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Myelin basic protein induces hexagonal phase formation in dispersions of diacylphosphatidic acid

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^{31}P nuclear magnetic resonance and low-angle X-ray diffraction measurements have shown that the basic protein of myelin caused diacylphosphatidic acid dispersions to change from a lamellar to a hexagonal lipid organisation. Several other basic proteins failed to effect a similar phase change, and had little influence on phospholipid headgroup structure and motion.

The belief that lipid-protein interactions are important in the functioning of biological membranes has spurred intensive research into the effects of intrinsic membrane proteins on lipid bilayers. Extrinsic membrane proteins have received less attention.

In work reported recently [1] ^{31}P-NMR was used to examine the association of several basic proteins with multilayers of diacylphosphatidylserine (diacylPS). Of the proteins studied, only myelin basic protein caused major changes in the lipid headgroups, inducing the formation of a second spectral component in slow exchange, on the NMR timescale, with the bulk lipid. Several other proteins, although apparently bound to a similar extent, had only minor effects on the chemical shift anisotropy, the spin-spin relaxation time, and the spin-lattice relaxation time of the phosphorus atom.

This study raised two questions: (i) Was the lack of effect of most of the proteins a result of their binding to the serine carboxylate group, with rotational isomerizations in the intervening bonds isolating the observed phosphorus atom from the effects of motional restriction? (ii) Was the spectral change observed with myelin basic protein attributable to changes in phospholipid headgroup structure or motion, or to a change in lipid organisation? Subsequent experiments with diacylphosphosphatidic acid have provided answers to these questions.

Diacylphosphatidic acid was prepared by phospholipase D digestion [2] of acetone-precipitated diacylphosphatidylcholine (diacylPC) isolated from egg yolks [3]. The crude diacylphosphatidic acid was purified by successive passage through columns (2.5 cm × 30 cm) of silica gel G (E. Merck, Darmstadt, F.R.G., 70–230 mesh) equilibrated with chloroform/methanol/ammonia (65 : 25 : 5, v/v) and chloroform/methanol/water (65 : 25 : 4, v/v). The lipid purity was examined by TLC in the same solvent systems. Myelin basic protein was prepared from fresh bovine brains [4]. Egg-white lysozyme (Grade I), calf thymus histone (Type IIS), and poly(L-lysine) hydrobromide (Type V, M_r 17000) were purchased from Sigma Chemical Co., MO, U.S.A. The lipid-protein recombinants were prepared as described previously [1].

Abbreviations: diacylPA, diacylphosphatidic acid; diacylPS, diacylphosphatidylserine; diacylPC, diacylphosphatidylcholine; diacylPG, diacylphosphatidylglycerol.

The sedimented lipid and lipid-protein pellets were transferred to plastic Beem standard electronmicroscopy embedding capsules using a spatula, and were then sealed under nitrogen. ^{31}P-NMR spectra were recorded at 121.44 MHz on a Bruker CXP300 spectrometer. Most spectra were obtained using a 90°-FID pulse sequence, with a 90° pulse of 8–10 μs, a repetition time of 2 s or 10 s and 25 kHz (a proton 90° pulse length of 10 μs) of proton decoupling field. Chemical shift positions were measured relative to phosphoric acid. Samples prepared for low-angle X-ray diffraction were loaded into capillary tubes of 0.5 mm diameter and 0.01 mm wall thickness (Karl Hilgenberg Glaswaren Fabrik, D-3509 Malsfeld, F.R.G.). The diacylphosphatidic acid X-ray diffraction patterns were recorded on film at 298 K using a Hilger-Watts focussed CuK_α X-ray source and a pin-hole camera. The myelin basic protein-diacylphosphatidic acid patterns were kindly obtained by Dr. P.M. Colman, C.S.I.R.O., Division of Protein Chemistry, Melbourne, using a rotating anode generator operated at 40 kV and 21 mA with a mirror monochromator camera having a specimen to film spacing of 400 mm and a 100 μm focussed beam.

