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NMR of Protein–Lipid Interactions in Model and Biological Membrane Systems

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1. INTRODUCTION

Membranes are composed predominantly of lipids, proteins, and sterol molecules and are responsible at least in part for a wide variety of biochemical processes such as respiration, vision, photosynthesis, cell–cell recognition, and nerve impulse transmission. Not surprisingly then, there have been considerable efforts spent in attempting to characterize the molecular structure of, and intermolecular interactions between, individual membrane components, in an attempt to relate the structures of membranes to their function. In this short review we discuss recent developments in our understanding of the structure of membranes obtained by means of NMR spectroscopic techniques. We show that protein–lipid interactions in both model and intact biological membranes are characterized by a dynamic disordering of boundary-lipid hydrocarbon chains, as viewed by high-field deuterium NMR spectroscopy, while ^{31}P spectra indicate significant disordering and/or immobilization within the phospholipid polar head group region due to association with protein. The effects are very different from those seen with cholesterol, and are in marked contrast to the old ideas of rigid, ordered "boundary-lipid" surrounding membrane proteins. We also present results suggesting that it will soon be possible to directly monitor the effects of lipid on protein dynamics by means of NMR spectroscopy.

2. DEUTERIUM NMR OF LIPIDS IN MODEL SYSTEMS

The idea that membrane proteins are solvated by a layer of immobilized boundary lipid was first put forward by Jost *et al.* (1973a–c, 1977) originally for the cytochrome oxidase system, and subsequently for cytochrome b_5 by Dehlinger *et al.* (1974) and sarcoplasmic reticulum ATPase by Jost and Griffith (1978), and similar concepts have been

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presented by many other workers (Caron *et al.* 1974; Grant and McConnell, 1974; Hesketh *et al.*, 1976; Marsh *et al.*, 1978; Warren *et al.*, 1974, 1975). In almost all instances, the evidence for rigid or immobilized "boundary lipid" has come from the "rigid-glass" (long correlation time) ESR spectrum of a nitroxide free radical introduced into the system, and it has been generally concluded that proteins increase order greatly for the first layer of lipid adjacent to protein, and that significant perturbations exist for the second and perhaps the third layers of lipid. Because it seemed to us plausible that nitroxides might not faithfully reproduce these interactions, due to their polar nature, we have used the nonperturbing techniques of ^{2}H and ^{31}P NMR spectroscopy to investigate these problems in more detail.

The theoretical background appropriate for consideration of the ^{2}H NMR spectra of lipid membranes is discussed in detail by Oldfield *et al.* (1978a,b, 1981) and by Seelig (1977) so we shall be content to briefly quote the principal result that for the ^{2}H nucleus (with spin $I = 1$ and in C–D bonds an asymmetry parameter $\eta = 0$) the allowed transitions correspond to $+1 \leftrightarrow 0$ and $0 \leftrightarrow -1$, and give rise to a "quadrupole splitting" of the NMR absorption line with separation between peak maxima of

$$\Delta\nu_Q = (3/2)(e^2qQ/h)[(3\cos^2\theta - 1)/2]$$

e^2qQ/h is the deuterium quadrupole coupling constant, which has been found to be about 170 kHz for C–D bonds by Derbyshire *et al.* (1969), and θ is the angle between the magnetic field, H_0, and the principal axis of the electric field gradient tensor at the deuterium nucleus. For a rigid polycrystalline solid, all values of θ are possible and one obtains a so-called "powder pattern" lineshape (see Fig. 1A) in which the separation between peak maxima is about 127.5 kHz, and the separation between the distribution steps is twice this value. In biological membranes, however, there is considerable motion of the C–D vector, due for example to *gauche–trans* isomerization along the hydrocarbon chain, to chain tilt, to diffusion, and to chain rotation; thus, we must take an average in time of $(3\cos^2\theta\text{-}1)$ for motions that are faster than 170 kHz. The result, in brief, is that the size of the quadrupole splitting decreases as the degree of order or "order parameter" (Seelig, 1977) of the system decreases.

