe that the latter is the more likely mechanism for reconstitution in the experiments we have described. Thus, the rate at which proteins become associated with the bilayer is much faster vs. rates of fusion of ULV. The data show, for example, that the event of association between bacteriorhodopsin and ULV of DMPC is completed at 18 °C within as short a time as a few seconds (Table I). The process of fusion of ULV is considerably slower, based on data reported by Kantor & Prestegard (1975) and our own observations. Moreover, by decreasing the concentration of myristate in ULV of DMPC, the rate of fusion of ULV is reduced as was the secondary rate of growth of lipid–bacteriorhodopsin vesicles. Yet, significant slowing of fusion between vesicles was not accompanied by a detectable change in the rate of incorporation of bacteriorhodopsin into vesicles, which remained faster than the techniques used in this paper for measuring the rate of this specific process. Finally, it is difficult to envision a mechanism by which fusion of ULV would trap integral membrane proteins within the bilayer of the fused vesicles in the absence of some type of specific interaction between the protein and the ULV. We believe for these reasons that the property conferred by so-called "fusogens" on ULV of DMPC not only catalyzes fusion between ULV but also catalyzes insertion of integral membrane proteins into the ULV. There is in this context an interesting parallel between the conversion of small ULV to large ULV on the one hand and the association between ULV and membrane proteins on the other hand. Large ULV are more stable than small ULV. Large integral membrane proteins are at a lower level of free energy when in bilayers vs. when aggregated in water. However, reactions 1 and 2 go to completion slowly

$$\mathrm{ULV_{small}} \rightarrow \mathrm{ULV_{large}} \quad (1)$$

$$\text{membrane protein}\text{–}n(\mathrm{H_2O})_n \rightarrow \text{membrane protein}\text{–}(\text{lipid})_n \quad (2)$$

and only under some conditions. In addition, no one has described conditions under which there is reversal of reactions 1 and 2. Both reaction 1 and reaction 2 must be constrained severely by kinetic factors. It seems that the kinetic barrier to these reactions is due to some property of the bilayer and that this is removed somehow by incorporation of fusogen. We want to propose, therefore, that fusogens in the bilayers catalyze fusion between ULV and integral membrane proteins.

Proteins are known to become associated with membranes in cells via a leader sequence that is hydrophobic (Blobel & Dobberstein, 1975). In the best studied systems, this association occurs between the membrane and the leader or signal sequence during translation of mRNA. It is obvious from studies in many systems, however, that proteins also become associated with membranes posttranslationally. Wickner (1980) has proposed a membrane-triggered folding model in

which the proteins insert as they fold. Our data suggest that this may not be necessary and that already folded integral membrane proteins may insert into bilayers without unfolding and refolding. This appears to be an especially interesting idea because bacteriorhodopsin, which "criss-crosses" the membrane bilayers 7 times (Henderson & Unwin, 1975), contains nine charged groups within the helical portions than span the membrane (Engelman & Zacai, 1980; McLachlan & Henderson, 1980). It is easier to visualize organization of this type achieved by fusion of prefolded protein with a bilayer vs. sequential "threading" of the protein through the membrane bilayer (Wickner, 1980). It is interesting too that studies of the posttranslational association between mitochondria and ADP/ATP transport protein, which is translated on soluble ribosomes, reveal that the newly synthesized protein is aggregated prior to uptake into the mitochondria (Zimmerman & Neuport, 1980). If aggregation of some hydrophobic, integral membrane proteins occurs in the cytosol of cells, then the conditions for association of aggregates with intracellular membranes in vivo could be similar to the conditions we have utilized for incorporating GT_{2P}, cytochrome oxidase, and bacteriorhodopsin into ULV.

Registry No. DMPC, 13699-48-4; EC 2.4.1.17, 9030-08-4; EC 1.9.3.1, 9001-16-5; hydrogen ion, 12408-02-5; myristic acid, 544-63-8.

REFERENCES

Alonso, A., Saez, R., Villena, A., & Goni, F. M. (1982) *J. Membr. Biol. 67*, 55–62.

Banerjee, R. K., Shertzer, H. G., Kanner, B. I., & Racker, R. (1977) *Biochem. Biophys. Res. Commun. 75*, 772–778.

Benz, R., Bochler-Kohler, B. A., Dieterle, R., & Boos, W. (1978) *J. Bacteriol. 135*, 1080–1090.

