J. Membrane Biol. 87, 211–215 (1985)

The Journal of
Membrane Biology

^2H and ^{31}P Nuclear Magnetic Resonance Studies of Membranes Containing Bovine Rhodopsin

Arlene D. Albert, Scott A. Lane*, and Philip L. Yeagle
Department of Biochemistry, SUNY/Buffalo School of Medicine, Buffalo, New York 14214

Summary. Purified, delipidated rhodopsin is recombined with phospholipid using octyl-glucoside (OG) and preformed vesicles. Normal egg phosphatidylcholine, phosphatidylcholine in which the N-methyl groups are fully deuterated, and dioleoyl phosphatidylcholine labeled with deuterium at carbons 9 and 10 were used. ^{31}P nuclear magnetic resonance (NMR) and ^2H NMR measurements were obtained of the pure phospholipids and of the recombined membranes containing rhodopsin. ^{31}P NMR of the recombined membrane (containing the deuterated phospholipid) showed two overlapping resonances. One resembled a normal phospholipid bilayer, and the other was much broader, representing a motionally restricted phospholipid headgroup environment. The population of phospholipids in the motionally restricted environment can be modulated by conditions in the media. ^2H NMR spectra of the same recombined membranes showed only one component. These experimental results agree with a theoretical analysis that predicts an insensitivity of ^2H NMR to lipids bound to membrane proteins. A model containing at least three different phospholipid environments in the presence of the membrane protein rhodopsin is described.

Key Words rhodopsin · NMR · lipid-protein interaction reconstitution

Introduction

Rhodopsin is the visual pigment of retinal rod outer segments and is found in high concentration in the disk membranes. Rhodopsin has been recombined with phospholipids in a manner which produces a low phospholipid-to-protein ratio. This procedure yields a highly homogeneous vesicle population suitable for studies of protein-phospholipid interactions (Albert, 1978; Albert & Yeagle, 1983). Use of this system in conjunction with the native rod outer segment disk membrane recently provided important new insight concerning the structure of the biological membrane. In ^{31}P nuclear magnetic resonance (NMR) spectra two overlapping resonances were observed. One was indicative of a highly motionally restricted environment for the phospholipid headgroups. The other resonance represented a slightly perturbed phospholipid bilayer (Albert et al., 1982; Albert & Yeagle, 1983).

In other recent studies using ^2H NMR of specifically deuterated, saturated phospholipids recombined with rhodopsin, two groups reported ^2H NMR data that apparently showed only a single resonance within the sensitivity of the experiment (Desse et al., 1981; Bienvenue et al., 1982). In these studies the deuterium label was located on the hydrocarbon chain of the phospholipid.

Here we present a multinuclear NMR study of the behavior of the phospholipid bilayer in the presence of rhodopsin, using both ^2H NMR and ^{31}P NMR. The data suggest that ^2H NMR is not sensitive to the motionally restricted phospholipid environment created by rhodopsin and detected by ^{31}P NMR. A model is described that reconciles all the NMR data.

Materials and Methods

Egg phosphatidylcholine and transphosphatidylated (from egg phosphatidylcholine) phosphatidylethanolamine were obtained from Avanti Polar Lipids. Dioleoyl phosphatidylcholine specifically deuterated at positions 9 and 10 was obtained from Cambridge Isotopes. Deuterated iodomethane was purchased from Merck and deuterium-depleted water from Aldrich. All other chemicals were reagent grade. Buffers were made from distilled, deionized water.

