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Rhodopsin–Phospholipid Reconstitution by Dialysis Removal of Octyl Glucoside†

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ABSTRACT: Recombinant membranes were prepared from phospholipid-free rhodopsin and egg phosphatidylcholine (PC) under a wide variety of conditions employing an octyl β-D-glucoside (OG) dialysis procedure. Two bands were consistently observed after sucrose density centrifugation of these recombinants. The major band, which was protein rich, had a molar phospholipid:protein ratio that was in the range of 30:1 to 50:1, even when the molar phospholipid:protein ratio of the solubilized solution prior to OG removal was as high as 300:1. Similar results were obtained when dioleoyl-PC, 1-palmitoyl-2-oleoyl-PC, disk lipids, or diphytanoyl-PC was used instead of egg PC. These results can be explained in terms of a lower stability of the OG–phospholipid micelles relative to the OG–phospholipid–rhodopsin micelles. Of the phospholipids that were used in the OG dialysis procedure, only saturated dimyristoyl-PC produced a protein-rich recombinant band with a phospholipid:protein ratio close to that of the initial solubilized solution. In contrast to the results obtained by using OG, when solubilized disks supplemented with egg PC were reconstituted from sodium cholate or dodecyltrimethylammonium bromide, the resulting recombinant membranes had initial and final phospholipid:protein ratios which were similar.

Reconstitution of biological membranes is a powerful method of studying protein-lipid interactions (Razin, 1972) as well as protein function (Racker, 1979). Often specific reconstitution conditions and/or techniques must be chosen to obtain a desired reconstitution product. The underlying processes which lead to the formation of specific reconstituted forms is not well understood. Detailed studies of the reconstitution process in a variety of systems is likely to result in the formulation of a general set of principles which would serve as a guide in the formulation of reconstitution experiments. Relatively few such studies have been carried out.

The properties of the nonionic detergent octyl β-D-glucoside (OG),[1] which include its being nondenaturing to proteins

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[1] Abbreviations: GLC, gas–liquid chromatography; OG, octyl β-D-glucoside; PE, phosphatidylethanolamine, PC, phosphatidylcholine; PS, phosphatidylserine; DMPC, dimyristoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; cmc, critical micelle concentration; EDTA, ethylenediaminetetraacetic acid; DTAB, dodecyltrimethylammonium bromide; TLC, thin-layer chromatography.

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(Stubbs et al., 1976), being a chemically well-defined species, and having a critical micelle concentration (cmc) of approximately 25 mM (Shinoda et al., 1961; Jackson et al., 1982), have resulted in its wide use as a detergent for reconstitution studies. Among the proteins that have been reconstituted into lipid bilayers by using an OG dialysis techniques are the glycoproteins of the Semliki forest virus (Helenius et al., 1977, 1981), glycophorin A (Mimms et al., 1981), rhodopsin (Davoust et al., 1979), the glycoprotein of vesicular stomatitis virus (Petri & Wagner, 1979), and bacteriorhodopsin (Dencher & Heyn, 1978).

The visual pigment rhodopsin is an integral membrane protein which spans the bilayer multiple times (Albert & Litman, 1978; Fung & Hubbell, 1978). It comprises 80–90% of the rod outer segment disk membrane protein (Papermaster & Dreyer, 1974; Smith et al., 1975) and can be obtained virtually lipid free in detergent solution, which allows complete replacement of disk lipids with other specified lipids. In addition, rhodopsin is extremely stable in OG (Stubbs et al., 1976). The above properties make rhodopsin an excellent protein for studying the mechanism of reconstitution of biological membranes.

In this paper, we have examined the OG dialysis reconstitution of rhodopsin in terms of the effect of such factors as the buffer, phospholipid, presence of a divalent metal ion, and the order of mixing of the components on the composition of the reconstitution product. For comparison, a bile salt and an ionic detergent were also employed to form recombinant membranes. Finally, we have undertaken a detailed examination of the reconstitution process itself for the rhodopsin–egg phosphatidylcholine (PC) and rhodopsin–disk lipid systems. Together, the results of these studies allow us to propose a model describing the reconstitution process.

Materials and Methods

Detergents. OG was synthesized as described by Noller & Rockwell (1938) with modifications given by Baron & Thompson (1975). Cholic acid was purchased from Eastman, recrystallized 3 times from 70% ethanol–water (Kagawa & Racker, 1971), solubilized in buffer, and adjusted to pH 7.5 with NaOH. Dodecyltrimethylammonium bromide (DTAB) was synthesized by reacting dimethyldodecylamine (80 g) with methyl bromide (30 mL at −10 °C) in anhydrous ether. Both the amine and methyl bromide were purchased from Eastman. The amine–methyl bromide mixture was stirred for 2 days at 0 °C; the solid product was dissolved in ethyl alcohol and recrystallized out of anhydrous ether. The recrystallization process was repeated twice more.

Phosphatidylcholines. Egg PC, dimyristoyl-PC (DMPC), and diphytanoyl-PC were purchased from Avanti Biochemicals; dioleoyl-PC (DOPC) and 1-palmitoyl-2-oleoyl-PC (POPC) were purchased from Fluka. Some egg PC was also purified according to Litman (1973). The purity of the PC preparations was examined by TLC, and all but the DOPC and POPC exhibited one spot. At loadings greater than 1 μmol, DOPC and POPC showed minor contamination by the corresponding lyso-PC; the POPC exhibited a small cap indicative of chain migration as well. An aliquot of the desired lipid in chloroform was transferred to a lyophilization tube and dried under a stream of N_2. This lipid film was either placed under vacuum overnight to remove residual solvent or dissolved in benzene or cyclohexane and lyophilized overnight.