In macroscopic appearance, sedimentation behaviour and the extent of protein binding, the protein-diacylphosphatidic acid complexes were similar to those prepared with diacylPS. After mixing the lipid and protein thoroughly, the proportion of protein bound to diacylphosphatidic acid was determined by sedimentation of the recombinants on a sucrose density gradient and analysis of the supernatants for unbound protein [5] and unsedimented lipid [6]. In the presence of the proteins no significant amounts of lipid were detected in the supernatants. When equal weights of protein and lipid were initially mixed the protein bound exceeded 75% of that added. Resuspension and recentrifugation of the pellets in protein-free buffers showed little dissociation of the proteins from the lipid.

As shown in Fig. 1, ribonuclease, lysozyme and poly(L-lysine) all increased the chemical shift anisotropy of the phosphorus nuclei at pH 7.5, yet had little effect on the NMR lineshapes. A summary of the chemical shift anisotropies is shown in Table I. Chemical shift anisotropy increases of similar magnitude were observed on binding of these proteins to diacylPS [1]. Although these proteins may bind to the carboxylate or phosphate groups of diacylPS, any electrostatic attraction can only to be to the phosphate group of diacylphosphatidic acid. Hence, even when the phosphate group is directly involved in binding the above three polypeptides, its structure and

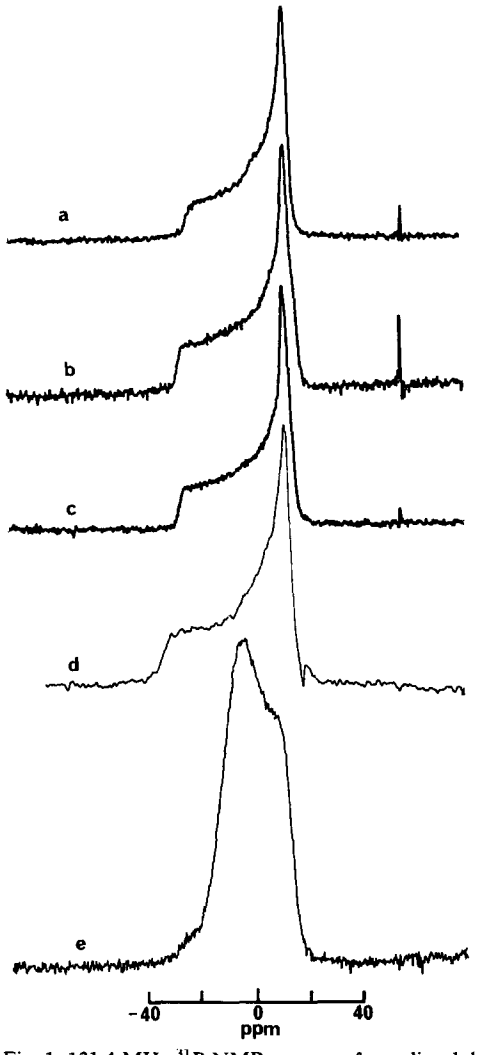

Fig. 1. 121.4 MHz ^{31}P-NMR spectra of egg diacylphosphatidic acid (diacylPA) in 0.1 M pH 7.5 Tris-HCl buffer containing 2 mM EDTA. (a) DiacylPA alone; (b) ribonuclease/diacylPA, 0.71:1 weight ratio; (c) lysozyme/diacylPA, 0.92:1; (d) poly(L-lysine)/diacylPA, 0.75:1; (e) myelin basic protein/diacylPA, 0.82:1. 1000 scans were accumulated at 293 K using 74–78 mg lipid: no linebroadening was employed in plotting spectra (a)–(c), but 50 Hz line broadening was used for (d), and 5 Hz for (e).

