Protein–Lipid Interactions at Membrane Surfaces: A Deuterium and Phosphorus Nuclear Magnetic Resonance Study of the Interaction between Bovine Rhodopsin and the Bilayer Head Groups of Dimyristoylphosphatidylcholine†

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ABSTRACT: Rhodopsin, isolated from bovine retinal rod outer segment disk membranes, has been reconstituted into bilayers of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine which was deuterated in the terminal methyl groups of the choline polar head group. By use of a mixed detergent system of cholate and octyl glucoside to solubilize the phospholipid and rhodopsin, 15 membrane complexes of predetermined phospholipid to rhodopsin mole ratios of between 350:1 and 65:1 have been produced by exhaustive dialysis and studied by a variety of techniques. Electron micrographs of replicas from freeze-fractured membrane complexes showed that the majority of the lipid, for all rhodopsin:phospholipid ratios, was contained in large bilayer vesicles with diameters in excess of 400 nm. Complexes produced with rhodopsin from frozen retina produced an absorption maximum at 478 nm after photobleaching whereas rhodopsin from fresh retina could be bleached more completely to an absorption maximum at 380 nm. Deuterium nuclear magnetic resonance (NMR) spectra from the lipid head groups of bilayers above the gel to liquid-crystalline phase transition temperature were shown to be sensitive in a systematic way to the presence of rhodopsin which could be bleached to 380 nm. The measured quadrupole splittings, taken as the separation of the turning points of the recorded NMR spectra, decreased from a value of 1.28 kHz for protein-free bilayers to approximately 0.40 kHz for bilayers containing 65 molecules of phospholipid for each rhodopsin at 32 °C. However, complexes formed by using rhodopsin from frozen retina or which had been fixed with glutaraldehyde displayed deuterium NMR spectra similar to protein-free phospholipid bilayers with no sensitivity to rhodopsin content and were thought to contain aggregated rhodopsin. Measurements of the total deuterium NMR spectral intensity recorded were compared with chemical and radiolabel determinations and showed that, within the error of the method, all the deuterated phospholipid was observed in the recorded spectra. No change in the high-frequency segmental motions of the lipid head groups was measured from deuterium NMR spin–lattice relaxation time measurements for the deuterated lipid groups. The phosphorus-31 NMR spectra of the lipid phosphate groups were typical for lipid bilayers at all rhodopsin:lipid ratios and were insensitive to the presence of rhodopsin, having a chemical shift anisotropy of approximately −45 ppm. For complexes which showed sensitivity to rhodopsin, the changes in the deuterium NMR spectra were shown, from control experiments, to be due only to the protein and not to residual detergents in the complexes. The NMR results suggest that nonaggregated rhodopsin in phospholipid bilayers may cause relatively small and nonspecific perturbations of the polar choline head group. Fast exchange of the lipid between the protein surface and the bulk lipid phase has also been demonstrated.

The wide diversity of lipids in natural membranes suggests some functional role for their interaction with membrane proteins, and considerable effort has been invested in the study of lipid–protein interactions in search of such a role [see Sanderman (1983), Marsh & Watts (1982), de Kruijff et al. (1985), and Watts & de Pont (1985) for reviews]. Studies of protein–lipid interactions have concentrated on the acyl chain associations of lipids with the hydrophobic surface of integral proteins (Seelig et al., 1982; Smith & Oldfield, 1984; Bloom & Smith, 1985), and little evidence for hydrocarbon chain ordering or specificity which depends upon the chemical nature of the chain has been obtained to date. However, the polar head group of phospholipids may play a special role in membranes since it is at the polar–apolar membrane interface that communication takes place with the cellular and extracellular environments and it is this chemical moiety of the commonly occurring diacylphospholipids which distinguishes their type. Molecular specificity in the interactions between lipids and proteins, and any effect they may have on membrane function, is most likely to occur at the bilayer interface where a high degree of chemical diversity is found.

Model membrane systems have played a significant role in discerning both functional and structural information about integral and peripheral protein interactions with lipids. In addition, spectroscopic methods, in particular spin-label electron spin resonance (ESR)[1] and nuclear magnetic resonance (NMR), have been used to provide dynamic information on the associations between various membrane components.

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[1] Abbreviations: NMR, nuclear magnetic resonance; ESR, electron spin resonance; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; CSA, chemical shift anisotropy; DSC, differential scanning calorimetry; $\Delta\nu_Q$, apparent deuterium quadrupole splitting; OG, *n*-octyl β-D-glucopyranoside; TEMPO-choline, 2,2,6,6-tetramethylpiperidine-1-oxyl-4-choline; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; ROS, rod outer segment(s); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; FID, free induction decay.

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Recently we have used ^2H NMR to study specific molecular interactions at the bilayer surface between different phospholipid types (Sixl & Watts, 1982, 1983), between charged phospholipids and a peripheral protein, the basic protein from myelin (Sixl et al., 1984), and between an integral protein, band 3, and the polar head groups of phosphatidylcholine (Dempsey et al., 1986). Using spin-label methods, we have also demonstrated molecular specificity of particular lipid types with cytochrome *c* oxidase (Knowles et al., 1981) and with (Na^+, K^+)-ATPase (Esmann et al., 1985).

