Interaction between Perdeuterated Dimyristoylphosphatidylcholine and Low Molecular Weight Pulmonary Surfactant Protein SP-C†

G. A. Simatos,‡ K. B. Forward,§ M. R. Morrow,§ and K. M. W. Keough*,‡,∥

Department of Biochemistry, Department of Physics, and Discipline of Pediatrics, Memorial University of Newfoundland, St. John's, Newfoundland, Canada A1B 3X9

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ABSTRACT: A low molecular weight hydrophobic protein was isolated from porcine lung lavage fluid using silicic acid and Sephadex LH-20 chromatography. The protein migrated with an apparent molecular weight of 5000–6000 on SDS–PAGE under reducing and nonreducing conditions. Gels run under reducing conditions also showed a minor band migrating with a molecular weight of 12 000. Amino acid compositional analysis and sequencing data suggest that this protein preparation contains intact surfactant protein SP-C and about 30% of truncated SP-C (N-terminal leucine absent). The surfactant protein was combined with perdeuterated dimyristoylphosphatidylcholine (DMPC-d_{54}) in multilamellar vesicles. The protein enhanced the rate of adsorption of the lipid at air–water interfaces. The ability of the protein to alter normal lipid organization was examined by using high-sensitivity differential scanning calorimetry (DSC) and ^{2}H nuclear magnetic resonance spectroscopy (^{2}H NMR). The calorimetric measurements indicated that the protein caused a decrease in the temperature maximum (T_m) and a broadening of the phase transition. At a protein concentration of 8% (w/w), the enthalpy change of transition was reduced to 4.4 kcal/mol compared to 6.3 kcal/mol determined for the pure lipid. NMR spectral moment studies indicated that protein had no effect on lipid chain order in the liquid-crystal phase but reduced orientational order in the gel phase. Two-phase coexistence in the presence of protein was observed over a small temperature range below the pure lipid transition temperature. Spin–lattice relaxation times (T_1) were not substantially affected by the protein. Transverse relaxation time (T_{2e}) studies suggest that the protein influences slow lipid motions.

Pulmonary surfactant is rich in phospholipid, most notably dipalmitoylphosphatidylcholine (DPPC),[1] and it also contains neutral lipid and at least four surfactant-associated proteins [e.g., see King et al. (1973), Whitsett et al. (1985), Persson et al. (1988), and Possmayer (1988)]. Preparations of natural surfactant readily reduce surface tension, preventing alveolar collapse in situ (Goerke, 1974). Synthetic surfactant mixtures also have the ability to reduce surface tension, but in the absence of surfactant proteins, the rate of adsorption at the air–water interface is slower than the rate found for the same amounts of natural surfactant [e.g., see Yu and Possmayer (1986)]. Surfactant is synthesized in the type II pneumocyte and is secreted into the hypophase where some of it exists in an unusual array referred to as tubular myelin. Ultimately, the surfactant lipid adsorbs as a surface-active monolayer at the air–water interface [e.g, see Efrati et al. (1987)]. The processes governing the progression of surfactant from newly secreted material to tubular myelin and the monolayer at the alveolar interface are not well understood. However, the observation that the rate of synthetic surfactant adsorption is enhanced in the presence of surfactant proteins suggests that these proteins play a role in altering the normal lipid organization of the host bilayer, thereby facilitating the production of surfactant monolayers.

Surfactant protein SP-A is an octadecamer with a reduced molecular weight of approximately 35K (Haagstrom, 1988). Two low molecular weight proteins are also present: SP-B with a molecular weight of approximately 8K (Curstedt et al., 1988) and an extremely hydrophobic protein which has a molecular weight of approximately 3.7K. Recently a collagenous protein designated CP4 (or SP-D) has been identified in primary cultures of rat pneumocytes which appears to be immunologically related to proteins in normal surfactant (Persson et al., 1988).

If pulmonary surfactant protein(s) function(s) by helping to disrupt the bilayer or promote the presence of nonbilayer phases in the surfactant, then ^{2}H nuclear magnetic experiments on bilayers containing lung surfactant protein might be expected to display changes in the lipid organization. Accordingly, we have studied the influence of a fraction containing the surfactant protein SP-C on bilayers of DMPC-d_{54} using ^{2}H NMR as well as high-sensitivity differential scanning calorimetry.

A variety of protein–lipid and polypeptide–lipid systems have been investigated using ^{2}H NMR. These include cytochrome *c* oxidase in a variety of lipids (Tamm & Seelig, 1983; Paddy et al., 1981; Kang et al., 1979), rhodopsin in DMPC (Bienvenue et al., 1982), sarcoplasmic reticulum ATPase in DMPC and DPPC (Rice et al., 1979), and synthetic polypeptides in DPPC (Huschilt et al., 1985). A number of recent reviews have commented on the results of such experiments (Davis, 1983, 1986; Bloom & Smith, 1985). Several observations

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* Correspondence should be addressed to this author at the Department of Biochemistry, Memorial University of Newfoundland, St. John's, Newfoundland, Canada A1B 3X9.

‡ Department of Biochemistry.

§ Department of Physics.

∥ Discipline of Pediatrics.

[1] Abbreviations: DMPC, dimyristoylphosphatidylcholine; DMPC-d_{54}, chain-perdeuterated dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSC, differential scanning calorimetry; ^{2}H NMR, ^{2}H nuclear magnetic resonance; M_1, first spectral moment; M_2, second spectral moment; SDS–PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; SP-B, pulmonary surfactant protein B; SP-C, pulmonary surfactant protein C; T_1, spin–lattice relaxation time; T_{2e}, transverse relaxation time; T_m, temperature maximum; τ_c, correlation time; τ, delay time.