Synthesis of N-Methyl Deuterated Phosphatidylcholine

Phosphatidylcholine was synthesized from phosphatidylethanolamine by methylation with deuterated iodomethane. 270 mg of transphosphatidylated phosphatidylethanolamine were added to a reaction mixture consisting of 50 ml dimethylformamide, 25 ml

* Deceased.

chloroform, 25 ml isopropanol and 50 ml 1 M $NaHCO_3$ containing 1 mM EDTA. This mixture was determined to be the most advantageous after trials with a number of other compositions. Five grams of deuterated iodomethane, precooled on dry ice, were added. The reaction mixture was stirred for 3 hr at 25°C. The reaction was monitored by thin-layer chromatography, using a solvent system containing chloroform, methanol and water (65 : 25 : 5). The primary biproduct was the dimethyl derivative of phosphatidylethanolamine, though smaller amounts of starting material and the monomethyl derivative were also present. The product was purified by silicic acid chromatography. The column was eluted with mixtures of chloroform, methanol and 20% aqueous ammonia. A step gradient was run, increasing in five steps the polarity of the solvent system. Several clear fractions were collected between the dimethyl derivative and phosphatidylcholine. The product co-migrated with native phosphatidylcholine when analyzed by thin-layer chromatography. The yield was 104 mg of phosphatidylcholine. This procedure is much more efficient than a previously described methylation procedure which required 14 days (Stockton et al., 1974).

Formation of Liposomes

Multilamellar liposomes were formed by drying the lipid out of chloroform into a film on a glass flask. Buffer was then added and the material vortexed to take up the lipid. Turbid suspensions were formed. To make small, unilamellar vesicles, the suspension was sonicated in a Branson W350 sonicator until clear in an ice bath. The vesicles were then centrifuged at 45,000 rpm for 45 min in a Beckman L5-50 ultracentrifuge and the supernatant harvested.

Preparation of Recombined Membranes Containing Rhodopsin

Dark adapted bovine retinas were obtained from J. Lawson, Inc., Lincoln, Nebraska. Osmotically intact rod outer segment disks were prepared by previously described procedures (Smith et al., 1975). Pure delipidated rhodopsin was prepared by Concanavalin-A chromatography as described previously (Albert & Litman, 1978; Litman, 1982). Recombined membranes were made by adding solubilized rhodopsin to sonicated vesicles such that the detergent concentration was below the critical monomer concentration (Albert, 1978; Albert & Yeagle, 1983). The recombined membranes were purified on a linear sucrose density gradient and formed a narrow band on the gradient. This was harvested, analyzed for rhodopsin and phospholipid content, and subjected to NMR analysis. Total protein was determined by the Lowry method. Rhodopsin concentration was determined by the change in absorbance at 500 nm (ΔA_{500}) upon bleaching in the presence of hydroxylamine. A molecular weight of 38,000 was used for rhodopsin and an extinction coefficient of 40,000. Phospholipid was determined by a modified (Litman, 1973) Bartlett assay (1959). Papain treatment of these membranes was performed as described previously (Albert & Litman, 1978). SDS gel electrophoresis demonstrated that the cleavage pattern in these membranes was the same as in the native disk membranes reported previously (Albert & Litman, 1978).

^{31}P NMR

109 MHz ^{31}P NMR spectra were obtained in 10-mm tubes with a JEOL FX270 Fourier transform spectrometer. A fully phase-cycled spin-echo sequence was used to eliminate spectral distortions, as previously described (Rance & Byrd, 1983). Data were collected prior to refocussing of the echo, and transformed from the point of focussing of the echo. No first-order phase corrections were employed. The decoupler was gated on only during acquisition to defeat any nuclear Overhauser effect and to prevent sample heating (Yeagle et al., 1975). 50 kHz spectra were obtained. Repetition rates of 1 sec were used and 2048 data points were collected. T_2 measurements were obtained by the Carr-Purcel-Meiboom-Gill sequence.

^{2}H NMR

^{2}H NMR spectra were obtained at 41.4 MHz on the same instrument using the same tunable broadband probe used for the ^{31}P NMR spectra. 50 kHz spectra were obtained using a quadrupole echo sequence and 100 msec repetition rates and 2048 data points. All spectra were obtained in the dark to prevent bleaching of the photopigment rhodopsin.

Spectral simulations were calculated on a computer using the equations of Seelig (Seelig, 1978). This consists of a function describing the transition probability convoluted with a Lorentzian lineshape. The combined function is integrated over the region of finite resonance intensity to produce the simulation. A constant linewidth was used, derived from an average of the T_2 values determined experimentally over the entire observed powder pattern. This restriction does not materially influence the results. The adequacy of fit was determined by visual comparison. A minimization function has been explored to digitally fit simulations with the observed spectra, with no significant difference in the results.