TABLE I

^{31}P CHEMICAL SHIFT ANISOTROPIES (CSA) FOR MULTILAMELLAR DISPERSIONS OF PHOSPHATIDIC ACID WITH BASIC PROTEINS, OBTAINED AT 300 K

The standard deviations of the CSA values were less than 2 ppm. The values for all proteins except myelin basic protein were for dispersions containing a protein-to-lipid weight ratio of 0.8 ± 0.2. All samples were dispersed in pH 7.5 0.1 M Tris-HCl/2 mM EDTA.

Protein added	CSA (ppm)
Pure phosphatidic acid	36
Ribonuclease	38
Lysozyme	38
Poly(L-lysine)	50
Myelin basic protein, 0.27 g/g lipid	43 [a]
Myelin basic protein, 0.57 g/g lipid	40 [a]
Myelin basic protein, 0.89 g/g lipid	40 [a]
Myelin basic protein, 1.43 g/g lipid	36 [a]

[a] The apparent chemical shift anisotropy of the remaining lamellar component only is given.

motion are not greatly altered. The results with diacylPS and diacylphosphatidic acid are consistent with the view that these polypeptides are bound by non-specific electrostatic attraction, effectively as counterions in an electrical double layer.

In contrast with the above proteins, ^{31}P-NMR spectra of myelin basic protein-diacylphosphatidic acid dispersions showed an additional spectral component that peaked 5.5–6 ppm downfield from phosphoric acid, and 3.7–4.2 ppm from the isotropic resonance of small single bilayer vesicles of diacylphosphatidic acid at pH 7.5. This peak was superimposed on the spectrum of pure diacylphosphatidic in buffer (Fig. 1e). As the protein content of the dispersions was raised from 0.27 to 1.43 g per g of diacylphosphatidic acid the intensity of the central peak increased (Fig. 2).

Dispersions of pure diacylphosphatidic acid display a similar spectrum near pH 4.5 (Fig. 3). As shown earlier by others [7,8], at pH 7.5 a characteristic lamellar phase spectrum is seen which at pH 3.5 develops into a narrower peak with reversed anisotropy, typical of a hexagonal phase lipid. At intermediate pH values the ^{31}P-NMR spectrum has a complex shape which appears to result from a superposition of the hexagonal and

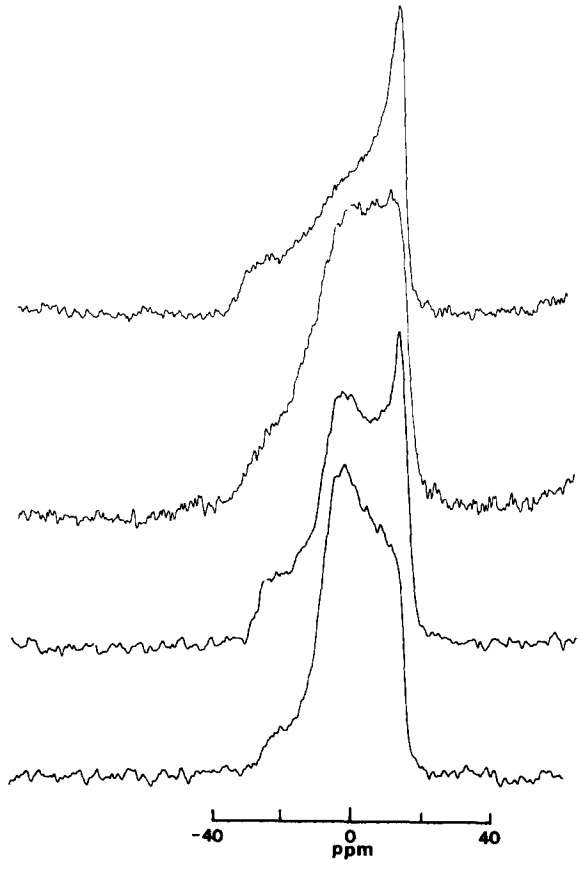

Fig. 2. Changes in the ^{31}P-NMR spectrum of diacylphosphatidic acid as a function of the amount of myelin basic protein bound at pH 7.5 in 0.1 M Tris-HCl buffer containing 2 mM EDTA. The bound protein/lipid weight ratios were, from top to bottom: 0.27, 0.57, 0.89 and 1.43. Line broadenings employed in plotting the spectra were 50 Hz (top two spectra) and 100 Hz. About 1000 scans were collected using 65–75 mg diacylphosphatidic acid. These spectra, and those in Fig. 1, were accumulated using a pulse repetition time of 2 s, but spectra recorded with a repetition time of 10 s were unchanged in shape.