Early studies demonstrated that addition of cholesterol to a deuterium-labeled dimyristoylphosphatidylcholine (DMPC) bilayer at a 1 : 1 mole ratio caused an almost twofold increase in order parameter of the lipid hydrocarbon chain, corresponding to an increase in ^{2}H quadrupole splitting from about 27 kHz to about 49 kHz (at 30°C; Oldfield *et al.*, 1971). Below the phase transition temperature of the pure lipid, the hydrocarbon chains were prevented by cholesterol from crystallizing into the rigid α-crystalline gel state, and the observed quadrupole splitting remained at about 50 kHz even at 10°C, some 13°C below the gel-to-liquid-crystal phase transition temperature. These effects seen with ^{2}H NMR were precisely those predicted on the basis of earlier calorimetric studies by Ladbrooke *et al.* (1968), and ^{1}H NMR of sonicated vesicles by Chapman and Penkett (1966).

The effects of proteins on the ^{2}H NMR spectra of hydrocarbon-chain-labeled phospholipids were predicted, using one simple version of the boundary-lipid idea, to be an increase in ^{2}H NMR quadrupole splittings (or order parameters) of lipid associated with protein (Dahlquist *et al.*, 1977; Hong and Hubbell, 1972; Jost and Griffith, 1978; Kleemann and McConnell, 1976; Marcelja, 1976; Marsh *et al.*, 1978; Owicki *et al.*, 1978; Schroder, 1977; Scott and Cherng, 1978). We show in Fig. 1 results we have obtained using cytochrome oxidase (EC 1.9.3.1) and sarcoplasmic reticulum ATPase (EC 3.6.1.3), the systems most frequently studied using physical techniques (Dahlquist *et al.*, 1977;

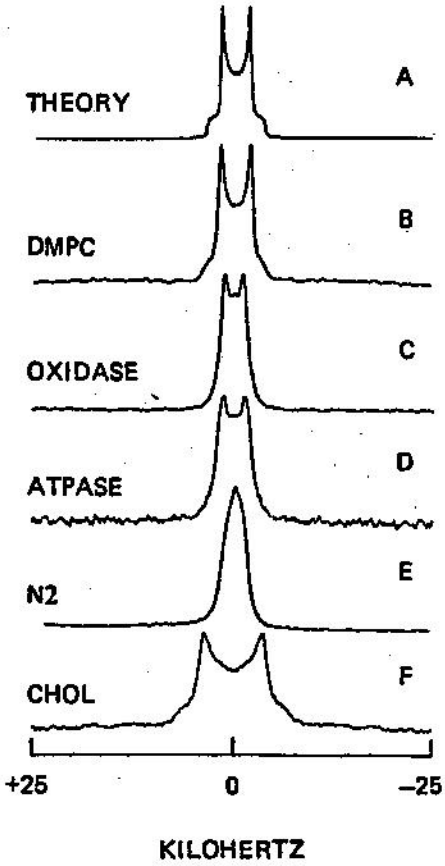

Figure 1. Theoretical and experimental deuterium NMR spectra of ^{2}H-labeled lipids showing the effects of proteins and cholesterol on hydrocarbon chain order. (A) Theoretical ^{2}H powder pattern; the splitting is arbitrary. (B) 1-Myristoyl-2-(14,14,14-trideutero)myristoyl-*sn*-glycero-3-phosphocholine (DMPC-d_3) in excess water at 30°C. (C) As in (B) but lipid contains 67 wt% cytochrome oxidase (cytochrome *c*: oxygen oxidoreductase, EC 1.9.3.1). (D) As in (B) but lipid contains 65 wt% sarcoplasmic reticulum ATPase (EC 3.6.1.3). (E) As in (B) but lipid contains 67 wt% beef brain myelin proteolipid apoprotein. (F) As in (B) but lipid contains 33 wt% cholesterol. Deuterium NMR spectra were obtained at 34 MHz.