Results

Recombined membranes containing rhodopsin and deuterated phospholipid were prepared as described above. The membranes formed were not detectably different from recombined membranes formed using the native, unlabeled phospholipid. The lipid/protein molar ratio was 40 : 1. A membrane with such a high protein content is an excellent candidate for studying the effects of a membrane protein on the phospholipids because the low lipid/protein ratio should magnify any such effects. The ^{2}H NMR spectra of the pure headgroup-labeled and hydrocarbon chain-labeled lipids were identical to spectra published previously (Stockton, et al., 1974; Seelig et al., 1981).

The ^{2}H NMR spectra of a membrane containing rhodopsin and deuterated phosphatidylcholines show no significant difference in the quadrupole coupling constant due to the presence of rhodopsin even in such high concentration. A modest broadening of the resonance is observable, as was noted previously in liquid crystalline phospholipids for deuterium probes (located in different parts of the molecule) recombined with rhodopsin (Deese et al., 1981; Bienvenue et al., 1982).

In the ^{31}P NMR spectra of membranes containing rhodopsin and deuterium labeled phospholipids, two overlapping resonances are observed. This agrees with results reported previously (Albert & Yeagle, 1983) with unlabeled phosphatidylcholine. As described in detail elsewhere (Albert & Yeagle, 1983) one spectral component is much broader than the other, and represents a motionally restricted phospholipid headgroup environment.

Papain treatment of rhodopsin in the rod outer segment disk membrane changes the relative populations of the two phospholipid environments as described in the Figure. For the normal membrane, the population of the motionally restricted environment is independent of temperature. For the papain-treated membrane, there is a sharp temperature dependence between 30 and 35°C, in 100 mM NaCl. In the absence of NaCl, this sharp, temperature-dependent transition occurs at a higher temperature, namely 40 to 45°C.

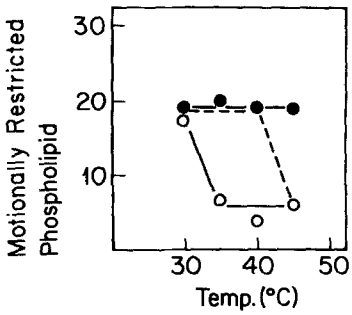

Fig. Temperature dependence of the population of the motionally restricted phospholipid environment in native rod outer segment disk membranes. This is presented as percent of total membrane phospholipid motionally restricted as indicated by the broad spectral component. Filled circles and solid line are the results for the native membrane. Open circles and solid line represent the papain-treated membranes, in 100 mM NaCl. Dashed line represents the results for the papain-treated membrane without NaCl. Otherwise, proteolysis conditions were as described elsewhere (Albert & Litman, 1978)

Discussion

Previous studies probing the interior of membranes containing rhodopsin with electron spin resonance (ESR) of lipid spin labels revealed the presence of two lipid environments, one more motionally restricted than the other (Watts et al., 1979). The population of the motionally restricted environment was proportional to the rhodopsin content, and thus was interpreted to represent lipid at the lipid-protein interface.

Subsequently, ^{2}H NMR measurements were made on recombined membranes containing rhodopsin and synthetic phospholipids that were deuterated on the hydrocarbon chains (Deese et al., 1981; Bienvenue et al., 1982). The spectra were sensitive to only one lipid environment.

A possible means of reconciling the ^{2}H NMR and ESR data was the difference in time scale between the two methods of measurement. The time scale of the ^{2}H NMR measurement is three to four orders of magnitude longer than the ESR measurement. Thus a model was suggested in which the lipids were exchanging between sites immediately adjacent to the protein and sites removed from the protein surface at a rate that was between the time scales of the two measurements (Deese et al., 1981; Bienvenue et al., 1982). This condition would give the appearance of two environments of the ESR time scale and one averaged environment on the ^{2}H NMR time scale.

