# Lung Surfactant Proteins, SP-B and SP-C, Alter the Thermodynamic Properties of Phospholipid Membranes: A Differential Calorimetry Study†

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ABSTRACT: The ability of the low molecular weight lung surfactant-associated proteins, SP-B and SP-C, to alter the thermotropic properties of synthetic multilamellar vesicles was tested using differential scanning calorimetry (DSC). The presence of either SP-B or SP-C in dipalmitoylphosphatidylcholine (DPPC) or dipalmitoylphosphatidylglycerol (DPPG) multilamellar vesicles broadened the DSC thermogram and reduced the enthalpy of transition in a concentration-dependent manner. With both proteins, the temperature at which the peak of the phase transition ($T_m$) was detected was shifted to a higher value. The increase in $T_m$ caused by both proteins was greater with DPPG than DPPC. We have interpreted these results as implying the presence of a protein-perturbed domain of lipid. Both SP-B and SP-C were found to influence the surface activity of the phospholipids in a concentration-dependent fashion. We speculate that instability of lipid packing predicted to occur at protein-created lipid domain boundaries may be important for the expression of surface activity in pulmonary surfactant.

Pulmonary surfactant is a mixture of lipids and proteins which helps to keep the lungs expanded by lowering surface tension at the air/fluid interface in the alveoli (Goerke & Clements, 1986). The lipids present in lung surfactant are rich in dipalmitoylphosphatidylcholine (DPPC)[1] and unsaturated phosphatidylcholine (PC) and contain significant amounts of phosphatidylglycerol (PG) and cholesterol in addition to other trace lipids (King & Clements, 1972). Three different surfactant-associated proteins have been characterized (Hawgood & Clements, 1990; Weaver & Whitsett, 1991).

A primary requirement for an adequate lung surfactant is the ability to greatly lower surface tension (<10 mN/m) when sufficiently compressed at an air/water interface at 37 °C (King & Clements, 1972). The main component of lung surfactant, DPPC, satisfies this requirement when spread on an aqueous surface, but pure DPPC does not adsorb and spread rapidly at an air/fluid interface at 37 °C. Rapid adsorption is a second important requirement of a functional lung surfactant (King & Clements, 1972). The other surfactant components, such as the unsaturated PC, PG, and all three of the surfactant-associated proteins, may contribute to the adsorption and spreading of the surfactant surface film and possibly modify the behavior of the interfacial film as it is cyclically compressed during respiration.

The largest surfactant protein, SP-A, binds readily to phospholipids (King, 1984) but has little effect on the surface activity of these phospholipids when tested in vitro in the absence of other proteins (King, 1984). The two smaller surfactant proteins, SP-B and SP-C, however, both have significant independent effects on phospholipid surface activity in vitro (Curstedt et al., 1987). SP-B has an apparent denatured molecular weight of 18 000 under nonreducing conditions and a molecular weight of approximately 9000 under reducing conditions (Hawgood et al., 1987). The processed active form of SP-B contains 79 amino acids in 3 loops constrained by 3 intrachain disulfide bonds (Johansson et al., 1991). The amino acid composition of SP-B is rich in cysteine residues, positively charged amino acids, and hydrophobic amino acids. SP-C has an apparent molecular weight of approximately 4000. SP-C is a cationic protein of only 35 amino acids. The carboxy-terminal tail is composed of 23 hydrophobic amino acids (Warr et al., 1987), and the protein is made even more hydrophobic by the addition of thioester-linked palmitic acid moieties on cysteines-5 and -6 in the human (Curstedt et al., 1990) and cysteine-5 in the dog (Stults et al., 1991). Both SP-B and SP-C are posttranslationally processed from larger precursors (Hawgood et al., 1987; Warr et al., 1987).

Although both SP-B and SP-C can dramatically increase the rate of phospholipid surface film formation in vitro (Curstedt et al., 1987; Hawgood et al., 1987; Warr et al., 1987), the precise mechanisms involved are not presently understood. As many membrane-associated proteins are known to affect the organization of the lipids around them, it is reasonable to suppose that the surfactant proteins may disturb the packing and order of the phospholipids in surfactant in ways that lead to bilayer instability and favor more rapid monolayer formation. Differential scanning calorimetry (DSC) is a technique that allows the direct examination of

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[1] Abbreviations: CD, circular dichroism; DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; DSC, differential scanning calorimetry; $\Delta H$, transition enthalpy; MLV, multilamellar vesicle(s); PC, phosphatidylcholine; PG, phosphatidylglycerol; $T_m$, temperature of main-phase transition peak; SDS, sodium dodecyl sulfate.

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the effect of membrane proteins on the thermodynamic properties of associated lipids (McElhaney, 1986). DSC does not provide molecular information on membrane structure or dynamics, but useful insight into the organization of the lipids into particular domains in response to the addition of protein can often be obtained. To begin an investigation of the effects of SP-B and SP-C on the physicochemical properties of various lipid membranes, we have performed DSC and surface activity measurements on multilamellar vesicles (MLV) containing either canine SP-B or SP-C. These results have been presented in preliminary form (Shiffer et al., 1988a).

