Pulmonary Surfactant-Associated Protein SP-B Has Little Effect on Acyl Chains in Dipalmitoylphosphatidylcholine Dispersions†

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ABSTRACT: Synthetic human pulmonary surfactant-associated protein SP-B has been interacted with chain-perdeuterated dipalmitoylphosphatidylcholine (DPPC-d_{62}) in aqueous dispersions, and the dispersions were investigated by magnetic resonance spectroscopy. The protein caused only small perturbations of the deuterium magnetic resonance spectra in the gel and liquid-crystal states. In an amount of 11% by weight in DPPC, it produced a small reduction in the magnitude of the first moments of the spectra in the gel and a small increase (~5%) in their magnitude in the liquid crystal. In the liquid crystal the protein was observed to cause a similar effect on all portions of the acyl chain, as observed by its proportional shifting of splittings obtained from "dePaked" spectra. Using data from circular dichroism spectra, the protein was found to be about 45% α-helical in methanol and in DPPC dispersions. α-Helical content was not significantly changed by the presence of 2 mM calcium or by the packing state of the acyl chains. The presence of the protein enhanced the adsorption rate of lipid into the air–water interface when dispersions of lipids or lipid plus SP-B were injected below the interface. The results could be consistent with the protein interacting with the lipid near the head groups or arranging itself around the edges of bilayer discs, or a combination of the two orientations.

Pulmonary surfactant is material which is synthesized and secreted by the type II pneumocytes in lung alveoli that acts to reduce surface tension at the air–water interface of the lung. The material reduces the work of breathing, prevents alveolar collapse, and reduces the driving force for edema in the air spaces. It is essential for proper physiological function of the lung, and if it is absent or compromised, serious pathological conditions may result.

Pulmonary surfactant is composed of approximately 90% lipids and 10% proteins. The lipids are mostly phospholipids, and dipalmitoylphosphatidylcholine (DPPC)[1] is the major phospholipid, such that it constitutes over 40% of the total weight of surfactant. It is generally considered that the surface properties of the air–water interface of the lung are contributed by a film of DPPC-enriched lipid that has arisen most likely via a process of selective exclusion of non-DPPC components during cycling compression and expansion of the lung surface during breathing.

The surfactant-associated proteins make important contributions to a number of surfactant functions. In particular, the hydrophobic surfactant proteins, SP-B and SP-C, appear to be necessary for the rapid movement of lipid from the aqueous subphase lining of the alveoli into the air–water interface, an essential transformation in surfactant. Some type of concerted mechanism must be involved in the cooperative transfer of lipids from the subphase into the interface (Keough, 1985; Goerke & Clements, 1966). The hydrophobic proteins are involved in promoting this process [e.g., Suzuki (1982), Tanaka et al. (1986), Takahashi and Fujiwara (1986), Yu and Possmayer (1986), Whitsett et al. (1986), Hawgood et al. (1987), and Smith et al. (1988)]. To understand how this process occurs, we need to understand how the proteins interact with lipids and in what ways they might disrupt lipid packing in the bilayer phase. In this work we have investigated the influence of a chemically synthesized human SP-B protein on the packing of dipalmitoylphosphatidylcholine using the technique of deuteron magnetic resonance spectroscopy. We have also investigated the structure of the SP-B in rigid and fluid lipid bilayers using circular dichroism spectroscopy.

MATERIALS AND METHODS

Chain-perdeuterated dipalmitoylphosphatidylcholine (DPPC-d_{62}) was obtained from Avanti Polar Lipids, Pellam, AL. The synthetic analogue of human SP-B, which was lacking the C-terminal methionine of the mature natural material, was made by solid-phase synthesis on a phenylacetamidomethyl resin, starting from the carbonyl terminal (Banany & Merrifield, 1980), and purified by reverse-phase high-performance liquid chromatography.