Disk Membrane Preparation. Disks were prepared from frozen bovine retinas (Hormel or American Stores) by the Ficoll floatation procedure of Smith et al. (1975). These disks were either used immediately or stored frozen (−20 °C) under N_2. Unless otherwise indicated, all procedures involving the visual pigment were performed in dim red light (Kodak red filter no. 1) at 4 °C.

Rhodopsin Purification. Disks were washed twice in buffer (Tris–acetate, pH 7.0) by pelleting in a Sorvall RC-2B with a SS-34 rotor at 15000 rpm for 30 min. The pellet from the second wash was solubilized in 50 mM OG dissolved in a Tris–acetate buffer. Small amounts of insoluble material were pelleted by centrifugation as described above. Rhodopsin was purified from the supernatant by affinity chromatography on a concanavalin A–Sepharose 4B column (Pharmacia Fine Chemicals) (Albert & Litman, 1978). The fractions containing rhodopsin were pooled and concentrated to 2–3.5 mg/mL in an Amicon ultrafiltration cell with a PM-10 or PM-30 membrane. The spectral ratio (A_{280}/A_{500}), i.e., the ratio of the absorbances of a solubilized solution of the protein at 280 and 500 nm, was routinely in the range 1.7–1.8. The rhodopsin concentration was measured by determining the difference in absorbance at 500 nm (ΔA_{500}) produced by bleaching an OG-solubilized sample with a high intensity microscope illuminator for 60 s. Absorption measurements were made with a Varian 219 spectrophotometer. A molecular weight of 38000 and an extinction coefficient of 40000 were taken for rhodopsin.

For some experiments, the mannoside concentration was reduced by dialysis. The column-purified rhodopsin was dialyzed against a 12–16-fold excess of 30–50 mM OG in buffer for 24–48 h. The spectral ratio of the solubilized rhodopsin was not affected by the removal of mannoside.

Buffer Preparation. Tris–acetate buffers were used for most of these experiments. The most frequently used buffer was 50 mM Tris–acetate, i.e., 50 mM Tris and 50 mM sodium acetate, adjusted to pH 7.0 by the addition of HCl. Prior to dialysis, buffers were degassed with N_2 to inhibit lipid and sulfhydryl oxidation.

Reconstitution with Egg PC. Since many variations were employed, only a general procedure is outlined here. Where variations and details are of importance, they are noted under Results. Two orders of mixing were most frequently employed. The first was to solubilize the lyophilized egg PC in an OG-containing buffer with subsequent addition of column-purified rhodopsin in OG. The second method was to add the following components in the order listed to a lyophilized egg PC sample: solid OG, buffer, and purified rhodopsin in OG. The initial rhodopsin concentration varied from 25 to 63 μM, while the calculated starting phospholipid:protein ratios (mol/mol) were varied from 100:1 to 300:1.

The total OG concentration before dialysis was between 67 and 300 mM, and the molar ratio of total OG:phospholipid (R_t) in the samples ranged from 7 to 66. This corresponds to a free micellar detergent:phospholipid ratio ($R_{eff(p)}$) (mol/mol) of at least 6.0. The $R_{eff(p)}$ was estimated by

$$R_{eff(p)} = \frac{[OG]_T - [OG]_{Rho}}{[PC]_T} \quad (1)$$

where $[OG]_T$ and $[PC]_T$ refer to the total OG and PC concentrations expressed in moles per liter and $[OG]_{Rho}$ is the amount of detergent in moles per liter required to solubilize the rhodopsin. The latter quantity was determined from the equation describing the solubilization of disk membranes (Stubbs & Litman, 1978):

$$[OG]_{Rho} = 0.0185 + 270[\text{rhodopsin}] \quad (2)$$

where $[OG]_{Rho}$ and [rhodopsin] are the concentrations expressed in moles per liter. It should be noted that the factor

0.0185 in this expression represents the cmc of OG in the presence of disk membranes. We have shown (Jackson et al., 1982) that for complete solubilization of egg PC membranes with OG, an effective detergent:phospholipid ratio (R_{eff}) of 3.0 is required:

$$R_{eff} = \frac{[OG]_T - 0.022}{[PC]_T} \quad (3)$$

where the quantities in brackets refer to the total concentrations in moles per liter and 0.022 is the cmc of OG expressed in moles per liter. Thus in these reconstitution experiments, we have at least twice as much detergent as is required for complete solubilization of the phospholipid.

The solubilized recombinant was incubated at 4 °C for a minimum of 4 h. Then dialysis was performed for approximately 48 h against at least a 150-fold excess of buffer with four changes at 8–19-h intervals. Sucrose density centrifugation and determination of phospholipid:protein ratios were performed as described below.

Sucrose Density Gradients and Phospholipid:Protein Ratio Determinations. After an aliquot was reserved, the recombinants were layered on top of 0–50% (w/w) continuous sucrose gradients prepared in the appropriate buffer and centrifuged in an SW-27 rotor at 25000 rpm for 6–17 h. The longer centrifugation times (12–17 h) were most frequently employed; however, no shift in the density of the bands was observed after just 6 h of centrifugation, which implies that equilibrium had been reached at that time. The resulting bands, which were observable in dim red light, were collected from the gradients by using a Pasteur pipet. Successive layers of sucrose were removed from the top of the gradient until the desired band was reached. These bands were dialyzed against an excess of buffer to remove the sucrose. When necessary, recombinants were concentrated in an Amicon ultrafiltration cell with a PM-10 or PM-30 membrane. Next the molar phospholipid:protein ratio was determined by phosphate (Bartlett, 1959) and total protein (Lowry et al., 1951) analysis. The ratio of the protein-rich recombinant band is also referred to as the final phospholipid:protein ratio. The phospholipid:protein ratio of the reserved aliquot was also determined as above and is referred to as the initial ratio.

