NMR STUDIES OF MEMBRANE SURFACES

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INTRODUCTION

Biological membranes are usually composed of a limited number of phospholipid types, often mixed with other, non-phosphate containing lipids and proteins. Magnetic resonance methods have proved particularly useful in the study of the acyl chain behaviour of phospholipids in membranes, in particular in the study of how lipids and proteins interact at the molecular level as reviewed by Marsh and Watts(1), Seelig et al.,(2) and in the series by Watts and De Pont(3). However rather less is known about the polar part of lipids which form the surface of biomembranes and are the first contact a living cell has with the external environment. The molecular conformation and motional properties of lipid head-groups, which identify the lipid type, are relatively well characterized for model membranes containing only one phospholipid species, as a result of proton, deuterium and phosphorus NMR* and neutron diffraction studies. However, very limited information is available on lipid structure at membrane surfaces if more than one species is in present in the membrane. We have therefore extended the NMR approach to study mutual lipid-lipid interactions and lipid-protein interactions which may occur at the membrane polar-apolar interface; this interface being the most probable site of any selective electrostatic or steric associations which may modulate membrane protein function.

D-NMR is a highly versatile approach to study membranes in that deuterium can substitute for protons, in a non-perturbing way, at many specific positions in a phospholipid to give information about almost any part of the molecule. In addition, together with the popular spin-label ESR approach, both methods can be used to give complimentary information by virtue of their range of applicability and different motional time-scales as discussed by Watts(4).

In the work to be described here, we have shown that the D-NMR spectra of phospholipids specifically deuterated in their polar head groups are very sensitive to the interactions at the bilayer surface between different phospholipid types as shown by Sixl and Watts(5,6). Also we have identified, for the first time, specific electrostatic interactions at a membrane surface, firstly to define the ionization state of phospholipid head-groups and of a partitioned anaesthetic, Watts and Poile(7), and secondly between a peripheral protein and different lipid types, Sixl et al.,(8), in addition to identifying conformatiopnal perturbations of lipid head-groups with integral proteins in Ryba et al., (9). We have also shown that the technique is capable of indicating that the functional and aggregation state of an integral

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* Abbreviations used: NMR, nuclear magnetic resonance; ESR, electron spin resonance; DMPC, 1,2-dimyristoyl-sn-glycero-3- phosphocholine; DMPG, 1,2-dimyristoyl-sn-glycero-3-phospho -rac-glycerol; CSA, chemical shift anisotropy; $\Delta\nu_Q$, deuterium quadrupole splitting.

protein in the bilayer membrane, Dempsey, et al.,(10).

2H-LIPID SYNTHESIS AND METHODS

The synthesis of head-group deuterated phospholipids used in our work has been described in detail elsewhere. Essentially, diacylglyerols are phosphorylated with phosphorus oxychloride followed by condensation of the required deuterated head-group alcohol according to the method of Eibl(11). In this way and with some modifications depending upon the lipid type, PC, PG, PE and PS can all be synthesised in good yields.

Deuterium NMR spectra have been recorded at 46.1 MHz on a Bruker WH-300 spectrometer employing single 90° pulses of 29μ sec duration. Phosphorus-31 NMR spectra were recorded at 121.5 MHz under broad-band proton decoupling at a power of 7 - 10 W. Temperatures were controlled by a nitrogen gas flow to an accuracy of $\pm 2^{\circ}C$.

One parameter of interest in D-NMR spectra of deuterated lipids in bilayer membranes is the quadrupole splitting, $\Delta\nu_Q$ measured in Hz. The powder spectrum arising from the deuteriums of a CD-bond results from a spherical distribution of two-line spectra ($m_I = 1$) from all of the individual orientations of the bilayer with respect to the applied magnetic field as discussed by Davis(12) and also Seelig and Seelig(13,14). Extending the measured parameter to give molecular detail is a little more complicated in that some information about the axis of CD motional averaging is required. If, as is the case for a lipid acyl chain, the axis of averaging and the symmetry of the CD orientation are the same, and in this case axial, then the familiar order parameter, S(CD), can be deduced directly from the quadrupole splitting, where S = (measured value/maximum value). In the cases to be discussed here, where the head-groups of the lipids are labelled with deuterium, the axis of CD-bond motional averaging is unknown and may or may not be the same as the molecular axis. We therefore are unable to deduce true molecular order parameters directly, since our measured experimental values relate to not only the amplitude of motion, but also the conformation of the CD-bond with respect to the bilayer surface. We therefore need more information to decipher and usually require other details to construct an exact picture of the head-group. In all the following discussions therefore, we have considered only the changes in the values of $\Delta\nu_Q$, the width of the D-NMR spectra.