Samples of either pure lipid or lipid plus protein were prepared by dissolving the materials in chloroform–methanol (2:1, v/v). The solvent was removed by rotary evaporation under reduced pressure followed by evacuation over P_2O_5 overnight. Samples contained 0, 5, and 11% (w/w) of the protein as a part of total solids. The sample with the higher

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[1] Abbreviations: CD, circular dichroism; DSC, differential colorimetry; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPC-d_{62}, chain-perdeuterated 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; ^{2}H NMR, deuterium nuclear magnetic resonance spectroscopy; SP-B, pulmonary surfactant protein B.

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protein concentration was prepared by extracting the sample of lower concentration by the method of Bligh and Dyer (1959), drying the lipid–protein extract obtained in the resulting lower phase, redissolving it, and enriching it with SP-B. The residual inorganic ions from the buffer in the original sample dissolved in the aqueous phase in this extraction. Weights of the NMR samples ranged from 18 to 26 mg. Each was placed in an 8-mm-diameter tube, hydrated with 250–300 μL of 50 mM phosphate buffer (pH = 7.0), and gently stirred with a fine glass rod. Samples were equilibrated in the spectrometer at 50 °C for 1–2 h.

Following equilibration, ^{2}H NMR spectra were collected for a series of temperatures beginning at 50 °C and descending to 4 °C. Temperatures were changed in 1° steps near the transition and 2° steps otherwise. Samples were allowed to equilibrate at each temperature for at least 20 min prior to the spectrum being obtained using 2000 transients with a repetition time of 0.9 s. NMR measurements were carried out in a superconductive solenoid at 23.2 MHz using a quadruple-echo pulse sequence (Davis et al., 1976) with a $\pi/2$ pulse length between 2.8 and 3.4 μs and a pulse separation of 35 μs. Other details of the spectrometer are reported in Simatos et al. (1990a). Oriented spectra were calculated from some powder spectra using the "dePaking" computer program developed and generously provided by Bloom and co-workers (Bloom et al., 1981; Sternin et al., 1983) and modified to run under UNIX on a MIPS M120 computer.

Samples for circular dichroism were prepared by dissolving the protein in methanol and the DPPC in chloroform–methanol, 2:1 (v/v), and mixing the two so that the protein constituted 10% of the total solids by weight. The solvents were evaporated under N_2, and the material was evacuated overnight. Water was added to give a final concentration of 0.5 mg of protein/mL. The samples were dispersed over 5–6 cycles of heating for about 10 min at 50 °C followed by vortexing for 30–60 seconds. The samples were transferred from their glass containers to small plastic tubes for an Eppendorf microcentrifuge, and they were sonicated at 0 °C using a Branson Sonifier 185 with a tapered microtip at a power setting of 3, employing 50–60 short (0.5–1 s) bursts of power. The sonicated samples were centrifuged in an Eppendorf Model 3200 microcentrifuge at 12000*g* for 1 min, and the clear supernatants were taken for circular dichroism analysis. The samples were analyzed in a 1.0-mm-path-length jacketed cell placed as close as possible to the photocell in a Jasco Model J-500A spectropolarimeter. Usually, 16 to 32 spectra were collected and averaged at each temperature.

Adsorption experiments were carried out at room temperature as described before (Simatos et al., 1990a). High-sensitivity differential scanning calorimetric scans of dispersions of DPPC-d_{62} and DPPC-d_{62} plus 11% SP-B used in the NMR studies were carried out in a Microcal MC-2 (Amherst, MA) as described before (Simatos et al., 1990a). Phosphorus was determined by a small modification of the method of Bartlett (1959) described previously (Keough & Kariel, 1987). Protein was estimated by weight and by the method of Bohlen et al. (1973).

RESULTS

Figure 1 shows adsorption isotherms from samples of dispersion of DPPC-d_{62} and the lipid containing 11% SP-B. The protein enhanced the rate of adsorption of the lipid into the interface. The sample with the lower lipid concentration plus 11% SP-B showed a lag before adsorption was started. There was no lag with the higher concentration.