Reconstitution with Several Phosphatidylcholines. The buffer employed for all procedures in these experiments was 50 mM Tris–acetate, pH 7.0. The column-purified rhodopsin sample was dialyzed to remove the mannoside, and its OG concentration was determined by the Anthrone test (Spiro, 1966). Solid OG, buffer, and rhodopsin were added in that order to the appropriate lyophilized lipid such that the OG concentration was 200 mM, the initial rhodopsin concentration was 60 μM, and the calculated starting phospholipid:protein ratio was 300:1. After the samples were completely solubilized as judged by the lack of visual turbidity and particulate matter, they were equilibrated for a minimum of 4 h. Then the samples were dialyzed against a 250-fold excess of buffer for approximately 48 h at 4 °C. There were four changes of buffer at 12–19-h intervals. The resulting recombinants were subjected to sucrose density gradient centrifugation. Phospholipid:protein ratios were determined for the collected bands. Both procedures were performed as described above. The percent recovery of rhodopsin (Hong & Hubbell, 1973), i.e., the percent of rhodopsin unbleached after recombinant formation, was estimated from

$$(A'_{280}/A'_{500})(A_{500}/A_{280}) \times 100 \quad (4)$$

where A'_{280} and A'_{500} are the absorbances of the column-purified rhodopsin at 280 and 500 nm and A_{280} and A_{500} are the corresponding absorbances obtained from rhodopsin in recombinant membranes after an aliquot was solubilized in 1.4% cetyltrimethylammonium bromide or 30 mM OG.

Reconstitution of Solubilized Disks with Several Detergents. For comparison, reconstitution was performed with the detergents DTAB and sodium cholate as well as OG. The procedure of Fung & Hubbell (1978) was employed with several modifications. Solubilized disks were supplemented with egg PC instead of an egg PC–PE mixture. Solubilization was aided by vortexing instead of homogenizing. Except for the above changes, recombinant preparation with sodium cholate was performed as the authors describe. For the recombinant prepared with DTAB, the initial detergent concentraton was 473 mM, and the buffer was 5 mM Hepes and 1 mM EDTA, pH 6.6. Dialysis was performed against approximately a 500-fold excess of buffer for 2 days with four changes of buffer. The initial OG concentration was 300 mM for the OG-prepared recombinant; the buffer employed was 50 mM Tris–acetate, pH 7.0. The solubilized material was dialyzed against a 250-fold excess of buffer for 2 days with four changes of buffer at 10–14-h intervals. The recombinant membranes were removed from dialysis and layered on 0–50% (w/w) sucrose gradients and centrifuged as previously described. All the above procedures were performed at 4 °C. The phospholipid:protein ratios were determined as described above.

Centrifugation Experiments. (A) Solubilized Disk Samples. Disks in Ficoll were washed twice in buffer. The final pellet was solubilized in 200 mM OG in a Tris–acetate buffer and centrifuged in an Sorvall SS-34 rotor at 15000 rpm for 30 min to remove particulate material.

(B) Rhodopsin–Egg PC Samples. Column-purified rhodopsin was dialyzed to remove the mannoside as described above. Solid OG was added to lyophilized Avanti egg PC; then buffer and solubilized rhodopsin were added such that the OG concentration was 200 mM and the phospholipid:rhodopsin ratio was 100:1. The sample was vortexed and then allowed to equilibrate at 4 °C overnight.

Three-milliliter aliquots of the solubilized rhodopsin-egg PC or disk samples were dialyzed against a fixed volume of degassed Tris–acetate buffer for 48 h to obtain samples having OG concentrations over the range of vesicle formation. After dialysis, the protein concentration was approximately 26 μM. The samples were analyzed for phospholipid, total protein, and OG. Subsequently the samples were centrifuged at 125000*g* for 45 min at 4 °C in a TY 65 rotor. Under these centrifugation conditions, bilayer material pellets while mixed micelles remain in solution. The supernatants were analyzed for phospholipid and protein content as described above. The percent phospholipid and protein remaining in the supernatant were calculated by dividing the quantities found in the supernatant by the corresponding values determined in the sample prior to centrifugation.

Determination of Number of Phospholipid Molecules per Rhodopsin Molecule in the Rhodopsin–Lipid–Detergent Micelles. The average number of lipid molecules per rhodopsin molecule in the rhodopsin–lipid–detergent micelles ($\bar{\nu}$) was calculated for recombinant solutions prior to dialysis as described by Stubbs & Litman (1978). The distribution coefficient of 400 mol of OG/mol of rhodopsin, which the authors determined for disks, was also used in our calculations for all lipids employed.

Electron Microscopy. Thin sections were prepared as previously described (Smith et al., 1975) except that the

samples were fixed in solution instead of in a pellet with 2% glutaraldehyde. The samples were kept dark at 4 °C through the fixation step. Electron micrographs were obtained on a Siemens IA electron microscope.

Results

Reconstitution with Egg PC. Rhodopsin was reconstituted with egg PC by solubilization in OG with subsequent removal of the detergent by dialysis. When the resulting recombinants were analyzed by sucrose density centrifugation, two populations were evident. One population, which was observed at or just below the top of the gradient, was phospholipid rich, while the second denser population was protein rich and generally banded at 30–35% sucrose. A typical example is the reconstitution performed in 50 mM Tris–acetate, pH 7.0, with a starting phospholipid:protein ratio of 300:1. Upon sucrose density centrifugation, the denser population exhibited a phospholipid:protein ratio of 48:1.