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found to apply generally to these systems have been summarized by Bloom and Smith (1985). For the liquid-crystalline phase, there is no evidence, from ^2H NMR, for a distinct long-lived class of boundary lipid at the protein–lipid interface. The average orientational order parameters for carbon–deuterium bonds in the chains are not significantly changed by the presence of protein. The spin–lattice relaxation time, T_1, is also changed very little by the presence of protein, indicating that the protein has very little effect on chain motions with correlation times shorter than about 10^{-8} s. The quadrupole echo decay time, T_{2e}, however, is significantly shortened by the presence of these intrinsic proteins, indicating that the relative importance of slow motions in the bilayer is enhanced in the presence of protein.

In the present work, spectra were collected over a range of temperatures spanning the pure lipid transition temperature for samples containing 1.0%, 2.2%, and 8.0% (w/w) protein in DMPC-d_{54}, as well as for the pure lipid. Inversion recovery experiments were used to determine T_1 for each sample above and below the pure lipid transition temperature. Quadrupole echo decay experiments were used to determine T_{2e} as a function of temperature for the pure lipid and the sample with the highest protein concentration. The results obtained are compared with those for a variety of membrane-spanning proteins and polypeptides. Part of these results were presented in preliminary form previously (Simatos et al., 1990).

Materials and Methods

Materials. ACS-grade chloroform and methanol were distilled before use. 2-Chloroethanol was purchased from Anachemia, Milwauke, WI. Electrophoresis equipment and materials were from Bio-Rad, Mississauga, Ontario. Low molecular weight electrophoresis marker was purchased from Bethesda Research Laboratories, Bethesda, MD. Chromatography-grade silicic acid (Keiselsäure) was obtained from Merck, Darmstadt, West Germany, and Sephadex LH-20 was from Pharmacia, Dorval, Quebec. The silver staining kit for SDS–PAGE was purchased from NEN, Mississauga, Ontario.

Isolation of Surfactant Protein. Surfactant from freshly excised pig lungs was prepared according to the method described by Keough et al. (1988). Lipid and hydrophobic protein were obtained by extraction of pig lung surfactant 3 times with chloroform and methanol according to the method of Bligh and Dyer (1959). The pooled chloroform phases were evaporated under N_2, resuspended in chloroform–methanol (CM) (2:1 v/v), and applied to a 1.5 × 75 cm silicic acid column. Elution was carried out using 3 bed volumes of 20:1 CM (v/v) followed by 2 bed volumes at 9:1, 4:1, 3:2, and finally 1:4. Fractions (2 mL) were collected and assayed for phosphorus (Bartlett, 1959; Keough & Kariel, 1987) and protein initially by using the fluorescamine method (Udenfriend et al., 1972). Protein determinations proved variable by using this method. A modification of the method described by Bradford (1976) was adopted. Samples were reduced under N_2 and solubilized in 0.05 mL of 2-chloroethanol followed by addition of 0.8 mL of H_2O and color reagent. Bovine serum albumin was used as a standard. There were no differences in the standard curves obtained for albumin in the presence or absence of 2-chloroethanol. Large amounts of phospholipids (>80 μg per assay tube) may affect the reliability of this assay so that the level of lipid in the sample must be controlled. Protein and phospholipid elution profiles were constructed. Two protein-enriched pools were detected followed by a large phospholipid peak. Protein from the first peak (pool 1) was electrophoresed on SDS–PAGE under reducing (using 5% DTT or 5% β-mercaptoethanol in sample buffer) and nonreducing conditions. On both types of gels, a major band was observed migrating at approximately 5 kDa, and a minor band corresponding to a molecular weight of about 12K was observed on heavily loaded gels. Pool 1 protein was applied to an LH-20 column equilibrated with CM (2:1 v/v) to remove residual lipid. Protein eluted from the column was dialyzed against three changes of CM (2:1 v/v) for 4 h and used for preparing the lipid dispersions containing 1% and 2.2% protein. For the preparation of the dispersion with 8% (w/w) protein, the dialysis was omitted due to excessive losses of protein in this step. Proteins prepared with or without dialysis contained less than 1% (w/w) phospholipid based on phosphorus and Bradford protein determinations.

Surface Activity. Samples of pure DMPC-d_{54} and DMPC-d_{54} containing 8.0% (w/w) surfactant protein in CM (2:1 v/v) were reduced to dryness under N_2 and resuspended in 0.15 mL of buffer containing 50 mM 3-morpholinoethansulfonic acid and 0.15 M NaCl at pH 6.9. Each sample contained 200 nmol of lipid. Dispersions were injected into a Teflon beaker containing 5 mL of stirred sample buffer in which surface pressure was measured by a platinum dipping plate suspended from a strain gauge.

Protein Sequencing. The sample was dissolved in 200 μL of dichloromethane/MeOH (1:1) solution. Twenty microliters of this solution was spotted on an 8-mm-diameter PVDF (Immobilon, Millipore) membrane and dried with a stream of nitrogen. This was then introduced into the reaction cartridge of a Porton Model 2090 gas-phase peptide sequencer equipped with an online PTH-amino acid analyzer. The membrane was covered with an 8-mm Whatman glass fiber (GF) which had been previously coated with about 1 mg of polybrene and precycled for two Edman cycles. The reagent delivery on the Porton sequencer took place from the bottom.

The PTH-amino acid analysis was carried out on a Hewlett Packard Amino Quant C18 column, 2.1 mm × 200 mm, using a sodium acetate solution/acetonitrile gradient at a flow rate 200 μL/min at 42 °C. The detection of PTH residues was carried out spectrophotometrically at 268 nm.

