^{2}H NMR Studies of the Effect of Pulmonary Surfactant SP-C on the 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine Headgroup: A Model for Transbilayer Peptides in Surfactant and Biological Membranes†

M. R. Morrow,‡ S. Taneva,§ G. A. Simatos,§ L. A. Allwood,§ and K. M. W. Keough*,§,‖

Department of Biochemistry, Discipline of Pediatrics, and Department of Physics, Memorial University of Newfoundland, St. John's, Newfoundland, Canada A1B 3X7

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ABSTRACT: Surfactant protein C (SP-C) was isolated from solvent extracts of porcine pulmonary surfactant by gel filtration chromatography. The surfactant protein was combined with dipalmitoylphosphatidylcholine deuterated at the α and β positions of the choline headgroup (DPPC-d_4) Deuterium nuclear magnetic resonance spectra were collected as a function of temperature for a series of protein concentrations. The splitting of the α-deuteron spectrum in the liquid-crystalline phase was insensitive to temperature but decreased with increasing protein concentration. The response of headgroup conformation to protein concentration was consistent with an interaction between the lipid headgroup dipole and the net positive surface charge associated with the protein. The observed effect per charge on the α splitting was less than that reported for singly-charged amphiphiles [Scherer, P. G., & Seelig, J. (1989) *Biochemistry 28*, 7720–7728] but was similar to that obtained using a multiply-charged amphiphilic polypeptide [Roux, M., Neumann, J.-M., Hodges, R. S., Devaux, P. F., & Bloom, M. (1989) *Biochemistry 28*, 2313–2321]. This comparison suggests that the charges on SP-C are located near the bilayer surface. The possibility that the headgroup response is sensitive to the degree of clustering of surface charge is discussed. The β-deuteron splitting in the liquid-crystalline phase decreased with increasing temperature but was relatively insensitive to protein concentration, suggesting that the torsion angle about the C_α–C_β bond might be sensitive to steric interactions between the lipid headgroup and the protein.

Pulmonary surfactant is a complex of lipids and proteins that is essential to lung function because it reduces surface tension in the air–water interface of the lung. The major lipid is phosphatidylcholine, most of which is saturated, so that 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC)[1] forms about half of the total mass of pulmonary surfactant. It also contains two hydrophobic proteins, SP-B and SP-C, reported to be at a concentration of 1-2%. Both have been implicated in the functioning of surfactant in the extracellular space, particularly in the transfer of lipids from the bilayer form, in which they are stored and secreted from the type II pneumocytes, to a putative monolayer at the air–water interface [reviewed in Keough (1992)].

SP-C is a highly conserved protein containing 35 amino acids. The C-terminal segment has 23 hydrophobic amino acids that form a helical segment which would span the hydrocarbon part of a bilayer of surfactant lipid. The N-terminal portion has three positively charged residues: arginine, at position 2, and arginine and lysine, at positions 11 and 12, just before the hydrophobic region. The N-terminal region also has vicinal cysteines at positions 5 and 6, both of which are palmitoylated. Canine SP-C has phenylalanine and a palmitoylated cysteine in these positions. The palmitates might form a binding site for some other protein. On the other hand, they may serve to "anchor" the N-terminal domain into the same bilayer as the α-helical C-terminal segment, or into an adjacent bilayer or monolayer. In the dispersions used in this study, which contain no other protein, we assume that the palmitates are embedded among the chains of the phospholipid.

The SP-C molecule is thus a well-anchored, transbilayer protein which, at pH 7, would have four positive charges on one side of the bilayer and a negative charge on the opposite side including the charges on terminal amino and carboxyl groups. Assuming that the orientation of the SP-C in the systems studied here is random, then each protein introduces an average net charge of +3 to one side of the bilayer.

While SP-C has functional significance for pulmonary surfactant, it also serves as a model peptide for a large number of transmembrane protein segments. Such segments usually contain a transmembrane portion (most often α-helical) with charged residues, especially basic ones, at one or both ends of the helix (e.g., Verner & Schatz, 1988; Reithmeier & Deber, 1992; Andersson et al., 1992; von Heijne, 1992). Thus, most transmembrane portions of proteins, including targeting sequences, have a generalized motif fulfilled by the SP-C molecule, so the study of its characteristics has general relevance to lipid–protein interactions in biological membranes as well as in pulmonary surfactant.

Previous studies (Simatos et al., 1990; Pérez-Gil et al., 1992) have shown that SP-C mixes well with the acyl chains of phosphatidylcholines in bilayers and monolayers. It perturbs the packing of the chains to approximately the same extent as most other transmembrane helices (Simatos et al., 1990). It could be expected that the charges on the SP-C molecule

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* Author to whom correspondence should be addressed at the Department of Biochemistry, Memorial University of Newfoundland.
‡ Department of Physics.
§ Department of Biochemistry.
‖ Discipline of Pediatrics.

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[1] Abbreviations: DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPS, 1,2-dimyristoyl-*sn*-glycero-3-phosphoserine; ^{2}H NMR, deuterium nuclear magnetic resonance; SP-C, pulmonary surfactant-associated protein.

