Reconstitution of Rhodopsin and the cGMP Cascade in Polymerized Bilayer Membranes

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ABSTRACT: The successful reconstitution of rhodopsin, the rod outer segment (ROS) G protein, and the ROS phosphodiesterase (PDE) into partially polymerized bilayer membranes is described. Purified bovine rhodopsin (Rh) was inserted into preformed partially polymerized lipid vesicles. Sonicated vesicles composed of approximately equal moles of dioleoylphosphatidylcholine (DOPC) (or 1-palmitoyl-2-oleoylphosphatidylcholine) and 1,2-bis(octadeca-2,4-dienoyl)phosphatidylcholine (DENPC) were photolyzed with 254-nm light to polymerize the DENPC and form domains of DOPC and polyDENPC in the vesicle wall. Rh–octyl glucoside (OG) micelles were slowly added to the vesicle suspension to give 15 mM OG (below the OG critical micelle concentration). The suspension was incubated and then dialyzed and purified on a sucrose gradient. Ultracentrifugation revealed a major Rh–lipid band which was harvested and found to contain a 100 ± 10 phosphatidylcholine to rhodopsin ratio (Rh–polyDENPC/DOPC). The orientation of Rh in the membrane was determined by limited proteolytic digestion of Rh and by competitive inhibition of monoclonal antibody binding to solubilized disk membranes. Results were compared with control membranes of Rh–DOPC (1:43) prepared by insertion and Rh–phospholipid membranes prepared by detergent dialysis. Visual inspection of thermolysin proteolytic patterns of Rh indicates one major population cleaved at the carboxy terminus, as is found in disk membranes with an asymmetric arrangement of Rh. In contrast, proteolysis of a Rh–egg PC/PE (1:50/50) membrane (detergent dialysis) produced two Rh populations, which indicates a symmetric arrangement of Rh. The Rh–polyDENPC/DOPC (1:100) membranes were allowed to compete with solubilized, immobilized disk membranes for the monoclonal antibody R2-15 (specific for the amino-terminal region of Rh). They were intermediate between the asymmetric ROS disk membranes and the symmetric dialysis membranes in their ability to bind the R2-15 monoclonal antibody. The data indicate ~80% of the Rh's in Rh–polyDENPC/DOPC are in the normal orientation found in disks. These Rh-containing polymerized bilayer membranes demonstrated functionality as determined by chemical regeneration, kinetic spectrophotometry, and cGMP cascade reconstitution experiments. In the latter experiments the peripheral proteins, ROS G protein and PDE, bound with comparable efficiency to both the polymerized PC bilayers and egg PC bilayers. Thus the biocompatibility of the phosphatidylcholine membrane surface was maintained after polymerization of DENPC.

Rhodopsin, the major integral protein of the light-harvesting and energy-transducing rod outer segment cell (ROS),[1] has been previously reconstituted into phospholipid membranes with retention of its chemical regenerability, photochemical functionality, and enzymatic functionality. Reconstitution of biological membranes from purified specific components has proven to be a convenient method for the study of protein–lipid interactions and protein functionality. Reconstitution allows the examination of specific surface and membrane interactions that are difficult to isolate in the more complex natural membrane and are sometimes not observed in detergent preparations of integral proteins.

Hubbell (1975) and his associates first demonstrated that purified delipidated rhodopsin in detergent could be recombined with lipid by detergent dialysis to give synthetic bilayer membranes. The properties of this type of membrane have been evaluated by several laboratories, and the functionality of the rhodopsin in these synthetic bilayers has been tested as well. First, the membranes were shown to be chemically regenerable following light exposure and bleaching of the rhodopsin (Hong & Hubbell, 1973). Second, rhodopsin–lipid bilayers show the normal photochemical intermediates with kinetics of relaxation comparable to that observed with ROS membranes (O'Brien et al., 1977; O'Brien, 1982). Third, light absorption by the 11-*cis*-retinylidene chromophore of the transmembrane rhodopsin initiates spectral and structural changes in the protein that enable it to interact with several outer segment enzymes.

Recombination of these ROS light-activatable enzymes with rhodopsin–lipid bilayers yields a system that displays the native activation sequence and a high level of chemical amplification (Fung et al., 1981; Tyminski & O'Brien, 1984), which results in the hydrolysis of $>10^5$ cyclic GMP molecules per photon. Recent reports indicate that this enzyme cascade directly

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[1] Abbreviations: ROS, rod outer segment(s); Rh, rhodopsin; P, phosphate; OG, octyl glucoside; TrTAB, tridecyltrimethylammonium bromide; PDE, phosphodiesterase; G protein, GTP binding protein; GTP, guanosine triphosphate; GDP, guanosine 5′-diphosphate; cGMP, guanosine cyclic 3′,5′-phosphate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; PC, egg phosphatidylcholine; PE, egg phosphatidylethanolamine; DOPC, dioleoylphosphatidylcholine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; DENPC, 1,2-bis(octadeca-2,4-dienoyl)phosphatidylcholine; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TLC, thin-layer chromatography; IgG, immunoglobulin G; ELISA, enzyme-linked immunosorbent assay; THF, tetrahydrofuran; BSA, bovine serum albumin.

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modulates the sodium permeability of the rod cell plasma membrane and controls visual excitation (Fesenko et al., 1985; Yau & Nakatani, 1985). Detergent dialysis membranes differ from native ROS in one important respect owing to the symmetrical arrangement of the asymmetric rhodopsin in the bilayer (Hubbell, 1975; Fung & Hubbell, 1978). However, Albert (1983) has reported the successful asymmetric insertion of rhodopsin into preformed bilayers.

In recent years several new classes of membrane bilayer forming amphiphiles have been introduced (O'Brien et al., 1984). These include lipids with polymerizable functionality, e.g., diacetylenes (Johnson et al., 1980; Hub et al., 1980; O'Brien et al., 1981), methacryloyl (Regen et al., 1980, 1982; Akimoto et al., 1981), dienoyl (Gros et al., 1981; Dorn et al., 1984), and vinyl (Tundo et al., 1982) groups. Certain of these lipids are attractive candidates for the formation of robust reconstituted protein–lipid membrane vesicles. In this report we describe the formation and characterization of rhodopsin in a bilayer membrane consisting of two phospholipids, one of which has been polymerized prior to insertion of the rhodopsin. The observed chemical, photochemical, and enzymatic functional rhodopsin behavior in these membranes demonstrates that sensitive vertebrate membrane proteins can be usefully incorporated into membranes that have been stabilized by polymerization reactions [preliminary report by Tyminski et al. (1985)].

Experimental Procedures

Materials. Frozen dark-adapted bovine retinae were obtained from J. Lawson Co. Buffers included *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (Hepes) from Calbiochem and tris(hydroxymethyl)aminomethane (Tris) from Sigma. Dithiothreitol (DTT) and thermolysin were purchased from Calbiochem. Phenylmethanesulfonyl fluoride (PMSF) and the nucleotides GTP, ATP, and cGMP were purchased from Sigma. Protein content was determined by the Bradford reagent, Bio-Rad Laboratories. Lipids for the preparation of phospholipid membrane vesicles were obtained from Avanti Biochemicals. Goat anti-mouse IgG conjugated with horseradish peroxidase was purchased from Hy-Clone. *o*-Phenylenediamine and appropriate buffers were part of an ELISA kit purchased from New England Nuclear. Hydrogen peroxide (30% in H_2O_2) was obtained from Kodak Laboratory Chemicals. Mouse monoclonal antibody R2-15, raised against bovine rhodopsin, was a gift of Paul Hargrave, University of Florida College of Medicine.