In this study, we have extended the investigation of phospholipid–protein interactions to the membrane surface while at the same time relating the structural and dynamic information from ^2H NMR experiments of selectively labeled phospholipids to the functional state of the protein, the photoreceptor protein rhodopsin from bovine retinal rod outer segment disk membranes. Most previous studies of rhodopsin–lipid interactions have concentrated on the acyl chain associations (Bienvenue et al., 1982; Watts et al., 1979; Kusumi et al., 1980) although this integral protein is thought to protrude from the bilayer hydrophobic interior (Osborne et al., 1978) and could interact either electrostatically or sterically with the membrane lipids. For this study, we have chosen to investigate the head group of phosphatidylcholine which has been extensively studied by ^1H NMR (Hauser, 1981), ^2H NMR, and ^{31}P NMR (Seelig et al., 1977; Gally et al., 1975; Skarjune & Oldfield, 1979), single-crystal X-ray diffraction (Pearson & Pascher, 1979), and neutron diffraction (Büldt & Wohlgemuth, 1981). This work has shown that structural and dynamic constraints are imposed by the interlipid interactions, and we have demonstrated that these constraints can be modulated by different lipid types in the same bilayer (Sixl & Watts, 1982, 1983) and by extrinsic proteins (Sixl et al., 1984). The indications from the present work are that the polar part of the phospholipid at the bilayer interface is sensitive to the presence only of functional, nonaggregated rhodopsin.

Materials and Methods

Phospholipid Synthesis. The phospholipid, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine was specifically deuterated (DMPC-d_9) or tritiated (DMPC-t_9) in the polar choline head group by methylation of 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine, prepared according to the phosphorylation method of Eibl (1980), using methyl iodide enriched in the specific hydrogen isotope (Eibl, 1980; Sixl & Watts, 1983). DMPC-d_4, deuterated in the choline C^α and C^β head-group segments, was produced by using ethanolamine-d_4 in the phosphorylation step followed by methylation with CH_3I. DMPC, either labeled or unlabeled, was purified by silica gel chromatography in basic solvents and appeared as single spots on thin-layer chromatograms. The optical rotation, $[\alpha]^{21}_{546}$, of DMPC-d_9 measured on a Perkin-Elmer 141 polarimeter in $CHCl_3$–CH_3OH (2:1 v/v) was +7.80°.

Lipid and Protein Analysis. Chemical analysis of phospholipid concentrations was carried out by the method of Eibl and Lands (1969), following sulfuric acid digestion of lipid phosphorus. More rapid estimates were obtained by measuring the specific activity of DMPC-t_9-doped complexes. The accuracy (though not the sensitivity) of both methods for phospholipid quantitation was very similar.

Protein was assayed by using a modified Lowry method (Markwell et al., 1981) and from the absorbance of rhodopsin at 500 nm, assuming a molar extinction coefficient of 40 000 cm^{-1} and a rhodopsin molecular weight of 40 000 (Daemen et al., 1972).

Isolation of Rhodopsin. Bovine rod outer segment (ROS) disk membranes were isolated from fresh and frozen (slowly in liquid N_2) retina from dark-adapted fresh ox eyes obtained from local slaughterhouses (Papermaster & Dreyer, 1974; Baehr et al., 1979). Isolated ROS were dissolved in *n*-octyl β-D-glucopyranoside (OG) (Sigma Chemical Co.) solution immediately, and rhodopsin was purified within 12 h (detergent-containing solutions were stored at 4 °C). The spectral ratio at 280 and 500 nm, $A_{280/500}$, of solubilized ROS after pelleting debris was less than 3.

Rhodopsin was isolated from ROS solubilized in 50 mM OG in 50 mM Tris–acetate buffer, pH 7.0, containing 1 mM $CaCl_2$ and 1 mM $MnCl_2$ by affinity chromatography over concanavalin A–Sepharose (Pharmacia) (Litman, 1982). Rhodopsin was washed free of endogenous lipid to a level of less than one phosphate per protein using 50 mM OG and was then eluted with 0.2 M methyl mannoside in buffer containing 30 mM OG. Rhodopsin was concentrated to approximately 4 mg/mL by using an Amicon ultrafiltration cell and PM-30 membrane and was then used immediately for reconstitution. The spectral ratio of rhodopsin ($A_{280/500}$) was below 1.75 (Litman, 1982). Polyacrylamide gel electrophoresis using 10% gels (Laemmli, 1970) showed rhodopsin as a single band of molecular weight 40 000. All manipulations involving rhodopsin were carried out in the dark or in dim red light.

Reconstitution of Rhodopsin with DMPC. A mixed detergent system for solubilization of rhodopsin and DMPC-d_9 was found to be essential for the production of complexes which after exhaustive dialysis had the desired lipid:protein ratio. Concentrated rhodopsin in approximately 60 mM OG was mixed with an equal volume of 75 mM cholate in dialysis buffer (see below) containing solubilized DMPC to produce a final concentration of rhodopsin of approximately 2 mg/mL (0.05 mM) and DMPC-d_9 with trace amounts of DMPC-t_9 between 3 mg/mL (2.5 mM) and 10 mg/mL (9 mM) depending upon the desired final protein:lipid mole ratio. This mixture was dialyzed against 2.5 L of buffer (50 mM Tris–acetate and 1 mM EDTA, pH 7.0) containing Amberlite XAD-2 (BDH Chemicals, Poole, U.K.) which had been washed first with acetone and then with boiling, deionized water. Dialysis for 7–9 days at 4 °C, with daily changes of buffer, reduced the concentration of cholate typically to less than 1 molecule per 1000 DMPC molecules as determined by the removal of [^{14}C]cholate (Amersham, England) from the reconstitution.

After dialysis, the sample was pelleted and resuspended in 10% sucrose in dialysis buffer. The material was loaded onto a continuous (10–50%) sucrose gradient and centrifuged (4 h, SW-40 rotor, 182000*g*, 4 °C). The single tight band observable in all cases was removed and washed twice with dialysis buffer to remove sucrose before resuspension in the same buffer (0.5–1.0 mL) made by using deuterium-depleted water. The final phospholipid:protein mole ratio after dialysis was always the same as the ratio before dialysis. The rhodopsin–DMPC complexes were then used immediately for NMR, ESR, and DSC and then for optical measurements and freeze–fracture electron microscopy.