LIPID-LIPID INTERACTIONS

Before protein-lipid interactions in mixed lipid type bilayers can be studied in detail, it is necessary to carry out control experiments of lipid-lipid associations, which may themselves be more important than the protein-lipid interactions. For this reason we have examined a combination of binary lipid mixtures to try and deduce some generalized information about lipid-lipid associations.

To summarise this earlier work, we have observed that:-

1). the α-segments of lipid head groups remain relatively unperturbed in most lipid mixtures;

2). the PE head-group is unperturbed by any other lipid, despite ethanolamine being smaller (35-42Å) than any other head-group (e.g.: PC = 47-54Å);

3). the α- and β-segments of PC reflect greater freedom of motion on the addition of any other lipid, all the others having smaller molecular volume compared to PC.

The rather rigid conformation of PE is particularly interesting in that a detailed molecular structure has been available for some time for this lipid from the single crystal X-ray diffraction studies of Hitchcock et al.,(15). It appears that long-range co-operative interactions are possible through H-bonds along the surface of the membrane bilayer, and the H-bond length (in PE, O - N = 2.2Å compared to 3.7Å for PC) suggests that these H-bonds are rather strong, perhaps approaching 20kcals/mole. An interpretation of the deuterium results is that these strong H-bonds maintain

the PE head-group in place such that even the bulky choline cannot perturb the surface structure.

IONIZATION STATES

To study the ioization states of phospholipid head-groups, bilayers of PE have been deuterated in the α- and β-segments of the ethanolamine group and the pH of the aqueous phase changed as described by Watts and Poile(7). The values of $\Delta\nu_Q$ are seen to reflect the protonation and deprotonation of the primary amino and phosphate groups of the lipid with pK_a's of 8.1 and somewhat less than 4.0 respectively as shown in Fig. 1. In addition, the ionization of the secondary amino group of the local anaesthetic, tetracaine, when partitioned into the bilayers, can also be determined from the changes in the PE head-group deuterons (Fig. 1).

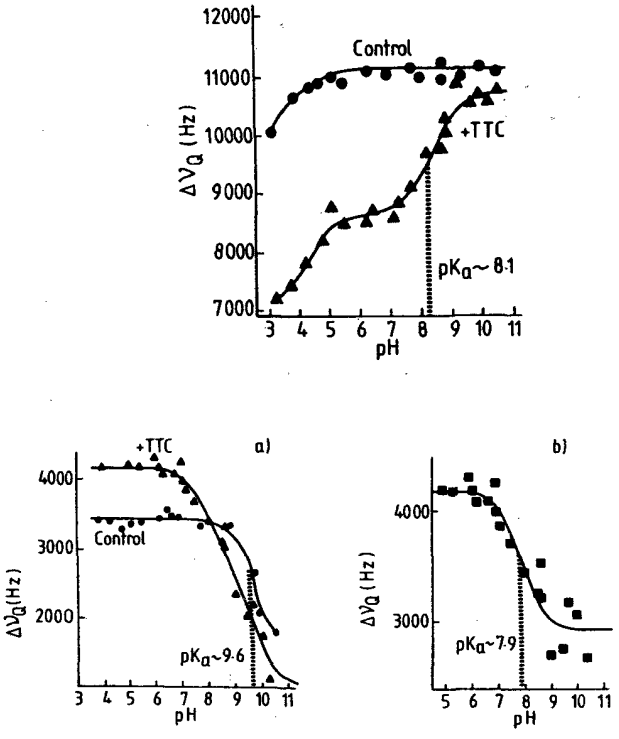

Figure 1: Changes in the values of $\Delta\nu_Q$ for the α-deuterons (upper curve) and β-deuterons (lower curves; b). is for TTC alone and is the difference of the control and +TTC in a).) as a function of the pH of the dispersion of PE-d_4/PC (1:1) bilayers with (Δ) and without (o) tetracaine.

