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^{31}P NUCLEAR MAGNETIC RESONANCE STUDIES OF THE ASSOCIATION OF BASIC PROTEINS WITH MULTILAYERS OF DIACYL PHOSPHATIDYLSERINE

R. SMITH [a,*], B.A. CORNELL [b], M.A. KENIRY [c] and F. SEPAROVIC [b]

[a] *Department of Biochemistry, La Trobe University, Bundoora, VIC 3083,* [b] *CSIRO Division of Food Research, North Ryde, NSW 2113 and* [c] *School of Chemistry, University of Sydney, Sydney, NSW 2006 (Australia)*

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Lysozyme, cytochrome *c*, poly(L-lysine), myelin basic protein and ribonuclease were used to form multilayer dispersions containing about 50% protein (by weight) with bovine brain diacyl phosphatidylserine (PS). ^{31}P nuclear magnetic resonance shift anisotropies, spin-spin (T_2) and spin-lattice (T_1) relaxation times for the lipid headgroup phosphorus were measured at 36.44 MHz. At pH 7.5, lysozyme, cytochrome *c*, poly(L-lysine) and ribonuclease were shown to increase the chemical shift anisotropy of PS by between 12–20%. Myelin basic protein altered the shape of the phosphate resonance, suggesting the presence of two lipid components, one of which had a modified headgroup conformation. The presence of cytochrome *c* led to the formation of a narrow spike at the isotropic shift position of the spectrum. Of the various proteins or peptides we have studied, only poly(L-lysine) and cytochrome *c* had any effect on the T_1 of PS (1050 ms). Both caused a 20–30% decrease in T_1 of the lamellar-phase phosphate peak. The narrow peak in the presence of cytochrome *c* had a very short T_1 of 156 ms. The possibility is considered that the cytochrome Fe^{3+} contributes to the phosphate relaxation in this case. The effect of all proteins on the T_2 of the phosphorus resonance was to cause an increase from the value for pure PS (1.6 ms) to between 2 and 5 ms. The results obtained with proteins are compared with the effects of small ions and intrinsic membrane proteins on the order and motion of the headgroups of lipids in bilayers.

Introduction

The interactions which occur between proteins and lipids within biomembranes determine not only the structural characteristics of the membranes but also the function of many of the membrane proteins. Studies of intrinsic membrane proteins have revealed that subtle changes in the lipid environment may be reflected in changes in the protein activity. For example, β-hydroxybutyrate dehydrogenase is activated only in the presence of phosphatidylcholines. The activities of several other intrinsic proteins depend on the physical properties of the bilayers to which they are bound. Water-soluble enzymes have also been shown to have modified activity when bound to membrane surfaces [1].

The effects of numerous intrinsic proteins on the order and mobility of lipid molecules in bilayers have been described. Many, but not all [2], of these proteins minimally perturb the rapid motions or order of either the acyl-chain or headgroup atoms. For most intrinsic proteins this result is

* To whom worrespondence should be addressed at (present address): Department of Biochemistry, University of Queensland, St. Lucia, QLD 4067, Australia.

Abbreviation: PS, phosphatidylserine.

understandable in that their retention in the bilayer depends primarily on nonspecific hydrophobic interactions with the bilayer hydrocarbon milieu. Extrinsic membrane proteins, by contrast, are assumed to bind through interactions with polar groups at the bilayer surface. Therefore, one might expect to find, particularly with highly basic polypeptides, considerable restrictions in the headgroup motion of acidic lipids.

In an initial attempt to characterize the interactions between lipids and extrinsic proteins, we have examined the binding to phosphatidylserine bilayers of several basic proteins, including the basic protein of myelin. Myelin basic protein is believed to bind directly to lipids in vivo, rather than to other membrane-bound proteins.

Materials and Methods

Diacylphosphatidylserine * (PS) was isolated by ion-exchange chromatography following Rouser et al. [3]. The material applied to the DEAE-cellulose column was either a chloroform/methanol (2 : 1) extract of fresh bovine brains or crude PS prepared from the 'cephalin' fraction of Folch [4]. Most of the PS was eluted with glacial acetic acid. The purity of the lipid was assessed by thin-layer chromatography on silica gel plates using chloroform/methanol/acetic acid/water (25 : 15 : 4 : 2, v/v) or chloroform/methanol/ammonia (60 : 35 : 5) as solvent. No impurity could be detected by charring with 25% sulphuric acid when typically 100 μg was applied to the plate. Colorimetric protein analyses of lipid dissolved in dodecyl sulphate showed that there was less than 0.01 g protein/g lipid.