Optical Measurements. ROS, rhodopsin, or reconstituted DMPC–rhodopsin complexes were solubilized in 1 wt % Ammonyx LO (Onyx Chemical Co.), and the absorbance at 280 and 500 nm was measured for calculation of the absorbance ratio, $A_{280/500}$, which was used as a criterion for determining the integrity of rhodopsin which should give values of less than 2.0 when pure, unbleached, and uncontaminated (Daemen et al., 1972). The complete optical spectrum between 270 and

600 nm was also measured for all rhodopsin–DMPC-d_9 complexes above 26 °C before and after photobleaching at 30 °C for 3 min with white light.

Electron Microscopy. Freeze fracturing of DMPC dispersions was carried out by using Balzers freeze-fracturing equipment. Replicas from the freeze-fractured samples were visualized on a Philips EM-400 electron microscope.

NMR Measurements. Deuterium NMR spectra were usually recorded at 46.1 MHz on a Bruker WH-300 spectrometer, employing single 90° pulses of 29-μs duration. One complex with a rhodopsin:DMPC-d_9 mole ratio of 105:1 was also examined by using a Bruker CXP-200 NMR spectrometer at a deuterium frequency of 30.7 MHz using quadrupolar echo pulse sequences (Davis, 1983) with 90° pulses of 4.5-μs duration.

T_1 measurements were made by standard inversion recovery pulse sequence experiments on the WH-300.

Phosphorus-31 NMR spectra were recorded by using the WH-300 at 121.5 MHz under broad-band proton decoupling at a power of 7–10 W using 60° pulses of 15-μs duration. The sample temperature in all the NMR experiments was controlled by air flow to an accuracy of ±0.5 °C.

Phospholipid Quantitation from NMR Spectral Intensities. The amount of deuterated phospholipid contributing to the recorded NMR spectrum was measured spectroscopically and then compared with both the chemical and radiolabel methods (see above). Quantitation of NMR spectra was carried out by first integrating the spectrum from the protein–phospholipid dispersion under examination in a sample tube containing an empty insert to give an integral I_2. Into the previously empty insert was added a known amount [C (milligrams)] of identically labeled DMPC-d_9; the spectrum of both dispersions was then recorded and integrated under identical instrumental conditions to give the integral I_1. The quantity of DMPC-d_9 contributing to the unknown spectrum is then given by $I_2C/(I_1 - I_2)$. The sample volumes and positions within the receiver coils were the same for all experiments, and spectra were integrated over 5 kHz.

In control experiments to test the NMR spectral integration method to quantify phospholipid concentration, a variety of DMPC-d_9 dispersions at a range of concentrations in the insert were examined and compared with similar protein-free DMPC-d_9 dispersions in the sample tube. In all cases, accuracies in determining the sample lipid concentration of ±6% were obtained between 26 and 41 °C when compared with chemical and radiolabel analyses, and with the total amount of lipid initially used.

Phase Transition Measurements. The main gel to liquid-crystalline phase transition temperatures of DMPC-d_9 bilayers and rhodopsin–DMPC-d_9 complexes were measured by DSC using a Perkin-Elmer DSC-2B.

Results

Rhodopsin was reconstituted into bilayers of DMPC-d_9 at 15 different phospholipid:protein mole ratios of between 350:1 and 65:1, the latter being close to the physiological ratio in the ROS disk membrane (Daemen, 1973). DSC experiments were carried out to determine the gel to liquid-crystalline phase transition temperature of DMPC-d_9 dispersions with and without rhodopsin. Pure DMPC-d_9 bilayer vesicles formed by detergent dialysis undergo the gel to liquid-crystalline phase transition between 23.7 and 23.9 °C which is close to that measured for hand-shaken dispersions of DMPC-d_9 (Sixl & Watts, 1983). For rhodopsin-containing complexes, the phase transition behavior was similar to that reported by Kusumi et al. (1980). At low protein concentrations, at protein:lipid mole ratios of less than 1:200, the phase transition temperature was centered at close to that for protein-free bilayers at approximately 24 °C, but slightly broadened in range. However, for dispersions containing more rhodopsin, with mole ratios of less than 108 lipids per protein, the DSC measurements of enthalpy change with temperature occurred over a somewhat broadened range than for other complexes. In all cases, the lipid was found to be in the fluid phase at temperatures above 28 °C. Opsin and rhodopsin in DMPC bilayers were also found to denature irreversibly at close to 57 and 72 °C, respectively (N. J. P. Ryba et al., unpublished results), as observed in native ROS membranes (Miljanich et al., 1985).

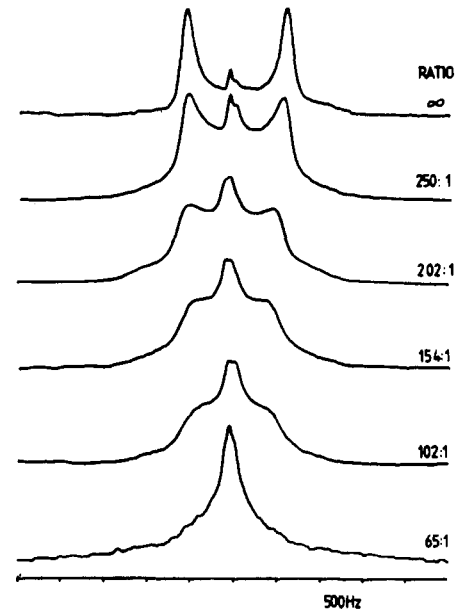

FIGURE 1: ^{2}H NMR spectra (46.1 MHz) of DMPC-d_9 (5–10 mg in 0.2–1.0 mL of deuterium-depleted buffer) for rhodopsin–DMPC-d_9 complexes at different mole ratios at 32 °C. Rhodopsin in these complexes could be bleached to give an absorption maximum at 380 nm (see text). The protein-free dispersion was produced by dialysis as for the protein-containing complexes. Spectra were recorded by using a 90° pulse of 29 μs, and 4000–12 000 scans were recorded with a spectral width of 10 kHz and a recycle time of 0.25 s.