INTERACTIONS BETWEEN A PERIPHAL PROTEIN AND LIPIDS

A number of proteins interact with a surface of membranes. One of these is the basic protein from myelin which comprises about 30 weight % of the total myelin protein, and although it is a peripherally bound protein, there is some evidence that it partially penetrates the membrane bilayer and associates preferentially with acidic phospholipids Boggs et al.,(16). We have studied the protein-induced perturbations of the head group motions in bilayers of the zwitterionic lipid DMPC and the negatively charged DMPG, both separately and in equimolar mixed lipid bilayers using the intact and two unequal fragments of myelin basic protein.

We observe that on increasing the protein concentration in the bilayer, reduces the quadrupole splittings of all three deuterated head-group segments of DMPG in liquid crystalline bilayers. Addition of basic protein to DMPG-d_5 membranes also affected the phosphorus-31 NMR spectra. For each protein concentration, the spectral line shape indicated the lipid molecules to be in a bilayer arrangement, Seelig(14), and single component.

When basic protein was added to DMPC-d_4 or DMPC-d_9 bilayers, neither the deuterium quadrupole splittings nor the phosphorus-31 CSA were altered, nor did the spectal line shapes differ from those of pure DMPC, indicating that no or only a very weak binding of the protein to the phosphatidylcholine membrane surface occurs. When basic protein was added to mixed bilayers of DMPC and DMPG (1:1) with either of the lipids containing the label, the changes in the spectra and in the values of the quadrupole splittings for DMPG-d_5 were similar to those in single component DMPG-d_5 bilayers with protein.

To gain information about the exchange rate of lipids between the bulk lipid phase and the complexes with basic protein, we have analysed our results in terms of a fast, two-site exchange of lipid between the protein-free lipid part of the bilayer

and the protein-associated phase, assuming that all the deuterated lipid contributes to the observed deuterium NMR spectra. Then $n_t = n_c + n_f$, where n_t, n_c and n_f are the number of total, complexed and free lipid molecules per mole of protein in a reconstituted sample respectively. The observed $\Delta\nu_Q$, Q_o, will then be a weighted average of the CD-bond $\Delta\nu_Q$ in each of the two environments such that $Q_o = (n_c/n_t)Q_c + (n_f/n_t)Q_f$. Substituting for n_f gives:

$$Q_o = n_c(Q_c - Q_f).1/n_t + Q_f \quad \text{(Equ 1)}$$

Therefore, if the assumptions about exchange are valid, plotting $1/n_t$ against Q_o will give a linear variation with an intercept at $1/n_t = 0$ of Q_f and a slope of $n_c(Q_c - Q_f)$.

The linearity we see in values of $\Delta\nu_Q$ with $1/n_t$ suggests that the ideas about fast lipid exchange between lipid-protein complexes and the bulk lipid are valid. Also, at $1/n_t = 0$, the extrapolated value of Q_f is close to the experimentally determined value of Q_o for pure lipid bilayers. The difference in slope, given by $n_c(Q_c + Q_f)$, for the three different sized proteins derived from myelin basic protein also demonstrates that this method is sensitive to the lipid:protein stoichiometry since the ratio of the slopes is in proportion to their molecular weights mol. wt. ratios are 12.6/18.4 = 0.69, ratio of slopes = 0.61; mol. wt. ratios are 5.8/18.4 = 0.32, ratio of slope = 0.28).

INTEGRAL PROTEIN INTERACTIONS WITH LIPID HEAD-GROUPS

Rhodopsin, from mammalian retinal discs, and band 3, from human erythrocytes, have been chosen as good examples of integral proteins with which to study lipid-protein interactions at membrane surfaces. Both proteins have been reconstituted in DMPC-d_9 at lipid:protein mole ratios between 65:1 and over 1000:1. From the D-NMR spectra of rhodopsin-DMPC-d_9 reconstitutions at 32°C, well above any lipid phase transition, it is clear that the motion of the terminal CD_3 groups of the choline, which are at the membrane surface, are very sensitive to the presence of the proteinas shown by Ryba et al.,(9). As the concentration of protein incorporated into the lipid bilayer membrane is increased, a considerable decrease in the measured quadrupole splitting is observed (to less than 0.3 kHz at a lipid:protein ratio close to that in disc membranes of 65:1). Concurrent with the changes in separation of the maxima of the spectra, the width of the lines also increases indicating spectral broadening indicative of inhomogeneity of the deuterium environment on the NMR time-scale. This may be due to a distribution in the vesicle sizes under examination. Indeed, freeze-fracture electron microscopy indicates that the protein containing vesicles are inhomogeneous both in size and in the concentration of rhodopsin seen in the membranes. However, measurement of the distribution of vesicle sizes shows that over 80% of the lipid is found in vesicles with radii greater than 200nm, these vesicles being smaller on average than those containing no protein and formed in a similar fashion. It can be readily calculated that vesicles must be smaller than one micron to average the small quadrupole splittings we detect through isotropic vesicles tumbling in solution. Thus it appears unlikely that the vesicle size contributes to a significant reduction in the quadrupole splitting.

