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^{1}H-NUCLEAR MAGNETIC RESONANCE STUDY OF THE ASSOCIATION OF THE BASIC PROTEIN OF CENTRAL NERVOUS SYSTEM MYELIN WITH LYSOPHOSPHATIDYLCHOLINE

Ross SMITH *

Biochemistry Department, La Trobe University, Bundoora, Victoria 3083, Australia

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High-resolution 270 MHz ^{1}H-nuclear magnetic resonance spectroscopy has been used to follow the interaction of myristoyllysophosphatidylcholine with bovine myelin basic protein. At lipid/protein ratios up to 30:1 it proved possible to follow changes in the spectra of both the protein and the lipid. Lysophosphatidylcholine induced several changes in the protein spectrum. Foremost amongst these changes were downfield shifts of histidine C_2 protons, and upfield shifts and broadening of the phenylalanine aromatic protons. Several other resonances assigned to nonpolar amino acid side chains also broadened. But even at a lipid/protein molar ratio of 30:1 the majority of the protein appeared to remain in a loosely coiled conformation. In the presence of the protein the lipid acyl chain peaks were moved upfield and broadened, whereas the resonances associated with the head-group protons were unaffected. These changes were consistent with partial immobilization of the acyl chain of lysophosphatidylcholine on binding to the basic protein, with hydrophobic interactions providing the predominant attraction between this lipid and the basic protein.

1. Introduction

Several proteins that are considered to be primarily extrinsic membrane proteins have been postulated to be capable of interacting hydrophobically with lipid bilayers. Examples include the basic protein from myelin [1] and cytochrome *c* [2]. Some of the controversy surrounding these proposals has arisen from the difficulty in demonstrating hydrophobic interactions by a small section of polypeptide. So, for example, the proposed hydrophobic interaction of the basic protein is supported by indirect evidence such as monolayer expansion [3], lowering of the gel-to-liquid-crystalline phase transition temperature [4], and NMR observations of the effect of this protein on the motion of lipid acyl chains in bilayers [5]. With these techniques the effect of the protein is inferred from changes in the average properties of the whole lipid population, which in each instance could arise from protein-induced alterations of lipid head-group packing rather than from a direct association with the hydrocarbon chains.

To provide more direct information on the nature of the interaction between lipid and protein the association of myelin basic protein with small numbers of molecules of MLPC has been examined. The relatively high critical micelle concentration of this amphiphile permits examination of the binding of monomers to the protein, an approach which is not possible with bilayer-forming lipids. At low lipid/protein ratios it is also possible to follow changes in the protein spectrum: this is not possible when the protein is bound to slowly diffusing, multilamellar vesicles, as the protein spectrum is then broadened beyond detection.

* Present address: Department of Biochemistry, University of Queensland, St. Lucia, Qld 4067, Australia.

Abbreviation: MLPC, myristoyllysophosphatidylcholine

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2. Methods

Bovine myelin basic protein was isolated using the standard technique of acid extraction of a chloroform/methanol precipitate from fresh white matter. It was purified by chromatography on Sephadex G-75 and carboxymethylcellulose [6]. MLPC was purchased from Sigma Chemical Co. (St. Louis, U.S.A.) and used without further purification. ^2H_2O (99.75%) was obtained from the Australian Atomic Energy Commission (Lucas Heights, Australia).

Protein and detergent were separately dissolved in ^2H_2O containing 2 mM sodium azide. These solutions were titrated to a p*D* of 7.7, with correction for the deuterium isotope effect on the glass electrode, and then freeze dried to remove the residual H_2O. After redissolution in ^2H_2O the protein and detergent solutions were mixed with additional ^2H_2O to obtain the desired concentrations and protein/detergent ratios. In some experiments the solutions also contained 0.005 M phosphate buffer.

^{1}H-NMR experiments were performed at 30°C on a Bruker HX 270 (at the National NMR Centre, Canberra) operating at 270 MHz with suppression of the water peak. Chemical shifts were referenced to the H^2HO resonance at 4.746 ppm relative to dioxane at 3.741 ppm. For each spectrum about 2000 transients were collected over 8192 data points using a pulse time of 10 μs, a spectral width of 3623 Hz and an acquisition time of 1.13 s. Each set of spectra was plotted using identical scaling factors to facilitate comparison of peak intensities and plotting of difference spectra. For many of the spectra resolution was also enhanced by computation of convolution-difference spectra.