The deuterium NMR spectra of typical rhodopsin–DMPC-d_9-reconstituted complexes at 32 °C, which is above the gel to liquid-crystalline phase transition temperature for all dispersions, are shown in Figure 1. The rhodopsin used to produce these complexes was isolated from fresh, not frozen, retina and could be photobleached to an absorption maximum at 380 nm. A protein-free dispersion of DMPC-d_9 produced by detergent dialysis (upper spectrum) gave very similar spectra to those from dispersions of DMPC-d_9 made by hand shaking the lipid in buffer above the phase transition temperature. The measured quadrupole splitting (1.28 kHz at 32 °C) and the line shape are both characteristic of a random dispersion of phospholipid bilayers [for example, see Seelig (1977)].

As the concentration of rhodopsin was increased in the DMPC-d_9 bilayers (Figure 1), a considerable decrease in the apparent ^{2}H NMR quadrupole splitting was observed (to less than 0.40 kHz at 32 °C at a 65:1 lipid:protein mole ratio). For spectra from complexes with a high protein content which were broad and the spectral maxima ill-resolved, the edges of the plateau were measured and taken as the apparent quadrupole splitting.

More than 90% of the labeled phospholipid was found to contribute to the observed deuterium NMR spectra of Figure 1 from comparison of the spectral integrals with both phos-

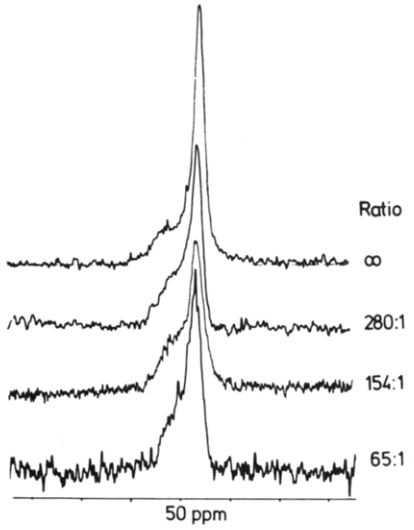

FIGURE 2: Proton-decoupled ^{31}P NMR spectra (121.5 MHz) for rhodopsin–DMPC-d_9 complexes at different mole ratios recorded by using 60° pulses of 15 μs and a line broadening of 80 Hz. Number of scans were 3000–5000 with a 1.2-μs recycle time and a spectral width of 37 kHz. Other conditions as for Figure 1.

phate and radiolabel analyses. These measurements suggest that the spectra recorded for all dispersions arise from all the lipid in the sample volume.

Representative ^{31}P NMR spectra from a DMPC-d_9 dispersion and from three rhodopsin–DMPC complexes are shown in Figure 2. All the spectra recorded were indicative of axially symmetric motion of the lipid phosphate group and were characteristic of phospholipid bilayers with a measured CSA for all dispersions of approximately −45 ppm at 32 °C within the limits of the experiment. All the protein-containing complexes gave ^{31}P NMR spectra which were essentially the same as for protein-free bilayers, within the sensitivity of the method. None of the ^{31}P NMR spectra recorded showed any evidence of a significant contribution from isotropically tumbling phospholipid complexes, although it is possible that the instrumental line broadening of 80 Hz employed to increase the spectral signal to noise ratio may have artificially reduced the recorded intensity of any narrow, low-intensity lines.

The spin–lattice (T_1) relaxation time for the deuterium NMR spectra for the choline methyl groups of DMPC-d_9 over the full temperature range studied (26–41 °C) was insensitive to the incorporation of rhodopsin into the phospholipid bilayers and was measured to be 51 ± 6 ms at 32 °C for protein-free and rhodopsin-containing complexes. The only inhomogeneity of T_1 in the powder spectra was at the center and resulted from the much longer T_1 of the residual HDO.

Deuterium NMR spectra for all DMPC-d_9–protein complexes were measured as a function of temperature. As the temperature was increased, the apparent quadrupole splitting decreased (Figure 3) for all complexes containing rhodopsin which could be bleached to an absorption maximum of 380 nm (see below). In Figure 4 are shown the experimental results from lipid–protein complexes similar to those shown in Figure 3, but replotted to show the change in apparent quadrupole splitting as a function of protein:lipid mole ratio. This approach to analyzing similar deuterium NMR results has been used by Kang et al. (1979) for acyl chain labeled phospholipids and by us to describe the fast exchange of lipids onto and off a peripheral membrane protein at the bilayer surface (Sixl et al., 1984). The observed quadrupole splitting (Q_o) is taken as the weighted mean of $\Delta\nu_Q$ for the head groups of DMPC-d_9 being perturbed by rhodopsin (Q_c) and $\Delta\nu_Q$ for

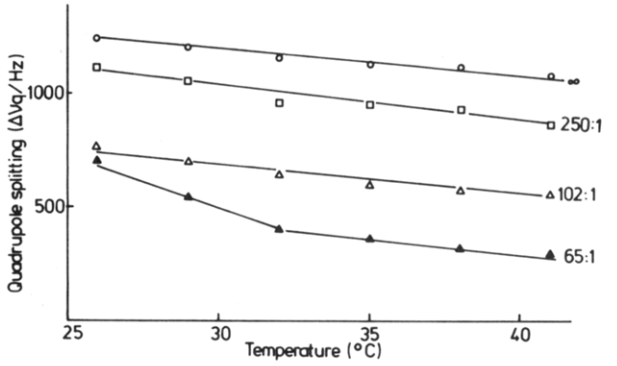

FIGURE 3: Measured quadrupole splittings ($\Delta\nu_Q$ in hertz), taken as the separation of the maxima or turning points in the ^2H NMR spectra, shown as a function of temperature for complexes of different DMPC-d_9:rhodopsin mole ratios. Conditions and bleaching characteristics of rhodopsin in the complexes to produce these data were the same as for Figure 1.