1,2-Bis(octadeca-2,4-dienoyl)-sn-glycero-3-phosphocholine (DENPC). Preparation of 2,4-Octadecadienoyl Chloride. A solution of 1.0 g (3.2 mmol) of 2,4-octadecadienoic acid (Ringsdorf & Schupp, 1981)[2] in 10 mL of dry benzene was treated with 1.0 mL of oxalyl chloride (14.5 mmol, 4.5 equiv) at 10 °C. The mixture was allowed to stir for 30 min beyond gas evolution, and then solvent and excess oxalyl chloride were removed under reduced pressure. The crude acid chloride was mixed twice with additional benzene and evaporated. The crude acid chloride (1.2 g) was used directly for the esterification.

Preparation of 1,2-Bis(2,4-octadecadienyl)-sn-glycerol. A solution of 1.5 g (4.4 mmol, 2.2 equiv) of 2,4-octadecadienoyl chloride in 10 mL of THF was added to a solution consisting of 0.55 g (2.0 mmol) of 2,3-dihydroxypropyl trichloroethyl carbonate, 3 mL of dry THF, and 1 mL (12.4 mmol, 3.1 equiv) of dry pyridine at 0 °C. The reaction was stirred at 20 °C for 12 h. The reaction mixture was then diluted with ether and washed with 3 N HCl, followed by water and saturated $NaHCO_3$. The organic phase was dried over $MgSO_4$ and filtered, and solvents were removed at reduced pressure to yield 2.5 g of crude product, which consisted of the diester carbonate and the starting acid. The crude diester carbonate was dissolved in 5 mL of THF, and 5 mL of glacial acetic acid was then added. This homogeneous solution was treated with 1 g (15 mmol, 7.5 equiv) of zinc dust and the mixture stirred at room temperature overnight. The pale yellow solution was filtered to remove the excess zinc salts, diluted with chloroform, and washed 3 times with saturated $NaHCO_3$ solution. The organics were dried over sodium sulfate and filtered, and the solvents were removed at reduced pressure to yield the crude diester monoalcohol, 2.2 g, which was purified by TLC in chloroform to yield a white semisolid, 1.2 g: R_f 0.3; NMR ($CDCl_3$) 0.5–2.5 (m, 54 H), 4.0–5.2 (m, 5 H), 5.8 (d, J = 15 Hz, 1 H), 6.1–6.3 (m, 2 H), 7.1–7.4 ppm (m, 1 H).

Preparation of Phosphatidylcholine. An ice-cold solution of 1.1 g (5 equiv) of 2-bromoethyl phosphoric acid dichloride in 10 mL of 1,1,2-trichloroethylene was treated with 1.3 mL of triethylamine followed by the dropwise addition of 0.4 g (0.5 mmol) of 1,2-bis(2,4-octadecadienoyl)-*sn*-glycerol in 5 mL of trichloroethane over 1 h. The reaction mixture was stirred for another 2 h at room temperature, and the disappearance of the starting diester alcohol was followed by TLC. The precipitated triethylamine hydrochloride was removed by filtration through Celite, and the sample was concentrated at reduced pressure and dissolved in 5 mL of THF. The sample was treated with 5 mL of 0.5 M NaOAc (pH 8.5) and 0.2 mL of 0.5 M EDTA (pH 10.5) and stirred for 4 h. The product was extracted twice with diisopropyl ether, and the combined organic phases were evaporated to dryness. The residue was dissolved in chloroform and dried over sodium sulfate, filtered, and concentrated under reduced pressure to yield an oil. The oil was dissolved in a small amount of chloroform, and the product was precipitated by the addition of acetone. After cooling to −20 °C, the solid was collected and dissolved in 2 mL of chloroform at 50 °C. Equal volumes (2 mL) of isopropanol and acetonitrile were added, followed by 7 mL of 24% trimethylamine in water. The mixture was capped tightly and stirred overnight. The solvents were removed under reduced pressure, and the mixture was diluted with water and extracted 3 times with chloroform. The combined organic extracts were dried over sodium sulfate, filtered, and concentrated under reduced pressure. The crude material was dissolved in a minimum volume of chloroform and precipitated with acetone. The solid was solubilized and applied to the top of a 15-g silica gel column. The column was eluted with chloroform followed by increasing amounts of methanol/water. The product eluted with chloroform/methanol/water (14:6:1) and was then chromatographed on thin-layer plates with the same solvent system to yield 0.2 g: R_f 0.2; NMR ($CDCl_3$) 0.5–2.5 (m, 54 H), 3.3 (s, 9 H), 3.5–4.6 (m, 8 H), 5.0–5.2 (m, 1 H), 5.8 (d, J = 15 Hz, 1 H), 6.0–6.3 (m, 2 H), 7.0–7.3 ppm (m, 1 H).

Isolation and Purification of Rod Outer Segments (ROS). All procedures concerning rhodospin and rhodopsin membranes were carried out at 4 °C under dim red light (Kodak safelight filter 2) or in complete darkness unless otherwise noted. All buffers were sterile filtered, and the preparations were handled and stored in sterilized tubes and containers.

[2] The dienoic acid was prepared by a modification of the condensation of the Grignard of 1-methoxy-1-buten-3-yne with tetradecanal, followed by reduction in situ with lithium aluminum hydride, hydrolysis, and then sodium chlorate oxidation.

Rod outer segments were isolated from thawed bovine retinae by sucrose flotation techniques as described previously by Tyminski and O'Brien (1984). After removal of the purified ROS from the sucrose gradient the spectral absorbance ratios of 280 nm/500 nm ranged from 2.5 to 3.0 and the yields were 15–17 nmol of rhodopsin/retina. Further purification was achieved by discontinuous sucrose gradient centrifugation to give a spectral ratio of 2.3–2.5.

Rhodopsin in Detergent. Purified rhodopsin in tridecyltrimethylammonium bromide (TrTAB) was prepared as described previously by Hong and Hubbell (1973) and O'Brien et al. (1977). Purified rhodopsin in octyl glucoside was obtained by concanavalin A affinity chromatography as described by Litman (1982). Appropriate column fractions were combined, concentrated, and then dialyzed to obtain rhodopsin in a buffer consisting of 30 mM octyl glucoside, 50 mM Tris–acetate, pH 7.0, and 100 mM NaCl (OG buffer).

Rhodopsin–Phospholipid Membrane Vesicles: Detergent Dialysis. Membrane vesicles were prepared by detergent dialysis from rhodopsin in tridecyltrimethylammonium bromide by the procedure described by O'Brien et al. (1977).

Rhodopsin Insertion. Membrane vesicles were prepared by sonication of the appropriate hydrated phospholipids. The lipids were hydrated with a buffer (50 mM Tris–acetate, pH 7.0, 100 mM NaCl) from thin films deposited in pear-shaped flasks by evaporation of a chloroform solution of the lipid(s). Sonication (Heat Systems Cuphorn) of the membranes for 45 min at 50 °C produced clear suspensions. Vesicle suspensions of DENPC/DOPC (1/1) or DENPC/POPC (1/1) were diluted with buffer to a lipid concentration of 9 mM and then placed in 1-cm quartz cells for photolysis and flushed with argon for 1 h. The cells were sealed under argon and stirred during a 3-h irradiation at 25 °C. The dienoyl absorbance was reduced to <7% of the original value. The photolyzed vesicles were concentrated to 20–25 mM for insertion of rhodopsin.

Rhodopsin in 30 mM OG buffer was added dropwise to a stirred vesicle suspension such that the octyl glucoside concentration slowly increased to 15 mM and the final OG:lipid ratio was 1.3. The initial rhodopsin:lipid ratio was 1:200. The membrane suspension in 15 mM OG was incubation overnight at 4 °C and then dialyzed with several changes against a 300-fold excess of buffer containing 0.5 g of Bio-Beads SM-2 (Bio-Rad). The rhodopsin-containing membranes were purified by continuous sucrose density gradient ultracentrifugation. The major bands were collected and assayed for rhodospin and phosphorus content.