Carr-Purcell A pulse sequences and pH titrations were used to identify some of the protein aromatic proton resonances.

3. Results

3.1. MLPC spectrum

A typical ^{1}H-NMR spectrum of MLPC is given in fig. 1. The resonance assignments, deduced from previous reports for related compounds [7,8], are listed in table 1.

Table 1

Chemical shift assignments for MLPC in ^2H_2O

Resonance: in fig. 1. Chemical shift: in ppm downfield from tetramethylsilane. The estimated error is 0.01 ppm except for values denoted by an asterisk which have an estimated error of 0.02. Assignments were based on published data [7,8].

Resonance	Assignment	Chemical shift
1	CH_2-O-P	4.33
2	CH_2-O-CO	4.17*
3	CH-OH	4.05*
4	CH-CH_2-O-P	3.92*
5	CH_2-N	3.70
6	N-CH_3	3.25
7	CH_2-COO	2.41
8	CH-CH_2-COO	1.62
9	$(CH_2)_n$	1.30
10	*t*-CH_3	0.88

The intensities of the two strongest resonances, the *N*-methyl protons and the acyl chain methylene protons, correlated closely with the MLPC concentration. More importantly, the chemical shifts of all resonances were invariant over the concentration range 1.1–16.7 mM. This range extends from near the critical micelle concentration to values well above it, showing that incorporation of these molecules into micelles did not alter the resonant frequencies.

Identical results were obtained in solutions containing additionally 0.05 M phosphate and 0.2 M sodium chloride, also at p*D* 7.7.

3.2. Effect of basic protein on the spectrum of MLPC

Spectra were recorded for the protein, MLPC and solutions containing both molecules at lipid/protein molar ratios from 0.25 : 1 to 30 : 1. To facilitate plotting of difference spectra the experiments were performed with identical amounts and concentrations of protein, and the same scaling factors were used in calculating the spectra.

At low MLPC/protein ratios several of the lipid resonances were obscured by those from the protein but the *N*-methyl proton resonance remained sharply resolved with its intensity ap-

proximately proportional to its concentration. The methylene resonance at low lipid/protein ratios (up to 4 : 1) was markedly diminished in intensity and moved upfield (fig. 2). At higher lipid concentrations two of the other head-group resonances, those arising from CH_2-O-P and CH_2-N, were detected at their usual chemical shifts. The methylene resonance was broadened and increased in intensity but remained upfield from its normal position: this is illustrated in fig. 3 which shows the difference spectra obtained at lipid/protein ratios from 3 : 1 to 30 : 1. At high lipid/protein ratios other peaks from the acyl chains (Nos. 7, 8 and 10 in table 1) also appeared at positions just upfield from their normal locations. The *N*-methyl peak remained unattenuated and unshifted.

Only a single methylene resonance was observed at all MLPC/protein ratios. This resonance was shifted upfield from the position in free MLPC (1.304 ppm), to 1.211 ppm at a 2 : 1 lipid/protein ratio and the chemical shift difference decreased to 0.057 ppm at the highest ratio used (30 : 1). Thus, the exchange of bound and free MLPC is rapid on the NMR time scale. If it is assumed that at a MLPC/protein ratio of 2 : 1, the lowest that yields a clearly assignable methylene resonance, the ob-