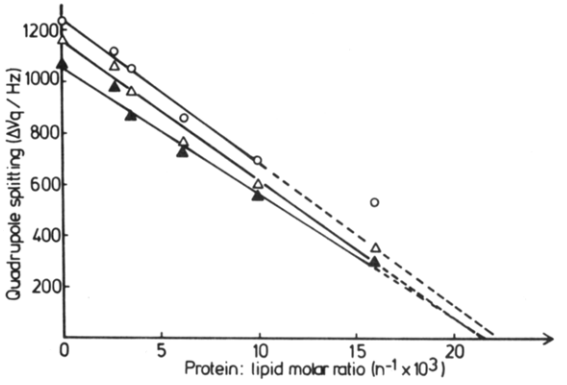

FIGURE 4: Variation in apparent quadrupole splittings ($\Delta\nu_Q$ in hertz), taken from data similar to those in Figure 3, as a function of rhodopsin:DMPC-d_9 mole ratio ($1/n_t$) at 26 (○), 32 (△), and 41 °C (▲).

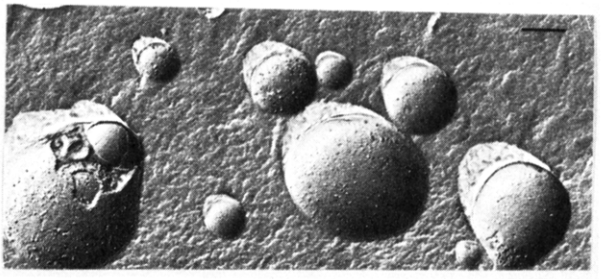

FIGURE 5: Freeze–fracture electron micrograph of a DMPC–rhodopsin complex at a mole ratio of 102:1 frozen from room temperature (ca. 15 °C). Bar represents 200 nm.

free and unperturbed choline methyl groups (Q_f). Then $Q_o = (n_c/n_t)Q_c + (n_f/n_t)Q_f$, where n_c, n_f, and n_t are the numbers of lipids being perturbed by rhodopsin, unperturbed by rhodopsin, and the total number of lipids in the complex, respectively, such that $n_t = n_c + n_f$. It can be shown that the experimentally measured quantities $1/n_t$ and Q_o should depend linearly on each other as is shown to be the case for the data presented in Figure 4 at three different temperatures.

Freeze–fracture electron micrographs (Figure 5) indicate that the protein-containing complexes produced are bilayer vesicles and are inhomogeneous in size. The concentration of rhodopsin in each vesicle, observed as pits or protrusions, appears inhomogeneous and may be the result of protein aggregation on freezing the complexes from 15 °C which is below the DMPC phase transition temperature of 23–24 °C (Hong & Hubbell, 1972). Measurements of the size distribution of

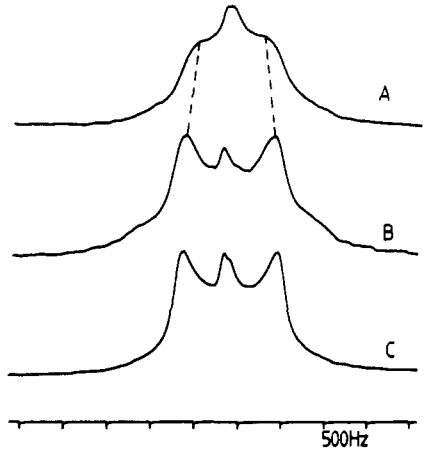

FIGURE 6: ^2H NMR spectra of DMPC-d_9 recorded from DMPC-d_9–rhodopsin complexes with mole ratios of 165:1 (spectra A and B) and 148:1 (spectrum C). Glutaraldehyde was added (10-fold molar excess of the protein concentration and incubated at 35 °C for 30 min) to the complex which displayed photobleaching characteristics as detailed in Figure 1 and gave spectrum a, to product spectrum b which is similar to that obtained from a protein-free complex (upper spectrum in Figure 1). Spectrum c was recorded from a complex which could not be subsequently photobleached to give an absorption maximum at 380 nm (see text); this deuterium NMR spectrum is similar to that from protein-free DMPC-d_9 bilayers at the same temperature. All other conditions as for Figure 1.

over 400 vesicles with a DMPC-d_9:rhodopsin mole ratio of 102:1 showed that over 80% of the lipid occurred in vesicles with diameters greater than 400 nm. These vesicles were found to be no smaller on average than protein-free vesicles, used to give the upper spectrum in Figure 1, formed in a similar fashion by dialysis. Both kinds of dispersions formed tight, single bands by sucrose density gradient centrifugation.