Myelin basic protein was prepared from fresh bovine brains [5]. Egg-white lysozyme (Grade I), calf-thymus histone (Type IIS), horse-heart cytochrome *c* (Type III) and poly(L-lysine) (Type II, M_r 100–4000) were all purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

* Initially this lipid was prepared by chromatography of bovine brain extract III (Sigma Chemical Co., St. Louis) on DEAE-cellulose. However, the material prepared in this manner was brown and gave short, variable spin-lattice relaxation times.

Formation of lipid-protein complexes

Purified PS was dried from chloroform solution, first by evaporation of solvent under nitrogen, and then by placing under vacuum for 4–6 h. Protein dissolved in 3 ml 0.1 M Tris (pH 7.5)/1 mM EDTA/2 mM sodium azide was added to the dry lipid and the mixture was dispersed by vortexing, and freezing and thawing, under nitrogen. Lipid and protein concentrations were both near 60 $g \cdot l^{-1}$ in the dispersions.

The dispersions were applied to the tops of 0–60% (w/v) linear sucrose density gradients made up in the Tris/EDTA buffer. The tubes, containing approximately 38 ml, were centrifuged at 4°C at $85000 \times g_{av}$ in a Beckman SW 27 rotor. In initial experiments, centrifugation was for 18–24 h, but equilibrium banding was achieved with the lipid-protein complexes (but not lipid alone) within 5 h and in later experiments centrifugation was for 4–5 h. Following centrifugation, the gradients were collected in 1 ml fractions. After addition of sodium dodecyl sulphate to a final concentration of 1%, aliquots of these fractions were assayed colorimetrically for protein [6] and for phosphorus [7]. Sucrose concentrations in protein- and lipid-free solutions were determined by refractometry.

Lipid and lipid-protein complexes taken from the density gradients were dialysed for 24 h at 4°C against several changes of Tris/EDTA buffer, to remove sucrose. The dialysed complexes were centrifuged in buffer at $105000 \times g_{av}$ in a Beckman 50 Ti rotor. After centrifugation for 1 h, the lipid-protein pellets contained 50–60% by weight of buffer, but the protein-free PS still contained 85% buffer after 12 h.

All samples were used immediately for NMR experiments, or stored under nitrogen at −30°C.

Nuclear magnetic resonance measurements

The sedimented samples were transferred to 10-mm NMR tubes either by using a spatula and then gently centrifuging the NMR tubes, or by simply sliding the cellulose nitrate centrifuge tubes into the NMR tubes. ^{31}P-NMR spectra were recorded at 36.44 MHz on a Bruker CXP100 spectrometer. The spectra were obtained using a (90° ~ τ ~ 180° ~ τ ~ FID) pulse sequence to prevent distortion of the ^{31}P signal by receiver dead-time. A 90° pulse of 8 μs, a repetition time of 4 s, and

15 W of proton decoupling power were employed to obtain the spectra.

Spin-lattice relaxation times were obtained using the inversion-recovery method with a waiting time of approximately $4T_1$ between pulses. Generally 12–20 delay times ranging from 1 ms to 2500 ms were used. As the spin-lattice relaxation time may be anisotropic, the peak heights were measured at the major ($\sigma_\perp$) and at the shoulder ($\sigma_\parallel$) peak positions. The peak heights and delay times were fitted to a single exponential function to obtain the corresponding relaxation times.

Spin-spin relaxation times were measured by varying the delay (τ) between pulses in a ($90° \sim \tau \sim 180° \sim \tau \sim$ FID) pulse sequence with delay times in the range 0.1–12 ms.

All of the spectra were accumulated at 300 ± 1 K. In several experiments spectra were obtained for identical masses of lipid with and without protein, to permit a comparison of the absolute spectral intensities.

Chemical shift anisotropies were calculated by fitting simulated spectra to those obtained experimentally.

Results

The lipid-protein recombinants differed substantially in macroscopic appearance from the pure lipid. Whereas removal of the solvent from the pure PS required extensive centrifugation, the protein-containing samples flocculated and sedimented readily. Myelin basic protein and lysozyme in particular gave very large aggregates which excluded excess buffer.