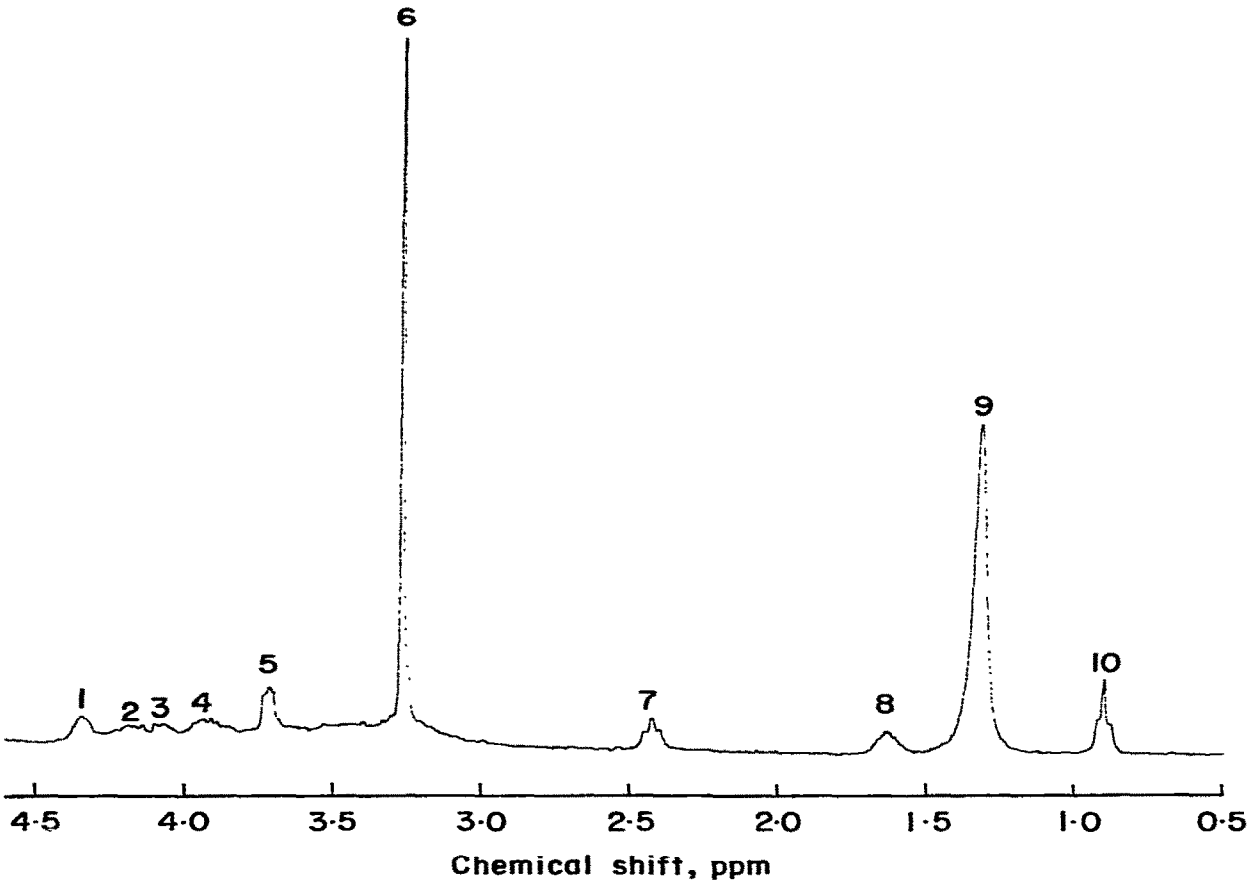

Fig. 1. 270 MHz ^{1}H-NMR spectrum of a 0.8 g/l solution of MLPC in ^2H_2O, p*D* 7.7. The peak assignments are presented in table 1.