^2H NMR. DMPC-d_{54} was synthesized according to the method described by Gupta et al. (1977) using fatty acid perdeuterated by the method of Hsiao et al. (1974). Stock solutions of DMPC-d_{54} and surfactant protein were prepared in CM (2:1 v/v). Appropriate volumes of each solution were mixed and then dried by rotary evaporation and placed under vacuum overnight at room temperature. Samples were prepared with protein concentrations of 1.0, 2.2, and 8% (w/w). A sample of pure DMPC-d_{54} was prepared in the same way from the same stock solution. Each sample contained about 35 mg of the perdeuterated lipid. After being dried, the samples were scraped into 8-mm NMR tubes and hydrated with 300 μL of 50 mM phosphate buffer at pH 7.0. The samples were gently stirred with a fine glass rod. Prior to a particular NMR experiment, each sample was equilibrated at 32 °C for approximately 2 h.

The ^2H NMR experiments were carried out in a superconductive solenoid at 23.2 MHz using a spectrometer constructed in-house. The signal was digitized and averaged by using a Nicolet 2090 oscilloscope interfaced to a Tandy 1200 microcomputer. The sample was located in a copper oven whose temperature was controlled by a second microcomputer. After the sample reached a particular temperature, it was allowed to equilibrate for at least half an hour before accumulation of the transients was initiated. Spectral moments were determined by using a phase-cycled quadrupole echo sequence (Davis et al., 1976) with a $\pi/2$ pulse length of be-

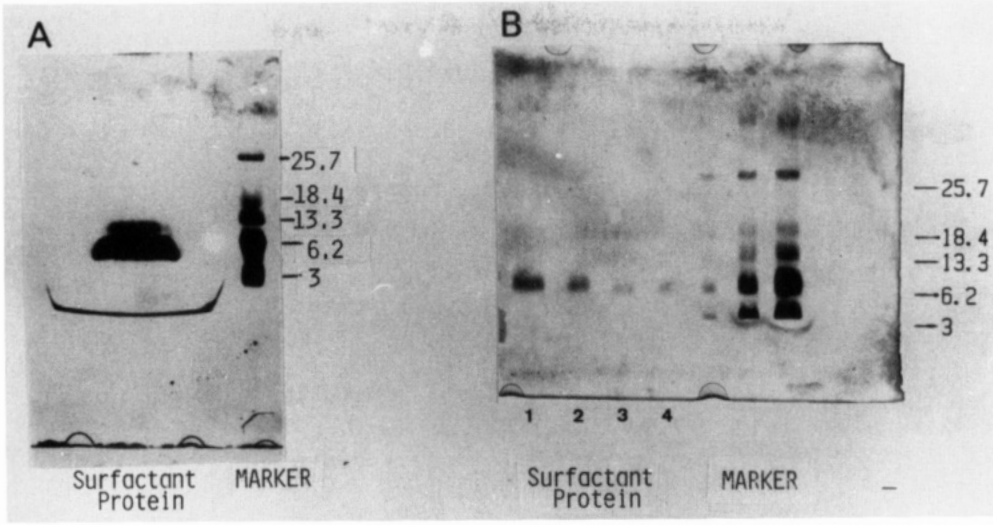

FIGURE 1: SDS–PAGE of surfactant protein isolated by silicic acid and LH-20 chromatography. (A) Twenty micrograms of protein under reducing conditions and (B) 10, 5, 2, and 1 μg of protein in lanes 1, 2, 3, and 4, respectively, under nonreducing conditions. Gels were visualized with silver stain.

tween 2.5 and 3.5 μs and a quadrupole echo delay time of 35 μs. For spectra in the liquid-crystalline phase, 4000 transients were normally collected with a dwell time of 2 μs and a repetition time of 1 s. For gel-phase spectra, 8000 transients were collected with a dwell time of 1 μs. To ensure that the Fourier transform began from the top of the quadrupole echo, it was generally necessary to shift the points in the accumulated echo signal by some fraction of a dwell time in order to leave one point at the top of the echo (Davis, 1983). To improve the signal to noise ratio in the current experiment, even and odd points in the accumulated signal were separated and shifted separately to give two free induction decays with points at the top of the echo. These were then added to yield contracted free induction decays with effective dwell times for the liquid-crystal and gel experiments of 4 and 2 μs, respectively.

The transverse relaxation time, T_{2e}, was measured for the pure lipid and the sample containing 8% (w/w) protein at a series of temperatures using quadrupole echo experiments with a series of delay times, τ, between the $\pi/2$ pulses of the sequence. The spectrometer conditions were as described for the spectral moment studies. For exponential decay, the signal intensity is expected to go as

$$A(2\tau) = A(0)e^{-2\tau/T_{2e}} \quad (1)$$

Measurements were also made of the spin–lattice relaxation time, T_1, above (22 °C) and below (16 °C) the gel to liquid-crystal transition temperature of the perdeuterated lipid. T_1 was measured by using an inversion recovery sequence consisting of a π pulse followed, after a delay, τ, by a quadrupole echo sequence. The inversion recovery sequence alternated with a normal quadrupole echo sequence, and the difference between the resulting transients was accumulated. The signal thus obtained, assuming exponential decay, is then given by

$$A(\infty) - A(\tau) = [A(\infty) - A(0)]e^{-\tau/T_1} \quad (2)$$

The ^{2}H NMR experiments were performed on samples containing lipids with perdeuterated chains. Because different positions along the chain display different relaxation rates, the resulting decays were not exponential. In addition, both transverse relaxation (Pauls et al., 1985) and spin–lattice relaxation (Brown et al., 1979) are expected to display a dependence on orientation which will also contribute to nonexponential decay for powder samples. While the use of a specifically deuterated lipid would have simplified the relaxation analysis, the limited amount of protein available precluded the preparation of additional samples with specific labels. In determining relaxation rates using the initial slopes of decay curves, we are implicitly averaging the relaxation behavior over orientation and position along the chain.