Band 3 seems to behave similarly to rhodopsin in bilayers containing little protein, that is in a dilute situation and $1/n_t$ with $\Delta\nu_Q$ is linear, as reported by Dempsey et al.,(10). However the slopes of this data, which give information about n_c the number of lipids interacting with the protein, changes dramatically with temperature. This suggests that a temperature dependent aggregation occurs, with higher aggregates at lower temperatures interacting with less lipids per protein monomer than at higher temperatures. A similar concentration and temperature dependent protein aggregation effect has been shown for bacteriorhodopsin in DMPC bilayers using optical methods described by Cherry(17) and for band 3 by Muhlebach and Cherry(18).

CONCLUSIONS

We have now extended the D-NMR method of studying membrane surfaces to such an level now that we feel our ultimate goal of studying structure-function relationships in membranes between lipids and proteins is in sight. No lipid-protein effects will be general and each protein will almost certainly be different from others. If the function of a protein can be related and modulated by a particular lipid type, then some rationale for the diversity and range of lipid types in biomembranes may have been deduced. If such functional associations are related to structural interactions, and whether they determine the life-time of a lipid at a protein interface or a specific allosteric control, then we can say that lipids do indeed control or modulate membrane function.

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REFERENCES

(1). Marsh, D., & Watts (1982) in Lipid-Protein Interactions (Jost. P.C. and Griffith, O.H. Eds.) Vol. II, Chapter 2, Wiley Interscience, New York.
(2). Seelig, J., Seelig, A., & Tamm, L. (1982) in Lipid Protein interactions (Jost, P.C. and Griffith, O.H. Eds.) Vol. II, Chapter 3, Wiley Interscience New York.
(3). Watts, A. and de Pont, J.J.H.H.M. eds. of "Progress in Protein-lipid Interactions", Vol. 1 & 2, Elsevier, Amsterdam.
(4). Watts, A. (1981) Nature 294, 512.
(5). Sixl, F., & Watts, A. (1982) Biochemistry 21, 6446.
(6). Sixl, F., & Watts, A. 81983) Proc. Natl. Acad. Sci. USA 80, 1613.
(7). Watts, A. and Poile, T.P. (1986) Biochim. Biophys. Acta. 861, 368-372.
(8). Sixl, F., Brophy, P.J. and Watts, A. (1984) Biochem. 23, 2032.
(9). Ryba, N.J.P., Dempsey, C.E. and Watts, A. (1986) Biochemistry, 25, 4818-4825.
(10). Dempsey, C.E., Ryba, N.J.P. and Watts, A. (1986) Biochemistry, 25, 2180-2187.
(11). Eibl, H. (1978) Proc. Natl. Acad. Sci. (USA) 75, 4074.
(12). Davis, J. (1983) Biochim. Biophys. Acta 737, 117-171.
(13). Seelig, J., & Seelig, A. (1980) Q. Rev. Biophys. 13, 190.
(14). Seelig, J. (1978) Biochim. Biophys. Acta 515, 105.
(15). Hitchcock, P.B., Mason, R., Thomas, K.M. and Shipley, G.G. (1974) Proc. Natl. Acad. Sci. (USA) 71, 3036.
(16). Boggs, J.M. Moscarello, M.A., & Papahadjopoulos, D. (1982a) in Lipid-protein Interactions (Jost, P.C. and Griffith, O.H. Eds.) Vol. II. Chapter 1, Wiley Interscience, New York.
(17). Cherry, R.J. (1979) Biochim. Biophys. Acta 559, 289.
(18). Muhlebach, T., and Cherry, R.J. (1985) Biochem. 24, 976.