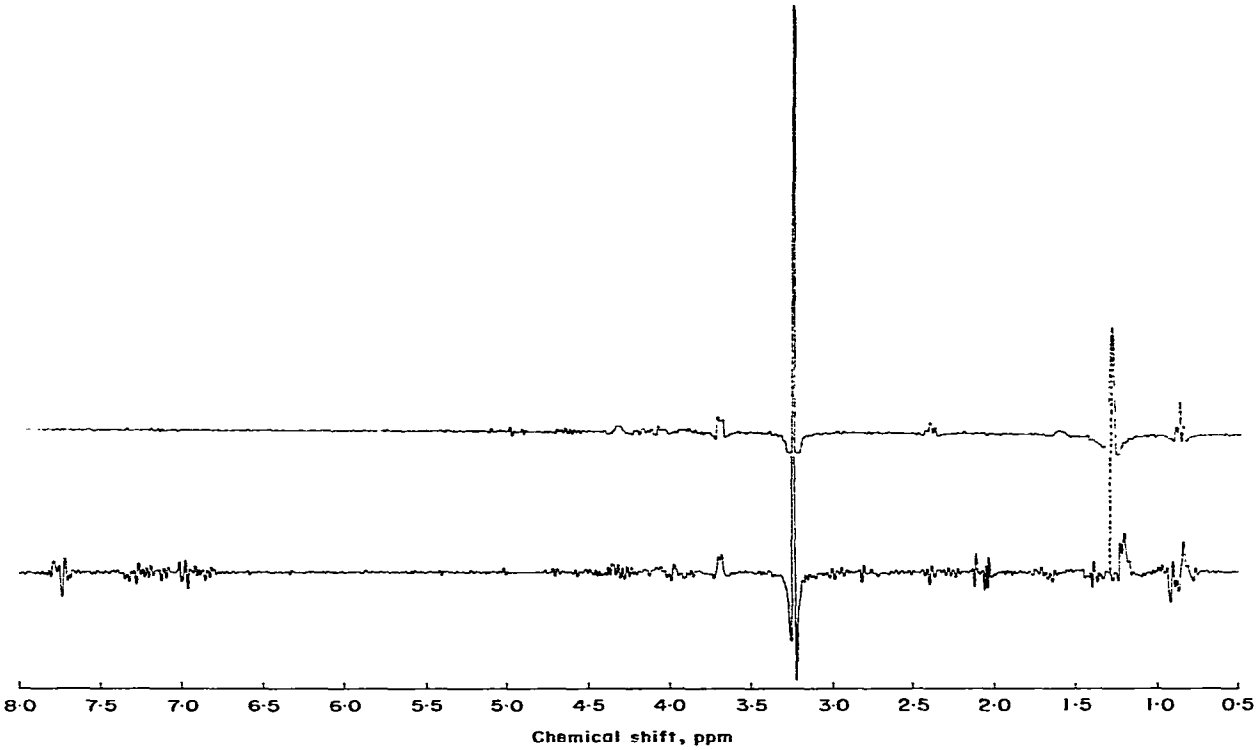

Fig. 2. Effect of basic protein on the spectrum of MLPC. At the bottom is the spectrum obtained by subtracting the resolution-enhanced spectrum for basic protein alone from that for 4:1 MLPC/basic protein: both samples contained an identical amount of protein. At the top, for comparison, is a resolution-enhanced spectrum of MLPC. The *N*-methyl resonances at 3.25 ppm have identical chemical shifts and are therefore superimposed in this diagram.

served chemical shift is predominantly that of bound MLPC then the chemical shift changes may be interpreted in terms of the proportion of added MLPC that is protein bound. At MLPC/protein ratios of 6:1 or less, greater than 80% of the lipid is bound, but a smaller proportion is bound at higher MLPC concentrations.

The methylene resonance of MLPC is in a spectral region which is free of intense protein resonances (cf. figs. 1 and 5). An indication of the changes in intensity of the methylene resonance on binding to the protein can therefore be obtained from difference spectra obtained by subtracting the unenhanced protein spectrum from that for the MLPC/protein solution. At low MLPC/protein ratios (up to about 3:1) the intensity of the lipid methylene resonance, with respect to the *N*-methyl peak, is far lower than in the free lipid. It appears that in the presence of the protein the environment of the lipid acyl chains is altered and their motion restricted. In contrast, the MLPC head group appears relatively unimpeded as none of its resolvable resonances is shifted or significantly broadened.

3.3. Effect of MLPC on the spectrum of basic protein

A resolution-enhanced spectrum of basic protein is given in fig. 4 with assignments in table 2 [10].

Addition of MLPC caused selective broadening of some of the peaks. Although MLPC enhances the self-association of the protein and should thus increase the rotational correlation time for the

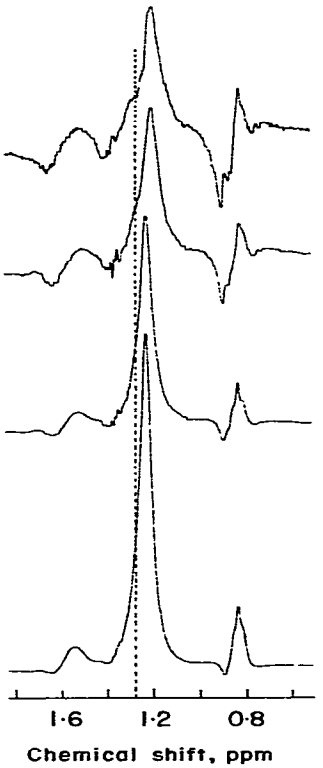

Fig. 3. Comparison of the lipid methylene regions of spectra obtained at MLPC/protein molar ratios of 3 : 1, 6 : 1, 15 : 1 and 30 : 1 (from top to bottom). These spectra were obtained by subtracting the unenhanced protein spectrum from equivalent spectra for lipid/protein solutions. All samples contained 10.1 g/l basic protein. The vertical dotted line marks the chemical shift of the methylene peak in the absence of the protein at the same p*D*. The spectra were scaled to give approximately equal heights for the *N*-methyl peak.

entire protein, a more general broadening of the protein spectrum occurred only at the highest lipid concentrations. Nor was there any overall loss in intensity of the protein spectrum, at least up to a lipid/protein ratio of 6 : 1.