DSC. Aliquots from the samples used for NMR were removed and prepared for DSC. The DSC samples (containing about 4 mg of DMPC-d_{54}) were dispersed in 2 mL of degassed 50 mM phosphate buffer at pH 7.0 and vortexed briefly at 25 °C. An aliquot of 1.2 mL of each dispersion was loaded into a MC-2 differential scanning calorimeter (Microcal Inc., Northhampton, MA) and run against buffer in the reference cell. Calorimetric scans were carried out at a rate of 30 °C/h and at an instrumental sensitivity of 1 and with a filtering constant of 15. Each sample yielded reproducible thermograms over three heating cycles from approximately 5 to 40 °C. Values for ΔH were calculated with the Microcal software by extending the base line from T_{onset} to T_{final} and determining the enclosed area under the transition. Residual sample dispersions were extracted as described above to recover the lipid and protein and remove the phosphate buffer. Aliquots of phosphate buffer were also extracted, and analysis showed that essentially all the phosphorus was recoverable in the aqueous phase with none detected in the organic phase. Extracted phospholipid from each dispersion was assayed for phosphorus content, and aliquots were evaluated for possible lipid hydrolysis using thin-layer chromatography. Approximately 0.5 mg of lipid was applied to a thin-layer chromatography plate and run in a solvent system containing chloroform–methanol–water, 65:25:4 (v/v), and visualized by charring with 70% H_2SO_4. No evidence of breakdown was observed. After the NMR and DSC studies, the samples were also assayed for protein content as described above and electrophoresed on SDS–PAGE.

Results

The delipidated surfactant protein was electrophoresed on SDS–PAGE under reducing and nonreducing conditions and visualized with silver stain (Figure 1). The sample displayed a major band with a mobility corresponding to a molecular weight of 5K–6K, with a minor band with a nominal molecular weight of 12K under reducing or nonreducing conditions on heavily loaded gels. Scanning densitometry indicated the

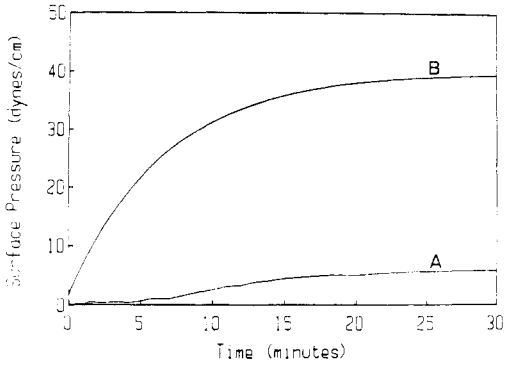

FIGURE 2: Adsorption isotherms for DMPC-d_{54} and DMPC-d_{54} containing 8% (w/w) surfactant protein. Dispersions containing 200 nmol of lipid were injected into the subphase for either (A) the pure lipid or (B) lipid plus protein. Temperature was 22 °C.

5–6-kDa component to be ≥90% of the material.

Amino acid analysis after a 24-h hydrolysis at 110 °C in 6 N HCl showed an amino acid composition consistent with that reported by Johansson et al. (1988) for SP-C under similar hydrolysis conditions. We also observed additional amino acids constituting less than 6% of the total which are not found in the surfactant protein SP-C but are constituents of SP-B (Possmayer, 1988). After 48 and 72 h of hydrolysis at 150 °C, we observed a modest increase in the valine content but not to the extent of that reported by Johansson et al. (1988). Another hydrolysis in 6 N HCl at 150 °C yielded a valine to leucine ratio of 2:1, consistent with the high ratio found by Johansson et al. (1988) from SP-C. Sequencing data for the first 15 amino acids showed the presence of a major sequence consisting of Leu-Arg-Ile-Pro-XXX-XXX-Pro-Val-Asn-Leu-Lys-Arg-Leu-Leu-Val-Val-. A minor sequence (30% based on the N-terminal amino acid) was also detected which was similar to the one above with the exception that the Leu at the N-terminal was absent. The major sequence and the truncated form are identical with those reported by Johansson et al. (1988) for porcine SP-C. The amino acids at position 5 and 6 are known to be cysteines (Johansson et al., 1988) which were not determined in the sequencing method used.

Isotherms at 22 °C of surface pressure versus time for dispersions of DMPC-d_{54} and DMPC-d_{54} containing 8% (w/w) protein are presented in Figure 2. In the dispersion containing protein, the surface pressure increased to 30 dyn/cm in the first 10 min and reached 40 dyn/cm after 30 min. In the dispersion of lipid alone, only a modest increase in surface pressure was observed, reaching 7 dyn/cm after 30 min. Similar rates of adsorption were observed when the dispersions were tested at 15 °C, below the transition temperature for the pure lipid.

Figure 3 shows the temperature dependence of the ^{2}H NMR line shape for DMPC-d_{54} in the region of the phase transition for the pure lipid, 2.2% (w/w) protein, and 8% (w/w) protein samples. For the pure lipid, there is a sharp transition from spectra characteristic of fast axially symmetric motion in the liquid-crystalline phase to typical gel-phase spectra at 19 °C and lower temperatures. In the presence of the protein, axially symmetric spectra persist to lower temperatures, and the change from liquid-crystal to gellike spectra is not as pronounced.

First spectral moments (M_1), from a series of such spectra as a function of temperature, are shown in Figure 4. The first spectral moment is proportional to the average quadrupole splitting. For the axially symmetric spectra characteristic of the liquid-crystalline phase, M_1 is thus proportional to the mean orientational order parameter (S_{CD}) where

$$S_{CD} = \tfrac{1}{2}(3 \cos^2 \theta_{CD} - 1) \qquad (3)$$

and θ_{CD} is the angle between the CD bond vector and the bilayer normal (Davis, 1979). In the gel phase, the interpretation of M_1 is complicated by the nonaxial symmetry of the motion but can still be taken as an indication of orientational order. It appears that, for the concentrations investigated, the presence of the protein in the bilayer has almost no effect on the spectral moment, and thus the orientational order, in the liquid-crystalline phase. In the gel phase, the protein reduces M_1, indicating a disordering influence on the surrounding lipid.