lamellar phase spectra. Such changes in the ^{31}P-NMR spectrum of diacylphosphatidic acid may have several origins: they may result from changes in the chemical shielding tensor of the phosphorus atom, from a conformational change of the phosphate group with respect to the remainder of the lipid molecule, from changes in the freedom of motion of the phosphate group, or from increased whole-lipid motion in a non-bilayer (e.g. hexagonal) phase.

A common origin for the change in the diacylphosphatidic acid spectrum on adding myelin

basic protein and on lowering the pH to 3–5 is apparent in the low angle X-ray diffraction results of Table II. Above pH 5 a weak lamellar diffraction pattern was obtained for diacylphosphatidic acid: the lipid appears to be organised into bilayers which stack with poor long-range order into multilayers, resulting in a weaker diffraction pattern than one obtains, for example, with diacylphosphatidylcholines. Below pH 5 a clear hexagonal II (H_{II}) phase pattern emerged with the characteristic $1:\sqrt{3}:\sqrt{4}:\sqrt{7}:\sqrt{9}$ spacing [9] of the diffracting planes.

With the initial addition of MBP the intensity of the lamellar phase diffraction pattern increased without a significant alteration of the repeat spacings observed for diacylphosphatidic acid dispersions. However, at pH 7.5 diacylphosphatidic acid dispersions containing a 1 : 1 lipid : protein (w/w) ratio gave a diffraction pattern that could not be indexed as Bragg reflections from one or more lamellar arrays. The line spacings were consistent with the co-existence of a lamellar phase and a hexagonal phase. Thus, the appearance of the second NMR spectral component, as with pure diacylphosphatidic acid at pH 4.5, is attributable to formation of hexagonal phase at pH 7.5 in the presence of myelin basic protein, with an exchange of lipids between the two phases that is slow on the ^{31}P-NMR timescale ($> 10^{-3}$ s). Two component spectra have also been obtained with very high concentrations of the hydrophobic gramicidin A peptide in diacylPC dispersions [13] and for cytochrome *c*/cardiolipin dispersions [18], again due to the formation of a hexagonal phase.

These results need to be contrasted with the outcome of X-ray scattering experiments performed on solutions of diacylphosphatidylglycerol (diacylPG) dispersed with myelin basic protein. It has been reported that myelin basic protein causes an enhancement of the long-range order in dispersions of diacylPG [10,11]. However, these authors found no evidence for a transition to a non-lamellar phase even for samples containing protein concentrations to 45 : 55 protein : lipid by weight. A similar conclusion was drawn from recent ^{31}P-NMR experiments on diacylPG-myelin basic protein dispersions [12].

Why, of these basic proteins, does myelin basic protein alone induce hexagonal phase formation in diacylphosphatidic acid (and probably diacylPS) dispersions? The headgroup area of diacylphosphatidic acid is small compared with the combined cross-sectional area of the two acyl chains. Reduction of the electrostatic repulsion between these headgroups further reduces their effective headgroup area, favouring the phase transition to H_{II}. The pK values of the diacylphosphatidic acid phosphate group are near 3.5 and 8 [14], hence at pH values approaching 3.5 the interheadgroup repulsion diminishes, favour-