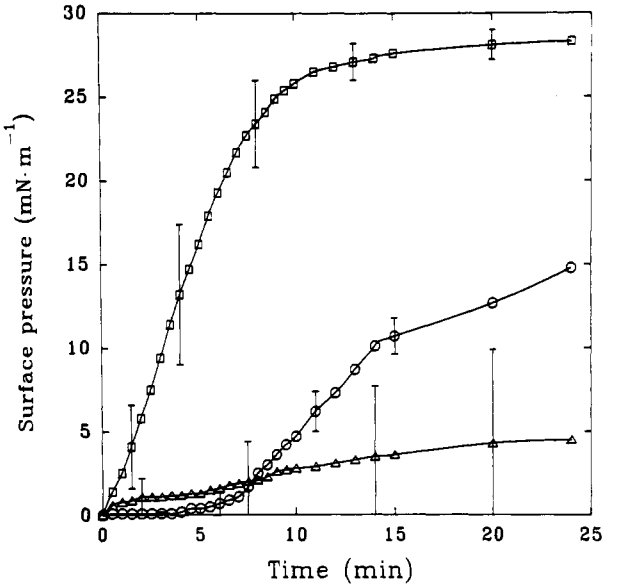

FIGURE 1: Adsorption of dispersions of DPPC-d_{62} and DPPC-d_{62} plus 11% (w/w) SP-B. Samples were injected into a total volume of 5 mL of 0.15 M NaCl plus 3 mM $CaCl_2$. △, 100 μg DPPC-d_{62}; ○, 56 μg DPPC-d_{62} + 6.9 μg SP-B; □, 111.2 μg DPPC-d_{62} + 13.8 μg SP-B. Values are means of 2–3 experimental runs, and the error bars represent ranges.

Figure 2 shows ^{2}H NMR spectra at a series of temperatures for DPPC-d_{62} and for DPPC-d_{62} with 11% (w/w) SP-B. The liquid crystal to gel transition for the pure chain-perdeuterated lipid is centered slightly above 37 °C in comparison to 41 °C for its hydrogen-containing analogue. Spectra for the sample containing SP-B were only slightly broader, in the liquid-crystalline phase, than corresponding spectra for the pure lipid sample. The spectra at 36 and 35 °C are superpositions of gel and liquid-crystal spectral components indicating that SP-B induces coexistence of gel and liquid-crystal domains over a narrow temperature range below the pure lipid transition temperature.

The first moment, M_1, is proportional to the average quadrupole splitting for spectra characteristic of axial symmetry and generally indicates average orientational order along chains. Figure 3 shows the temperature dependence of M_1 for three samples, pure lipid and lipid containing 5 and 11% (w/w) SP-B. Values of M_1 for the sample containing 11% (w/w) SP-B in the liquid-crystalline phase are about 5% higher than those for the pure lipid, indicating a slight increase in chain ordering due to the presence of SP-B. This sample also shows a slightly less abrupt change in M_1 at the transition due to the coexistence of gel and liquid-crystal domains over a small temperature range. It is apparent, however, that SP-B does not have a large effect on orientational order in either the gel or liquid-crystal phases or on the gel to liquid crystal phase transition itself.

Figure 4 shows oriented spectra, obtained by "dePaking" powder spectra, for DPPC-d_{62} and for the sample containing 11% (w/w) SP-B, both at 40 °C. Corresponding splittings, are between 6 and 7% larger for the sample containing SP-B, but there is no qualitative difference in the distribution of order along the chain. This suggests that the perturbation due to SP-B is not localized at a particular depth in the bilayer but is distributed along the chain. This is consistent with a recent report (Lafleur et al., 1990) that the shape of the order parameter profile is correlated with order parameter magnitudes and, thus, is not sensitive to localized perturbation.