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would influence the packing of the lipid polar headgroups. In this ^2H NMR investigation, we have used DPPC that was deuterated in the α and β positions of the choline portion of the headgroup to investigate the influence of the protein on headgroup orientation (Seelig et al., 1987). This technique, in which changes in quadrupole splittings of the α- and β-deuteron resonances given information about headgroup orientation, has been used to investigate the effect on lipid headgroups of adsorbed cations and anions (Akutsu & Seelig, 1981; Roux & Bloom, 1990; Akutsu & Nagamori, 1991; Macdonald & Seelig, 1988; Rydall & Macdonald, 1992a), charged amphiphiles (Scherer & Seelig, 1989; Macdonald et al., 1991; Marassi & Macdonald, 1992), and charged polypeptides and proteins (Kuchinka & Seelig, 1989; Roux et al., 1989; Dempsey et al., 1989). Roux et al. (1989) studied the effect of a synthetic polypeptide having the sequence Lys-Lys-Gly-Leu_{20}-Lys-Lys-Ala-$CONH_2$ which they denote as $K_2GL_{20}K_2A$. This polypeptide has a transmembrane helix and positive charges at both ends of the helix. The effect of SP-C will be compared with this polypeptide and other amphiphiles.

MATERIALS AND METHODS

Materials. DPPC deuterated at the α and β positions of the choline headgroup (DPPC-d_4) was purchased from Avanti Polar Lipids (Alabaster, AL). The labels α and β refer to the choline methylene groups nearest and next-nearest, respectively, to the phosphate group. In addition to the expected α and β-deuterium signals, ^2H NMR on this sample showed a minor spectral component with a small quadrupole splitting which was assumed to arise from partial deuteration of the γ-methyls of the choline headgroup. This did not interfere significantly with the rest of the spectrum, and the lipid was used without further purification.

Isolation of Surfactant Protein. Hydrophobic protein was prepared from lipid extracts of porcine surfactant prepared as described previously (Keough et al., 1988). Surfactant protein C (SP-C) was isolated by gel filtration chromatography as described by Pérez-Gil et al. (1992). One portion of SP-C was prepared as described by Simatos et al. (1990). The protein gave one band of expected mobility on reducing and nonreducing SDS–PAGE. There was little lipid contamination (below the detection limit at $\leq$5 mol % lipid in the protein preparations) as indicated by the SDS–PAGE gels or analysis of phosphorus levels in the protein. The estimation of protein was done by the method of Bohlen et al. (1973) and amino acid analysis (Pérez-Gil et al., 1992).

Mixing of Protein and Lipid and Determination of Composition. Samples of pure DPPC-d_4 and DPPC-d_4 containing SP-C concentrations of 9%, 14%, 18%, and 30% (w/w) were prepared. For the three highest protein concentrations, samples consisted of between 10 and 15 mg of material. The other two samples consisted of about 35 mg of material. Samples of lipid and protein were mixed in chloroform–methanol 2:1 (v/v). The solvent was removed by rotary evaporation and evaporation under N_2, followed by evacuation overnight.

^2H NMR. Dried protein–lipid mixtures were placed in a 500-μL NMR tube and hydrated with about 250 μL of 50 mM phosphate buffer at pH 7.0. The samples were then gently stirred with a fine glass rod. Phosphate buffer was chosen to allow direct comparison with previous experiments on this system (Simatos et al., 1990). Comparison of the α and β splittings in the liquid-crystalline phase with previously reported values (Akutsu & Seelig, 1981) indicated that the buffer had little effect on headgroup conformation. The water in the buffer was not deuterium-depleted.

^2H NMR spectra were collected in a superconducting solenoid (Nalorac Cryogenics, Martinez, CA) at 23.2 MHz using a locally-built spectrometer previously described elsewhere (Simatos et al., 1990). Spectra were obtained using a phase-cycled quadrupole echo sequence (Davis et al., 1976) with a $\pi/2$ pulse length of between 2.5 and 3.8 μs and a pulse separation of 50 μs. For all samples, free induction decays were collected with a repetition time of 0.45 s. For the two largest samples, between 8000 and and 12 000 transients were averaged to produce each spectrum. For smaller samples, up to 48 000 transients were averaged with 26 000 transients being typical. The digitizer dwell time was normally 2 μs per point. The resulting signal was split into four free induction decays which were independently smoothed and symmetrized, as described by Prosser et al. (1991), before being recombined into a free induction decay corresponding to an effective dwell time of 8 μs. Comparison with unsymmetrized spectra was used to ensure that symmetrization did not add any artifacts. For the pure lipid sample, liquid-crystal spectra were collected with a digitizer dwell time of 5 μs and an effective dwell time of 10 μs.

After hydration, each sample was warmed to 55 °C and allowed to equilibrate for about 1 h before the start of the data collection. Temperatures were lowered in 2 °C steps except near the main liquid-crystal to gel transition for which 1 °C steps were used. After each temperature change, the sample was allowed to equilibrate for at least 20 min before the beginning of data collection.