Interpretation of some of the spectral changes

Table 2

Chemical shift assignments for bovine myelin basic protein in ^2H_2O at p*D* 7.7

Chemical shift: in ppm downfield from tetramethylsilane.

Resonance assignment	Chemical shift
1 histidine, C_2-H	7.70–7.80
2 tryptophan, indole C_4-H	7.60
3 tryptophan, indole C_7-H	7.40
4 phenylalanine, aromatic	7.30
5 tyrosine, C_2-H and C_6-H (*meta*)	7.09
6 histidine, C_4-H	6.96
7 tyrosine, C_3-H and C_5-H (*ortho*)	6.81
8 lysine, ε-CH_2; phenylalanine, β-CH_2; tyrosine, β-CH_2	2.97–2.99
9 arginine, N^G-methyl	2.79
10 methionine-19, methyl	2.04
11 leucine β-CH_2 and γ-CH; lysine, δ-CH_2 and β-CH_2	1.63–1.68
12 leucine, methyl	0.90

was complicated by overlap of lipid and protein resonances. However, the methyl peaks arising primarily from leucine (resonance 12 in fig. 4) and methionine-19 methyl (resonance 10 in fig. 4) were consistently broadened even at a 1 : 1 MLPC/protein ratio. These changes are manifested in fig. 5 as decreases in the heights of these resonances relative to the other peaks in the spectrum.

In the aromatic region the histidine C_2 proton peaks near 7.8 ppm were shifted downfield by about 0.03 ppm at a 6 : 1 lipid/protein ratio, the shift increasing to 0.14 ppm at a ratio of 30 : 1, and the phenylalanine peaks at 7.3 ppm were lowered in intensity. The C_2-H and C_6-H protons of the four tyrosine residues give a pair of resonances centred at 7.09 ppm and the C_3 and the C_5 protons contribute two more peaks of similar intensity centred at 6.81 ppm. Although these pairs of peaks are doublets arising from spin-spin coupling, the downfield peak in each set was consistently reduced in intensity on addition of MLPC, presumably because of chemical shift heterogeneity of the tyrosine protons.

Several other minor changes were observed. Two low-intensity doublets centered at 7.60 and 7.67 ppm were removed by the lipid: on the basis of the assignments of McDonald and Phillips [9] the