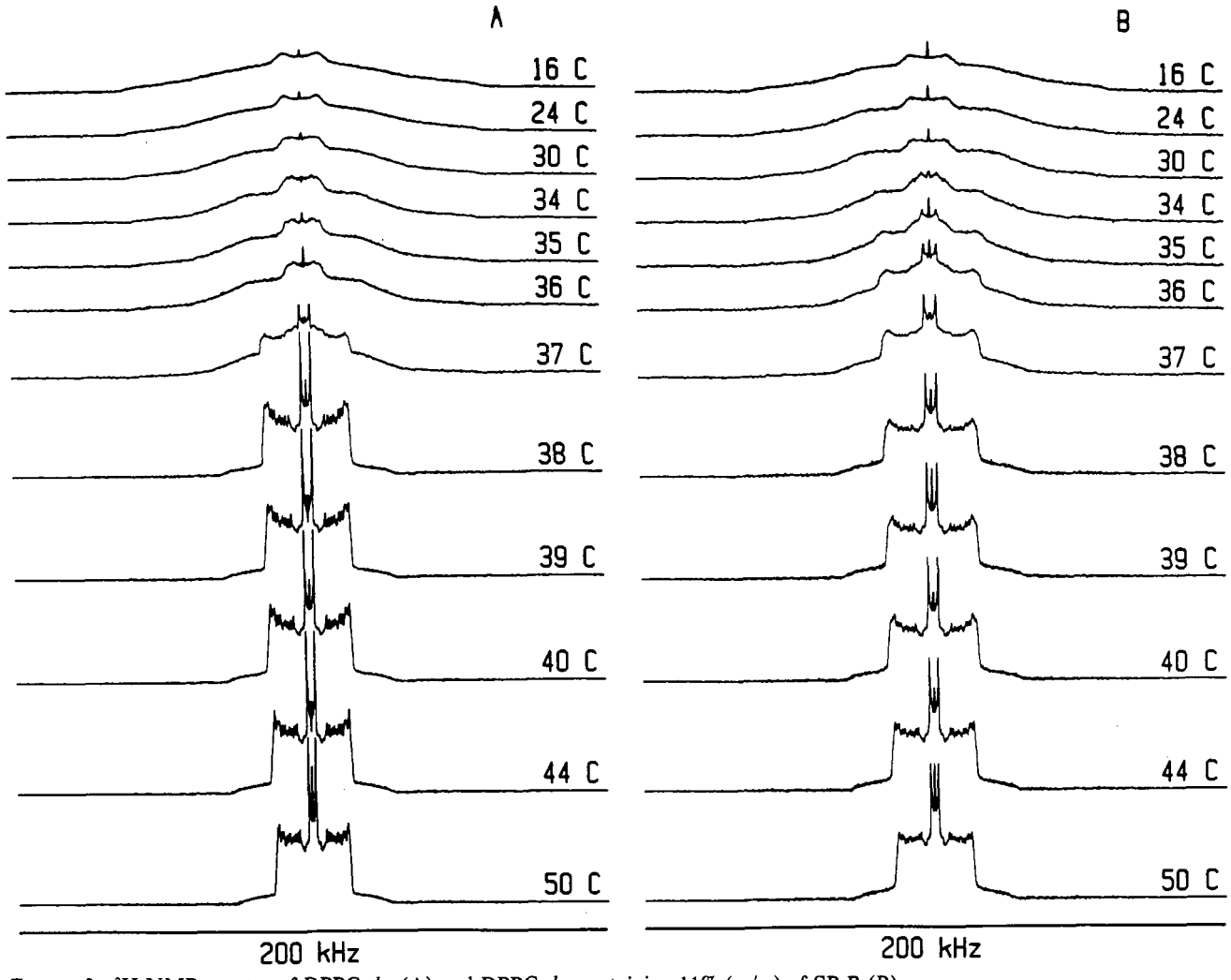

FIGURE 2: ^2H NMR spectra of DPPC-d_{62} (A) and DPPC-d_{62} containing 11% (w/w) of SP-B (B).