Hesketh *et al.*, 1976; Jost *et al.*, 1973a,b,c, 1977; Longmuir *et al.*, 1977; Marsh *et al.*, 1978; Moore *et al.*, 1978; Oldfield *et al.*, 1978a; Warren *et al.*, 1974, 1975), together with the much smaller model system beef brain myelin proteolipid apoprotein (N2) used by Curatolo *et al.* (1977) and by Papahadjopoulos *et al.* (1975). All systems were "complexed" or "reconstituted" with DMPC bilayers specifically deuterated at the terminal methyl position of the *sn*-2 chain, using standard techniques used by Curatolo *et al.* (1977), Oldfield *et al.* (1978a), and Warren *et al.* (1974). For comparison, also included is a ^{2}H NMR spectrum showing the effect of cholesterol, the sample again being prepared with standard techniques used by Oldfield *et al.* (1978b).

As may be seen from the spectra in Fig. 1, the effect of incorporating protein into the lipid bilayer is to cause a *decrease* in order parameter (quadrupole splitting) of the ^{2}H-labeled methyl group, a *disordering* rather than an ordering effect. Similar results shown by Oldfield *et al.* (1978a) are obtained with a wide range of other lipids and proteins. Cholesterol, on the other hand, causes a large *increase* in order parameter, consistent with its well-known "condensing" or ordering effect (Fig. 1F). Similar ordering effects are seen with cholesterol using lipids labeled at other chain positions, although the disordering effect of protein is by far the most pronounced at the methyl end of the hydrocarbon chain. Above the lipid gel-to-liquid-crystal phase transition temperature, therefore, it appears that proteins may tend to disorder the packing of lipid hydrocarbon chains somewhat, while cholesterol orders their packing. Notably, ESR studies of such protein–lipid samples in our

laboratories have confirmed that *highly immobilized* nitroxide spin-label spectra are nevertheless obtained, suggesting that differences in time scale between the two resonance experiments, or specific interactions between the nitroxide probes and the protein surfaces, may be involved. In any case, because no two-component spectra are seen above the pure lipid phase transition temperature over a wide concentration range, exchange between "bound" and "free" lipid must be very fast ($\gtrsim 10^3$–10^4 sec^{-1}), suggesting that boundary lipid does not stay bound very long (Kang *et al.*, 1979a).

At temperatures below that of the gel-to-liquid-crystal phase transition, we find that both cholesterol *and* proteins prevent lipid hydrocarbon chains from crystallizing; however, cholesterol-containing samples are far more ordered than those containing protein.

The effects described above must clearly be related to the structures of the perturbing molecules—the proteins and cholesterol. It seems most likely that the rough, irregular surfaces of proteins (with their ~ 20 different amino acid side chains) may tend to cause lipid hydrocarbon chains to pack in a slightly disordered way into "vacancies" created by the side chains, and perhaps created in a time-dependent way by rotation and internal motions of the protein. Cholesterol, on the other hand, is a rigid tetracyclic structure, and inspection of molecular models reveals that it has rather planar sides, at least on the scale of a C–C bond length. Cholesterol thus acts as the rigid boundary assumed in the theoretical calculations of protein–lipid interaction discussed by Kleemann and McConnell (1976), Marcelja (1976), and Scott and Cherng (1978). Overall, our results suggest that boundary lipid is slightly more disordered than pure lipid, but that the rates of motion are probably slowed down due to protein–lipid steric interactions.