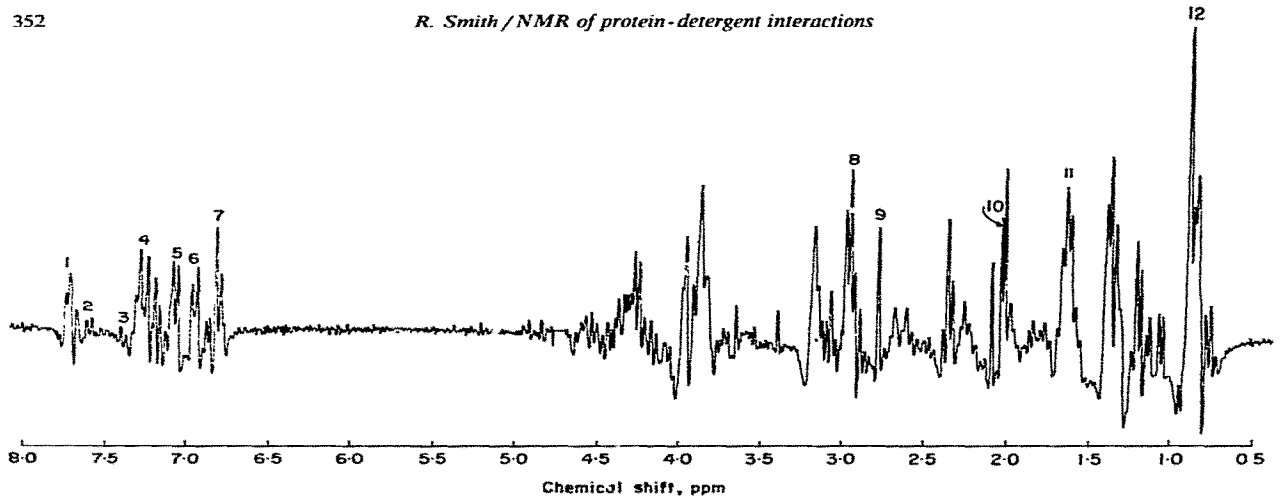

Fig. 4. ^{1}H-NMR spectrum for a 10.2 g/l bovine myelin basic protein at p*D* 7.7. The resolution in this spectrum was enhanced using convolution difference methods. The assignments of the numbered peaks are given in table 2.

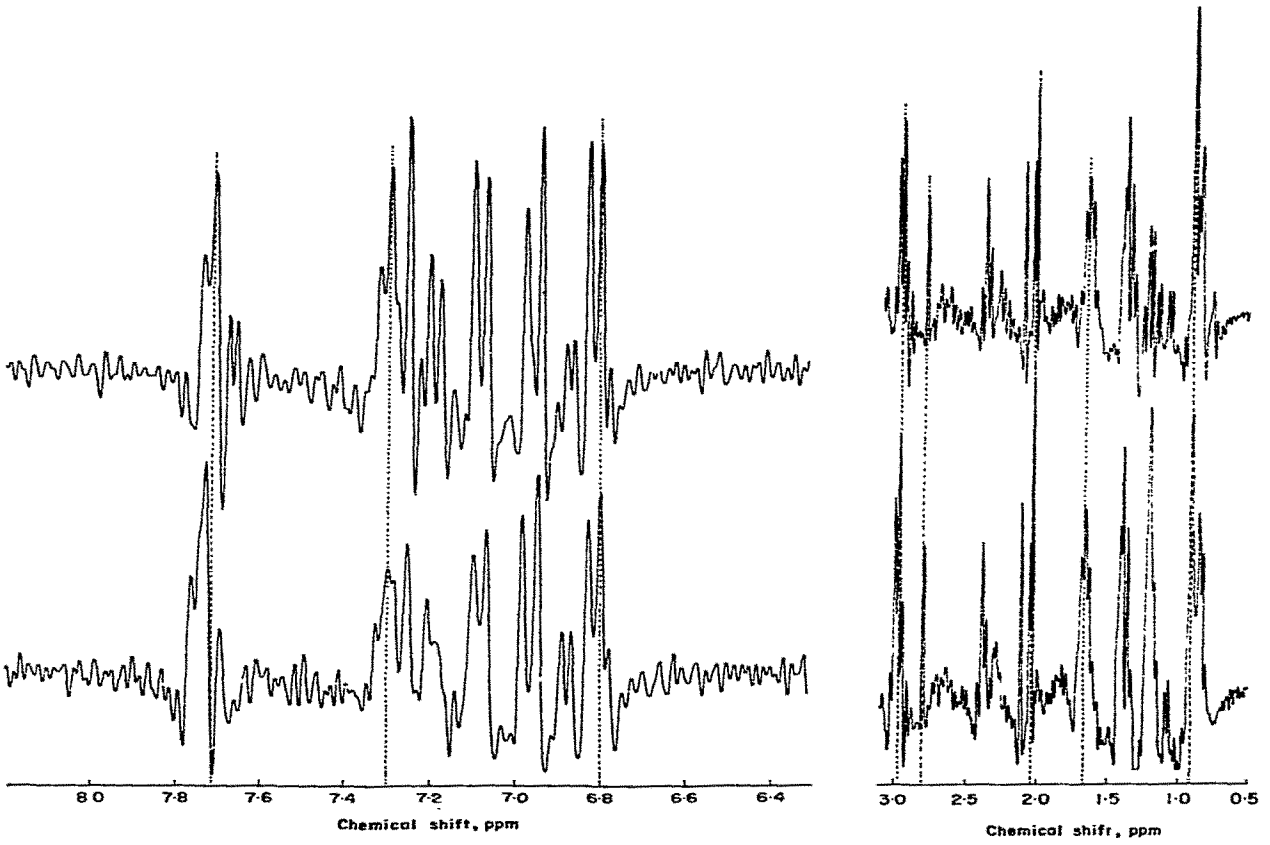

Fig. 5. The effect of MLPC on the resonances of basic protein. At left the aromatic regions are shown, with the aliphatic region at right. In both sets the upper spectrum is for basic protein alone and the lower for 4:1 MLPC/basic protein. The vertical dotted lines are included to assist comparison of the spectra.