Bleaching and Regeneration of Rhodopsin. Suspensions of rhodopsin–lipid membranes were bleached in the presence of 0.2 M hydroxylamine and 10 mM Hepes, pH 6.6. The excess hydroxylamine was then removed by washing the membranes (3 times) with buffer. An absorption spectrum was determined for a portion of the bleached membrane after solubilization in 100 mM TrTAB, 200 mM hydroxylamine, and 10 mM Hepes, pH 6.6. Regeneration of the bleached membrane suspension was accomplished by addition of a 2-fold excess of 11-*cis*-retinal in ethanol, followed by overnight incubation in the dark at 4 °C. The spectrum of the regenerated rhodopsin was again obtained in 100 mM TrTAB.

Isolation and Purification of the ROS Peripheral Proteins, G Protein and PDE. The G protein and PDE were isolated by a modification of the method of Kuhn (1982). The method utilizes the light-induced binding of the G protein to the rhodopsin membranes to separate it from the PDE which is soluble under hypotonic conditions. The procedure for isolation and the purification of these two proteins is the same as described previously by Tyminski and O'Brien (1984).

PDE Assay. The assay for PDE activity is adopted from Yee and Liebman (1978) and Liebman and Evanczsuk, (1982). All operations during the assay were shielded from the room safelights. Membranes stored in the dark under argon at 4 °C were diluted in a moderate ionic strength buffer, 10 mM Hepes, pH 7.9, 120 mM NaCl, 2 mM $MgSO_4$, and 1 mM DTT, and combined with selected amounts of PDE and G protein. The sample was allowed to equilibrate at 25 °C in a thermostated bath. Aliquots of GTP and cGMP were added to give a sample volume of 0.5 mL. The sample pH was monitored with a Microelectrodes MI-410 and amplified by a Keithley electrometer. After addition of saturating levels of substrate (~1 mM cGMP), the dark activity was determined, and the sample was exposed to a 1-ms light flash (Vivitar Model 283 with a Corning GS5-60 filter) 15 cm from the sample. The flash output was attenuated by calibrated neutral density filters (Optical Industries).

Thermolysin Proteolysis of Membrane Vesicles. The buffer for proteolysis was 10 mM Tris–acetate, pH 7.5, and 6 mM $CaCl_2$. Thermolysin was solubilized in buffer by raising the pH to 11, and then returning to pH 7.5 after complete solubilization. The proteolysis was initiated by combining the rhodopsin–lipid membranes with thermolysin in buffer at 20 °C at a weight ratio of 20 for rhodopsin to thermolysin. Aliquots of the reaction mixture were removed at selected times, and the reaction was quenched with an equal volume of 36 mM EDTA and 10 mM Tris–acetate, pH 7.5, and then immediately frozen in dry ice. Control reactions consisted of membranes in $CaCl_2$ buffer mixed 1:1 with EDTA buffer and then treated with thermolysin. Solubilization of membranes with sodium dodecyl sulfate (SDS) prior to thermolysin treatment exposed all available rhodopsins to proteolysis. Samples were prepared for electrophoresis by solubilizing the frozen samples in 1% SDS buffer.

Sodium Dodecyl Sulfate (SDS)–Polyacrylamide Gel Electrophoresis (PAGE). SDS–PAGE was performed in a slab gel apparatus (Bio-Rad) by the discontinuous method of Laemmli (1970). The gel thickness was 0.75 mm, and the acrylamide and *N,N'*-methylenebis(acrylamide) concentrations were 15% and 0.08%, respectively. The protein bands (20 μg of protein/well) were visualized with Coomassie brilliant blue dye R-250.

Electron Microscopy: Negative Stain. Samples of the membrane vesicle suspensions were observed by electron microscopy after negative staining with uranyl acetate or ammonium molybdate.

Freeze–Fracture. Small droplets of glycerol (30% v/v) containing samples were deposted on conventional Balzers gold planchettes and then frozen from room temperature in melting Freon 22 (−160 °C). Fracturing and replication was done with a Balzers BAF 301 apparatus. The replicas were cleaned in a sodium hypochlorite solution, washed with distilled water, and observed in a JEOL 1000X electron microscope.

Immunochemistry. For ELISA, 96-well plates (Costar) were coated with solubilized, bleached disk membranes. The disks were purified from bovine rod outer segment membranes by flotation in 5% Ficoll and then washed with distilled H_2O several times and resuspended to a rhodopsin concentration of 2 mg/mL. One milliliter of purified disks was diluted with an equal volume of 0.01 M phosphate buffer, pH 7.4, 0.14 M NaCl, and 0.003 M KCl (PBS) and solubilized with 1 mL of 1% (v/w) Triton X-100 in PBS, pH 7.4. The solubilized disks were diluted 500-fold with PBS, pH 7.4, giving a final rho-

dopsin concentration of 1.3 μg/mL and 0.001% Triton X-100. Each well was filled with 100 μL of this dilute rhodopsin solution and allowed to incubate at 4 °C overnight. The plates were washed 3 times with 0.05% Tween in PBS, pH 7.4 (wash buffer). Any nonspecific binding sites were masked by the addition of 100 μL of 1% BSA in PBS, pH 7.4. After incubation at 25 °C for 1.5 h, excess BSA was removed by washing 3 times with wash buffer, followed by H_2O. The plates were stored at −20 °C.

The binding of monoclonal antibody to immobilized rhodopsin was assayed directly by the addition of 100 μL of goat anti-mouse IgG conjugated with horseradish peroxidase (diluted 1:10 000 with 1% BSA in PBS, pH 7.4). After 1–2 h, free goat anti-mouse IgG was removed by washing 5 times with wash buffer. The enzyme substrate, *o*-phenylenediamine (OPD), was added in the presence of H_2O_2 (100 μL of 0.19% OPD, 0.014% H_2O_2, and 17 mM citrate in 65 mM phosphate, pH 6.3). The oxidation of OPD, producing a colored product, was allowed to proceed for ~10 min and then stopped with 100 μL of 4.5 M H_2SO_4. The amount of oxidized OPD was quantitated by measuring the optical density at 492 nm with a Titertek Multiskan MCC/340. All incubations were at 25 °C.

Competition Assays. Direct competition assays between immobilized rhodopsin and rhodopsin-containing membranes were performed at a monoclonal antibody concentration that gives 50–65% saturation of immobilized binding sites available, as determined by saturation binding experiments using the above-described ELISA procedure. Bleached membranes ranging in concentration from 2×10^{-9} to 0.5×10^{-3} μg/mL rhodopsin in 1% BSA/PBS, pH 7.4, were added to rhodopsin-coated wells, followed by 50 μL of the diluted monoclonal antibody. After 2 h of incubation, the plates were washed 5 times with wash buffer. The amount of monoclonal antibody bound to the plates was determined by an ELISA.

Kinetic Spectrophotometry. A single-flash kinetic spectrophotometer was utilized to observe the relaxation of the photochemical intermediates of rhodopsin bleaching in the membrane vesicles. The instrument has been previously described by O'Brien (1982). The electrical output from the photomultiplier tubes was connected to a differential amplifier of a Nicolet Model 1090 digital oscilloscope for display of the kinetic record. The information could be photographed and/or transferred to magnetic tape for permanent storage.

Results and Discussion

Membrane Vesicle Formation and Polymerization. The polymerizable lipid (DENPC) was prepared by the Eibl method (Eibl & Nicksch, 1978). The previously reported 2,4-octadecadienoic acid (Ringsdorf & Schupp, 1981)[2] was condensed with the racemic monotrichloroethyl carbonate of glycerol via the acid chloride. The trichloroethyl carbonate was reductively cleaved with Zn in THF/acetic acid. After chromatography the diester monoalcohol was condensed with 2-bromoethyl phosphoric acid dichloride, hydrolyzed, and aminated with trimethylamine by the procedure developed by Eibl and Nicksch (1978). The racemic phosphatidylcholine was purified by preparative TLC.