The protein-rich band on the sucrose density gradient was generally broad, and protein and lipid were frequently detected between the phospholipid-rich and protein-rich bands. Although the recovery was not determined in all experiments, in three experiments where the starting phospholipid:protein ratio as 300:1, the recovery of protein from the protein-rich band was approximately 30% of that loaded on the gradient. This low recovery is consistent with the observed heterogeneity of the recombinant membranes on the gradient. The deviation in the final phospholipid:protein ratio of several recombinant samples prepared under similar conditions was 10% or less.

A series of conditions were varied to determine what factors affect the reconstitution of egg PC with rhodopsin. First a variety of buffers were tried. Buffers that were used included the following: 10 mM Hepes, pH 7.0; 10 mM Tris–acetate and 50 mM KCl, pH 7.0; and 50 mM Tris–acetate, pH 7.0. The above buffers yielded recombinants with final phospholipid:protein ratios between 32:1 and 48:1, even though the initial phospholipid:protein ratios were between 100:1 and 300:1. Since it has been reported that the presence of divalent cations is important for reconstitution (Razin, 1972), we investigated the effect of the addition of 3 mM $MgCl_2$ to the 50 mM Tris–acetate, pH 7.0, buffer. In a typical set of experiments where the starting phospholipid:protein ratio was 300:1, the recombinant prepared without Mg^{2+} exhibited a final phospholipid:protein ratio of 48:1, while the final ratio of the recombinant prepared with Mg^{2+} was 49:1. Furthermore, the addition of 200 mM KCl to the 50 mM Tris–acetate buffer had no significant effect on the final phospholipid:protein ratio.

Another variable that was examined was the rate of detergent removal. Two aliquots of a solubilized rhodopsin–egg PC mixture with a phospholipid:protein ratio of 200:1 were prepared. The first was dialyzed against a small volume of buffer for 36 h so that the OG concentration was slowly lowered to 18 mM. The purpose here was to determine if time was needed near the detergent's cmc for rearrangement or transfer of lipid and protein between micelles. Dialysis was continued against a 160-fold excess of buffer for 2 days with four more changes. The OG was more rapidly removed from the second sample which was dialyzed against a 160-fold excess of buffer for the first 24 h followed by five additional changes of buffer at 9–15-h intervals. The resulting final phospholipid:protein ratios were 47:1 and 41:1, respectively.

In the preparation of recombinants, the order of mixing of the components had no apparent effect on the final phospholipid:protein ratio. One recombinant was mixed as described under Materials and Methods, i.e., solid OG was added to lyophilized PC, followed by an OG-buffer solution, and finally rhodopsin in OG. A second recombinant was prepared by adding solid OG to lyophilized lipid; subsequently, rhodopsin in an OG solution was added. In the first case, both OG–rhodopsin and OG–phospholipid micelles may initially be present. However, the order of mixing of the second recombinant ensures that phospholipid will be directly incorporated into micelles containing rhodopsin. Both recombinant solutions were incubated overnight prior to removal of the detergent by dialysis. The two recombinants formed nearly identical bands on sucrose density gradients, consistent with a phospholipid:protein ratio of 50:1. As an additional check, two recombinants with a calculated starting phospholipid:protein ratio of 300:1 were prepared by using the 2 orders of addition described above. In this experiment, the two samples were incubated only 4 h before dialysis was started, yet the final phospholipid:protein ratios were 49:1 and 48:1.

Table I: Comparison of Several Phospholipids

lipid	phospholipid:protein ratio[a]	spectral ratio[a]	no. of double bonds/lipid molecule
DOPC	61:1	2.23	2.0
egg PC	48:1	2.71	1.7[b]
POPC	58:1	2.09	1.0
DMPC	233:1	2.37	0
diphytanoyl-PC	27:1	2.02	0

[a] Determined after sucrose density centrifugation. The calculated starting phospholipid:protein ratio was 300:1. [b] Based on an average number determined by fatty acid analysis by GLC.

The number of phospholipid molecules per rhodopsin molecule ($\bar{\nu}$) in the rhodopsin–lipid–OG micelles prior to dialysis was calculated for several recombinant solutions with various starting phospholipid:protein ratios. Using starting phospholipid:protein ratios of 100:1, 200:1, and 300:1 yielded the following values of $\bar{\nu}$ and final phospholipid:protein ratios: 7, 30:1 and 19, 43:1; 22, 45:1 and 33, 44:1; 30, 49:1 and 39, 48:1, respectively. Thus, the slight variation in the final phospholipid:protein ratio (30:1 to 49:1) appears to be due to differences in the initial phospholipid:protein ratio and not the initial mixed micelle composition.

$R_{eff(p)}$ was also calculated for the above samples. The samples with an initial phospholipid:protein ratio of 200:1 had an $R_{eff(p)}$ of 10 and 16, while the samples whose initial phospholipid:protein ratio was 100:1 had an $R_{eff(p)}$ of 55 and 17. This illustrates that the final phospholipid:protein ratio was not dependent on $R_{eff(p)}$ in the range of values studied.

For some experiments, the mannoside was dialyzed out of the column-purified rhodopsin. While this did not affect the final phospholipid:protein ratio of the resultant recombinant, it did make possible a more accurate determination of the OG concentration of the rhodopsin solution.

Finally, freeze–fracture electron micrographs of an egg PC–rhodopsin recombinant, with an $R_{eff(p)}$ of 12 (R_t = 14), revealed that the recombinant membranes were large and predominantly multilamellar.