When mixed in equal amounts by weight, the proteins were found to be largely bound to lipid. Unbound protein, which remained at the top of the density gradients on centrifugation, rarely exceeded 20% of that originally in the dispersions and it was typically less than 10%. No significant amount of free lipid was found on the gradients containing lipid-protein complexes. These observations were consistent with the results of the protein and lipid analyses, which showed the recombinants taken from the gradients to contain 39–52% protein by weight of total solids: only recombinants with close to 50% protein were used for NMR studies.

The lipid-protein complexes banded with apparent densities in the range 1.15–1.17 $g \cdot l^{-1}$. These densities were in accord with the measured lipid and protein contents.

There was no evidence of dissociation of protein from the lipid-protein recombinants. The final compositions were similar after 4 h or 24 h centrifugation in the density gradients, and no significant amount of protein was lost in the subsequent dialysis and centrifugation.

All available evidence suggests that the lipid and proteins were intimately mixed in these complexes. This point has been addressed specifically for the myelin basic protein [8], but in the NMR spectra (see below) no unperturbed PS was detected in the presence of any of the proteins.

In some of the NMR experiments recombinants were used which were prepared with the omission of the density gradient centrifugation. The amounts of the proteins remaining in the supernatants during the final centrifugation were in agreement with those observed at the top of sucrose density gradients in other experiments.

Chemical shift anisotropies

The pure lipid, PS-lysozyme, PS-ribonuclease and PS-poly(L-lysine) spectra all had the axially symmetric powder patterns characteristic of phospholipid in the liquid-crystalline bilayer phase. Examples of these spectra are seen in Figs. 1a and 1d for pure PS and PS-lysozyme, respectively. On occasions the PS spectrum also contained a minor peak located at the isotropic chemical shift position which was thought to arise from the smaller lipid aggregates. Due either to internal molecular diffusion or to whole body tumbling, these aggregates have the chemical shift anisotropy of their phosphate groups averaged to zero. This isotropic peak was removed by the addition of protein, except with cytochrome *c*.

In all instances the addition of a protein or peptide caused an increase in the measured chemical shift anisotropy (Table I). Comparison of the spectral intensity of the phosphorus resonance failed to show any loss of signal due to the introduction of the proteins. In these spectra, the electronic asymmetry about the ^{31}P nucleus and its orientation to the average molecular long axis combine to determine the observed chemical shift anisotropy.