At selected temperatures, quadrupole echo experiments were performed with a series of pulse separations between 50 and 600 μs. Least-square fits to semilog plots of the echo decay were used to estimate the transverse relaxation time, T_{2e}.

RESULTS AND DISCUSSION

Figure 1 shows spectra at selected temperatures for DPPC-d_4 and for DPPC-d_4 containing 18% (w/w) SP-C. At higher temperatures, the spectra are superpositions of sharp powder pattern doublets characteristic of fast, axially symmetric motion in a liquid-crystalline phase. The broader spectrum seen at low temperature is characteristic of the slower reorientation in the gel phase. For the pure lipid at the highest temperature, the doublet corresponding to the α site in the choline has a slightly greater splitting than that for the β site. An oriented spectrum corresponding to the observed powder spectrum can be obtained using the "dePakeing" algorithm developed by Bloom and co-workers (Bloom et al., 1981; Sternin et al., 1983). Transforming the pure lipid spectrum for 51 °C in this way (not shown) resolves the α signal into two doublets of equal intensity differing in splitting by about 300 Hz. This inequivalence of the α-deuterons was previously noted by Gally et al. (1975) and by Scherer and Seelig (1989). For the sample containing 18% (w/w) SP-C, the α splitting is smaller than the β splitting. In each case, the central doublet is assumed to arise from a partial deuteration of the γ-methyl groups. The isotropic component at zero frequency presumably arose from natural abundance deuterium in the water used for the buffer. For both series of spectra shown in Figure 1, the α splitting in the liquid-crystalline phase is relatively insensitive to temperature while the β splitting increases with decreasing temperature. Comparison of the two series of spectra shows that the α splitting decreases noticeably with increasing protein concentration while the β splitting is relatively insensitive to protein concentration. It can also be

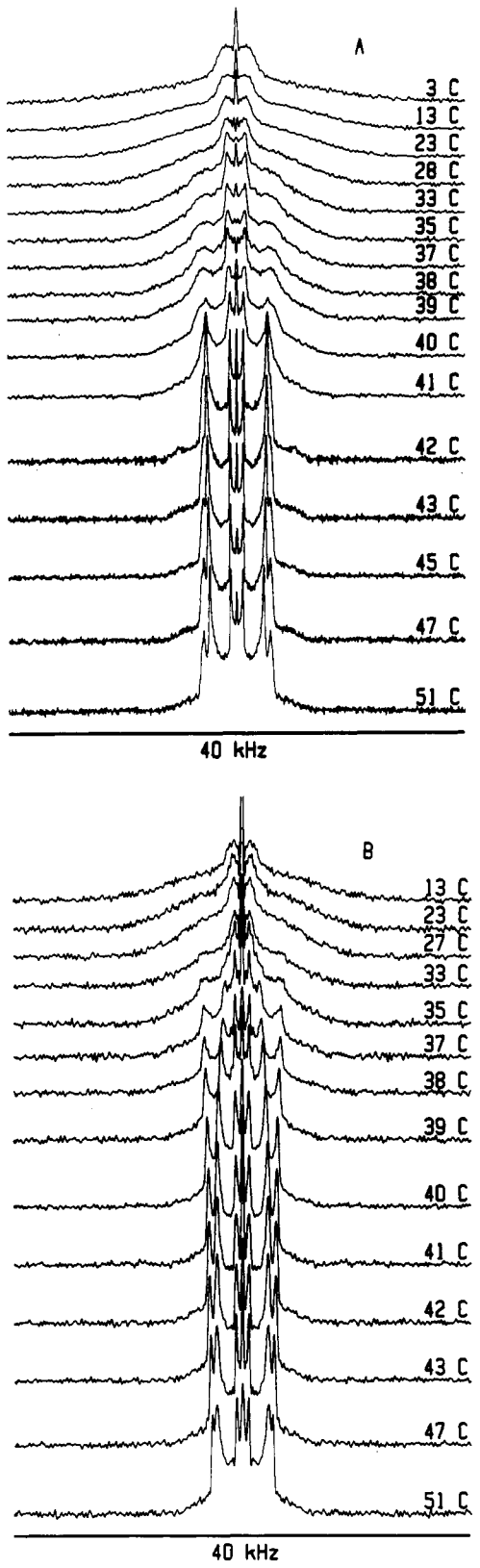

FIGURE 1: Temperature dependence of the ^2H NMR spectrum for (A) DPPC-d_4 and (B) DPPC-d_4 containing 18% (w/w) SP-C.

seen that in the presence of the protein, liquid-crystalline features persist to somewhat lower temperatures, and there is a range of a few degrees over which gel and liquid-crystal components coexist.