The dienoyl chromophore of the DENPC (Figure 1) absorbs at 260 nm (3×10^4), which allows photoinitiation of the polymerization of the lipid in membrane bilayers by the light (254 nm) from a low-pressure mercury lamp. The polymerization proceeds satisfactorily above or below the lipid phase transition, T_c; thus the lipids may be polymerized when the DENPC is in the liquid analogous state, whereas lipid diacetylene bilayers are polymerized only below the T_c (O'Brien et al., 1981). Previous studies of dienoyl lipids have shown they form unilamellar vesicles upon careful hydration, and the size may be controlled by application of shear forces. Polymer formation appears to proceed by the 1,4 addition of the diene units of dienoyl compounds as shown by ^{13}C NMR spectroscopy, infrared spectroscopy, and X-ray crystallography (Tieke & Wegner, 1981; Tieke, 1984; Tieke & Chapius, 1984), as well as studies of the lipid phase behavior. Photogeneration of free radicals (as opposed to thermal initiation) is the most efficient method of initiating the polymerization of dienoylphosphatidylcholine. Since polymerization requires a bilayer arrangement, the vesicles must first be formed and then photopolymerized before rhodopsin can be incorporated into the bilayer environment.

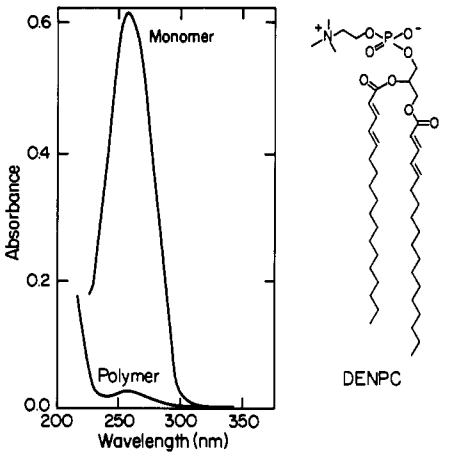

FIGURE 1: Absorption spectra of sonicated bilayer membranes consisting of DENPC/DOPC (1/1). Irradiation with UV light initiates the polymerization in bilayers and results in loss of the dienoyl chromophore.

In order to provide a nonpolymerized domain for accommodation of the rhodopsin in the membrane, the DENPC was mixed with DOPC or POPC in a mole ratio of 1:1.2 in chloroform solvent prior to lyophilization and hydration of the bilayers. The mixed-lipid system was sonicated to form lipid vesicles, which were then flushed with argon and irradiated with ultraviolet light to induce polymerization of the dienoyl groups. The DENPC monomer absorbance at 260 nm was reduced by photolysis to less than 7% of the starting absorbance. Conversion to polymer was followed by thin-layer chromatography on silica gel. Previous reports for dienoyl lipids and dimyristoylphosphatidylcholine (DMPC) or dipalmitoylphosphatidylcholine (DPPC) show the existence of phase-separated domains of these lipids in the mixed bilayers (Gaub et al., 1985; Tsuchida et al., 1986). Generally, polymerization enhances phase separation. Examination of 1:1 mixtures of DENPC and POPC by differential scanning calorimetry indicates the lipids are miscible until polymerization of the DENPC induces phase separation (data not shown). Pure DENPC bilayers exhibited a phase transition at 18 °C, and no transition was observed for pure polyDENPC membranes from −20 to 50 °C. A mixed membrane of DENPC/POPC shows a transition near 10 °C. After photopolymerization, the transition of the polyDENPC/POPC membranes moves to a lower temperature, near that of POPC bilayers (~2 °C). These data indicate that domains enriched in POPC were formed during the polymerization of DENPC.

Rhodopsin Insertion. The membrane vesicles which are composed of domains of 1,4-polymerized DENPC, nonpolymerized DOPC, and a mixture of the two were utilized as the target for rhodopsin insertion. The procedure was adapted from that of Albert (1983), who first applied it to the

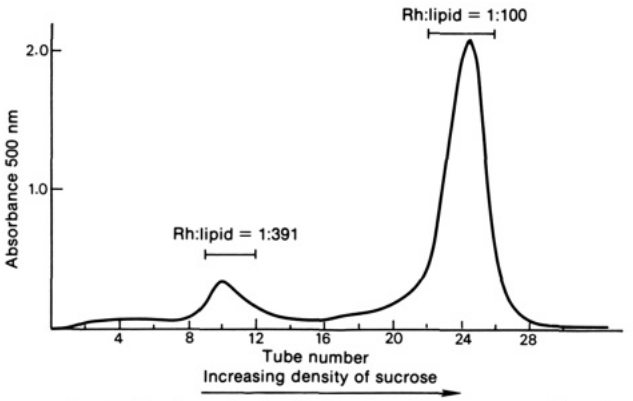

FIGURE 2: Profile of rhodopsin distribution after ultracentrifugation through a 5–45% sucrose gradient. Rh–polyDENPC/DOPC gradients were fractionated from the top of each gradient into 1-mL volumes. Quantitation of rhodopsin was determined by absorption spectroscopy of each fraction. Rhodopsin:lipid ratios were calculated after combining major protein fractions and analyzing for phosphorus content. Combined protein fractions and corresponding protein:lipid ratios are indicated above the major peaks.

insertion of rhodopsin into egg PC vesicles. The method utilizes rhodopsin solubilized in octyl glucoside micelles at an OG concentration of 30 mM, which is above the critical micelle concentration (cmc) of OG (22 mM in 50 mM KCl) (Jackson et al., 1982). The solubilized rhodopsin is added to the suspension of membrane vesicles while stirring at 4 °C until the final OG concentration is 15 mM (below the cmc). The OG to lipid mole ratio was 1.3, which is below the reported saturation level for OG in ROS membranes of 1.4 mol of OG per mole of phospholipid. Thus the lipid membranes are not solubilized by the procedure, yet sufficient detergent has been added to nearly saturate the membranes and thereby alter the bilayer packing in a manner that allows incorporation of the transmembrane protein, rhodopsin, into the membrane. After the overnight incubation at 4 °C the membranes were dialyzed to remove the OG. The resulting Rh-containing membranes were collected and found to include 83% and 78% of the originally added Rh, respectively, for the polyDENPC/DOPC and DOPC membranes. An attempt to insert Rh into pure polyDENPC membranes by a similar procedure was largely unsuccessful.

The rhodopsin-containing membranes were purified on a 5–45% sucrose density gradient by overnight ultracentrifugation at 90000*g*. The gradient (Figure 2) shows a major narrow band of membranes that contains 100 nmol of the original 175 nmol of rhodopsin. In different preparations the phosphorus to rhodopsin ratio varied from 90 to 110 for this major band. Secondary bands of higher lipid to rhodopsin ratios were also found on the gradients which accounted for an additional 50–60 nmol of rhodopsin. Some free lipid was found at the top of the gradient. Therefore, approximately 60% of the rhodopsin is incorporated into membrane vesicles which are homogeneous in density and uniform in composition. Similarly, the gradient of the Rh–DOPC membrane shows a major band that accounts for >75% of the rhodopsin. This membrane has a greater density due to the higher protein content (phosphorus to rhodopsin ratio of 43) and is similar in composition to Rh–egg PC membranes reported by Albert (1983).