Reconstitution with Several Phosphatidylcholines. The OG technique (see Materials and Methods) was used to reconstitute rhodopsin with POPC, DOPC, and diphytanoyl-PC as well as egg PC. The calculated starting phospholipid:protein ratio of each recombinant was 300:1. The phospholipid:protein ratios reported in Table I were determined after sucrose density gradient centrifugation. Although POPC, egg PC, and DOPC have an average number of double bonds per lipid molecule ranging from 1 to 2, the final phospholipid:protein ratios showed no trend based on the degree of unsaturation. For all

three, the final phospholipid:protein ratio was between 48:1 and 61:1. Only the recombinant prepared with saturated DMPC had a final phospholipid:protein ratio close to the calculated starting ratio. Reconstitution with diphytanoyl-PC whose fatty acid chains are 3,7,11,15-tetramethylhexadecanoic acid yielded recombinants with the lowest phospholipid:protein ratio. This is likely attributable to the presence of the four bulky methyl groups which might be expected to interfere with the favorable packing of the diphytanoyl-PC side chains with rhodopsin. Thus the products of reconstitution appear to be dependent on the packing properties of the lipid as well as its degree of saturation.

The spectral ratio (A_{280}/A_{500}) of the column-purified rhodopsin was in the range 1.7–1.8. Thus, on the basis of the spectral ratios in Table I, the percent recovery of the rhodopsin after recombinant membrane formation was between 65 and 90. These values are in the same range as those determined by Hong & Hubbell (1973) and O'Brien et al. (1977) by using alkyltrimethylammonium bromide detergents. A comparison of Lowry and ΔA_{500} determinations of the rhodopsin concentration indicated that the change in the spectral ratio upon recombinant formation is largely the result of bleaching or denaturation of rhodopsin as opposed to being due to lipid peroxidation.

Reconstitution of Solubilized Disks with Several Detergents. The effect of the detergent on the reconstitution process was also examined. Reconstitution with OG, sodium cholate, and DTAB was performed with solubilized disks supplemented with egg PC as described under Materials and Methods. The calculated starting phospholipid:protein ratio was 117:1; chemical analysis of the recombinant samples prior to centrifugation yielded an initial ratio of 94:1. The DTAB and sodium cholate recombinants had final phospholipid:protein ratios of 76:1 and 88:1, respectively, while the OG recombinant had a final phospholipid:protein ratio of 32:1. This latter value is consistent with the ratios obtained by using either solubilized disk or egg PC–rhodopsin samples. The recombinant prepared with DTAB was the most homogeneous as judged by the tight, narrow band formed on the gradient. No protein was detected elsewhere on the gradient. The least homogeneous recombinant was the one prepared with OG. However, an advantage of OG, is that recombinants prepared with this detergent usually had lower spectral ratios than those prepared with DTAB or sodium cholate (B. Litman, unpublished results).

Thin-section electron micrographs illustrate the morphological differences in the recombinants prepared with the three different types of detergents. The recombinant prepared with OG was multilamellar and had a diameter in the range of 8000 Å. The R_t and $R_{eff(p)}$ of this sample prior to dialysis were 24 and 20, respectively. On the other hand, the membranes prepared with sodium cholate or DTAB were predominantly unilamellar with mean vesicle diameters of 1500 and 1900 Å, respectively. Within each preparation there was a wide distribution in the vesicle diameters.

Centrifugation Experiments. The reconstitution process was studied in further detail to gain insight into the origin of the low phospholipid:protein ratios obtained when rhodopsin was reconstituted with egg PC solubilized in OG. Aliquots of disks solubilized in OG were dialyzed to various fixed OG concentrations. The phospholipid and protein concentrations of each aliquot were determined before and after centrifugation at 125000*g* for 45 min. Figure 1A is a plot of the percent phospholipid and protein in the supernatant after centrifugation vs. the total OG concentration for disks in 10 mM Tris–acetate and 50 mM KCl, pH 7.0. At a given OG concentration in the range of recombinant formation (26–37 mM), a greater percentage of the protein, relative to the phospholipid, remained solubilized. Similar curves were obtained for the inverse process, i.e., solubilization of the disk membrane with OG (Stubbs & Litman, 1978). The phospholipid:protein ratio of the supernatant material at OG concentrations from 38 to 27 mM was estimated from the curves in Figure 1A; the ratio decreased from 55:1 at 38 mM to 28:1 at 30 mM, below which it remained constant.