3. DEUTERIUM NMR OF LIPIDS IN BIOLOGICAL SYSTEMS

Specifically deuterated fatty acids have been incorporated into a variety of cell membrane systems over the past 10 years (e.g., by Davis *et al.*, 1979; Kang *et al.*, 1979b; Oldfield *et al.*, 1972; Stockton *et al.*, 1977), and ^{2}H NMR spectra have been obtained as a function of temperature for various membrane fractions and for isolated lipids dispersed in water. A general observation that may be made is that there is as yet no evidence for a "condensing" or "ordering" effect of protein on membrane lipid in any of the "intact" biological membranes examined. Cholesterol, on the other hand, again exhibits an ordering effect on lipid, even in the presence of membrane protein as shown by Stockton *et al.* (1977). Kang *et al.* (1980) have shown that in the *Acholeplasma laidlawii B* membrane system, protein has little effect on lipid order, due perhaps in part to the low protein-to-lipid ratio. For example, ^{2}H quadrupole splittings and linewidths observed for 4-, 6-, 8-, and 14-^{2}H-labeled tetradecanoic acid-enriched *A. laidlawii* cell membranes are within experimental error the same as those observed with the corresponding lipid extracts, and are also the same as those seen previously by Oldfield *et al.* (1978a) with bilayers of pure 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) when examined immediately above the end of the solid-to-fluid phase transition.

In contrast, ^{2}H NMR spectra of hexadecan-1-oic acid-enriched *Escherichia coli* L48-2 cell membranes have in our laboratory shown considerable line broadening compared with spectra of their lipid extracts, and $\Delta\nu_Q$ values were slightly decreased. Results with these *E. coli* cell membranes showed essentially the same NMR lineshapes as those seen previously by Rice and Oldfield (1979) with the DMPC–gramicidin A′ system: these include collapsed terminal methyl group quadrupole splittings and large (4–6 kHz) linewidths of methylene-segment chain resonances, emphasizing that a wide range of types of protein-

lipid interactions may be observed in microbial membrane systems. The results with intact *E. coli* membranes suggest that large-amplitude fluctuations of the methylene chain segments may be caused by protein–lipid interaction in some systems.

4. PHOSPHORUS NMR OF LIPIDS IN MODEL AND BIOLOGICAL MEMBRANES

^{31}P has a spin $I = ½$ and a 100% natural abundance, so that it is a particularly attractive species for studying the dynamic structures of lipid membranes. ^{31}P NMR studies of the gel and liquid-crystalline states of a variety of phospholipids, together with the effects of cholesterol on head-group molecular motion, have already been conducted by a number of investigators (Barker *et al.*, 1972; Brown and Seelig, 1978; Cullis and deKruyff, 1976; Griffin, 1976; Griffin *et al.*, 1978; Kohler and Klein, 1976; Niederberger and Seelig, 1976; Seelig, 1978; Yeagle *et al.*, 1975), so it is therefore of some interest to compare these results with ^{31}P NMR studies of protein–lipid interactions.

We show in Fig. 2 selected results from a series of ^{31}P NMR experiments recently carried out in our laboratory revealing that addition of protein to a lecithin bilayer causes in all cases a decrease in $\Delta\sigma$ of the lipid phosphate head group. This is accompanied by increased T_1 and T_2 relaxation rates shown by Rajan *et al.* (1980), which suggests that in many cases lipid polar head groups may interact directly with membrane proteins, and that