Samples of the purified Rh–polylipid (1:100) membranes and the recovered lipid from the top of the gradient were analyzed by TLC. Both samples show a similar pattern of spots on the TLC plates with R_f values of 0.33, ~0.1, and the origin, which are ascribed to monomeric DOPC, oligomers, and polymers of DENPC, respectively. Samples of photo-

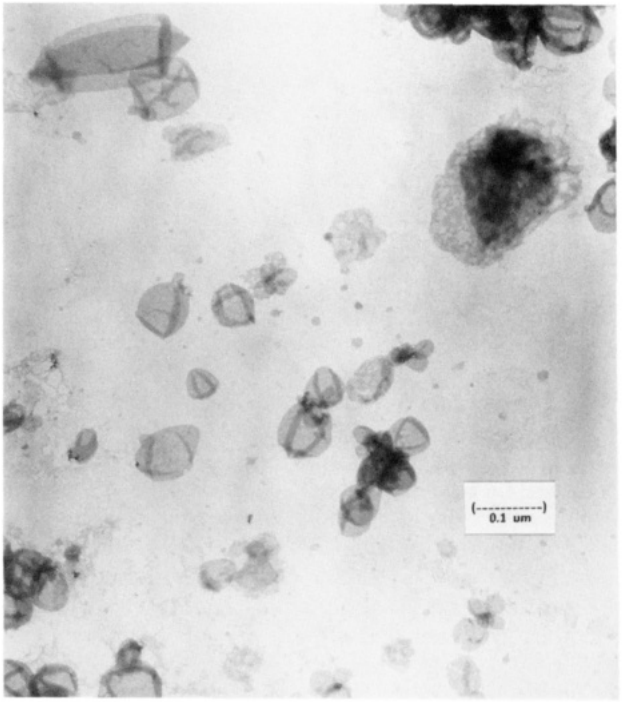

FIGURE 3: Electron microscopy of Rh–polyDENPC/DOPC (1:100) membranes. Purified membranes isolated from a 5–45% sucrose density gradient (Figure 2) were diluted to 0.1–0.2 wt % lipid and then stained with 2% uranyl acetate.

polymerized DENPC/DOPC vesicles prior to Rh insertion show the same TLC pattern. Thus the lipid composition (polyDENPC to DOPC ratio) is similar for both the Rh-containing and Rh-free membrane vesicles separated on the gradient. These data indicate that the lipid composition of the vesicles after Rh insertion is not significantly changed from the composition of the starting vesicles (mole ratio DENPC/DOPC of 0.45). Therefore, the purified Rh membranes consist of Rh, polyDENPC, and DOPC and will be referred to throughout the rest of this report as Rh–polyDENPC/DOPC (1:100).

Upon addition of the rhodopsin in OG to the sonicated bilayer vesicles, the turbidity of the suspension increased dramatically. This observation was also reported by Scotto and Zakim (1985), who successfully incorporated three different integral membrane proteins into preformed vesicles that contain a small amount of cholate or myristate, both of which induce fusion of phospholipid vesicles. Fusion of sonicated vesicles occurred during this insertion process in the presence of OG. Negative stain electron micrographs of such preparations reveal large, unilamellar vesicles with diameters ranging from 0.2 to 0.9 μm (Figure 3). Sonicated preparations of polyDENPC/DOPC before the addition of rhodopsin in OG were about 0.1 μm in diameter (micrographs not shown).

Freeze–Fracture Electron Microscopy. The organization of rhodopsin in isolated disk membranes and in synthetic membranes prepared by detergent dialysis has been studied by using the technique of freeze–fracture electron microscopy. In this technique, membranes are rapidly frozen and then fractured along the interior plane of the bilayer, exposing the hydrophobic surfaces of the bilayer. When isolated disk membranes are fractured, rhodopsin preferentially adheres to the concave fracture face of the vesicles as evidenced by the presence of a uniform distribution of particles covering the surface of this fracture face (Chen & Hubbell, 1973). The convex fracture face contains no particles but is somewhat textured in appearance. These results are characteristic of many integral membrane proteins asymmetrically arranged

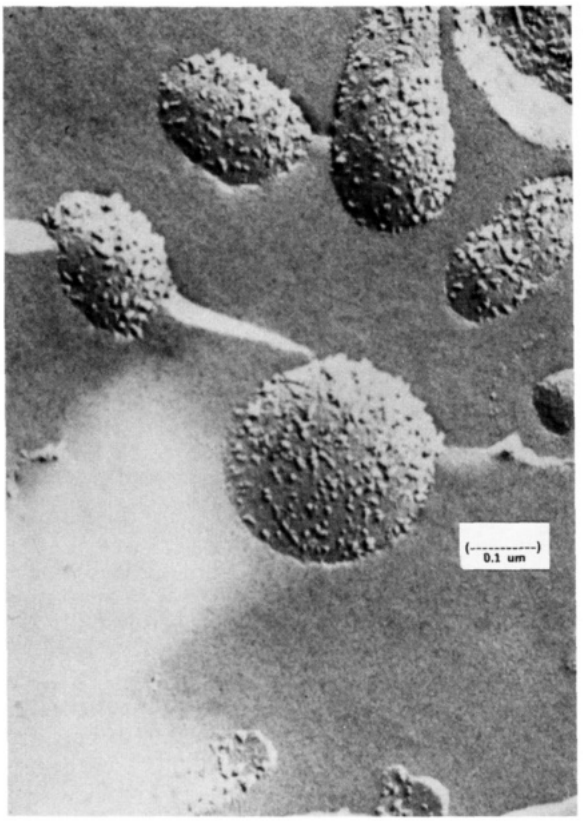

FIGURE 4: Freeze–fracture replicas of Rh–polyDENPC/POPC (1:100) membranes in 30% glycerol/water frozen from room temperature.

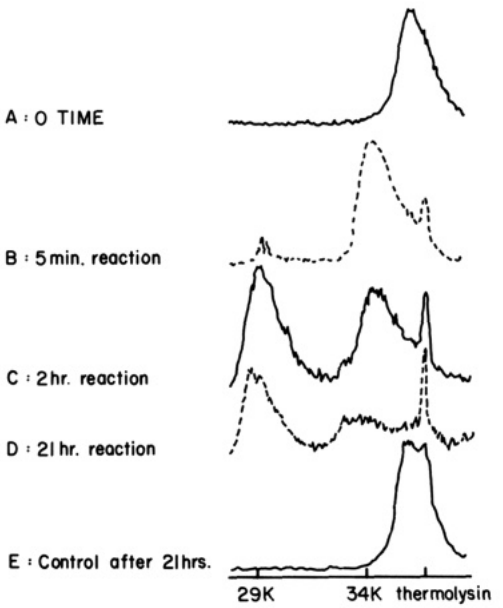

FIGURE 5: Densitometer traces of rhodopsin gel patterns after treatment of Rh–polyDENPC/DOPC (1:100) with thermolysin for (A) 0 time (before addition of thermolysin), (B) 5 min, (C) 2 h, or (D) 21 h. Trace E is the 21-h control reaction (membranes plus EDTA, then thermolysin addition).

in their native lipid environment. In contrast, membranes prepared by detergent dialysis techniques show particles on both fracture faces, presumably indicative of a symmetric arrangement of rhodopsin in these bilayer membranes.

In Rh–polyDENPC/DOPC (1:100) (prepared by insertion), rhodopsin is best accommodated in areas enriched in DOPC, as attempts to insert rhodopsin into 100% polyDENPC vesicles resulted in loss of most of the rhodopsin after dialysis to remove OG. Purification of these membranes on a 5–45% sucrose gradient produced a rhodopsin-rich band at the bottom of the gradient which was estimated to have a lipid:protein ratio of less than 20:1. The rhodopsin in this polymeric environment exhibited greatly reduced thermal stability as compared with other synthetic bilayers containing rhodopsin.

Freeze–fracture replicas of Rh–polyDENPC/DOPC (1:100) membranes demonstrate the presence of rhodopsin-rich and lipid-rich areas as shown in Figure 4. A nonrandom distribution of particles is apparent, with irregularly shaped zones devoid of protein particles. A count of the particle-free domains between 350 and 1500 nm^2 in area (500–2000 lipids per domain) shows these large zones comprise about 30% of the surface area. If the Rh-free areas are polyDENPC, then 70% of the original DENPC is found in these domains. The lipid in the rhodopsin-rich domains is presumably primarily DOPC. When a preparation of Rh–eggPC (1:100) (detergent dialysis) is fractured under identical conditions, a uniform distribution of particles is observed on all fracture faces.