Figures 2 and 3 show the temperature dependence of the spectral first moment (M_1) and the mean transverse relaxation time (T_{2e}) for DPPC-d_4 and for the samples with the three

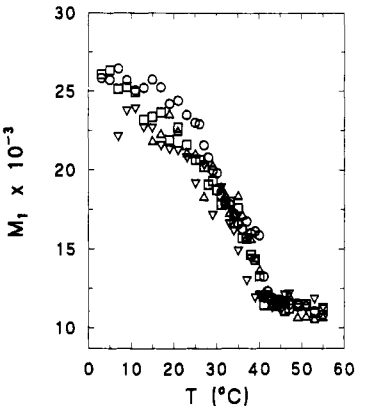

FIGURE 2: Temperature dependence of the first spectral moment, M_1, for DPPC-d_4 (○), 9% (w/w) SP-C in DPPC-d_4 (□), 14% (w/w) SP-C in DPPC-d_4 (△), and 18% (w/w) SP-C in DPPC-d_4 (▽).

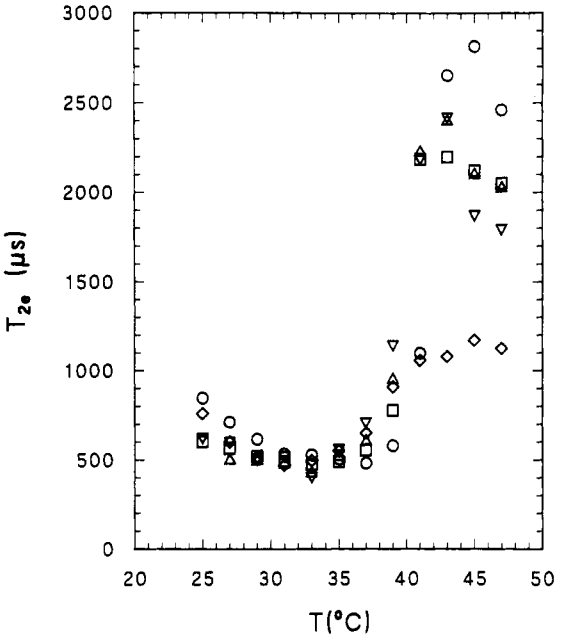

FIGURE 3: Temperature dependence of T_{2e} for DPPC-d_4 (○), 9% (w/w) SP-C in DPPC-d_4 (□), 14% (w/w) SP-C in DPPC-d_4 (△), 18% (w/w) SP-C in DPPC-d_4 (▽), and 30% (w/w) SP-C in DPPC-d_4 (◇).

lowest SP-C concentrations. It can be seen that the protein has only a small effect on the mean splitting, and thus M_1, in the liquid-crystalline phase. Using M_1 as an indicator of phase behavior, the protein is seen to broaden and lower the transition slightly, which is consistent with the effect seen using lipid labeled on the fatty acid chains (Simatos et al., 1990). In the gel phase, the presence of protein reduces M_1, suggesting some disordering effect. The average transverse relaxation time, T_{2e}, of the headgroup deuterons in the liquid-crystalline phase is reduced by the addition of SP-C. This protein has a similar effect on the transverse relaxation of chain deuterons (Simatos et al., 1990). It is likely that the presence of the protein increases the correlation times of motions which are limiting T_{2e} and that its effect on these motions is not specific to either the chain the or headgroup region.

The effect of SP-C on the DPPC-d_4 headgroup is illustrated in Figure 4 which shows spectra at 51 °C for DPPC-d_4 (Figure 4A) and for samples with 9%, 14%, 18%, and 30% SP-C by weight. Again, in the pure lipid spectrum, the doublet corresponding to the α site in the choline displays a slightly greater splitting. As the protein concentration is increased,

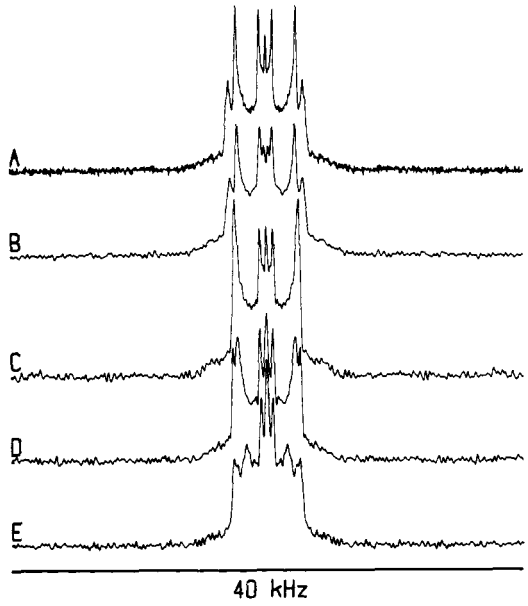

FIGURE 4: ^{2}H NMR spectra at 51 °C for (A) DPPC-d_4, (B) 9% (w/w) SP-C in DPPC-d_4, (C) 14% (w/w) SP-C in DPPC-d_4, (D) 18% (w/w) SP-C in DPPC-d_4, and (E) 30% (w/w) SP-C in DPPC-d_4.