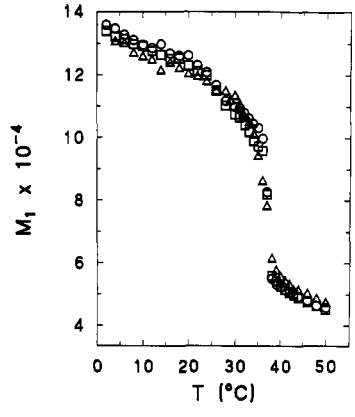

FIGURE 3: First moments of the ^2H NMR spectra of DPPC-d_{62} (○), DPPC-d_{62} containing 5% (w/w) SP-B (□), and DPPC-d_{62} containing 11% SP-B (△).

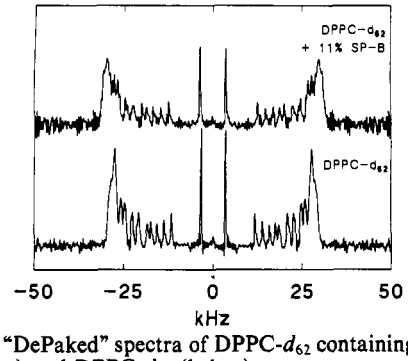

FIGURE 4: "DePaked" spectra of DPPC-d_{62} containing 11% (w/w) SP-B (above) and DPPC-d_{62} (below).

Figure 5 shows the circular dichroism spectra of SP-B in DPPC above and below the phase transition temperature. The secondary structure contributions were calculated using the program CDPROT (Menédez-Aries et al., 1988) and a number of data bases (Chen et al., 1974; Bolotina et al., 1980; Yang & Kubota, 1985; Yang et al., 1986). Because of the limitations in the range of the spectra, and since the base data are for soluble proteins, we regard such calculations with caution, especially with regard to the amounts of secondary structure other than α-helix. The data in Table I represent a consensus from various calculations, and they suggest that the α-helix content of SP-B is 40–45% in DPPC and that it changes only very little as the lipid phase changes. As the figure, shows, there was only a slight difference in the two spectra taken below and above the gel to liquid crystalline phase transition temperatures of DPPC. Circular dichroism spectra of the protein dissolved in methanol were essentially the same as shown for the lipids, and calculations of α-helical content indicated that essentially the same amount of α-helix was present in the peptide when it was dissolved in methanol or when it was interacting with lipid in aqueous suspension (Table I). The addition of 2 mM Ca^{2+} had essentially no effect on the spectra under these conditions.

While SP-B had little effect on chain ordering in the liquid-crystalline phase, its effect on the thermotropic behavior