Two other possibilities should also be considered. One is that the deuterated phospholipid has sufficiently different properties from the normal phospholipid that interactions with the protein are inhibited. This seems unlikely, but cannot be absolutely ruled out. Another possibility is that the ^{2}H NMR resonance from the restricted environment cannot be seen. The latter possibility has not been adequately tested.

Recent measurements using ^{31}P NMR detected the presence of two separable phospholipid environments in the native rod out segment disk membrane and in recombined membranes containing rhodopsin. One environment is motionally restricted relative to the other. The two environments were detected both indirectly (Albert et al., 1982) and directly (Albert & Yeagle, 1983). The ^{31}P NMR data further require two phospholipid headgroup environments between which phospholipids exchange slowly on the msec time scale (Albert & Yeagle, 1983). More recently, in another membrane, we have directly measured (using magnetization transfer techniques) the exchange rate at 1 sec^{-1} (Selinsky & Yeagle, 1985). Therefore, postulating an exchange rate of 10^{6} to 10^{7} sec, as suggested elsewhere (Deese et al., 1981; Bienvenue et al., 1982), is clearly ruled out in the case of the motionally restricted component detected by the ^{31}P NMR data.

To understand the apparent differences in the results obtained from ^{2}H NMR and ^{31}P NMR experiments, it must be considered whether ^{2}H NMR measurements are insensitive to phospholipid environments that reflect phospholipids bound to membrane proteins. Theoretical considerations argue in favor of such a suggestion. The intensity of the ^{2}H NMR resonance obtained by quadrupole echos suffers from a sensitivity to the frequencies of motion in the membrane. If intense fluctuations are experienced by the labeled compound in the range of cor-

relation times which correspond to the observed quadrupole couplings for the deuterated lipids, the observed resonance intensity is artificially attenuated (Spiess & Sillescu, 1981). This is due to the enhanced relaxation leading to a loss of observable magnetization during the formation of the echo.

The rotational correlation time for rhodopsin is in the range of 10 to 100 μsec (Cone, 1972). If a phospholipid binds to rhodopsin, it would assume the rotational correlation time of the protein for the period of time it is bound. (The ^{31}P NMR data indicate that the residence time of the phospholipid on the protein is much greater than the protein rotational correlation time.) Therefore phospholipids bound to rhodopsin would experience rotational motion with a correlation time of 10 to 100 μsec. Quadrupole couplings for protein-bound deuterated lipids would be expected to be in the range of about 10 to 100 kHz, depending upon position of labeling and extent of motional restriction. Therefore the frequency corresponding to the quadrupole couplings and the frequency corresponding to the expected correlation times for lipids bound to proteins, overlap. Consequently one would predict that the ^{2}H NMR resonances from phospholipids tightly bound to rhodopsin would exhibit reduced intensities and be difficult to observe, consistent with the reported data. ^{31}P NMR does not suffer from the same limitation, because the frequency corresponding to rhodopsin rotation is higher than the frequency corresponding to the effective chemical shift anisotropy of the ^{31}P powder pattern.

These arguments then suggest that the less-restricted bilayer component of the ^{31}P NMR spectra reports on the same phospholipid environment as does the observed ^{2}H NMR spectra. T_2 of the two nuclei, as well as the observed quadrupole splittings and the ^{31}P chemical shift anisotropy (of the less-restricted bilayer component), behave in a completely analogous manner in the presence of membrane proteins. This lends further support to the suggestion that the ^{2}H NMR spectra and the less-restricted bilayer component of the ^{31}P NMR spectra report on the same phospholipid domain.

An analogous explanation is advanced for the results of recent magic angle spinning cross polarization (CPMAS) ^{13}C NMR spectra of membranes containing rhodopsin (Sefcik et al., 1983). While resonances from the phospholipids are readily seen, no resonances are seen from the protein. The decoupling field for the CPMAS experiments is in the range of 10 to 50 kHz. This is of similar frequency to the rotation rate of rhodopsin quoted above. Such a match will produce $T_{1\rho}$ values so short that the resonances from the protein cannot be seen in a CPMAS experiment. This theoretical prediction matches the experimental observation, and demonstrates the powerful effect of rhodopsin rotation on the NMR experiments.