former derived from the C_4 indole protons of the single tryptophan residue. Several side-chain methylene resonances near 2.98 and 1.65 ppm were also slightly perturbed. But at MLPC/protein ratios up to 6:1 there was no change in the N^G-methyl arginine peak at 2.79 ppm: this is in contrast to the results of Littlemore and Ledeen [11], obtained at high MLPC concentrations.

Many of the above changes are evident in the spectra in fig. 5. Several of the changes involve non-polar side chains of the protein which may interact with the lipid acyl chain, but modifications in the environments of these groups could also have resulted from the conformational change induced by lipid.

3.4. Effect of sodium chloride

Changes in the lipid spectrum parallel to those described above were obtained in the presence of 0.03 M phosphate and 0.1 M sodium chloride at p*D* 7.7. But the protein spectrum was less well resolved, obscuring the spectral effects of MLPC. Addition of 0.2 M sodium chloride at the protein concentrations used (10 g/l) caused partial precipitation of the protein-lipid complex.

4. Discussion

It has recently been shown that MLPC at low lipid/protein molar ratios promotes protein self-association [6] and induces a coil-to-helix transition in part of the polypeptide chain [15]. Hydrophobic interactions clearly dominate the attraction between protein and lipid. Only the resonances assigned to the lipid acyl chain were noticeably modified by the interaction. The lack of restriction of the head-group protons is consistent with the observation of Littlemore and Ledeen [11] that, at high lipid/protein ratios, the average MLPC phosphorus atom was more mobile in the presence of basic protein.

Many of the protein resonances influenced by MLPC arise from non-polar amino acids. Similar involvement of aromatic or non-polar amino acids has been seen on interaction of basic protein with dodecyl sulphate [1,12,13] and phosphatidylserine vesicles [14]. Some of these non-polar residues could be in contact with the acyl chains of these lipids. But, on the basis of existing evidence, it cannot be determined whether they interact directly with lipid molecules or are involved in the intramolecular and intermolecular protein-protein interactions that are promoted by lipids [6,15].

In accord with previous monolayer studies [16], it was the N-terminal half of the protein that appeared to interact hydrophobically with MLPC: methionine-19 (labelled methionine-20 in ref. 10) but not methionine-166 was modified at low MLPC/protein ratios. But, whereas Boggs et al. [17] have reported that both N- and C-terminal fragments generated from basic protein by cleavage at the sole tryptophan residue are able to bind to lipid vesicles through hydrophobic bonds, a recent ^{13}C-NMR study has been interpreted as locating both methionine residues at the surface of lipid vesicles [18,19].

Although the C_2 proton resonances of histidine moved progressively downfield as the MLPC/protein ratio increased, the association of the uncharged imidazole group, which is generally described as hydrophilic [22] or weakly hydrophobic [23], with the hydrocarbon chain of MLPC appears unlikely. Given that the self-association of the protein and the aggregation of protein-coated phosphatidylcholine vesicles have been shown to be dependent on deprotonation of histidine residues [20,21] they may instead be influenced by binding to other protein molecules.

The relatively small changes in the protein spectrum show that the majority of the polypeptide retains the coil-like conformation that it adopts in the absence of lipid [10,15,24], with MLPC causing only a small segment to become helical [15]. The high affinity of the basic protein for MLPC is possibly associated with this small segment of the molecule, which would also be likely to interact with the hydrophobic core of lipid bilayers.

In conclusion, the use of low concentrations of single-chain amphiphile offers advantages over lipid micelles or vesicles. At low molar ratios the protein spectrum may be observed with minimal interference from the lipid spectrum. Also, it is possible to detect modifications to the lipid spectrum which are not averaged over a large popu-

lation of perturbed and unperturbed molecules as in large lipid aggregates. In the present study this has afforded a strikingly clear demonstration of the involvement of the hydrocarbon chain of the lipid in the binding between MLPC and the basic protein.

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