In Figure 4 the particles appear on both concave and convex fracture faces with equal distributions of particles on each face. Although this result suggests symmetry of rhodopsin in these membranes, other evidence to be discussed (thermolysin proteolysis and competitive binding experiments with R2-15 monoclonal antibody to rhodopsin) indicates the major population of rhodopsin molecules to be asymmetrically oriented. Since integral membrane proteins interact extensively with the hydrophobic chains of lipid molecules, perhaps the lack of a specific preference of rhodopsin for the outer half of these bilayers is the result of a symmetric lipid distribution.

Proteolysis of Rhodopsin–Lipid Membranes. Thermolysin proteolysis of native ROS proceeds from the carboxy terminus of the rhodopsin molecule (Hargrave & Fong, 1977). Initially, a small fragment of 1200 daltons is removed from the rhodopsin, which causes an apparent decrease of 4 kdaltons in the molecular mass estimated by SDS–PAGE. This is followed by further cleavage to produce subunits of apparent molecular mass of 29 and 12 kdaltons (Fung & Hubbell, 1978). PAGE provides a convenient method of determining the rate of formation of these cleavage products. Since thermolysin (37.5 kdaltons) is a too large a water-soluble protein to penetrate through the vesicle bilayer, proteolysis occurs preferentially on the outside surface of sealed rhodopsin–lipid vesicles. Thermolysin requires Ca^{2+} as a cofactor and is deactivated by EDTA. This conveniently allows complete deactivation of the enzyme membrane reaction mixture before and during PAGE. Therefore, proteins exposed to the outside surface of the vesicle may be proteolyzed, the reaction quenched with EDTA, and the membranes solubilized with SDS prior to PAGE without proteolysis of the freshly exposed protein surfaces. Control experiments with membranes solubilized in SDS and then treated with EDTA-quenched thermolysin demonstrate that the rhodopsin is not cleaved.

Proteolysis of Rh–eggPC/eggPE (1:50/50) membrane vesicles prepared by detergent dialysis shows cleavages of some but not all of the rhodopsin. The electrophoretic patterns for samples proteolyzed 21 h contain a 38-kdalton band (starting rhodopsin) along with slightly heavier stained bands of the normal rhodopsin fragments. These data are similar to those published by other groups and are consistent with about 35–40% of the rhodopsin remaining after proteolysis (Fung & Hubbell, 1978). This is interpreted to mean that this fraction of the rhodopsin is oriented with the carboxy terminus inside the vesicle (retrograde orientation).

Visual inspection of proteolytic gel patterns of rhodopsin in the insertion membranes indicates the majority of the 38-kdalton band is cleaved to smaller fragments. The cleavage

pattern observed is similar to that of disk membranes; therefore, the accessibility of thermolysin to rhodopsin in these insertion membranes is comparable. To quantitate the distribution of proteolytic products, electrophoretic gel patterns stained with Coomassie blue R-250 were scanned by using a laser densitometer. The traces are shown in Figure 5. Accurate quantitation of the rhodopsin 38-kdalton band is difficult because thermolysin (molecular mass 37.5 kdaltons) is only slightly resolved from the broad rhodopsin band. It is estimated that 10–20% of the rhodopsin 38-kdalton monomer remains after a 21-h incubation with thermolysin. The control of rhodopsin and EDTA, followed by thermolysin addition (trace E in Figure 5), shows no evidence of proteolysis to smaller fragments.

Membranes of DENPC/DOPC (1:2) with entrapped self-quenched calcein dye remain able to sequester the dye during and after photopolymerization. The polymerization induces phase separation without an enhancement of dye leakage. This observation gives confidence that the larger thermolysin molecule is unable to leak into partially polymerized membranes during the proteolysis experiment.

Competition Binding Assays with Monoclonal Antibody R2-15 to Rhodopsin. Monoclonal antibodies have been utilized as site-specific probes in accessing the structure, function, and topography of rhodopsin. The antibody, designated as R2-15, exhibits anti-amino-terminal binding specificity. A tentative site of binding has been assigned to the amino-terminal 3–8 segment of rhodopsin (Hargrave et al., 1986). The binding specificity of monoclonal antibodies makes them ideal probes for the determination of the orientation of rhodopsin in membranes. If the rhodopsin is asymmetric or in the normal orientation as is found in native rod outer segments, R2-15 will not recognize or bind to asymmetric membranes as the amino terminus is within the disk interior, shielded by the membrane bilayer. Upon disruption of the membrane (detergent solubilization), the amino terminus becomes available for recognition by R2-15. A symmetric orientation of rhodopsin in synthetic membranes will produce recognition of approximately half of the rhodopsin molecules. Upon disruption of the membrane, additional amino-terminal sites (those within the membrane interior) become available for binding.

Competition experiments were performed by using an enzyme-linked immunoassay to study the antigenic properties of isolated disk membranes and synthetic phospholipid membranes containing purified rhodopsin to the R2-15 monoclonal antibody. The results of competitive binding studies with disks, Rh–egg PC (1:179) (detergent dialysis), and Rh–polyDENPC/POPC (1:100) prepared by insertion are shown in Figure 6. The protein concentration required to produce 50% inhibition of antibody binding to immobilized rhodopsin was determined from these inhibition curves. Bleached, solubilized disk membranes had a 1500-fold greater ability to bind antibody than isolated disk membranes. Isolated disks do compete at very high concentrations (50% inhibition at 100 μg/mL), suggesting the availability of a few rhodopsins to the antibody. Disk membranes were stored in small aliquots at −20 °C immediately after isolation, removed as necessary, and thawed once before use. This process probably caused some of the disks to become inverted or leaky. The inhibition curves generated on all disk preparations were very reproducible, and identical curves were obtained for all solubilized, bleached rhodopsin-containing synthetic membranes prepared by dialysis or the insertion method. For clarity, data are shown only for solubilized disks.

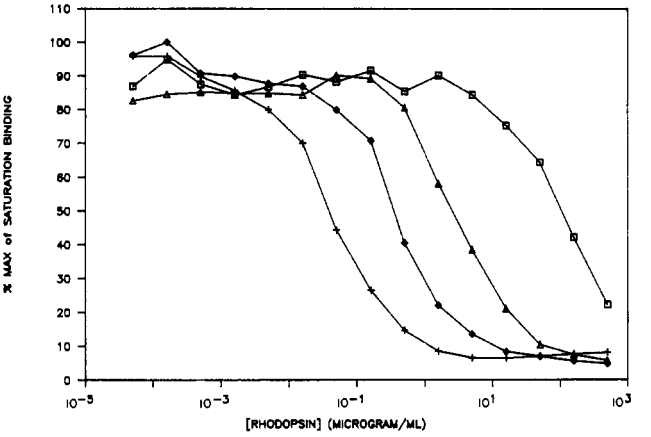

FIGURE 6: Competitive inhibition of R2-15 monoclonal antibody binding to bleached, solubilized immobilized disk membranes. Serial dilutions of competing membranes were added to the rhodopsin-coated wells in 1% BSA/PBS, pH 7.4. The monoclonal antibody was then added. The amount of monoclonal antibody bound to immobilized rhodopsin after 2 h was determined by the method described under Experimental Procedures. Competing membranes, reported in μg/mL rhodopsin, were solubilized disks (+), Rh–PC (1:179) (◇), Rh–polyDENPC/POPC (1:100) (△), and disk membranes (once frozen and thawed) (□).

Solubilized Rh–eggPC (1:179) was 10 times more competitive than membranes of Rh–eggPC (1:179) prepared by detergent dialysis. Upon solubilization of these membranes, twice as many sites become available for antibody binding; therefore, a 2-fold increase in binding ability was expected. The greater increase observed upon solubilization of these membranes is probably a result of the presence of multilamellar structures. Detergent dialysis techniques usually produce a population of multilamellar vesicles which would prevent the antibody from finding some of the rhodopsin molecules until detergent solubilization of the membranes.