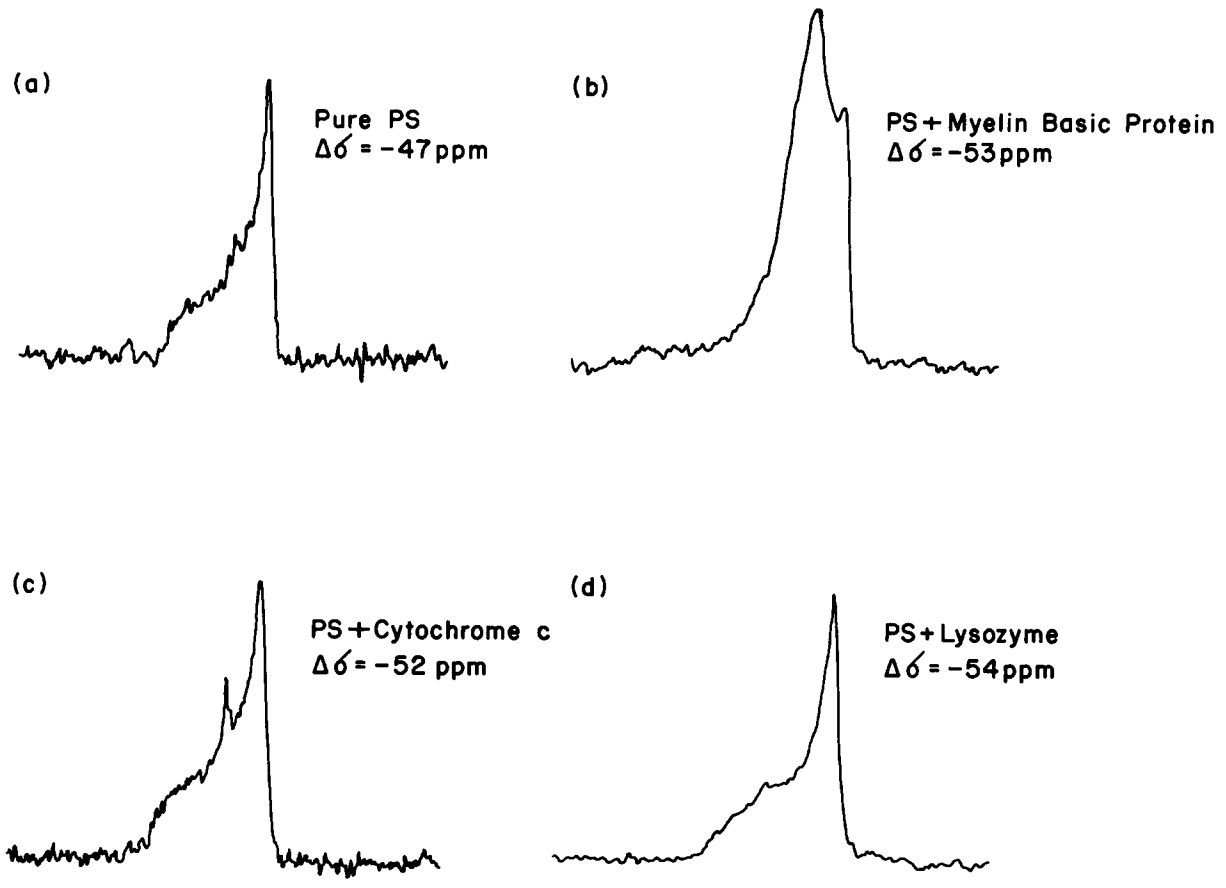

Fig. 1. Examples of ^{31}P spectra recorded at 36.44 MHz using 15 W of proton decoupling power and a 90° pulse duration of 8 μs. The pulse repetition rate was one per 4 s. All samples were run at 300 K and were dispersed in at least an equal weight of water to total solids. (a) Pure PS; (b) PS plus 52% by weight of myelin basic protein; (c) PS plus 48% cytochrome *c* by weight; (d) PS plus 48% lysozyme by weight.

Myelin basic protein induced a substantial change in the phosphorus resonance causing the appearance of an intense broad peak in the centre of the spectrum (Fig. 1b).

Cytochrome *c* had a smaller overall effect on the spectral shape (Fig. 1c), but, as mentioned in the next section, caused large changes in the spin-lattice relaxation time. De Kruijff and Cullis [9] have found little change in the chemical shift anisotropy on mixing cytochrome *c* with phosphatidylserine, phosphatidylglycerol, phosphatidylethanolamine or phosphatidylcholine.

Relaxation times

Despite the effect of lysozyme, ribonuclease and myelin basic protein on the chemical shift anisotropy of PS, they produced little change in the spin-lattice relaxation time of the phosphorus nuclei (Table I).

By contrast, in the presence of cytochrome *c* or poly(L-lysine), T_1 was found to shorten. In the presence of cytochrome *c*, PS showed two populations, both with substantially reduced relaxation times. Of the two populations, one possessed the chemical shift anisotropy similar to that of pure PS and the other a chemical shift anisotropy which was narrowed beyond the resolution of the spectrometer. Of all the proteins studied, cytochrome *c* had the greatest effect on the relaxation rate of the phosphorus atoms.

Considering the geometry of cytochrome *c*, the nearest distance of approach of the paramagnetic

TABLE I

^{31}P CHEMICAL SHIFT ANISOTROPIES (CSA), SPIN-LATTICE RELAXATION TIMES (T_1) AND SPIN-SPIN RELAXATION TIMES (T_2) FOR MULTILAMELLAR DISPERSIONS OF PHOSPHATIDYLSERINE WITH BASIC PROTEINS AND POLYPEPTIDES, OBTAINED AT 300 K

Protein added	CSA [a] (ppm)	T_1 [a] (ms)	T_2 [a,b] (ms)
Pure phosphatidylserine	-47 ± 1 (6)	1145 ± 180 (11)	1.61 ± 0.49 (4)
Bovine myelin basic protein	-53 (3)	1005 ± 160 (3)	4.04 ± 0.2 (2)
			2.1 ± 0.2 (central component)
Ribonuclease	-54 (3)	1080 ± 60 (3)	5.0 ± 1.0 (3)
Poly(L-lysine)	-57 (2)	790 ± 80 (2)	3.5 (1)
Lysozyme	-54 (2)	998 ± 44 (2)	4.0 ± 0.28 (2)
Cytochrome *c*	-52 (3)	693 ± 102 (3)	2.3 ± 0.14 (2)
		156 ± 13 (narrow component)	

[a] ± 1 S.D. based upon the number of experiments indicated in parenthesis.

[b] The values given are for the $\sigma_\perp$ component.