Glutaraldehyde was added to a reconstituted DMPC-d_9–rhodopsin complex with a mole ratio of 165:1 and to a protein-free DMPC-d_9 dispersion. The spectrum recorded from the rhodopsin-free complex did not change, either in line separation or in line width, upon the addition of a large excess of glutaraldehyde up to a glutaraldehyde:DMPC-d_9 mole ratio of 10:1. In the rhodopsin-containing complex, however (Figure 6), the normally collapsed spectrum (with an apparent quadrupole splitting of 0.80 kHz and typical of those shown in Figure 1) reverted to a narrow spectrum (with a quadrupole splitting of 1.00 kHz at 38 °C) which is similar to that for a protein-free bilayer dispersion with a value for $\Delta\nu_Q$ of 1.15 kHz at the same temperature. In addition, some samples, those produced with rhodopsin isolated primarily from frozen retina, displayed unusual bleaching characteristics in that a large absorption maximum at 478 nm was formed with subsequently no decay to an absorbing species at 380 nm. These samples also produced narrow deuterium NMR spectra very similar to those from protein-free dispersions and rhodopsin-containing complexes fixed with glutaraldehyde, the deuterium NMR spectra of such complexes being independent of the initial rhodopsin:DMPC-d_9 mole ratio (Figure 6). From both glutaraldehyde-fixed complexes and complexes which could subsequently only be partially bleached, there was no evidence of any ^2H NMR spectral components outside the spectral width used for the integration method.

The integrity of rhodopsin in the DMPC complexes after all NMR experiments was assessed by measuring the spectral ratio $A_{280/500}$. The absorbance ratio was routinely found to be 1.7–1.8, even for rhodopsin in complexes which did not bleach to an absorption maximum of 380 nm, indicating that rhodopsin had not been photobleached during the NMR experiments since these ratios were similar to those determined before the NMR measurements which never lasted for more than 12 h on any one complex. In addition, no complex was used more than one for NMR measurements, and subsequent experiments by other techniques were always performed within 2 days of complex preparation.

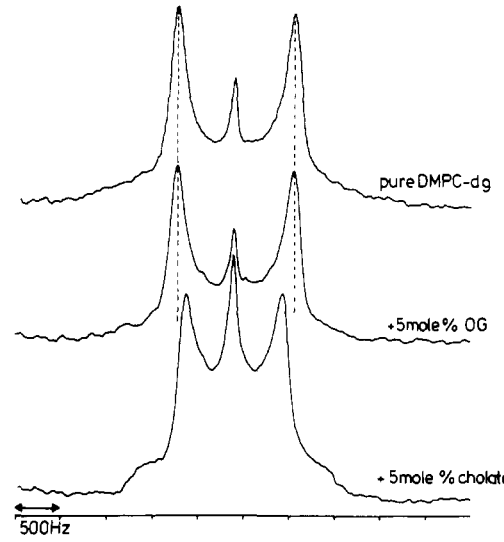

FIGURE 7: ^2H NMR spectra of DMPC-d_9 bilayers in the absence (upper spectrum) and presence (middle and lower spectra) of octyl glucoside and cholate used to produce the rhodopsin–DMPC-d_9 complexes. Conditions as for Figure 1.

The residual cholate in the complexes formed by dialysis of rhodopsin and DMPC-d_9 was monitored by the removal of radiolabeled tracer and showed that this impurity was below a 1:10 detergent:rhodopsin mole ratio for all recombinants. To test the effects of detergents on the ^2H NMR spectra of DMPC-d_9 bilayers, either OG or cholate was added to hand-shaken dispersions of protein-free DMPC-d_9 at a non-solubilizing concentration of 5 mol g of the phospholipid (Figure 7). Although OG had no detectable effect on the head-group properties of DMPC-d_9, cholate above about 2 mol % caused a relatively large decrease in the measured quadrupole splitting and an increase in the central isotropic spectral component. No broadening of these spectra was apparent. Subsequent mixing of the detergents by adding 2 mol % OG to the 5 mol % cholate–lipid complex reduced the central spectral component while at the same time failed to affect the quadrupole splitting or the line width. Similarly, addition of cholate to a bilayer dispersion containing OG produced a reduction in the measured quadrupole splitting with no increase of intensity in the central line.

DISCUSSION

Bilayer membrane recombinants of bovine rhodopsin and dimyristoylphosphatidylcholine, made with predetermined mole ratios of lipid and protein by exhaustive dialysis using a mixed detergent system, have been studied by a variety of techniques. The photobleaching behavior of rhodopsin in these complexes was measured from the optical absorption spectra before and after exposure to light at temperatures above the DMPC-d_9 bilayer gel to liquid phase transition temperature. Two types of reconstituted complex were formed. One displayed an absorption maximum shift from 500 nm for rhodopsin to 480 nm on bleaching. The other bleached from rhodopsin to a species with an absorption maximum at 380 nm. Only the second type of complex showed systematic changes in the ^2H NMR apparent quadrupole splittings with rhodopsin content in the bilayers.

Electron microscopy of a variety of complexes indicated that the distribution of vesicle sizes and their degree of heterogeneity were independent of the level of protein incorporation. At 30 °C, the contribution to the correlation time (τ_c) of a phospholipid in a bilayer vesicle with a diameter of 400 nm, due to vesicle rotation in water and lateral diffusion in the bilayer of 6.5×10^{-8} cm^2·s^{-1}, is approximately 8×10^{-4} s (Burnell et al., 1980). Vesicles with diameters greater than 400 nm therefore are large enough that little or no reduction of the deuterium quadrupole splitting of 1.2 kHz will occur simply through vesicle rotation or phospholipid lateral diffusion in the bilayer (Stockton et al., 1976). Freeze–thawing, to disrupt any small vesicles in one reconstituted sample at a DMPC-d_9:rhodopsin mole ratio of 105:1, showed no detectable change in the spectral line shape. The broad central regions of the ^{2}H NMR spectra from protein-containing complexes may, however, result from some degree of sample inhomogeneity.