The Rh–polyDENPC/POPC (1:100) membranes prepared by insertion were intermediate between the ROS disks and Rh–egg PC membranes in their capability to bind antibody. These membranes were 15 times more competitive than the asymmetric disks but were 10-fold less competitive than the symmetrical membranes prepared by detergent dialysis. Treatment of the Rh–polyDENPC/POPC (1:100) membranes with detergent increases their ability to bind antibody to a level comparable to that of solubilized disks. This is presumably due to solubilization of the DOPC domains to allow antibody access to the Rh amino terminus. The data indicate that most but not all of the rhodopsins in the intact Rh–polyDENPC/POPC (1:100) membranes have their amino terminus protected from interaction with the antibody. If 90–95% of the rhodopsins in disks were in the normal orientation, then these data suggest that about 80% of the rhodopsins in Rh–polyDENPC/POPC (1:100) are in the same orientation. This high degree of asymmetry is in substantial agreement with the proteolysis data.

Regenerability of Rhodopsin–Lipid Membranes. High chemical regenerability is a characteristic of native rhodopsin, and the yields are dependent on the molecular environment of the protein. In our experiments the procedures of Hong and Hubbell (1973) were used, and purified ROS membranes were selected as a standard of comparison. In these experiments the regeneration of bleached rhodopsin in the ROS membranes was 92%, which compares to the reported values of 83 ± 5%. Previous publications have reported that rhodopsin in most detergents is either not regenerable or very poorly regenerable. However, rhodopsin in lipid bilayers prepared by detergent dialysis ranges in regenerability from

Table I: Kinetic Data for Metarhodopsin I Relaxation of Rhodopsin Membranes at 25 °C, pH 7.0

membranes	flash conversion to meta I	no. of exponentials	k_1 (s^{-1})
ROS	(1)	single	140
Rh–polyDENPC/DOPC (1:100)	0.7	multiple	160
			50
Rh–DOPC (1:43)	0.3	double	90
			35

57% to 97%, depending on the lipid composition and whether EDTA and/or DTT was present during the formation of the membrane (Hong & Hubbell, 1973).

No regeneration yields are published for the rhodopsin–lipid membranes prepared by rhodopsin insertion, although Albert has privately communicated that the yield for Rh–egg PC (1:40) membranes is about 40%. We observed the regeneration of photolyzed rhodopsin in the Rh–DOPC (1:43) was 47%. The regeneration was 42% for the polymerized membranes of Rh–polyDENPC/DOPC (1:100). Thus about half of the rhodopsins inserted into the polyDENPC/DOPC vesicles are in a lipid environment that maintains the native, regenerable configuration of rhodosins upon bleaching.

Kinetics of Relaxation of Rhodopsin Photointermediates. Freshly prepared and sonicated ROS membranes in Hepes buffer (pH 7.0) were examined by flash photolysis for comparison with the rhodopsin membrane vesicles. In each case the sample was excited with a pulse from a frequency-doubled neodymium laser (pulse width 20 ns at 4 kV), which initiates a transient increase in absorbance at 470 nm followed by a decrease over several milliseconds at 25 °C. These absorbance changes are associated with the formation and decay of metarhodopsin I. The decay at 470 nm is accompanied by an increase at 380 nm, which is assigned to metarhodopsin II formation. Typical data for ROS, Rh–DOPC (1:43), and Rh–polyDENPC/DOPC (1:100) membranes are shown in Table I.

The ROS data display a single exponential decay of meta I, which is similar to that observed and reported previously by this laboratory and others. The relaxation is first order for at least three half-lives and has a $k_1 = 140 \pm 5\ s^{-1}$.

The data for the Rh–polyDENPC/DOPC (1:100) membranes show rapid formation of the 470-nm intermediate (meta I) and a multiexponential relaxation of meta I. The flash excitation induced increase in the meta I absorbance of these membranes was 0.7 that observed with ROS membranes, which indicates some of the rhodopsin in the polymerized membranes does not readily form meta I. The initial relaxation of the light-stimulated metarhodopsin I in the Rh–polyDENPC/DOPC (1:100), $k_1 = 160\ s^{-1}$, was similar to that observed with the ROS, but it is accompanied by at least one slower component not usually found in ROS. The ROS-like component accounts for 55–60% of the signal. Since the membranes are isolated after sucrose density ultracentrifugation, they are unlikely to have a large variation in the mole ratio of lipid to protein. Therefore, the multiphasic relaxation kinetics suggests the rhodopsin is inserted into lipid domains of different sizes within the same vesicle.

The Rh–DOPC (1:43) membranes also show a rapid formation of meta I, but the intensity of the signal is only 0.3 of that found with the ROS membranes. The relaxation of metarhodopsin I to meta II is slower than observed with ROS and consists of at least two exponentials. The faster component has a k_1 of 90 s^{-1} and accounts for about 35% of the signal. This retarded relaxation of meta I is suggestive of the type

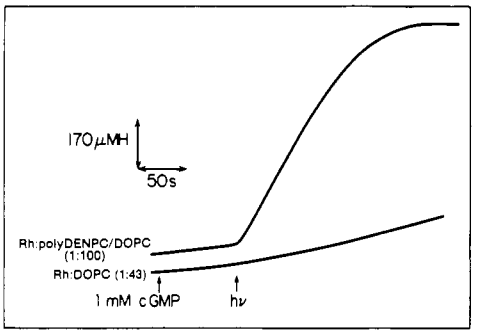

FIGURE 7: Time course of pH for light-stimulated PDE activity of purified Rh–polyDENPC/DOPC (1:100) and Rh–DOPC (1:43) (2 μM Rh) membrane vesicles recombined with purified ROS G protein (0.8 μM) and PDE (0.02 μM) in moderate ionic strength buffer, pH 7.9, and 250 μM GTP at 25 °C. An aliquot of substrate was added in the dark to give 1 mM cGMP. The sample was then exposed to a 1-ms light flash, which bleached 8% of the Rh. The sample pH changed until the cGMP was hydrolyzed. Controls without GTP, cGMP, or enzymes did not change pH upon light exposure of the Rh membranes.

of behavior reported for Rh–egg PC (1:100) at temperatures below 20 °C. We have also found that as the lipid content of the membrane is increased from 1:100 Rh–egg PC to 1:500, the relaxation of meta I becomes progressively faster and more complete. This is interpreted to mean that at high rhodopsin content as found in the Rh–DOPC (1:43) membranes the membrane is either too stiff to allow complete relaxation of the protein intermediates or some of the rhodopsins are not adequately solvated by the available lipid to permit relaxation.

Reconstituted PDE Activity. The ability of rhodopsin in recombined membranes to catalyze the activity of the ROS phosphodiesterase (PDE) serves as a test of its enzymatic functionality. Reconstitution of the light-induced PDE activity requires the recombination of both the G protein and the PDE in isotonic media to Rh–lipid membranes (Tyminski & O'Brien, 1984). Furthermore, the G protein must interact with bleached rhodopsin and bind GTP to its α-subunit in order to communicate with the PDE associated with the phospholipid membrane surface.

Initially, we demonstrated that the freshly purified PDE in isotonic buffer binds to the phospholipid surface of the membranes prepared above. The PDE associates with Rh–DOPC (1:43), Rh–polyDENPC/DOPC (1:100), and Rh–PC/PE (1:50/50) membrane vesicles to a similar extent as estimated by equilibration of the PDE with the membrane. The membranes were pelleted by a 30-s spin in an airfuge, and the PDE content in the membrane-rich pellet and in the supernatant was determined by trypsin activation of the PDE.