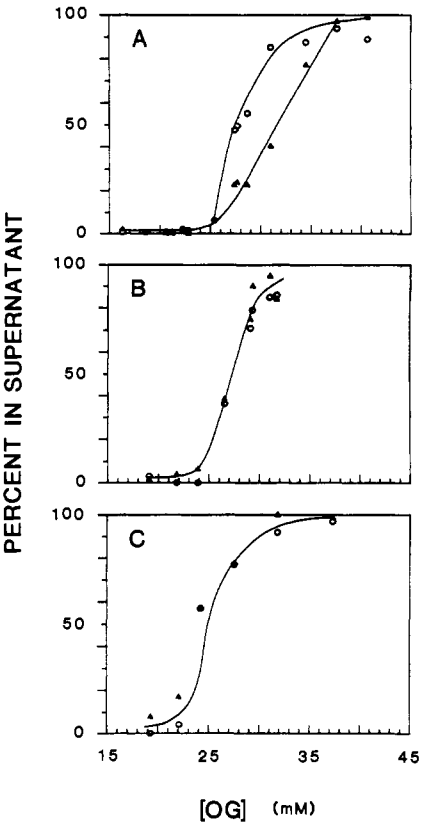

FIGURE 1: Dependence of the percent phospholipid (△) and protein (○) remaining in the supernatant after centrifugation as a function of the OG concentration. The samples were centrifuged at 125000*g* for 45 min. (A) The rhodopsin–disk lipid system in 10 mM Tris–acetate and 50 mM KCl, pH 7.0. Prior to centrifugation, the rhodopsin concentration was 1.26 mg/mL, and the phospholipid:protein ratio was 55:1. (B) The rhodopsin–egg PC system in 10 mM Tris–acetate and 50 mM KCl, pH 7.0. Prior to centrifugation, the rhodopsin concentration was 0.80 mg/mL, and the phospholipid:protein ratio was 103:1. (C) The rhodopsin–egg PC system in 50 mM Tris–acetate and 200 mM KCl, pH 7.0. Prior to centrifugation, the rhodopsin concentration was 0.98 mg/mL, and the phospholipid:protein ratio was 99:1.

When the detergent was completely dialyzed away from an aliquot of the solubilized disk solution and centrifuged on a 0–50% (w/w) sucrose density gradient, two major recombinant populations were obtained. One was slightly below the top of the gradient, and the other was a broad band between 38 and 41% sucrose with a phospholipid:protein ratio of 22:1. Typically the sucrose between the bands contained protein which indicated that there is considerable variability in the phospholipid:protein ratio of the recombinants that were formed.

The same experiments were repeated substituting 50 mM Tris–acetate and 200 mM KCl, pH 7.0, for 10 mM Tris–acetate and 50 mM KCl, pH 7.0. Analogous curves were obtained except that they were shifted to lower OG concentrations by approximately 5 mM. The protein-rich population banded at 39–42% sucrose and had a phospholipid:protein ratio of 18:1.

Parts B and C of Figure 1 are plots of the results obtained when an egg PC–rhodopsin system was substituted for the disks. The buffers used and the starting phospholipid:protein ratios are indicated in the figure legend. In contrast to the experiments with solubilized disks, the protein and phospholipid exhibited identical pelleting behavior, suggestive of the formation of a single population of vesicles with a phospholipid:protein ratio identical with that of the starting material. However, when recombinants prepared from these systems were placed on gradients, two populations were again obtained. The phospholipid:protein ratios of the protein-rich band for 10 mM Tris–acetate and 50 mM KCl, pH 7.0, and for 50 mM Tris-acetate and 200 mM KCl, pH 7.0, were 32:1 and 27:1, respectively. Once again the curve in 50 mM Tris–acetate and 200 mM KCl, pH 7.0, was shifted about 3.5 mM lower in OG concentration.

Discussion

The goals of this paper were to obtain information concerning the mechanism of membrane protein–phospholipid reconstitution and to examine the effect of various experimental parameters such as the buffer, phospholipid, detergent, order of mixing of the components, and the rate of dialysis on this process. The results obtained here allow us to propose a model explaining the generation of the two vesicle populations and the low phospholipid:protein ratios observed in the OG dialysis reconstitution.

Before discussing the model, it is first helpful to establish a few important points. The order of mixing of components for many of our reconstitution experiments was such that the phospholipid was not directly incorporated into OG–rhodopsin micelles. According to Mimms et al. (1981), this may lead to the formation of pure phospholipid vesicles and aggregated protein, especially if the protein is not very water soluble. However, this effect was not responsible for the formation of the two populations, phospholipid-rich and protein-rich vesicles, obtained in our experiments. When two recombinants were prepared, one such that OG–egg PC micelles were added to OG–protein micelles and the other such that egg PC was directly solubilized in an OG–protein solution, nearly identical results were obtained. These samples were incubated approximately 15 h prior to dialysis. When a similar set of recombinants were incubated for only 4 h prior to dialysis, nearly identical results were obtained. Therefore, there must have been rapid equilibration, i.e., movement of rhodopsin and/or phospholipid between micelle populations, in our solubilized system. Second, the number of lipid molecules per rhodopsin molecule in the solubilized protein–lipid–OG micelles before dialysis varied from 7 to 39, yet the corresponding change in the final phospholipid:protein ratio was only from 30:1 to 49:1. Thus, the final phospholipid:protein ratio is not merely a reflection of the initial mixed micelle composition. The above results also clearly indicate that in order to obtain the final phospholipid:protein ratios we report, there must be a coalescence of the lipid–OG micelles with the protein–lipid–OG micelles as they are depleted of detergent. The most striking example of this is the recombinant prepared with DMPC. In the initial protein–lipid–OG micelles, there were approximately 39 lipid molecules per rhodopsin molecule, yet the final phospholipid:protein ratio was 233:1.

The reconstitution study with disk lipids (Figure 1A) employed disks (1.26 mg of rhodopsin/mL) that were solubilized in 200 mM OG. In the solubilized sample prior to dialysis, there were approximately four phospholipid molecules per rhodopsin molecule in the solubilized rhodopsin–lipid–detergent micelles. Since the phospholipid:protein ratio of the solubilized disks was 55:1,[2] most of the phospholipid was present as lipid–detergent micelles. As the OG concentration is lowered, detergent is removed from micelles, leading to their aggregation or coalescence and subsequent vesicle formation. If the detergent is randomly removed from the protein–lipid–detergent micelles and lipid–detergent micelles, then the vesicles formed by this aggregation process should have a final phospholipid:protein ratio similar to the initial ratio. The results from the reconstitution study with solubilized disks demonstrated that at higher OG concentrations, a greater percentage of the lipid relative to the protein pelleted. Thus, it appears that the vesicles which initially formed were rich in phospholipid. From 30 mM down to 27 mM OG, the phospholipid:protein ratio remained constant at 28:1. This implies that within this range of OG concentrations, only rhodopsin–lipid–detergent micelles, whose average phospholipid:protein ratio was 28:1, remained in solution.