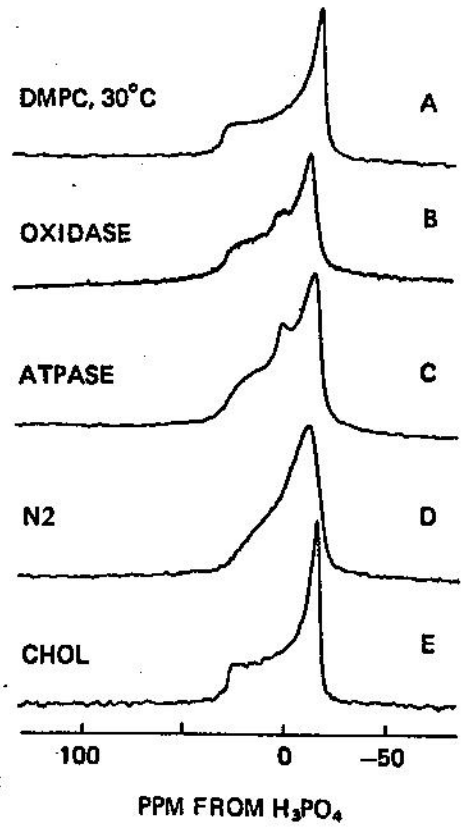

Figure 2. ^{31}P NMR spectra of DMPC in the presence of a variety of proteins, and of cholesterol. (A) DMPC in excess water, 30°C. (B) As (A) except lipid contains 67 wt% cytochrome oxidase. (C) As (A) except lipid contains 65 wt% sarcoplasmic reticulum ATPase. (D) As (A) except sample contains 67 wt% beef brain myelin proteolipid apoprotein. (E) As (A) except sample contains 33 wt% cholesterol. The weak features at 0 ppm in (B) and (C) arise from small phosphate molecule impurities. Spectra were obtained at 60.7 MHz under conditions of proton-decoupling.

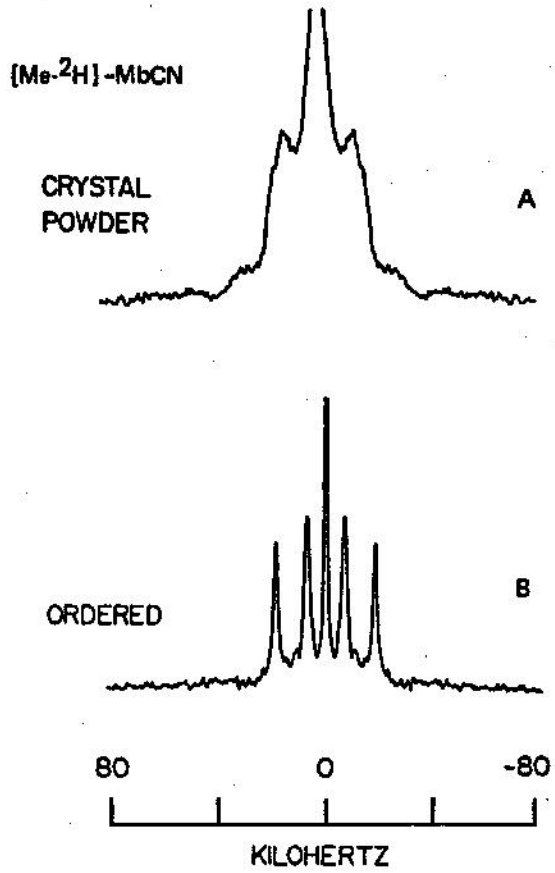

Figure 3. Deuterium NMR spectra of labeled protein crystals and membrane proteins obtained at 55.3 MHz. (A) Solid powder sample of methionine-labeled sperm whale cyanoferrimyoglobin hydrated with 90% saturated $(NH_4)_2SO_4$. (B) Magnetically ordered cyanoferrimyoglobin suspended in ~90% saturated $(NH_4)_2SO_4$. (C) [β-$^2H_1$]valine, 23°C. (D) [β-$^2H_1$]valine-enriched *Halobacterium halobium* purple membranes, in H_2O at 37°C. The sharp central peaks in (A), (B), and (D) are due to HO^2H.

as a result the head-group phosphate motion may become slower and more isotropic. Cholesterol also decreases $\Delta\sigma$ as shown by Brown and Seelig (1978) and Rajan *et al.* (1980), but in this case T_1 remains the same and there are slight increases in T_2 relaxation times from the values found in pure lipid bilayers, suggesting that cholesterol simply acts as an inert spacer molecule.