The available magnetic resonance data concerning the interaction between phospholipid and rhodopsin in a membrane can be summarized. ESR measurements detect about 24 phospholipids perturbed by rhodopsin (Watts et al., 1979). Because of the short time scale of the measurement, one would expect that the whole surface of the protein that is exposed to the lipid would influence the ESR results. In agreement with this expectation, it was suggested that the number of lipids in the perturbed environment detected by ESR would constitute one complete layer of lipid around the protein (Watts et al., 1979). Since this is at the protein-lipid interface, these are appropriately referred to as interfacial lipids.

ESR detects lipids in fast and in slow exchange with the protein surface. Fast exchange refers to the time scales between 10^4 sec^{-1} (which is "fast" for the NMR time scale) and 10^7 sec^{-1} which is slow for the ESR time scale). ^{31}P NMR only detects those in slow exchange. Therefore ^{31}P NMR should detect a smaller perturbed component than ESR. This agrees with the measured data. In fact, the ^{31}P NMR spectra suggest that about 15 phospholipids are motionally restricted by each rhodopsin (Albert & Yeagle, 1983). These phospholipids may be bound to the protein at the interface between the lipid and the protein and they constitute a special subset of interfacial lipid. Models for rhodopsin show clustering of charged amino-acid residues at the interface, which might provide sites of interaction for the phospholipid headgroups (Hargrave et al., 1983).

The remainder of the phospholipid could be considered to inhabit a "normal" bilayer. However, even this is perturbed by the presence of the protein. ^{2}H NMR and ^{31}P NMR spectra both show a difference between the "normal" bilayer phospholipids in rhodopsin-containing membranes, and pure phospholipid dispersions. This difference is strongly evident in the T_2 measurements. This may reflect an increase in the intensity of low frequency motions. These may be cooperative motions induced in the lipid bilayer by rhodopsin rotation. The protein surface is rough due to the various sizes of the amino-acid residues protruding from the transmembrane alpha helices. Protein rotation will then cause alternate compression and expansion of the bilayer at a particular point, thereby propogating relatively low frequency collective motions through this suprannular lipid (or lipid outside the lipid-protein interface).

In summary, then, the model which is consistent with all the magnetic resonance data predicts at

least three phospholipid environments in rhodopsin-containing membranes. Protein-bound lipids are the smallest class, and are a subset of the second environment, the interfacial lipid. Suprannular lipid contains the majority of the phospholipids in a biological membrane, and represents essentially a perturbed lipid bilayer.

Finally it is interesting to note that the number of phospholipids bound to rhodopsin can be modulated. A transition in that number is seen as a function of temperature in the papain-treated membrane. Such a transition is not seen in the normal disk membrane (Fig. 1). Papain treatment leaves most of the rhodopsin molecule in the membrane as two pieces which remain associated (Albert & Litman, 1978). Therefore the change in the population of the motionally restricted component may result from a temperature-dependent change in the association of the two domains of the protein. Ionic interactions may play a role, since the transition is salt dependent. Interhelical interactions of this sort have been hypothesized to play a role in bacteriorhodopsin structure (Engelman, 1982).

This work was supported by grants from the National Institutes of Health to A.D. Albert (EY03328) and to P.L. Yeagle (HL23853). P.L.Y. is a Research Career Development Awardee of the National Institutes of Health (HL00937). This paper is dedicated to the memory of Scott Lane (1961–1983).