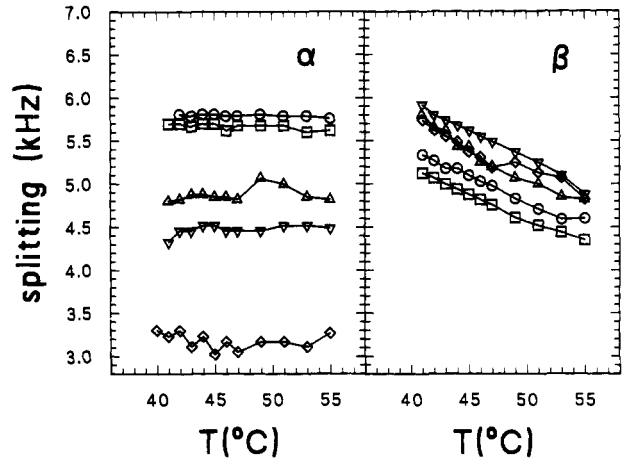

FIGURE 5: Temperature dependence of the α- and β-choline deuteron doublet splittings for DPPC-d_4 (○), 9% (w/w) SP-C in DPPC-d_4 (□), 14% (w/w) SP-C in DPPC-d_4 (△), 18% (w/w) SP-C in DPPC-d_4 (▽), and 30% (w/w) SP-C in DPPC-d_4 (◇).

the α splitting decreases noticeably while the β splitting increases more slowly. For the highest SP-C concentration, the α and β doublets are still identifiable, but their shapes are not characteristic of equivalent deuteron pairs undergoing axially symmetric motion. Both the α and β signals can be resolved into a pair of doublets by "dePakeing" (not shown). The components of the α signal are of similar intensity and could arise from some inequivalence of the α-deuterons. The components of the β signal are not equivalent. If, however, the doublet shapes reflect some axial asymmetry, "dePakeing" might be misleading. The apparent α and β splittings for spectra from the sample containing 30% SP-C by weight are generally consistent with the concentration dependence displayed by the samples containing less SP-C. At this time, however, we have not attempted a more detailed analysis of spectra for the highest SP-C concentration.

Figure 5 shows the temperature dependence of the α and β splittings for the five samples. The splittings have been measured between maxima in the powder spectra rather than from "dePaked" spectra in order to facilitate comparison with

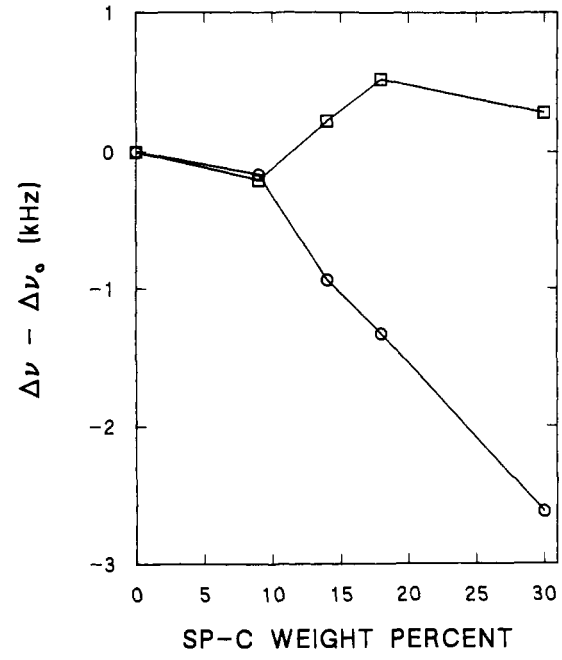

FIGURE 6: SP-C concentration dependence of the α (○) and β (□) choline deuteron doublet splittings at 46 °C. $\Delta\nu$ is the observed splitting, and $\Delta\nu_0$ is the splitting in the absence of SP-C.

splittings reported elsewhere. It is apparent that the α splitting is very sensitive to protein concentration and largely insensitive to temperature. The β splitting decreases with increasing temperature but does not vary consistently with increasing protein concentration. Figure 6 shows the concentration dependence of the doublet splitting for the α- and β-deuterons at 46 °C. Similar behavior is seen at other temperatures above the liquid–crystal–gel transition.

It has been well established that the ^{2}H NMR spectra of methylene deuterons in polar headgroups display splittings which are influenced by the presence of bilayer surface charges (Seelig et al., 1987). Changes in α- and β-deuteron splittings for phosphatidylcholine bilayers have been observed in the presence of adsorbed cations (Akutsu & Seelig, 1981; Roux & Bloom, 1990; Akutsu & Nagamori, 1991) and anions (Macdonald & Seelig, 1988; Rydall & Macdonald 1992a), charged amphiphiles (Scherer & Seelig, 1989; Macdonald et al., 1991), and charged proteins or polypeptides (Kuchinka & Seelig, 1989; Roux et al., 1989; Dempsey et al., 1989). It is generally found that the presence of positive (negative) charge on the bilayer surface results in a decrease (increase) of the α splitting and an increase (decrease) in the β splitting. A striking aspect of the headgroup response to surface charge is the linear relationship between changes in the α and the β splitting. Scherer and Seelig (1989) show that for a phosphatidylcholine bilayer perturbed by a variety of positive and negative amphiphiles, a plot of the change in β splitting versus the change in α splitting gives a straight line with a negative slope. The magnitude of the slope is nearly twice as large for negative amphiphiles as for positive ones. Because of the counterdirectional effect on the α and β sites, it has been argued that the observed changes in splitting result from a change in headgroup orientation rather than a change in flexibility which would be expected to change both splittings in the same direction (Seelig et al., 1987). While a detailed description of the conformational change resulting from the presence of surface charge has not yet been presented, a number of models have been proposed to account for certain aspects of the observed behavior and corresponding effects on