With intact biological membranes, spectral signal-to-noise ratios have been lower than those obtained with model systems and no differences between ^{31}P NMR spectra of membranes and their lipid extracts supporting the above ideas have yet been reported. Work in the area of intact biomembrane ^{31}P NMR has concentrated on observation and characterization of "isotropic" signal components, sometimes generated by the presence of protein or polypeptide as shown by Burnell *et al.* (1980), Rajan *et al.* (1980), and Stier *et al.* (1978). These results may be linked to the presence of "lipidic particles" in the lipid bilayer as discussed by Burnell *et al.* (1980) and Miller (1980).

At present, a plausible model of protein–lipid interaction is, therefore, one in which proteins "immobilize" lipid head groups, while simultaneously disordering somewhat the lipid hydrocarbon chains, due to the rough nature of the protein surface.

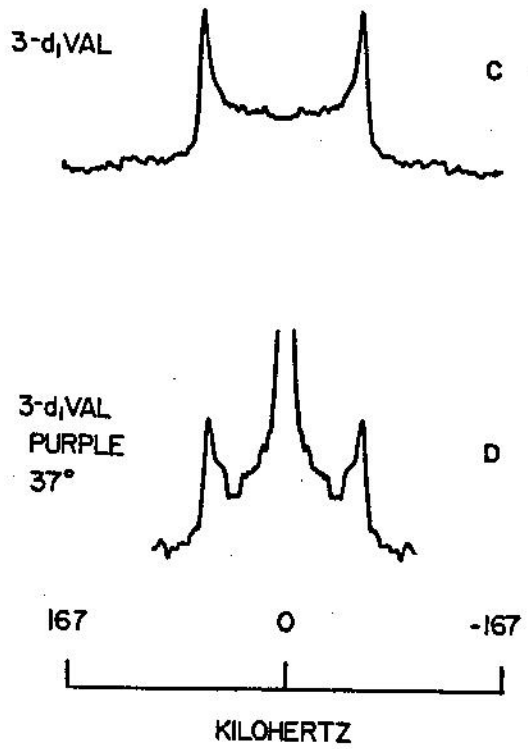

Figure 3. continued

5. DEUTERIUM NMR OF PROTEINS IN MODEL AND BIOLOGICAL SYSTEMS

The ideal way to monitor protein–lipid interactions would be to observe the effects of various membrane lipid constituents on protein static and dynamic structure directly, via observation of the NMR signals originating from the protein itself. Recently, considerable progress has been made in exploring this possibility.

First, it has been shown by Oldfield and Rothgeb (1980) and Rothgeb and Oldfield (1981) that it is possible to detect, resolve, and assign individual groups in (paramagnetic) protein crystals, a new type of "model system," using high-field ^{2}H NMR, as shown in Figs. 3A and B. Shown in Fig. 3A is a ^{2}H NMR spectrum of a random powder distribution of [*methyl*-^{2}H] methionine-labeled sperm whale cyanometmyoglobin microcrystals, while in Fig. 3B we show the spectrum obtained after suspension of the microcrystals in a strong magnetic field, which causes sample ordering as shown by Rothgeb and Oldfield (1981). Both ^{2}H-labeled sites are resolved in Fig. 3B, and have recently been assigned as shown by Rothgeb and Oldfield (1981). In principle, a variety of methods may be used to orient, or order, diamagnetic biological membranes, and thus resolve individual sites, if adequate spectrometer sensitivity is available to detect them. Again, the first steps in this area have been taken by Oldfield *et al.* (1981). ^{2}H NMR spectra of the pure amino acid [β-^{2}H$_1$]valine, in the solid state, and of [β-^{2}H$_1$]valine-labeled purple membranes from the extremely halophilic organism *Halobacterium halobium* (Figs. 3C and D) show the very rigid nature of the purple membrane protein, bacteriorhodopsin, and indicate that it should now be possible to study the dynamics of amino acid residues in many different biomembrane systems. It seems certain, therefore, that in the next 5 years a much more detailed picture of the

effects of membrane lipid constituents on protein structure (and function) will be acquired using highfield NMR and isotopic labeling methods.

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