TABLE II

LONG SPACINGS DEDUCED FROM LOW-ANGLE X-RAY DIFFRACTION PATTERNS OF DIACYLPHOSPHATIDIC ACID DISPERSIONS

Sample	Diffraction band (nm)							
Diacylphosphatidic acid [b]								
pH 3.5	7.11	–	4.11	3.56	–	–	–	–
pH 4.0	6.16	–	3.63	3.12	–	–	–	–
pH 4.5	6.66	– [a]	3.79	3.24	– [a]	–	–	–
pH 7.5	–	9.24		–	4.62	–	–	–
Myelin basic protein (pH 7.5)								
lamellar		5.60			2.82			1.87
hexagonal	6.62		3.80	3.30		2.50	2.24	
Ratio of spacings								
lamellar		1			1/2			1/3
hexagonal	1		$1/\sqrt{3}$	1/2		$1/\sqrt{7}$	1/3	

[a] Although the presence of lamellar phase lipid was indicated by NMR, the diffraction lines for this phase were not observed.
[b] Dispersions were prepared in 0.1 M acetate, 2 mM EDTA, 2 mM sodium azide titrated with HCl or acetic acid to pH 3.5, 4.0 or 4.5 and in 0.1 M Tris-HCl, 2 mM EDTA, 2 mM sodium azide, pH 7.5.

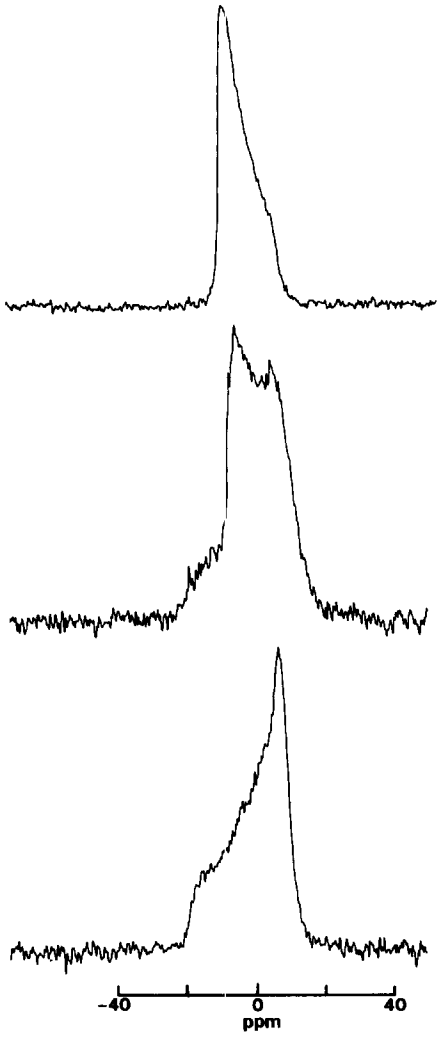

Fig. 3. Effect of pH on the spectrum of diacylphosphatidic acid. Each sample contained 75 mg of diacylphosphatidic acid in 0.1 M acetate buffer containing 2 mM EDTA at pH 3.5 (top), pH 4.5 (middle) or pH 5.5 (bottom). The spectra were obtained with 800–1100 pulses and plotted with 25 Hz line broadening. Diacylphosphatidic acid is unstable at low pH. However, ^{31}P-NMR spectra recorded within two hours of adding pH 3.5 buffer showed a clear hexagonal phase spectrum which was unaltered after several hours near room temperature. TLC in chloroform/methanol/ammonia (see Methods) showed no lipid degradation after two hours at pH 3.5.

ing the observed phase transition (see above, and Refs. 7 and 8). At pH 7.5 myelin basic protein mimics the effect of lowering the pH of pure diacylphosphatidic acid dispersions, suggesting that it too lowers the electrostatic repulsion between the phosphate groups. There is evidence from X-ray diffraction [15] and ESR [16,17] studies that myelin basic protein penetrates the headgroup region of phospholipid bilayers, which may result in positioning of basic amino acid side chains within the plane of the phosphate groups. By contrast, the other basic proteins may be anchored in an electrical double layer above the plane of the phosphate groups and be ineffective in screening the electrostatic repulsions between lipid headgroups.

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