phosphorus chemical shift anisotropy (Akutsu & Seelig, 1981; Scherer & Seelig, 1989; Roux et al., 1989; Macdonald et al., 1991; Akutsu & Nagamori, 1991). A common feature of these models is a tilting of the choline headgroup as a unit in response to a torque exerted on the phosphorus–nitrogen dipole of the choline by charges located near the plane containing the phosphorus atoms. It has been suggested that the rotation giving rise to the tilt occurs about the carbon–oxygen bond linking the glycerol backbone to the phosphate (Scherer & Seelig, 1989; Macdonald et al., 1991). The conformation around the C_α–C_β bond is reported to be gauche (Akutsu & Nagamori, 1991). Such a conformation would be consistent with a counterdirectional change of the α and β splittings on tilting of the choline group.

In the liquid-crystalline-phase spectra presented here, the α splitting decreases with increasing SP-C concentration but is insensitive to temperature. The β splitting is less sensitive to protein concentration but decreases with increasing temperature. Rydall and Macdonald (1992b) recently reported a similar response of the phosphatidylcholine headgroup to the neutral amphipathic polypeptide staphyloccal δ-toxin. Because of the insensitivity of the β splitting to polypeptide concentration, they attributed the observed response of the headgroup deuteron splittings to polypeptide-induced disordering of the headgroup rather than to an electrostatic response to charges on the polypeptide. The β splitting, however, is known to be more sensitive to steric interactions than the α splitting (Akutsu & Seelig, 1981). It is not clear, then, that the α-deuteron could be significantly disordered without there being at least a similar reduction in splitting for the β-deuteron. Given the charges present on SP-C, it would seem reasonable to explore the possibility that the α splitting reflects an electrostatic interaction and that the lack of response observed for the β splitting reflects a competition between electrostatic effects, which would increase the β splitting, and other effects which would disorder part of the headgroup and thus decrease the β splitting.

It should first be noted that because of the small protein concentrations accessible in this work, the effective surface charge examined was relatively low compared to that used in systematic studies of the change in headgroup deuteron splitting with charge (Seelig et al., 1987; Scherer & Seelig, 1989). Scherer and Seelig (1989) report that in plots of the change in β splitting versus the change in α splitting, there is a change in slope near the point of zero surface charge. It is possible that the correlation between the α and β splittings is less well determined at low surface charge. Indeed, in a plot of the change in headgroup deuteron splitting as a function of amphiphile mole fraction, presented by Scherer and Seelig (1989), the β splittings for a number of samples with small concentrations of positively charged amphiphiles fall below the smooth curve which extrapolates the behavior at high concentration back to the origin. It is thus possible that because of screening or other effects, there might be a threshold charge concentration which must be attained before the striking correlation between the α and β splittings is established.

It is also possible, however, that the insensitivity of the β splitting to protein concentration is a result of competition between the headgroup's response to surface charge and steric effects. The difference in sensitivity to temperature displayed by the α and β splittings is striking and has been observed previously by others. Gally et al. (1975) attributed this behavior to the temperature dependence of torsional oscillations about the C_α–C_β bond of the choline group. Akutsu and Seelig (1981) found that the α and β splittings also responded differently to the concentration of neutral amphiphiles. In particular, addition of cholesterol resulted in a decrease in the β splitting. They suggested that the change in separation of the polar groups brought about by inclusion of a neutral amphiphile caused a change of the torsion angle about the C_α–C_β bond. The addition of cholesterol had no effect on the α splitting. It is thus possible that as the SP-C concentration is increased, the α splitting is sensitive only to the change in surface charge while the β splitting responds, in opposite senses, to the overall choline tilt and to a change in the torsion angle about the C_α–C_β bond brought about by a change in steric constraints. If this is the case, the α splitting likely indicates more directly the extent of any response to surface charge.