The influence of the protein on the nature of the transition is illustrated by Figure 5. This figure shows pairs of spectra, for pure lipid, 2.2% (w/w) protein, and 8% (w/w) protein samples at the temperatures between which M_1 displays the largest change with temperature. For the pure lipid, the transition is abrupt. There is no evidence, in the gel-phase spectrum at 19 °C, of spectral features like the sharp 90° edges characteristic of the liquid-crystalline phase. The sample with 2.2% (w/w) protein displays an axially symmetric spectrum characteristic of the liquid-crystalline phase at 19 °C. At 18 °C, the spectrum is a superposition of an axially symmetric component and a gellike component. The observation of an unambiguous two-component spectrum of this sort implies that exchange between domains of the two phases takes place with a characteristic time that is long compared with T_{2e} for the various spectral components involved. The temperature de-

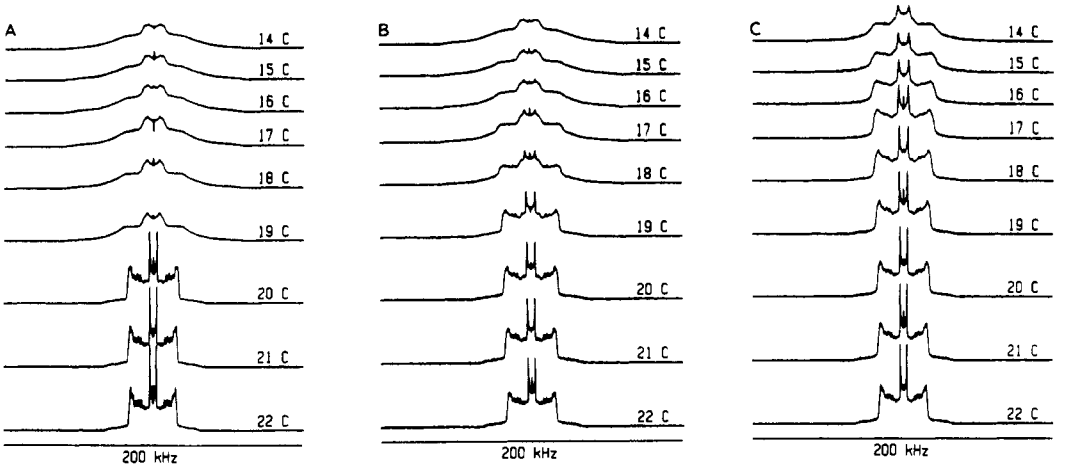

FIGURE 3: Temperature dependence of the ^{2}H NMR spectrum of DMPC-d_{54} near the pure lipid transition for (A) the pure lipid, (B) 2.2% (w/w) protein in DMPC-d_{54}, and (C) 8.0% (w/w) protein in DMPC-d_{54}.

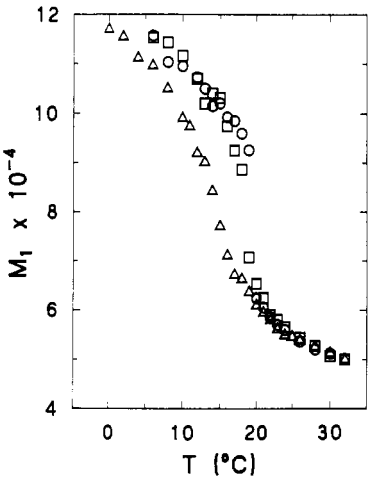

FIGURE 4: Temperature dependence of the first spectral moment, M_1. Pure DMPC-d_{54} (○); 2.2% (w/w) protein in DMPC-d_{54} (□); Δ8% (w/w) protein in DMPC-d_{54} (△).

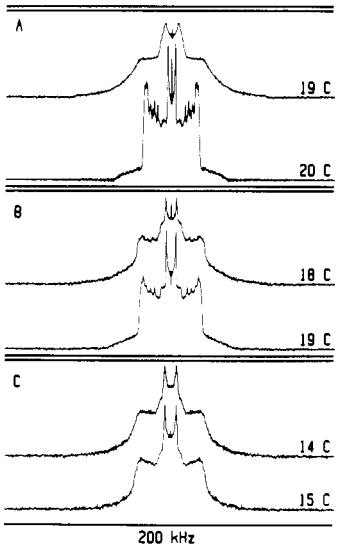

FIGURE 5: ^{2}H NMR spectra at temperatures between which M_1 undergoes the largest change on cooling. (A) Pure DMPC-d_{54}; (B) 2.2% (w/w) protein in DMPC-d_{54}. The spectrum at 18 °C is a superposition of liquid-crystal and gellike spectra having the sharp edges characteristic of the axially symmetric liquid-crystalline spectrum in addition to the broad wings characteristic of the gel-phase spectrum; (C) 8% (w/w) protein in DMPC-d_{54}.

pendence of M_1 in this region thus reflects the changing proportion of gel and liquid crystal as the two-phase coexistence region of the binary phase diagram is traversed. In the spectra for the 8% (w/w) protein sample, two-phase coexistence is not as apparent in the transition region. As the temperature is lowered to 15 °C, the spectra suggest the presence of a single phase with its orientational order becoming more gellike as the temperature is lowered. If the spectrum at 14 °C does consist of two components, their spectra are rather similar, and neither is distinctly liquid crystal or gellike. In this sample, roughly half of the change in M_1 associated with the transition occurs before the broad wings characteristic of gel-phase spectra appear. Little of the change in M_1 is due to shifting proportions of liquid-crystal and gellike contributions in two-component spectra.