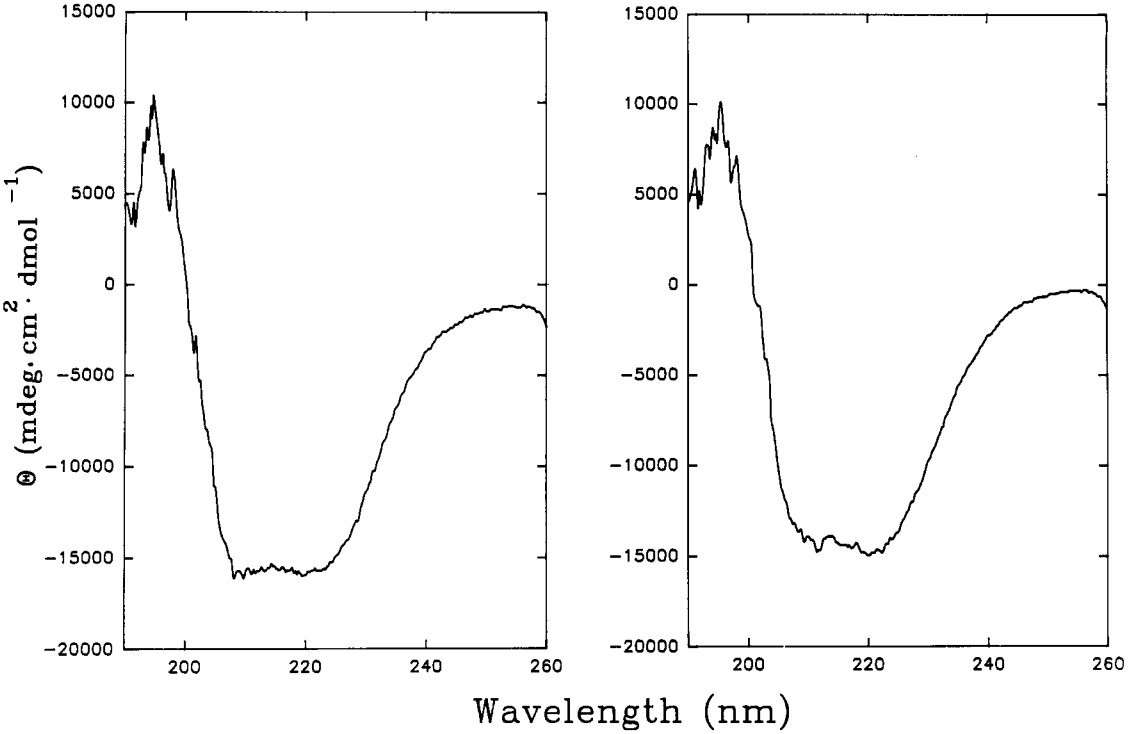

FIGURE 5: Circular dichroism spectra of SP-B in sonicated DPPC vesicles at 23 °C (left) and 56 °C (right).

Table I: Estimates of Secondary Structure for SP-B[a]

structure[b]	SP-B in methanol	SP-B in DPPC		SP-B in DPPC			
				23°		56°	
		23°	56°	+Ca^{2+}	−Ca^{2+}	+Ca^{2+}	−Ca^{2+}
α	47	47	42	37	45	40	41
β	1	3	10	5	6	8	10
T	8	10	12	7	11	13	13
R	44	40	36	51	38	39	36

[a] The values were taken as those which gave the best fits between experimental and calculated curves using the various base data sets.[b] α, α-helix; β, β-structure; T, β-turn; R, random.

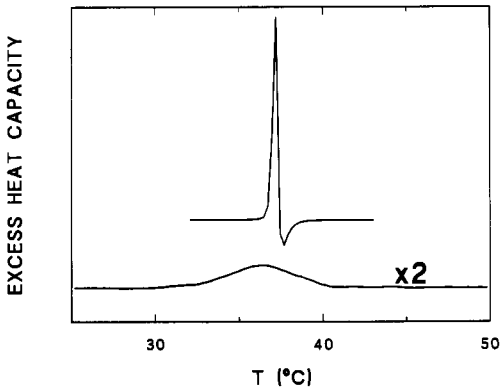

FIGURE 6: DSC traces. The upper scan is DPPC-d_{62}. The baseline was fit by a cubic polynomial and subtracted. The lower scan is DPPC-d_{62} with 11% (w/w) SP-B. The baseline was fit by a quadratic polynomial and subtracted. Excess specific heat has been normalized to lipid content in each sample.

indicated a significant association of the protein with the bilayer. The spectra in Figure 2B show coexistence of gel and liquid-crystalline phases over a range of at least 2°. Broadening of the transition is also seen in the temperature dependence of M_1 as shown in Figure 3. This broadening is particularly apparent in the differential scanning calorimetry traces shown in Figure 6. It is interesting to note that, despite the broadening, the transition enthalpies for the pure lipid

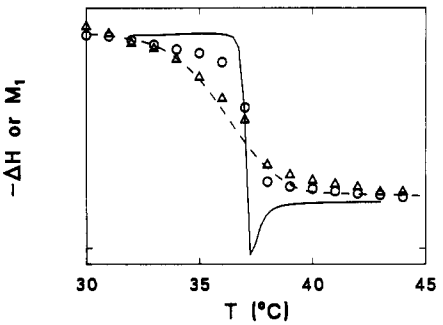

FIGURE 7: Comparison of NMR and DSC results for the main transition. Open symbols: ^2H NMR first spectral moment (M_1) versus temperature for DPPC-d_{62} (○) and DPPC-d_{62} containing 11% (w/w) SP-B (△). Lines: Minus the integral of excess heat capacity for DPPC-d_{62} (solid line) and DPPC-d_{62} containing 11% (w/w) SP-B (dashed line). Curves were obtained by integrating the traces of Figure 6 and multiplying by −1. The $-\Delta H$ axis was shifted and scaled so as to match the negative DSC integrals to the high- and low-temperature values of M_1.

and the sample containing 11% (w/w) SP-B were approximately equal.