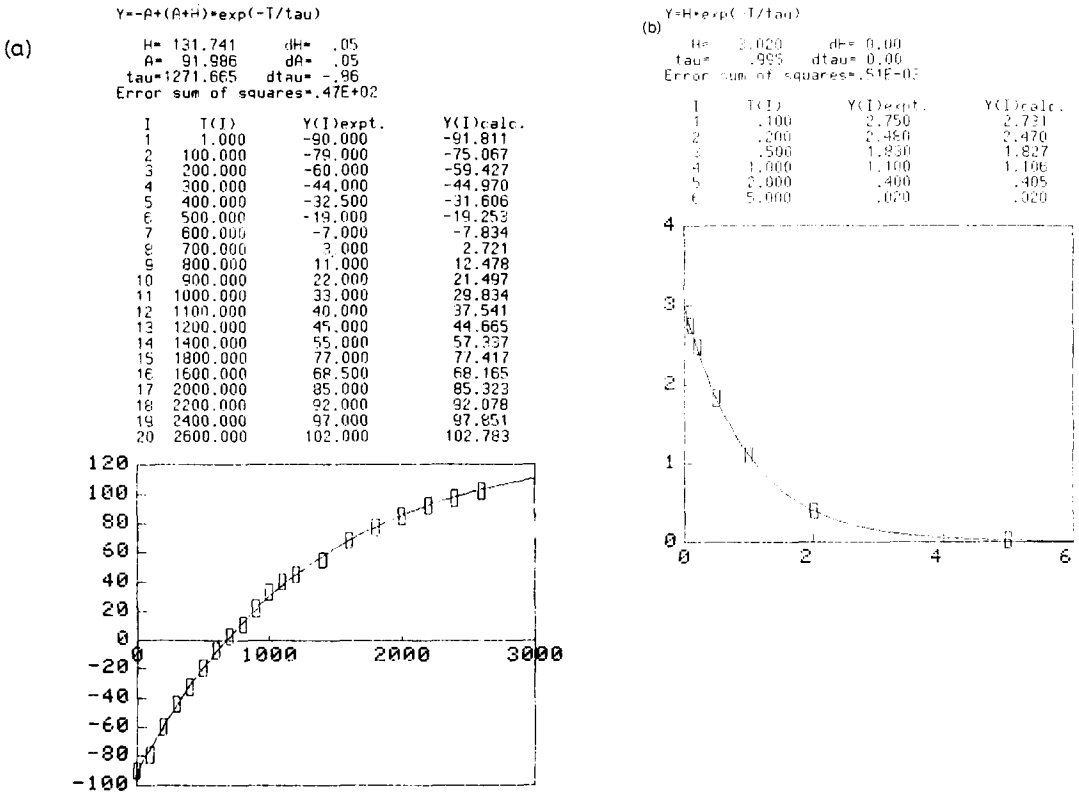

Fig. 2. Examples of ^{31}P relaxation functions obtained using the same conditions described in the caption to Fig. 1 but using either an inversion-recovery or spin echo pulse sequence. (a) Pure PS spin-lattice (T_1) relaxation function obtained using $180° \sim \tau \sim 90°$ inversion-recovery sequence; (b) pure PS spin-spin (T_2) relaxation function obtained for the $\sigma_\perp$ component of the spectrum using a $90° \sim \tau \sim 180° \sim \tau \sim$ FID pulse sequence.

Fe^{3+} ion to the phosphate group is of the order of 1 nm. Assuming a spin diffusion coefficient [10] of 10^{-17} m^2/s, the effects of spin diffusion are expected to be felt on relaxation times longer than approx. 50 ms.

This suggests that the shortened values of T_1 observed with the addition of cytochrome *c* to PS derive a substantial contribution from the paramagnetic relaxation of the Fe^{3+} centre which may dominate any effects arising from a change in conformation or the rate of reorientation of the phosphate group. This interpretation is supported by the insensitivity of T_2 to the addition of cytochrome *c*. The spin-spin relaxation time would be expected to shorten if the variation in T_1 arose from a dynamic effect. The reverse is not necessarily true, in that quite substantial changes may occur in T_2 or the chemical shift anisotropy and not be reflected in a change in T_1. This reflects the sensitivity of T_1 to motion on the timescale of 10^{-8} s, whereas T_2 is dominated by the slowest motion present in the system. The increases seen in T_2 with the addition of protein to PS indicate an increase in the orientation rate of the slower molecular motions undergone by the headgroup. In the case of poly(L-lysine), there is the additional effect that the intensity of motion on the shorter timescale of 10^{-8} s is slightly reduced. Examples of the spin-spin and spin-lattice relaxation functions are shown in Fig. 2.