The spectra obtained do not suggest the presence of any protein-induced nonbilayer phase. Figure 6 shows the spectra at 32 °C for the pure lipid and for the sample containing 8% (w/w) protein. Shown, at the same scale, is the difference between these spectra. The presence of the protein induces small differences in the splitting of particular deuteron com-

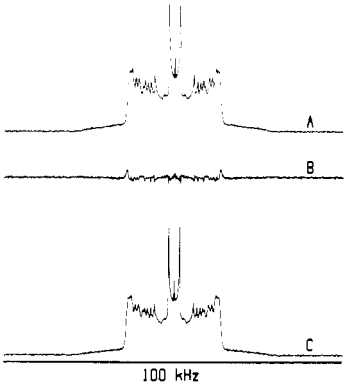

FIGURE 6: (A) ^{2}H NMR spectrum for pure DMPC-d_{54} at 32 °C. (B) The difference between spectra A and C. (C) ^{2}H NMR spectrum for 8% (w/w) protein in DMPC-d_{54}.

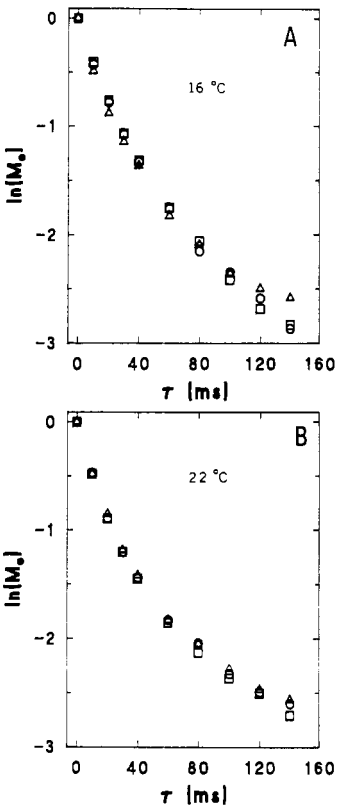

FIGURE 7: Inversion recovery results plotted as ln $[A(\infty) - A(\tau)]$ versus τ at (A) 16 °C and (B) 22 °C. DMPC-d_{54} (○); 2.2% (w/w) protein in DMPC-d_{54} (□); 8% (w/w) protein in DMPC-d_{54} (△).

ponents, and some residue is seen in the subtraction at the 90° edges. There is, however, no evidence of an additional axially symmetric spectral component in the protein-containing sample. It is estimated that such a component having about 5% of the overall signal intensity would be detected in the subtraction. A nonbilayer component with a narrow spectrum compared to that of the bilayer, such as the hexagonal H_{II} phase, should be even more apparent, and the limit of detectability should decrease accordingly.

Figure 7 shows the result of the inversion recovery experiments, at 16 and 22 °C, for the pure lipid and the two higher protein concentrations. There is essentially no dependence on protein concentration in this concentration range. The initial decay, which represents an average over all of the deuterons, gives a T_1 of 28 ms at 22 °C and 30 ms at 16 °C. The slower decay, at larger values of τ, reflects the more slowly relaxing deuterons on the chain. Spectra taken at longer τ (not shown) indicate that it is deuterons with a smaller quadrupole splitting,

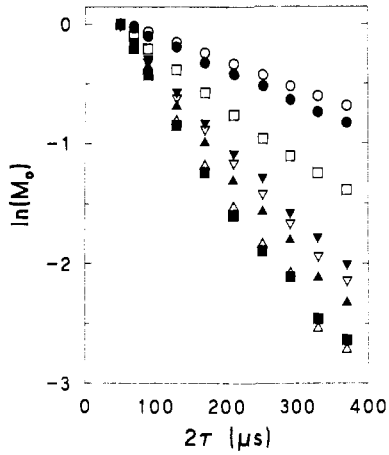

FIGURE 8: Quadrupole echo decay results plotted as ln intensity versus 2τ for 8% (w/w) protein in DMPC-d_{54} at 26 (○), 22 (●), 18 (□), 14 (■), 10 (△), 6 (▲), 2 (▽), and −2 °C (▼).

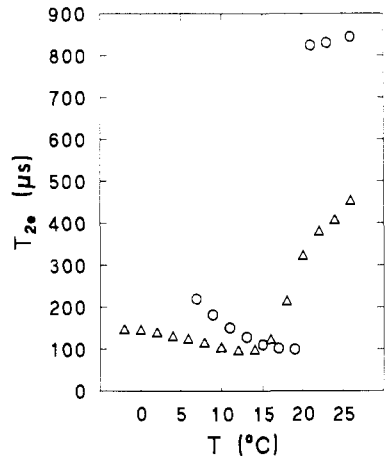

FIGURE 9: T_{2e} versus T for DMPC-d_{54} (○) and 8% (w/w) protein in DMPC-d_{54} (△).

particularly the methyl deuterons at the bilayer center, which relax more slowly.

Figure 8 displays some of the quadrupole echo decay results for the sample with 8% (w/w) protein. The initial slope yields $\langle T_{2e}^{-1} \rangle$ where the average is over all the sites along the chain. Over the range of 2τ employed, the departure from exponential behavior is small.

Figure 9 displays T_{2e} as a function of temperature for pure DMPC-d_{54} and for the sample with 8% (w/w) protein. In the gel phase of the pure lipid, T_{2e} decreases as the temperature is raised. At the transition, T_{2e} increases discontinuously by about a factor of 8. In the presence of protein, T_{2e} displays a minimum below the pure lipid transition temperature and undergoes a more continuous increase as the temperature rises into the liquid-crystalline regime where T_{2e} is about half of the pure lipid value. The effect of the protein on T_{2e} in the liquid-crystalline phase is very similar to that seen for a variety of protein–lipid systems (Bloom & Smith, 1985). Overall, the effect of the protein on T_{2e} is very similar to that seen with gramicidin (Morrow, 1990).