It is important to recognize that the broadening seen in the DSC experiment is consistent with that shown by NMR. Since M_1 is a measure of chain order, its temperature dependence should correspond approximately to minus the integral of the DSC trace if most of the transition enthalpy is associated with chain disordering (Morrow & Davis, 1987). Figure 7 shows the temperature dependence of M_1 for DPPC-d_{62} and for the sample containing 11% (w/w) SP-B along with negative integrals of the corresponding DSC curves from Figure 6. The enthalpy axis has been shifted and scaled so that the end points of the integrated DSC curves correspond to high- and low-temperature values of M_1. The relationship of the DSC curves, however, has been conserved, and similarity in the size of the step at the transition reflects the similar transition enthalpies for the two samples. The pure-lipid integral is somewhat distorted by the negative overshoot on the DSC trace of the pure lipid. (This overshoot is a consequence of

the instrument operating conditions and is accounted for in the calculations of ΔH.) For the protein-containing sample, NMR and DSC yield very similar shapes and widths for the step at the transition. This observation confirms that the association of protein with the bilayer indicated by DSC is also present in the NMR sample despite the insensitivity of the liquid-crystalline chain ordering to the pressure of SP-B.

DISCUSSION

The addition of the synthetic SP-B enhanced the rate of adsorption of material into the air–water interface. The significance of the lag in the adsorption at the lower concentration remains to be established. At the higher concentration, the adsorption of lipid plus 11% protein is rapid at first, but slows considerably after the initial adsorption period. This effect was seen with SP-C and DMPC (Simatos et al., 1990a,b) and with SP-C and DPPC (Pérez-Gil et al., 1992). The significance of this reduction in the rate is not yet understood. The limiting surface pressure for an air–water interface fully filled with lipid would be about 45 mN/m. When acidic lipids such as phosphatidylglycerol are present in addition to proteins, near-equilibrium surface pressures are reached more rapidly (e.g. Pérez-Gil et al., 1992).

The results presented here indicate that SP-B causes only a slight perturbation of the packing of the acyl chains of DPPC. The other hydrophobic surfactant protein, SP-C, has been found to perturb acyl chain packing of saturated PC in a fashion similar to that found for other bilayer-spanning proteins and polypeptides (Simatos et al., 1990a,b; Bloom & Smith, 1985; Huschilt et al., 1985). SP-C was found to disorder the chains in the gel phase but to have little or no effect on the chain orientational order in the liquid-crystalline phase (Simatos et al., 1990a,b). At 5% by weight in saturated PC it caused the occurrence of gel and liquid crystal coexistence over a narrow temperature range just below that of the transition of the pure lipid. An amount of 8% (w/w) of SP-C in lipid was found to produce a continuous phase change from liquid crystal to gel, with no detectable region of two-phase coexistence (Simatos et al., 1990a,b). This is an effect similar to that of gramicidin in a lipid bilayer (Morrow & Davis, 1988; Morrow & Whitehead, 1988).

The protein SP-B caused a smaller pertubation of the chain packing in PC bilayers than did SP-C. While the sample containing 11% protein (w/w) produced spectra with some evidence of two-phase coexistence over a very narrow temperature range (Figure 1), there was only a very small effect of the protein on the orientational order, especially in the gel phase.