The following model emerges. The lipid–OG micelles are less stable than the rhodopsin–lipid–OG micelles so that as the OG concentration is initially lowered by dialysis, OG is perferentially removed from the lipid–OG micelles, resulting in the formation of the phospholipid-rich vesicle population. As the OG concentration is further decreased to 30 mM, some micelles containing protein are also disrupted, and the vesicles that pellet upon centrifugation become increasingly protein rich. This explains the observation of recombinant vesicles between the phospholipid-rich and protein-rich bands on the sucrose density gradients. Finally, at 30 mM OG and below, the remaining rhodopsin–lipid–detergent micelles, which have an average phospholipid:protein ratio of 28:1, are depleted of detergent and form the protein-rich population. This limiting phospholipid:protein ratio of 28:1 is in good agreement with the final phospholipid:protein ratio of 22:1 obtained for the recombinant formed from solubilized disks. The 28:1 ratio also correlates well with the results of the Stubbs & Litman (1978) study of the solubilization of disk membranes with OG. These authors found that the initially solubilized protein–lipid–detergent micelles contained 25–30 mol of phospholipid/mol of rhodopsin. This corresponds to approximately a boundary layer of lipid being associated with each rhodopsin molecule in the protein-rich recombinant bands (Stubbs et al., 1976; Watts et al., 1979).

Although the phospholipid and protein showed identical pelleting behavior for the rhodopsin–egg PC system, both a phospholipid-rich population and a protein-rich population were evident on the sucrose density gradients. Therefore, it is likely that the egg PC–rhodopsin reconstitution proceeds in a manner analogous to that described for the disks. A comparison of the centrifugation curves obtained for the egg PC–rhodopsin and disk lipid–rhodopsin systems (Figure 1A,B) indicates that the protein and lipid centrifugation curve of the egg PC–rhodopsin system corresponds to the protein centrifugation curve of the disk system. Thus, the disk lipid vesicles must have a higher apparent sedimentation coefficient than the egg PC vesicles. This could be due to one or more of the following factors. The sedimentation coefficient is proportional to $M(1 - \bar{v}\rho)$, where ρ is the density of the buffer and M and $\bar{v}$ are the molecular weight and partial specific volume of the vesicles, respectively. The same buffer (10 mM Tris–acetate and 50 mM KCl, pH 7.0) was employed for the two systems being compared (Figure 1A,B), so ρ does not contribute to

[2] This ratio was determined after the solubilized disks were centrifuged to remove particulate matter and, therefore, is lower than the 65:1 to 70:1 phospholipid:protein ratio routinely obtained for disks.

the difference in sedimentation coefficients. Since the disk lipids are 42% PC, 46% PE, and 10% PS (Anderson & Sperling, 1971) and PE and PS have a lower $\bar{v}$ than egg PC (Steele et al., 1978; Litman, 1973), this could account for the higher sedimentation coefficient observed for the disk lipid vesicles. In addition, if the disk lipid vesicles are larger than the egg PC vesicles, then the former would have the larger sedimentation coefficient.

With both the disk and egg PC–rhodopsin systems, the pelleting curves were shifted to lower OG concentrations when the buffer with the higher salt concentration was used (50 mM Tris–acetate and 200 mM KCl, pH 7.0). Although OG is a nonionic detergent, its cmc is somewhat salt dependent. This is in qualitative agreement with the results of Shinoda et al. (1961), who observed a depression of the cmc of OG with increasing ionic strength of a given salt. DeGrip & Bovee-Geurts (1979) also reported the same trend for both octyl glucoside and nonyl glucoside. However, the shift in cmc with ionic strength is not as large as that observed with ionic detergents and bile salts (Helenius et al., 1979).

The results obtained from the reconstitution of rhodopsin with other phospholipids may also be interpreted in terms of micelle stability. Apparently DMPC interacts more favorably with OG than the unsaturated lipids egg PC, POPC, or DOPC. In fact the DMPC–OG micelles must have a stability comparable to that of the DMPC–rhodopsin–OG micelles since their final phospholipid:protein ratio is near the calculated starting ratio. The diphytanoyl-PC–OG micelles appear to behave like the unsaturated phospholipids, which is likely attributable to the presence of the bulky methyl groups distributed along the acyl chain.

Reconstitution of solubilized disks supplemented with egg PC using an ionic detergent (DTAB) and a bile salt (sodium cholate) as well as the nonionic detergent (OG) demonstrated that the detergent plays an important role in determining the reconstitution product. The final phospholipid:protein ratio obtained, as well as the morphology of the recombinant membranes, was dependent upon the detergent employed. Our findings with DTAB are similar to those of Hong & Hubbell (1972) in that for a phospholipid:protein ratio of approximately 100:1, there is an excellent correlation between the initial and final phospholipid:protein ratios.

It is interesting to compare our results with those obtained by OG dialysis reconstitution of other proteins. When the transmembrane protein glycophorin was reconstituted with egg PC, the resultant vesicles were unilamellar and contained only 25–50% of the protein that was added. This is in contrast to our results where we were unable to incorporate all of the lipid into protein-containing vesicles. Helenius et al. (1981) obtained two classes of vesicles when they reconstituted the spike glycoprotein of Semliki forest virus into phospholipid bilayers. The phospholipid-rich population that they obtained was probably formed from detergent depletion of mixed micelles, while the protein-rich population was formed by an entirely different mechanism. This second mechanism is typical of that found when proteins containing both hydrophilic and hydrophobic portions, which allow them to form aggregates or oligomers in solution, are reconstituted. In these cases, there is an asymmetric insertion or partitioning of protein into preformed lipid vesicles. Examples of this are cytochrome b_5 (Roseman et al., 1977) and the glycoprotein of vesicular stomatitis virus (Petri & Wagner, 1979).