Heating thermograms obtained for the dispersions of pure DMPC-d_{54} and DMPC-d_{54} plus 1.1, 2.2, and 8% protein are shown in Figure 10. A decrease in the enthalpy change of the phase transition was apparent with increasing protein concentration. Generally, a low-temperature broadening was seen in the thermograms for protein-supplemented dispersions with a corresponding increase in the half-height peak widths. Analytical data for the thermal transitions of pure DMPC-d_{54}

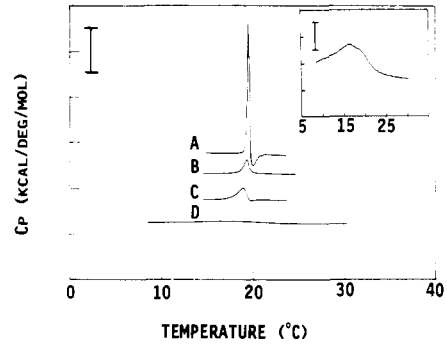

FIGURE 10: Heating thermograms for (A) DMPC-d_{54} and DMPC-d_{54} plus (B) 1.0%, (C) 2.2%, or (D) 8% (w/w) surfactant protein (bar = 10 kcal deg^{-1} mol^{-1}). Expanded scale for 8% (w/w) surfactant protein in DMPC-d_{54} (bar = 1 kcal deg^{-1} mol^{-1}, inset).

and lipid–protein dispersions are summarized in Table I. The onset of the phase transition was depressed from 18.8 °C for the pure lipid to 15.7 °C for the dispersion with 2.2% protein. A graded drop in T_m with increasing protein concentration was also evident. The T_m for the pure lipid was found to be 19.5 °C, and addition of protein yielded reduced T_m values of 19.3, 18.9, and 17.0 °C for dispersions containing 1.0, 2.2, and 8% protein, respectively. A reduction in the enthalpy change of transition was also evident from 6.3 kcal/mol for the pure lipid to 4.4 kcal/mol for the dispersion with 8% protein.

Discussion

Biophysical properties including dynamic surface tension lowering and rapid adsorption are considered important in the efficient function of pulmonary surfactant. Though surfactant is rich in phospholipid, it is now considered that the low molecular weight, hydrophobic, surfactant proteins play an important role in normal surfactant function by increasing the rate of monolayer formation. When either or both of the hydrophobic surfactant proteins, SP-B and SP-C, have been added to DPPC or mixtures of DPPC and other lipids, they have enhanced the rate of monolayer formation (Yu & Possmayer, 1986; Fujiwara et al., 1987; Efrati et al., 1987; Curstedt et al., 1987; Smith et al., 1988). It has been suggested that the rapid movement of lipid from the subphase to the air–water interface might involve the production of irregularities in bilayer packing, or even the production of nonbilayer phases. The protein studied here, which we consider to be primarily SP-C, caused an increase in the adsorption rate of DMPC to the air–water interface both above and below the phase transition temperature for the pure lipid, consistent with the properties of these hydrophobic proteins observed by other workers.

These studies have included systems containing 0–8% by weight of the hydrophobic protein SP-C as estimated by the Bradford technique. It is not easy to establish the exact lipid to protein ratios for the hydrophobic protein in true natural surfactant, and there are potential technical difficulties with the estimation of surfactant proteins. Many estimations of total hydrophobic protein content (about 1–2% by weight of lipid) have been based on the amount of proteins found in lipid extract by either amino acid analysis or Lowry protein assays [e.g., see Yu et al. (1983)]. However, color production in the Lowry protein procedure is dependent primarily on tyrosine residues, of which there are none in SP-C. We could not detect protein in our material by the Lowry procedure, whereas we could with the fluorescamine or Bradford methods. The SP-C protein is very resistant to conventional amino acid analysis, so its content in surfactant might be underestimated by this procedure also. Thus, the levels of protein used in these studies

Table I: Differential Scanning Calorimetry for DMPC-d_{54} plus Surfactant Protein SPC[a]

	T_m (°C)	T_{onset} (°C)	T_{final} (°C)	$\Delta T_{1/2}$ (°C)	ΔH (kcal mol^{-1})
DMPC-d_{54}	19.5	18.8	19.9	0.3	6.3
DMPC-d_{54} + 1.0% protein	19.3	16.7	20.2	0.7	6.6
DMPC-d_{54} + 2.2% protein	18.9	15.7	19.8	1.1	6.0
DMPC-d_{54} + 8.0% protein	17.0		24.6	7.8	4.4

[a] T_m, temperature of maximum excess heat capacity; T_{onset}, temperature of transition onset; T_{final}, temperature of transition completion; $\Delta T_{1/2}$, width of transition at half-maximum excess heat capacity; ΔH, enthalpy change of transition.

seem to be within the range which may be relevant to the amount present in natural pulmonary surfactant.

Our calorimetric measurements indicate that the proteolipid caused a small decrease in the T_m of the pure lipid and some broadening of the transition with concomitant lowering of the onset temperature. At a protein concentration of 8%, we observed a significant reduction in the enthalpy change of transition from 6.3 kcal/mol for the pure lipid to 4.4 kcal/mol for the system with 8% (w/w) protein. The decrease in the enthalpy change of the transition in the presence of the protein was similar to that observed by Shiffer et al. (1987) using DPPC and SP-C. For the pure lipid, they observed an enthalpy change of transition of 9.1 kcal/mol, and at a protein concentration of 4% and 20% w/w, they observed a reduction in the transition enthalpy changes to 5.4 and 3.3 kcal/mol, respectively (Shiffer, personal communication). These calorimetric data indicate that the protein interacts with the bilayer array and causes a loss of cooperativity in the melting behavior. If it is assumed that the protein molecular weight is 3.7K (Johannson et al., 1988), the sample containing 8% protein would have 1.7 mol % protein. In our experiment, the reduction in the enthalpy change of transition caused by this amount of protein was 30%. If it is assumed that the protein influences the lipid so as to remove all the enthalpy change of transition associated with an individual lipid molecule, then each protein molecule appears to influence approximately 18 lipid molecules.