Blobel, G., & Doberstein, B. (1975) *J. Cell Biol. 67*, 835–851.

Carroll, R. C., & Racker, E. (1977) *J. Biol. Chem. 252*, 6981–6990.

Date, T., Goodman, J. M., & Wickner, W. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4669–4673.

Dencher, N. A., & Heyn, M. P. (1979) *FEBS Lett. 108*, 307–310.

Dencher, N. A., & Heyn, M. P. (1980) *Methods Enzymol. 88*, 5–35.

Duck-Chong, C. G. (1979) *Lipids 14*, 492–497.

Engleman, D. M., & Zacai, G. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5894–5898.

Enoch, H. G., & Strittmatter, P. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 145–149.

Enoch, H. G., Fleming, P. J., & Strittmatta, P. (1977) *J. Biol. Chem. 252*, 5656–5660.

Eytan, G. D., & Racker, E. (1977) *J. Biol. Chem. 252*, 3208–3213.

Eytan, G. D., & Broza, R. (1978) *J. Biol. Chem. 253*, 3196–3202.

Eytan, G. D., Matheson, M. J., & Racker, E. (1976) *J. Biol. Chem. 251*, 6831–6837.

Henderson, R., & Unwin, P. (1975) *Nature (London) 257*, 28–32.

Hinkle, R. C., Kim, J. J., & Racker, E. (1972) *J. Biol. Chem. 247*, 1338–1339.

Hochman, Y., & Zakim, D. (1983) *J. Biol. Chem. 258*, 4143–4146.

Hwang, S. B., & Stoeckenius, W. (1977) *J. Membr. Biol. 33*, 326–350.

Jahnig, F. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3691–3695.

Jain, M. K., & de Hass, G. H. (1983) *Biochim. Biophys. Acta 736*, 157–162.

Jain, M. K., Egmond, M. R., Verheij, H. M., Spitz-Castro, R., Dijkman, R., & de Hass, G. H. (1982) *Biochim. Biophys. Acta 688*, 341–348.

Jain, M. K., Streb, M., Rogers, J., & de Hass, G. H. (1984) *Biochem. Pharmacol. 33*, 2541–2551.

Kagawa, Y., & Racker, E. (1971) *J. Biol. Chem. 246*, 5477–5487.

Kagawa, Y., Kandrach, A., & Racker, E. (1973) *J. Biol. Chem. 248*, 676–684.

Kantor, H. L., & Prestegard, J. H. (1978) *Biochemistry 17*, 1790–1795.

Klausner, R. D., Bridges, K., Tsumov, H., Blumenthal, R., Weinstein, J. N., & Ashwell, G. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5087–5091.

Lawaczeck, R., Kainosho, M., & Chan, S. I. (1976) *Biochim. Biophys. Acta 443*, 313–330.

Maccecchini, M. L., Rudin, Y., Blobel, G., & Schatz, G. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 343–347.

Mason, T. L., Poyton, R. D., Wharton, D. C., & Schatz, G. (1973) *J. Biol. Chem. 248*, 1346–1354.

McLachlan, A. D., & Henderson, R. (1980) *Biochem. Soc. Trans. 8*, 677–678.

Nakae, T. (1976) *J. Biol. Chem. 251*, 2176–2178.

Peterson, G. L. (1977) *Anal. Biochem. 83*, 346–356.

Prestegard, J. H., & Fellmeth, B. (1974) *Biochemistry 13*, 1122–1126.

Racker, E. (1973) *Biochem. Biophys. Res. Commun. 55*, 224–230.

Racker, E., & Stoeckenius, W. (1974) *J. Biol. Chem. 249*, 662–663.

Ross, E., & Schatz, G. (1976) *J. Biol. Chem. 251*, 1997–2004.

Schindler, H., & Rosenbusch, J. P. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3751–3755.

Vogel, H. (1981) *FEBS Lett. 134*, 37–42.

Vogel, H., Jahnig, F., Hoffmann, V., & Stumpfel, J. (1983) *Biochim. Biophys. Acta 733*, 201–209.

Wickner, W. (1980) *Science (Washington, D.C.) 210*, 861–868.

Wong, M., Anthony, F. H., Tillack, T. W., & Thompson, T. E. (1982) *Biochemistry 21*, 4126–4132.

Zimmerman, R., & Neuport, W. (1980) *Eur. J. Biochem. 109*, 217–229.