References

Albert, A.D. 1978. Rhodopsin: Its Substructure and Incorporation into Phospholipid Vesicles. Ph.D. Thesis. University of Virginia, Charlottesville

Albert, A.D., Litman, B.J. 1978. Independent structural domains in the membrane protein bovine rhodopsin. *Biochemistry* **17:**3893–3900

Albert, A.D., Litman, B.J., Yeagle, P.L. 1982. ^{31}P NMR investigation of rhodopsin-phospholipid interactions in bovine rod outer segment disk membranes. *Biophys. J.* **37:**34–36

Albert, A.D., Yeagle, P.L. 1983. Phospholipid domains in bovine rod outer segment disk membranes. *Proc. Natl. Acad. Sci. USA* **80:**7188–7191

Bartlett, G.R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234:**466–468

Bienvenue, A., Bloom, M., Davis, J.H., Devaux, P.F. 1982. Evidence for protein-associated lipids from deuterium NMR studies of rhodopsin-DMPC recombinants. *J. Biol. Chem.* **257:**3032–3038

Cone, R.A. 1972. Rotational diffusion of rhodopsin in the visual receptor membrane. *Nature New Biol.* **263:**39–43

Deese, A.J., Dratz, E.A., Dahlquist, F.W., Paddy, M.R. 1981. Interaction of rhodopsin with two unsaturated phosphatidylcholines: A deuterium NMR study. *Biochemistry* **20:**6420–6427

Engelman, D.M. 1982. An implication of the structure of bacteriorhodopsin. *Biophys. J.* **37:**187–188

Hargrave, P.A., McDowell, J.H., Curtis, D.R., Wang, J.K., Juszczak, E., Fong, S., Mohana Rao, J.K., Argos, P. 1983. The structure of bovine rhodopsin. *Biophys. Struct. Mech.* **9:**235–244

Litman, B.J. 1973. Lipid model membrane characterization of mixed phospholipid vesicles. *Biochemistry* **13:**2545–2550

Litman, B.J. 1982. Purification of rhodopsin by concanavalin A affinity chromatography. *Methods Enzymol.* **81:**150–153

Rance, M., Byrd, R.A. 1983. Obtaining high fidelity spin $\frac{1}{2}$ powder spectra in anisotropic media: Phase cycled Hahn echo spectroscopy. *J. Magn. Reson.* **52:**221–240

Seelig, J. 1978. ^{31}P nuclear magnetic resonance and the headgroup structure of phospholipids in membranes. *Biochem. Biophys. Acta* **515:**105–138

Seelig, J., Tamm, L., Hymel, L., Fleischer, S. 1981. Deuterium and phosphorus NMR and fluorescence depolarization studies of functional reconstituted sarcoplasmic reticulum vesicles. *Biochemistry* **20:**3922–3932

Sefcik, M.D., Schaefer, J., Stejskal, E.O., McKay, R.A., Ellena, J.F., Dodd, S.W., Brown, M.F. 1983. Lipid bilayer dynamics and rhodopsin-lipid interactions: A new approach using high resolution solid state ^{13}C NMR. *Biochem. Biophys. Res. Commun.* **114:**1048–1055

Selinsky, B.S., Yeagle, P.L. 1985. Phospholipid exchange between restricted and non-restricted domains in sarcoplasmic reticulum vesicles. *Biochim. Biophys. Acta* **813:**33–40

Smith, H.G., Jr., Stubbs, G.W., Litman, B.J. 1975. The isolation and purification of osmotically intact disks from retinal rod outer segments. *Exp. Eye Res.* **20:**211–217

Spiess, H.W., Sillescu, H. 1981. Solid echoes in the slow motion region. *J. Magn. Reson.* **42:**381–389

Stockton, G.W., Polnaszek, C.F., Leitch, L.C., Tulloch, A.P., Smith, I.C.P. 1974. A study of mobility and order in model membranes using ^{2}H NMR relaxation rates and quadrupole splittings of specifically deuterated lipids. *Biochem. Biophys. Res. Commun.* **60:**844–848

Watts, A., Volotovski, I.D., Marsh, D. 1979. Rhodopsin-lipid associations in bovine rod outer segment membranes. Identification of immobilized lipid by spin labels. *Biochemistry* **18:**5006–5013

Yeagle, P.L., Hutton, W.C., Huang, C., Martin, R.B. 1975. Headgroup conformation and lipid-cholesterol association in phosphatidylcholine vesicles: A ^{31}P (^{1}H) nuclear Overhauser effect study. *Proc. Natl. Acad. Sci. USA* **72:**–3477–3480

Received 5 February 1985; revised 18 June 1985