The ^{31}P NMR spectra of DMPC–rhodopsin complexes (Figure 2) were characteristic of phospholipid bilayers and did not appear to change significantly with rhodopsin concentration within the experimental limits. This observation is in contrast to our previous results in which specific electrostatic binding of myelin basic protein from bovine spinal cord to negatively charged phospholipid bilayers caused an appreciable decrease in the CSA of the ^{31}P NMR spectra, as well as in the ^{2}H NMR quadrupole splittings from head group labeled negatively charged phospholipids (Sixl et al., 1984). The lack of observable isotropic ^{31}P NMR lines in the spectra of Figure 2 indicates that the sharp central peaks of the corresponding ^{2}H NMR spectra (Figure 1) are due not to small vesicles, but probably to residual HDO; this result confirms the electron microscopic observation (see above) and similar results with band 3–DMPC complexes studied by us (Dempsey et al., 1986).

The NMR spectra were recorded on a spectrometer with a maximum spectral width of 50 kHz. Thus, any deuterated lipids which give rise to quadrupole splittings somewhat larger than 50 kHz were not observed. Furthermore, the single-pulse method of excitation used by us suffers the disadvantage that a programmed delay between pulsing and data acquisition is required to prevent a spurious signal arising from the electronic recovery of the spectrometer after pulsing. Thus, due to the programmed delay times and the long 90° pulse length, the first 50–75 μs of the FID was not recorded. As a consequence, any deuterons with a very short T_2 which would give rise to extremely broad spectra would only contribute extensively to the initial, but in our case excluded, part of the FID. Broad spectral components would therefore not be recorded even if much of the signal intensity was within the observable spectral width of the WH-300 spectrometer. To control and investigate such possible instrumental problems, we measured the deuterium NMR spectral intensity over the whole temperature range studied and confirmed that the majority of the lipid in the recombinants was indeed visible by NMR in our spectra and under our instrumental conditions for all samples. This strongly suggests that no deuterium spectral component with large quadrupole splittings, or with a very broad line width, arising from any rhodopsin–DMPC-d_9 recombinant, was or could have been recorded. It has been proposed that rhodopsin incorporation into lipid membranes causes the immobilization (on the ^{31}P NMR time scale) of some phospholipids and hence generation of rather broad spectra in addition to the usual bilayer powder pattern (Albert & Yeagle, 1983). Although the signal to noise ratios of our ^{31}P NMR spectra (Figure 2) do not enable us either to confirm or to refute this proposal, it has not been confirmed by others (Ellena et al., 1986; Smith & Ekiel, 1984). The reorientation time needed to average and reduce the anisotropy of a deuterium quadrupole splitting of 1.2 kHz is close to the reorientation time which is needed to average the ^{31}P NMR CSA of 45 ppm (about 6 kHz), although the motional properties of a phospholipid phosphate group may be very different from that of the polar head group. There are no indications from our present deuterium NMR data of two-component spectra or of any immobilization of a significant proportion of the deuterated DMPC choline head groups by rhodopsin, as discussed earlier with respect to the lipid quantitation and below with respect to the fast, two-site exchange analysis.

In comparative experiments carried out on the same lipid–protein complex, with a DMPC:rhodopsin mole ratio of 105:1 and using a high-power CXP-200 NMR spectrometer with a quadrupole echo sequence (Davis, 1983) as well as the high-resolution WH-300 NMR spectrometer using single radio-frequency pulses, it was possible to exclude instrumental spectral distortion as an explanation for the broadening and reduction in measured quadrupole splittings with increasing rhodopsin content in DMPC-d_9 bilayers. The quadrupole splittings obtained were identical from both machines on the same sample and at the same temperatures.

From the control experiments to determine the effect of detergents on DMPC-d_9 bilayers, it was shown that residual amounts of either of the detergents used to produce the complexes were not responsible for the spectral changes observed for DMPC-d_9 bilayers with rhodopsin.

We therefore conclude that the incorporation of rhodopsin into DMPC-d_9 bilayers is itself largely responsible for the observed systematic changes in the ^{2}H NMR quadrupole splittings. Furthermore, protein aggregation induced by addition of glutaraldehyde showed that a relationship exists between the amount of protein surface exposed to lipid and the extent of these ^{2}H NMR spectral changes. It has been demonstrated that a measured quadrupole splitting can be reduced by spectral broadening independently of any change in C–D bond order parameter (Seelig, 1977). In addition, rhodopsin reconstituted into bilayers of DMPC-d_{54} caused no change in the *average* order of fluid-deuterated lipid acyl chains from moment analysis of the recorded spectra, despite a pronounce change in the spectral shape (Bienvenue et al., 1982). Therefore, it remains unclear whether any change in lipid order per se is needed to account for our experimental observations, although the systematic changes in the separation of the maxima of the ^{2}H NMR spectra strongly indicate that rhodopsin–DMPC interactions do indeed occur at the bilayer membrane polar–apolar interface.

The analysis and presentation of the experimental results in Figure 4 show that the relationship between the directly measured apparent quadrupole splitting (Q_o) and the chemically determined DMPC:rhodopsin ratio ($1/n_t$) for the complexes studied is linear. The single-component spectra recorded therefore indicate that lipids exchange onto and off the rhodopsin interface fast enough to average the quadrupolar anisotropy measured for the choline methyls of between 1.2 and about 0.4 kHz, giving a limit to the rate of lipid exchange of faster than around 10^3 Hz. The decrease in the slopes of Figure 4 at different temperatures is due only to the small change in the value of $\Delta\nu_Q$ for protein-free bilayers, from 1.24 kHz at 26 °C to 1.08 kHz at 41 °C, and not a significant change in n_c, the number of DMPC head groups interacting with rhodopsin [see Results and Sixl et al. (1984), Watts et

al. (1985), and Dempsey et al. (1986)]. It is suggested, therefore, that rhodopsin does not change its state of aggregation significantly [probably being monomeric from saturation transfer spin-label ESR studies (Watts, 1982)] in a temperature-dependent manner. This is in contrast to band 3 from human erythrocytes in erythrocyte membranes as studied by fluorescent methods (Mühlebach & Cherry, 1985) and in DMPC bilayers as studied by us using ^2H NMR methods similar to those described here (Dempsey et al., 1986).