Discussion

Ribonuclease, poly(L-lysine), lysozyme and cytochrome *c* all produce an increase in the chemical shift anisotropy of the ^{31}P headgroup resonance of PS. This indicated that the presence of these proteins causes a change in the conformation or the amplitude of motion of the lipid headgroup. In addition to changing the chemical shift anisotropy, they also produce an increase in T_2 although only poly(L-lysine) and cytochrome *c* have any significant effect on T_1. The changes produced by the protein are in all cases quite subtle and suggest a slight restriction in the amplitude of motion undergone by the headgroup in the presence of protein. This restriction is associated with a general increase in the rate at which the slower modes of reorientation undergone by the headgroup are occurring. By contrast, the more rapid rates of reorientation measured by T_1 are essentially unaltered by these proteins. The dramatic reduction in T_1 seen with cytochrome *c* is more likely to reflect the paramagnetism of the Fe^{3+} centre and not an effect on the dynamics of the lipid headgroup.

As shown in Fig. 1b, myelin basic protein induced a major conformational change in the PS phosphate group: in some spectra the central peak had a more clearly defined positive chemical shift anisotropy. Similar spectral changes are induced on formation of a hexagonal II phase structure [11] and on changing the phosphorus orientation in lamellar phase phospholipids [12]. The component of the phosphorus signal which remained with a negative chemical shift anisotropy and by implication as a largely unperturbed lamellar phase dispersion, behaved in much the same manner as did the PS dispersions mixed with the other proteins studied. Both the chemical shift anisotropy and T_2 increased. Again as with the PS-lysozyme and PS-ribonuclease dispersions, T_1 was essentially unaltered. The reason for the two-phase behaviour of dispersions containing the myelin basic protein is unclear. It is unlikely these effects arise from a gross inhomogeneity in the mixing of the lipid and protein [8].

The effects of these basic proteins may be compared with those of particular divalent ions (e.g., Ca^{2+}) which transform the spectrum to that expected from a rigid lattice and dramatically increase the value of T_1 [13]. The proteins we have studied approach more closely in their behaviour the monovalent cations and some divalent cations that act largely as counterions within the electrical double-layer [14–16].

The results for these water-soluble proteins may also be compared with those for intrinsic proteins. Several integral proteins have been shown to have small effects on the order and high frequency motion of lipid acyl chains [17–19]. But although similarly minor effects were observed at 121.4 MHz for the headgroups of dioleoylphosphatidylcholine and dielaidoylphosphatidylcholine in the presence of the proteins of sarcoplasmic reticulum [19], Oldfield and colleagues [17,20] have reported a 3–4-fold reduction in the 60.7 MHz ^{31}P T_1 and T_2 values for dimyristoylphosphatidylcholine in the presence of several intrinsic proteins.

Packing constraints, due to the presence of large amounts of intrinsic protein, may be more important in deciding the dynamic modes of the lipid headgroup than are ionic interactions between lipid and protein.

In conclusion, we compare the effects of the basic proteins on the motion of the lipid headgroup with their effects on other bilayer properties. Ribonuclease and poly(L-lysine) are widely considered to be held at the membrane surface solely by coulombic attractions to the negative lipids (see, however, Ref. 21). If this is so, then the NMR data suggest that their interaction parallels that of a counterion held in a potential well at the charged lipid bilayer surface, without long-lived associations between individual basic amino-acid sidechains and lipid headgroups. From the NMR data, the same conclusion could be reached for lysozyme, although under other conditions it has been described as interacting hydrophobically with lipids [22–24]. From the present results, the polar interactions appear to dominate the behaviour of the phosphate group.

Cytochrome *c* and myelin basic protein both lower the gel-to-liquid-crystalline phase-transition temperatures, increase ion permeation of bilayers and expand lipid monolayers [25]. These and other [23,24,26] results have been interpreted as deriving from hydrophobic interactions between lipids and these proteins, even though some studies have led to contrary conclusions [27]. The major changes in spectral shape induced by these proteins are indicative of a substantial difference in the mode of interaction of the myelin basic protein and the other basic proteins with lipids.

Finally we come to the paradoxical conclusion that the ionic interactions which occur between the basic proteins and the PS headgroups appear to have far less influence on the conformation and dynamics of these groups than do intrinsic membrane proteins which interact hydrophobically with the membrane lipid.

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