Next, the light-stimulated PDE activity was determined for rhodopsin (2 μM Rh) membrane samples reconstituted with 0.02 μM PDE and 0.8 μM G protein in isotonic 10 mM Hepes buffer, pH 7.9. The Rh–polyDENPC/DOPC (1:100) (Figure 7) displays low dark activity, which increases upon a millisecond flash excitation. The enzymatic activity responsible for the change in sample pH has a V_m of 4.9 μM H^+ s^{-1} (μM rhodopsin)$^{-1}$. The light to dark ratio of 17 is on the low end of the normally observed range for other reconstituted Rh–lipid membranes (Tyminski & O'Brien, 1984) and suggests that there is a very small amount (<0.2%) of optically bleached rhodopsin in the membrane sample. The observed V_m is similar to that found with other Rh–lipid membranes (Tyminski & O'Brien, 1984) and can be increased by the use of additional G protein. For comparison, the same amounts of enzymes were recombined with a Rh–egg PC/egg PE (1:50/50) membrane prepared by detergent dialysis as reported previously

(Tyminski & O'Brien, 1984). Light stimulation of this membrane yields activity with a V_m of 4.7 μM H^+ s^{-1} (μM rhodopsin)$^{-1}$. In contrast, the recombination of the enzymes with Rh–DOPC (1:43) membranes (prepared by insertion) results in a sample that displays little light-activated PDE activity, approximately 0.1 that found with the Rh–polyDENPC/DOPC membranes.

The maximum light-induced PDE activity in a reconstituted system is dependent on the concentration of both the G protein and the PDE and the membrane surface area. Thus, increases in the concentration of G protein in the sample will increase the measured V_m. In favorable circumstances, addition of sufficient G protein will yield a light-induced PDE activity comparable to the turnover number obtained by trypsin activation of the PDE. In the present experiments with Rh–polyDENPC/DOPC the observed activity is about half the maximum value expected from the PDE turnover number of 1000 s^{-1}. Initial experiments demonstrated that further increases in the G protein content of these reconstituted samples result in increases in the PDE activity.

Although more extensive evaluation of these insertion membranes is necessary to probe the behavior of rhodopsin in the membranes and its enzymatic functionality, some preliminary conclusions should be noted. Polymerization of the DENPC does not appear to inhibit the binding of G protein or PDE to the lipid membrane surface. Some of the rhodopsin embedded in the bilayer matrix composed of monomeric DOPC and polyDENPC is able to interact with the G protein and catalyze the binding of GTP. The activated G protein with bound GTP activates nearly all of the PDE associated with the membrane vesicle surface in a manner similar to that found with Rh–lipid membranes prepared by dialysis. Thus a large proportion of the PDE, whether initially bound to the surface domains of DOPC or polyDENPC, is activable. This suggests that the activation sequence is facilitated by movement of the G protein and perhaps the PDE along the surface of the membrane.

The inability of bleached rhodopsin in the Rh–DOPC (1:43) to function as an activator for the PDE could be due to the low conversion of rhodopsin to meta II (see Kinetics of Relaxation of Rhodopsin Photointermediates) which would decrease the efficiency of binding of the G protein by metarhodopsin II and inhibit the subsequent exchange of GTP for GDP on the G protein.

Summary

Membrane vesicles were prepared from the polymerizable lipid DENPC and the nonpolymerizable lipid DOPC and subsequently polymerized by UV irradiation. Rhodopsin in OG was incubated with the partly polymerized membrane vesicles, which are composed of domains of 1,4-polymerized DENPC, nonpolymerized DOPC, and a mixture of the two. The conditions were chosen to favor protein insertion. The rhodopsin-containing vesicles were purified by sucrose density ultracentrifugation, and approximately 60% of the rhodopsin was incorporated into membrane vesicles which are homogeneous in density and are presumed to be uniform in composition. The purified Rh–polyDENPC/DOPC had a protein to lipid ratio of 1:(100 ± 10), and the lipid in the membrane shows TLC spots due to monomeric PC and polymerized and oligomeric lipid. Thermolysin proteolysis of the Rh–polyDENPC/DOPC (1:100) membrane vesicles and competitive binding experiments using R2-15 monoclonal antibody specific for the amino terminus of rhodopsin indicate about 80% of the Rh molecules are in the normal orientation found in ROS disk membranes.

Chemical regeneration of photolyzed rhodopsin in Rh–polyDENPC/DOPC (1:100) indicates that about half of the rhodopsins inserted into the polyDENPC/DOPC vesicles are in a lipid environment that maintains the native, regenerable configuration of rhodopsin upon bleaching. The photochemical relaxation of the meta I intermediate in the Rh–polyDENPC/DOPC (1:100) shows a fraction of the rhodopsin behaves like that in ROS membranes and that this accounts for about 55–60% of the excited rhodopsins. The additional slower relaxations are likely due to rhodopsin that is inserted into lipid domains that are too small to accommodate the rhodopsin in a normal manner.

The observed successful reconstitution of light-induced activation of PDE with Rh–polyDENPC/DOPC (1:100) is likely to involve the ~50% of the rhodopsins that behave in a normal ROS manner. Both ROS peripheral proteins, G protein and PDE, associate with the Rh–polyDENPC/DOPC membranes as efficiently as nonpolymerized Rh membranes. Therefore, polymerization of the PCs does not effect the biocompatibility of the membrane surface. Light exposure of 8% of the Rh appears to activate nearly all of the PDE bound to the membrane surface, which suggests that the activation process involves movement of the G protein and/or the PDE rather than exclusively membrane diffusion of the Rh.

A significant proportion of the Rh inserted into preformed and polymerized lipid vesicles is comparable to ROS Rh in its chemical regenerability, photochemical formation of metarhodopsin II, and ability to catalyze the exchange of GTP for GDP on the G protein and activate PDE. These experiments demonstrate that sensitive vertebrate proteins may be combined with stabilized bilayer assemblies in a manner that preserves protein functionality.

Acknowledgments

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Registry No. PDE, 9068-52-4; DENPC, 76282-07-0; polyDENPC, 103719-14-8; DOPC, 10015-85-7; POPC, 6753-55-5; 2,4-octadecadienoyl chloride, 113380-22-6; 2,4-octadecadienoic acid, 76282-17-2; oxalyl chloride, 79-37-8; 1,2-bis(2,4-octadecadienoyl)glycerol, 76282-06-9; 2,3-dihydroxypropyl trichloroethyl carbonate, 38835-14-2; 2,3-bis(2,4-octadecadienoyl)propyl trichloroethyl carbonate, 113380-23-7; (2-bromoethyl)phosphoric acid dichloride, 4167-02-6; triethylamine, 121-44-8.

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Structure of Phosphate-Free Ribonuclease A Refined at 1.26 Å

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ABSTRACT: The structure of phosphate-free bovine ribonuclease A has been refined at 1.26-Å resolution by a restrained least-squares procedure to a final R factor of 0.15. X-ray diffraction data were collected with an electronic position-sensitive detector. The final model consists of all atoms in the polypeptide chain including hydrogens, 188 water sites with full or partial occupancy, and a single molecule of 2-methyl-2-propanol. Thirteen side chains were modeled with two alternate conformations. Major changes to the active site include the addition of two waters in the phosphate-binding pocket, disordering of Gln-11, and tilting of the imidazole ring of His-119. The structure of the protein and of the associated solvent was extensively compared with three other high-resolution, refined structures of this enzyme.

Refinement of a protein structure appears to be a never-ending task, since it is not as yet possible to describe the structure sufficiently well to ensure complete agreement of the model with the diffraction data. Even for the best refined protein structures, and final crystallographic R factors ($R = \sum|F_{obsd} - F_{calcd}|/\sum F_{obsd}$) are over 10% (for example, for crambin 11.1% at 0.945-Å resolution—Hendrickson, personal communication, 1986), although the quality of experimental data should allow the final numbers to be only half as large. Only a few proteins have been refined at high resolution with

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