The fast, two-site exchange analysis, although probably not a complete description of the lipid–protein interaction, has some use in an analytical form to indicate molecular associations at the membrane bilayer surface. When rhodopsin was artificially aggregated by using glutaraldehyde, the measured quadrupole splitting was not dependent on the protein concentration, and spectra were very similar to unperturbed DMPC. Futhermore, in complexes where rhodopsin failed to bleach beyond a species with an absorption maximum at 478 nm, the measured ^2H NMR spectrum was also independent of protein content. It is suggested, therefore, that rhodopsin which could only be bleached to a species which absorbed light at 478 nm was aggregated. This conclusion agrees with previous reports on the aggregation of rhodopsin in rod outer segment membranes (Olive et al., 1978; Watts et al., 1981) and suggests that a significant number of lipids are not trapped in between aggregates of rhodopsin, which is similar to the case in bilayers containing the calcium ATPase (East et al., 1985) and band 3 (Dempsey et al., 1986).

The exact physical origin of the observed ^2H NMR spectral changes of DMPC-d_9 upon interaction with rhodopsin is not clear. A number of factors may be involved, some of which may include orientational and possibly, in addition, rate changes in the motion of the $-(CD_3)_3$ group. However, this uncertainty at this stage appears not to detract from the usefulness of the approach in detecting lipid–protein interactions in a semiquantitative manner and, in the case of rhodopsin, its functional and aggregation state.

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Effect of Cholesterol on Viscoelastic Properties of Dipalmitoylphosphatidylcholine Multibilayers As Measured by a Laser-Induced Ultrasonic Probe†

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ABSTRACT: Using a novel laser-induced ultrasonic probe, we have examined the bulk viscoelastic properties of fully hydrated dipalmitoylphosphatidylcholine (DPPC) aligned multibilayers in terms of the anisotropic in-plane elastic stiffness (C_{11}) and viscosity (η_{11}). Our measurements of C_{11} are in accord with those reported on Brillouin light scattering on a similar system. Our measurements on viscosity are the first of their kind and are, on the average, a factor of 10 lower than microviscosities estimated by spectroscopic techniques. We report the first comprehensive study of the effects of cholesterol on the bulk mechanical properties of DPPC multibilayers. At temperatures above the phase transition temperature of DPPC (T_c), an increase in both C_{11} and η_{11} is noticed when cholesterol is incorporated in the multibilayers. However, at temperatures below T_c, no measurable changes are detected in either C_{11} or η_{11}. These results, reflecting changes in the bulk viscoelastic properties of the multibilayers, differ from the changes reported by local fluidity parameters in that the latter indicate a decrease in the bilayer fluidity in the presence of cholesterol above T_c and an *increase* below T_c ("dual effect" of cholesterol). Our data suggest that the "dual effect" of cholesterol is noticeable only on a molecular scale. Increasing cholesterol concentrations higher than 20 mol % cease to further affect C_{11} or η_{11} of the DPPC multibilayers. This agrees with various results reported in the literature, by techniques measuring the local effects of cholesterol, and supports the changes in molecular organization postulated to occur when cholesterol concentration reaches 20 mol % in the lipid bilayers.

Cholesterol is an important and abundant constituent of most eukaryotic membranes. In cell membranes, cholesterol constitutes up to 50 mol % of the lipid. The physiological significance of cholesterol and its effect on membrane fluidity has been the subject of many recent articles and reviews (Shinitzky et al., 1983a,b). The interactions of cholesterol with phospholipid molecules (the other major lipid component of cell membranes) have been actively examined through model systems (bilayers, vesicles, and micelles) for the past decade (Jain, 1975; Phillips, 1972; Demel & De Kruijff, 1976). Various techniques including electron spin resonance (ESR) spectroscopy (Recktenwald & McConnell, 1981; Shimshick & McConell, 1973, NMR spectroscopy (Cullis, 1976; Tilcock et al., 1982), electron microscopy (Copeland, & McConnell, 1980; Lentz et al., 1980), IR spectroscopy (Cortijo & Chapman, 1981), fluorescence spectroscopy (Vanderkooi, 1974; Shinitzky & Barenholz, 1978), and differential scanning calorimetry (DSC) (Papahadjopoulos et al., 1973; Mabrey et al., 1978; Estep et al., 1978) have been used to study the effect that cholesterol has on the mechanical properties and fluidity of the cholesterol/phospholipid model system. All these techniques have different definitions of the membrane fluidity. The term "membrane fluidity" has been used in the literature to describe two different types of motion: (1) the vibrational or rotational movement of a group on a molecule, often measured by NMR and ESR; (2) the diffusivity or translational motion of a molecule. Although both classes of properties depend on the "free space" available in a two-dimensional lattice (Blank, 1962; Blank & Britten, 1965), when different samples are compared, the trends can be easily masked by a large variety of competing processes (Schreier et al., 1978). Furthermore, all these techniques measure changes in the molecular environment of the bilayer rather than bulk changes in the bilayer plane.

More recently, viscosity and lateral compressibility measurements have been performed on multibilayers according to the theories of wave propagation through lipid multibilayers

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