The small amount of protein available limited the number of SP-C concentrations which could be studied, and thus the precision with which the dependence of α splitting on protein concentration could be measured. Nevertheless, it is possible to make a rough comparison between the effect of charges on SP-C and the effect of charges on other amphiphilic species if it is assumed that the α-deuteron splitting does indeed reflect an electrostatic interaction between the headgroup dipole and SP-C. If SP-C inserts into the bilayer with random orientation, then it is expected to contribute four positive charges and one negative charge in the neighborhood of the bilayer surface. Using a molecular mass of 4.2 kDa for SP-C, a concentration of 18% (w/w) SPC in DPPC-d_4 corresponds to a protein mole fraction of about 0.038. Recently, Marassi and Macdonald (1992) demonstrated that in a mixture of cationic and anionic amphiphiles, the observed quadrupole splitting of phosphocholine deuterons was the sum of effects due to the charges present rather than a response to the net surface charge. Using charged amphiphiles, Scherer and Seelig (1989) found that the change in α-deuteron splitting was about −30 kHz per unit mole fraction for positively charged amphiphiles and about +15 kHz per unit mole fraction for negatively charged amphiphiles. If the charges on SP-C were equally effective, the net effect of 3.8 mol % SP-C on the α splitting of DPPC-d_4 would be a reduction by about 4 kHz. In the present work, this concentration of SP-C is actually found to reduce the α splitting by about 1.4 kHz which suggests that the effect of charges on SP-C is less than half of that due to charges on the amphiphiles used by Scherer and Seelig (1989).

It is interesting to compare the change in α splitting due to SP-C with that observed by Roux et al. (1989) for DMPC bilayers containing the synthetic peptide $K_2GL_{20}K_2A$. This polypeptide has three positive charges near the N-terminal end and two positive charges near the C-terminal end. They report that at a peptide mole fraction of 0.032, the DMPC α splitting is reduced by 2.1 kHz. If the positive charges on $K_2GL_{20}K_2A$ had the same effect as those on the charged amphiphiles used by Scherer and Seelig (1989), the expected net effect of the five positive charges would be a reduction in the α splitting of about 4.8 kHz. In the case of $K_2GL_{20}K_2A$, the effectiveness of the charges is also less than half that of the charges on the amphiphiles used by Scherer and Seelig (1989).

On the basis of an order of magnitude comparison and the direction of the change in splitting due to the charged proteins, the reduction in α splitting seen in the presence of SP-C results does appear to be consistent with an electrostatically-induced tilting of the choline group. The effect per charge, due to SP-C, on the α splitting is only slightly less than that due to the multiply-charged $K_2GL_{20}K_2A$. This similarity suggests that the charges on SP-C and the polypeptide exist in roughly

analogous locations relative to the plane of the lipid headgroups. This, in turn, suggests that the N-terminus of SP-C is not significantly further from the interface than charges on $K_2GL_{20}K_2A$.

The effect per charge on the α splitting due to either SP-C or on the synthetic peptide $K_2GL_{20}K_2A$ is significantly less than that due to singly-charged amphiphiles (Scherer & Seelig, 1989) in which the charges are presumably located at the interface between the hydrophobic and polar regions of the bilayer. In the case of SP-C and $K_2GL_{20}K_2A$, some of the charges are located on residues which may extend beyond this interface. The smaller observed effect might indicate that these charges exert less torque on the headgroup dipole and thus that the effect is very sensitive to charge location. If this were the case, however, the relative similarity of the effect per charge due to SP-C and $K_2GL_{20}K_2A$ would seem somewhat fortuitous given the rather different distribution of charges in the two cases.

An alternate possibility is that the effect of charges on headgroup deuteron splitting might be sensitive to grouping of the charges. Marassi and Macdonald (1992) have shown that the change in deuteron splitting does not reflect the overall surface charge but is, rather, a net response to local intermolecular interactions. Over the characteristic time for a deuterium NMR experiment, lipids in a liquid-crystalline bilayer can diffuse distances on the order of 10 nm and thus can extensively sample their environment (Davis, 1983). The observed splitting reflects an average over the resulting conformations. For a bilayer of headgroup-labeled lipid containing charged guest molecules, the observed change in the headgroup deuteron splitting will depend both on the frequency with which a diffusing lipid encounters a charged entity and on the magnitude of the conformational change during the encounter. Charges on neighboring residues of a polypeptide or protein will affect nearby lipids in a manner which must be correlated to some extent, and a particular lipid will not encounter the neighboring charges independently. One would thus expect the frequency of encounters with a given amount of charge organized in clusters to be lower than would be the case if the same charge were dispersed on independent, singly-charged amphiphiles. Whether or not the resulting effect on the observed headgroup deuteron splitting differs, in these two cases, would then depend on the extent to which the perturbation, during an encounter, was linearly proportional to the amount of charge involved in that encounter. The fact that the change in splitting is known to saturate at high charge concentration (Scherer & Seelig, 1989; Macdonald et al., 1991) suggests that a nonlinear local response is at least a possibility. If the instantaneous perturbation of the headgroup conformation saturates for relatively small local charge groupings, then the observed change in splitting might depend primarily on the concentration of charge clusters rather than on the total charge present, and the average effect of charges localized in clusters might be smaller than the net effect of the same number of charges acting independently.