The effect of the surfactant protein on the first spectral moment, M_1, is very similar to that observed for a synthetic amphiphilic polypeptide in DPPC (Huschilt et al., 1985). Like the bilayer-spanning polypeptide studied by Huschilt et al. (1985), this surfactant protein has little effect on M_1 in the liquid-crystalline phase, reduces the discontinuity in M_1 at the transition, and lowers M_1 in the gel phase. In the presence of the synthetic polypeptide, however, the change in M_1 across the transition is primarily due to changing proportions of superposed gel and liquid-crystal spectral components. In the present system, little of the change in M_1 occurs over spectra showing two-phase coexistence. A significant part of the change in M_1 at the transition is due to the increased splitting, on cooling, of nearly axially symmetric spectra. These lack the broad wings characteristic of the gel phase. Two-phase coexistence in a binary system is indicative of first-order transition behavior. The apparent absence, for the highest protein concentration studied here, of two-component spectra in the range accounting for most of the change in M_1 at the transition suggests that the protein may promote a shift from first-order behavior to a more continuous phase change. This possibility has been discussed for other systems (Morrow & Whitehead, 1988; Morrow & Davis, 1988).

As observed for other proteins, there is essentially no effect on the lipid T_1, suggesting that the surfactant protein does not perturb lipid chain motions with characteristic times in the range 10^{-9}–10^{-8} s (Bloom & Smith, 1985). There is, however, a profound effect on motions with correlation times long enough to influence T_{2e}. Recently, Bloom and Sternin (1989) used a quadrupole Carr–Purcell–Meiboom–Gill experiment to demonstrate that transverse relaxation in the liquid-crystalline phase contained a significant contribution from slow motions which they interpreted as diffusion of lipid molecules around curved vesicle surfaces. Such motion is too slow to contribute to motional narrowing.

The observation that in the liquid-crystalline phase the surfactant protein reduces T_{2e} without affecting M_1 suggests that the protein is mostly influencing a slow motion. The contribution to the quadrupole echo relaxation rate, from such a motion, should increase with decreasing correlation time (Pauls et al., 1985). If the slow motion is diffusion around the curved vesicle surface, then the protein effect in the liquid crystal may be interpreted as a reduction in average vesicle diameter or an increase in diffusion along the bilayer surface.

Below the gel to liquid-crystal transition, the sample with 8% (w/w) protein displays a minimum in T_{2e}. This implies that one of the motions contributing to transverse relaxation is passing through the regime in which the correlation time is approximately $(\Delta M_2)^{-1/2}$ where ΔM_2 is that portion of the second moment modulated by the motion (Pauls et al., 1985). In general, cooling should increase the correlation times for all motions. Since the diffusive motion is in the slow motion regime even in the liquid-crystalline phase, the minimum likely reflects slowing of a faster motion. Although the T_{2e} minimum is not observed for the pure lipid sample, the trend in T_{2e}, up to the intervention of the transition, suggests that the minimum value of T_{2e} is not too different from that observed in the presence of protein. Within the proposed interpolation scheme of Pauls et al. (1985), this would suggest that the protein does not significantly alter ΔM_2 for the motion dominating the transverse relaxation. The shift in temperature of the minimum does, however, suggest that the protein alters the temperature dependence of the correlation time τ_c for the motion by allowing shorter τ_c to persist to lower temperatures. This is very similar to the influence of gramicidin on transverse relaxation in the gel phase of DMPC (Morrow, 1990). Meier et al. (1987) have recently studied the effect of myelin proteolipid apoprotein on the lipid motions in specifically labeled DMPC using a comprehensive model to simulate line shapes and relaxation. They are able to account for the effect of the protein on the lipid in their highest protein concentration samples by increasing the correlation times for all of the motions in their model by about an order of magnitude over those for the pure lipid.

The hydrophobic protein appears to influence the lipid acyl chains in a manner not too dissimilar from that for other proteins which penetrate the lipid bilayer. To that extent, the studies carried out here do not suggest any special mechanism by which the hydrophobic protein would enhance transfer of lipid from bilayers in the hypophase of lung alveoli to the air–water interface. It is possible that such a transfer could be effected by the induction of nonbilayer phases (e.g., hexagonal or micellar phases) by the hydrophobic proteins. It is thus useful to know what would be the lower limits of detection of such phases by the NMR techniques used here. The analysis presented in Figure 6 suggests that the likelihood of such phases being present in the sample with 8% protein is low. Micellar or hexagonal phases would have been expected to produce signals of much narrower splittings than observed in the subtracted spectra (protein plus lipid, minus lipid) seen in Figure 6. No such signals were seen so that the amount

of nonbilayer phases present above the T_c of the lipid was small. While the hydrocarbon chains in the bilayer were influenced only to a limited extent by the surfactant protein, it may be possible that the protein could cause significant perturbation in the head group region. Roux et al. (1989) found that such a differential perturbation of the different regions of a lipid bilayer could be produced by a synthetic cationic integral membrane peptide. Currently, experiments are in progress to check the possibility that SP-C might act in the same fashion.

Nevertheless, we estimate that the lower limit of detectability of such phases by the method is about 5% of the total lipid. Therefore, the studies do not preclude the possibility that induction of such phases in small amounts by the hydrophobic proteins may be a potential mechanism by which these proteins act. Also, one cannot yet totally discount the possibility that the influence of SP-C on a more complex mixture of lipids in the presence of calcium could be different from the behavior seen in the system studied here.

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