We have previously reported on the formation of egg PC vesicles by an OG dialysis technique (Jackson et al., 1982). In these studies, it was demonstrated that it was necessary to use the effective OG to PC ratio (R_{eff}) rather than the total OG to PC ratio (R_t) when samples containing different PC concentrations were compared. Furthermore, it was shown that egg PC vesicles prepared with an R_{eff} of 6.0 were unilamellar, and the lipid was predominantly in vesicular form. Thus, in the current reconstitution experiments with rhodopsin, we used an $R_{eff(p)}$ of at least 6.0 (and as high as 65.0). Although we did not examine the reconstituted membranes by electron microscopy as a function of $R_{eff(p)}$, the final phospholipid:protein ratio was found to be independent of $R_{eff(p)}$. The egg PC–rhodopsin recombinant that was examined by electron microscopy was predominantly multilamellar. This preparation had an $R_t = 14$ and $R_{eff} = 12$, well above the values of these parameters which were found to produce single lamellar vesicles in a pure phospholipid system. It, therefore, appears that the structure of the egg PC–rhodopsin recombinants is a result of the protein and not a reflection of the OG to egg PC ratio.

In summary, the final phospholipid:protein ratio of the recombinant membranes that we prepared was not dependent upon the buffer employed, including the salt concentration, the addition of a divalent metal ion (Mg^{2+}), or the order of mixing of the components. The final phospholipid:protein ratio was only slightly dependent on the initial ratio. Although the products of reconstitution are clearly dependent upon the particular protein, lipid, and detergent used and their interactions with each other, it is seen that certain generalizations can be made for samples containing a specified type of protein and detergent. It is also seen that obtaining meaningful quantitative measurements on a homogeneous reconstituted membrane population first requires isolating the desired vesicle population.

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Size Dependence of the Translational Diffusion of Large Integral Membrane Proteins in Liquid-Crystalline Phase Lipid Bilayers. A Study Using Fluorescence Recovery after Photobleaching†

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ABSTRACT: The translational diffusion of bovine rhodopsin, the Ca^{2+}-activated adenosinetriphosphatase of rabbit muscle sarcoplasmic reticulum, and the acetylcholine receptor monomer of *Torpedo marmorata* has been examined at a high dilution (molar ratios of lipid/protein ≥ 3000/1) in liquid-crystalline phase phospholipid bilayer membranes by using the fluorescence recovery after photobleaching technique. These integral membrane proteins having molecular weights of about 37 000 for rhodopsin, about 100 000 for the adenosinetriphosphatase, and about 250 000 for the acetylcholine receptor were reconstituted into membranes of dimyristoylphosphatidylcholine (rhodopsin and acetylcholine receptor), soybean lipids (acetylcholine receptor), and a total lipid extract of rabbit muscle sarcoplasmic reticulum (adenosinetriphosphatase). The translational diffusion coefficients of all the proteins at 310 K were found to be in the range (1–3) × 10^{-8} cm^2/s. In consideration of the sizes of the membrane-bound portions of these proteins, this result is in agreement with the weak dependence of the translational diffusion coefficient upon diffusing particle size predicted by continuum fluid hydrodynamic models for the diffusion in membranes [Saffman, P. G., & Delbrück, M. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3111–3113]. Lipid diffusion was also examined in the same lipid bilayers with the fluorescent lipid derivative *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)dimyristoylphosphatidylethanolamine. The translational diffusion coefficient for this lipid derivative was found to be in the range (9–14) × 10^{-8} cm^2/s at 310 K. In consideration of the dimensions of the lipid molecule, this value for the lipid diffusion coefficient is in agreement with the continuum fluid hydrodynamic model only if a near-complete slip boundary condition is assumed at the bilayer midplane. Alternatively, kinetic diffusion models [Träuble, H., & Sackmann, E. (1972) *J. Am. Chem. Soc. 94*, 4499–4510] may have to be invoked to explain the lipid diffusion behavior.

In recent years considerable effort has been dedicated to the study of the diffusion of membrane components in model phospholipid bilayer membranes [for reviews see Cherry (1979), Jovin et al. (1981), and Vaz et al. (1982)]. The purpose of such model membrane studies is primarily to understand the diffusion of membrane components in terms of the physical parameters governing such diffusion in the quasi-two-dimensional lipid bilayer matrix and, eventually, to have a base line to which diffusion studies in the far more complex biological membranes may be compared.

Recent studies on the translational diffusion of membrane-bound peptides and proteins (Wu et al., 1978; Smith et al., 1979a, 1980; Vaz et al., 1979, 1981) in reconstituted model membrane systems have indicated that the diffusion of these entities in liquid-crystalline phase phospholipid bilayers is rapid, with translational diffusion coefficients (D_t)[1] in the

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[1] Abbreviations: AchR, acetylcholine receptor from *Torpedo marmorata*; DMPC, dimyristoylphosphatidylcholine; D_t, translational diffusion coefficient; FRAP, fluorescence recovery after photobleaching; NBD-DMPE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)dimyristoylphosphatidylethanolamine; SR-ATPase, the Ca^{2+}-activated adenosinetriphosphatase from rabbit skeletal muscle sarcoplasmic reticulum.

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