In the case of SP-C, two positive charges exist on neighboring residues at positions 11 and 12. The other two positive charges are located near the N-terminus. The 2 charge clusters are thus separated by about 11 residues and presumably interact independently with diffusing lipids. The negative charge is situated on the opposite side of the bilayer. In the case of the synthetic polypeptide $K_2GL_{20}K_2A$, the five positive charges occur in a group of three and a group of two. If the change in α splitting were sensitive to the extent of charge clustering, both SP-C and $K_2GL_{20}K_2A$ would be expected to show a smaller effect per charge than singly charged amphiphiles as, in fact, is observed.

The maximum reduction in the effect per charge on the headgroup would occur in the limiting case where the headgroup conformational change is saturated by interaction with a single charge. This would be equivalent to assuming that the headgroup switches between two conformational states depending only on whether or not the lipid interacts locally with a charge group. The observed response then depends on the number of independent charge groups carried by the protein or polypeptide but not on the magnitude of the charge in each group. If this were the case, then the change in α splitting observed by Scherer and Seelig (1989) could be interpreted as per unit mole fraction of charge clusters rather than per unit mole fraction of charge, and the response of the α splitting to a positively charged cluster would be about -30 kHz per unit mole fraction of positive clusters and $+15$ kHz per unit mole fraction of negative clusters. If $K_2GL_{20}K_2A$ is then taken to have two independent positive clusters, this clustering-sensitive picture would predict a change in α splitting of -1.9 kHz for a polypeptide mole fraction of 0.032. This agrees well with the reported change of -2.1 kHz (Roux et al., 1989). A reduction in the effectiveness of clustered charge could also account for the observation, by Roux et al. (1989), that the DMPC splitting is relatively unaffected by the addition of DMPS and $K_2GL_{20}K_2A$ in relative amounts for which the net membrane charge is zero. Without taking charge-clustering into account, this result would appear to be inconsistent with either the observations reported by Marassi and Macdonald (1992) for ternary mixtures or the observation (Scherer & Seelig, 1989; Marassi & Macdonald, 1992) that positive charge generally has a significantly larger effect on headgroup deuteron than negative charge.

If SP-C can be taken to have two independent positive clusters and one negative cluster then a clustering-sensitive picture would predict a change in α splitting, at 3.8 mol % SP-C, of -1.7 kHz, which is close to the observed shift of -1.4 kHz. The consistency of this simple clustering-sensitive picture with the observed results for the two multiply-charged molecules is rather striking. It suggests the need for a systematic examination of the effect of charge-clustering on charge-induced headgroup conformational change. This could be done, for example, by comparing the effects of structurally similar amphiphiles having single or multiple charges in the headgroup region. If the effect of charge on the observed α splitting is indeed sensitive to clustering in this simple way, it would appear that the positive charge on the N-terminus of SP-C influences diffusing lipids independently and is thus spatially separated, on average, from the positive charges on residues 11 and 12. It should also be noted that if the charges at the N-terminus of SP-C were simply neglected, this clustering-sensitive picture would predict a change in α splitting, at 3.8 mol % SP-C, of about -0.6 kHz, which is clearly inconsistent with the observed result. This comparison supports the suggestion that the positive charge associated with the N-terminus resides near the bilayer surface.

CONCLUSIONS

Protein-induced changes in the quadrupole splitting of deuterons on the α- and β-carbons of the choline group are consistent with at least two, and probably all four, of the potential positive charges and the negative charge on the SP-C molecule being located in or close to the plane of the choline moiety of the headgroup. Increasing the positive charge density in that region by increasing the protein concentration

causes a tilt of the headgroup upward from the surface of the bilayer away from the usual orientation of the phosphorus–nitrogen dipole, which for phosphatidylcholine is usually nearly parallel to the surface (Seelig et al., 1987).

Two of the 4 positive charges on SP-C, on the side chains of lysine and arginine at positions 11 and 12, occur just before the start of the 23-residue hydrophobic C-terminal portion. The hydrophobic portion has been suggested to fold into a hydrophobic α-helix which spans the DPPC bilayer. Given the location of the charges in the SP-C sequence, the behavior of the headgroup deuteron splittings is consistent with the hydrophobic portion of SP-C being embedded in the hydrocarbon portion of the bilayer and with the negative charge near the C-terminal end of the molecule being near a bilayer surface.

If we include all of the charges on SP-C, its effect on the lipid headgroup is consistent with that found for the transmembrane peptide $K_2GL_{20}K_2A$ with positive charges at both termini (Roux et al., 1989). This suggests that the positive charge near the N-terminus of SP-C plays a role in changing the headgroup conformation and thus that it resides close to the bilayer surface. We assume that SP-C is randomly oriented in the bilayers in this study. It is worthwhile contemplating the possibility that *in vivo* SP-C is not randomly oriented in the bilayer of lamellar bodies, but is inserted with a specific polarity. Were that to be the case, the asymmetric distribution of charge across the bilayer produced by the protein could modify headgroup packing in a different way on either side of the bilayer. It could be associated with asymmetric lipid distribution across the bilayer also. Such influences may have important biological consequences. Additional studies with multilayers prepared with a Langmuir–Blodgett system would shed further light on this idea.

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