A potential explanation for the small effect of SP-B on M_1 might have been that the protein was incompletely associated with the lipid. The data presented in Figures 6 and 7 tend to strongly discount this suggestion. The effect of SP-B to broaden and reduce the maximum in excess specific heat of the endotherm seen by DSC would suggest a significant interaction between the protein and the lipid. As noted in the Results section, the integral of the DSC curve and the M_1 curve are closely matched, indicating that both techniques are "seeing" the same influence of the protein. There is, therefore, a significant influence of the protein on the transition. Both the M_1 and the DSC data indicate that the protein has little effect on the enthalpy change associated with the overall transition from the gel to the liquid-crystalline state. Assuming that the enthalpy change is primarily due to changes in the acyl chains, these observations would be consistent with only a small influence of the protein on the chains.

The monomer molecular mass for SP-B is about twice that of SP-C (8.6 vs 4.2 kDa). The SP-B monomer contains seven cysteines, which in the natural protein form inter- and intrachain linkages (Johansson et al., 1991), so that the naturally occurring form is a dimer. The extent and the location of disulfides in the synthetic protein are not known. SDS–PAGE gels of the synthetic SP-B indicated that the majority of the material appeared to be monomeric, but on highly loaded gels, ladders showing small amounts of higher molecular weight polymers were observed. Comparisons between the magnitudes of effects of the two proteins on the basis of molarity in the samples are of only limited value without specific details of their three-dimensional structures, data which is unavailable at the current time. Structural predictions (Waring et al., 1990; Takahashi et al., 1990) and our CD results on SP-B here and on SP-C (unpublished), together with Fourier transform infrared studies of SP-C (Pastrana et al., 1991; Vandenbussche et al., 1992), suggest that SP-B has less α-helix than SP-C. Comparisons per unit of mass and per amino acid residue show SP-C to perturb acyl chain packing much more than SP-B.

SP-B has only a small effect on the orientational order and dynamics of the acyl chains. In the amounts used it caused almost no effect in the gel state, but it did increase overall order very slightly in the liquid-crystal state (Figures 2 and 3). Baatz et al. (1990), studying bovine SP-B in DPPC–PG (7:1) mixtures in the presence of fluorescent lipid probes, concluded that the protein interacted at or near the head-group region of the phospholipid bilayer and that it introduced ordering of the lipid in that region. Baatz et al. (1991), also employing fluorescence probe studies, observed that synthetic human SP-B caused a slight ordering of acyl chains of DPPC–DPPG (7:1) in the region of the head group and no substantial effect deeper in the bilayer. Such an interpretation would be consistent with our observation of a very small effect on the acyl chains. These data do not appear to be consistent with the perturbation of acyl chains by hydrophobic parts of SP-B to any great extent, although artificial peptide models of portions of SP-B may interact in such a way (Cochrane & Revak, 1991).

Other orientations of the SP-B with respect to the lipid may be consistent with the results to date, also. Williams et al. (1991) have recently shown that DPPC plus PG plus SP-B can form discs. An orientation of SP-B around the edges of lipid bilayer discs, reminiscent of the interactions between phospholipids and apolipoprotein (Morrisett et al., 1977), could be possible. Hydrophobic faces of the calculated amphipathic helices in the protein could interact with the hydrophobic edges of the lipid discs (Waring et al., 1989). Charge interactions between lipid head groups, for example, PG polar groups, and the positive charges on the protein could help orient the lipid–protein association. Besides agreeing with the electron microscopic evidence (Williams et al., 1991), this arrangement would be consistent with the relatively small magnitude of the effect of SP-B on the lipid chains seen by ^{2}H NMR.

With the evidence to date, it is not possible to discount either model. Evidence is consistent with SP-B interacting with the lipid primarily in the head-group region or with the head group and at the edges of discs. It should be noted, however, that a slight increase in chain ordering in the liquid-crystalline phase, induced by the presence of SP-B, suggests that the protein causes a slight reduction in area per lipid in the liquid-crystalline phase. Such an observation may be more easily accommodated in a model involving interaction around

disc edges than in a model involving interaction primarily at the head groups. The latter type of interaction might be expected to separate head groups slightly and thus reduce chain-packing density.

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