## MATERIALS AND METHODS

Dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylglycerol (DPPG), and egg phosphatidylglycerol (egg PG) were obtained from Avanti Polar Lipids (Birmingham, AL). The lipids were found to be chromatographically pure by thin-layer chromatography (Touchstone et al., 1979) and were therefore used as supplied. Thin-layer chromatographic plates were from Whatman (type LK 5D, Clifton, NJ), L-histidine, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (Tes), and ethylenediaminetetraacetic acid (EDTA) were from Sigma (St. Louis, MO), and Sephadex LH-60 was from Pharmacia (Piscataway, NJ). Chloroform and methanol (Mallinckrodt, Inc., Paris, KY) were redistilled prior to use, and butanol (Mallinckrodt, Inc.) was used as supplied. Water was double-distilled from glass.

*Preparation and Analysis of Lung Surfactant.* Lung surfactant was isolated from the lung lavage fluid of adult dogs as previously described (Hawgood et al., 1985). Briefly, the lungs from exsanguinated dogs were removed, degassed, and lavaged 3 times at 4 °C with 1000 mL of 5 mM Tris-HCl/100 mM NaCl, pH 7.4, per lavage. The pooled lung washings were spun at $150g_{av}$ for 20 min to remove cellular material. The supernatant was then centrifuged at $20000g_{av}$ for 2 h, and the resulting pellet (approximately 90–92% of the total phospholipid from the supernatant in the previous step) was dispersed in buffer containing 5 mM Hepes/1.64 M NaBr, pH 7.4. After equilibration for 1 h, this suspension was spun at $100000g_{av}$ for 15 h. The pellicle was resuspended in isotonic saline, dounced several times, and then centrifuged at $100000g_{av}$ for 1 h. This step was repeated, and then the final pellet was resuspended in double-distilled water and used as purified lung surfactant. Phospholipid concentrations were calculated from the phosphorus content (Bartlett, 1959). For protein determination (Lowry et al., 1951), 1% sodium dodecyl sulfate (SDS) was added to all samples, and bovine serum albumin was used as the standard.

*Purification of SP-B and SP-C from Surfactant.* The lipids and proteins were isolated from purified lung surfactant as previously described (Warr et al., 1987). In order to separate surfactant lipids from SP-B and SP-C, purified surfactant was resuspended in double-distilled water (approximately 10–15 mg of phospholipid/mL), injected into rapidly stirred 1-butanol (1-butanol/water, 50/1, v/v), and then stirred at room temperature for 30 min. After centrifugation at $10000g_{av}$ for 20 min, the supernatant (butanol phase containing surfactant lipids plus SP-B and SP-C) was dried under vacuum at 42 °C and then dissolved in chloroform/methanol/0.1 N HCl (1/1/0.05, v/v). After centrifugation at $10000g_{av}$ for 10 min, the chloroform/methanol-soluble fraction was chromatographed on Sephadex LH-60 using the same acidified chloroform/methanol mixture. The eluted fractions were monitored for protein by the fluorescamine assay (Böhlen et al., 1973) in the presence of 1% SDS and by SDS–polyacrylamide gel electrophoresis without disulfide reducing agents utilizing silver-stained 15% gels (Morrissey, 1981), and for inorganic phosphorus (Bartlett, 1959). Fractions containing protein of the appropriate molecular weight but no detectable phospholipid were pooled and used as SP-B or SP-C. Final protein concentrations of the pooled samples were determined by weighing aliquots of the purified proteins (Cahn electrobalance, Ventron Instruments, Corp., Paramount, CA). These weights corresponded well with the values obtained by fluorescamine analysis and by quantitative amino acid analyses, but were consistently 10 times higher than the protein concentrations obtained in our modified Lowry assay. Lipid/protein molar ratios were calculated using molecular weights of 10 176 for canine SP-B and 4364 for canine SP-C (Hawgood et al., 1987; Warr et al., 1987). To detect fatty acids in the protein samples, 50 μg of each protein was hydrolyzed in 5% HCl in methanol for 18 h at 70 °C. The samples were extracted with hexane and the methyl esters separated on a Hewlett-Packard gas–liquid chromatograph. Methylated nonadecanoic acid was used as a standard for quantitation.

*Preparation of Liposomes.* Multilamellar liposomes (MLV), with or without SP-B or SP-C, were prepared at temperatures above the phase transitions of the lipid mixtures employed. An aliquot of DPPC or DPPG (in chloroform) and the SP-B or SP-C (in 0.1 N HCl/chloroform/methanol, 0.05/1/1, v/v) were dried under nitrogen, vacuum-evaporated for an additional hour, and then rehydrated in buffer (155 mM NaCl, 2 mM L-histidine, 2 mM Tes, and 1 mM EDTA, pH 6.9) at 65 °C for 15 min. Since calcium can significantly influence the thermodynamic properties of both PC and PG, it was not included in the buffer used to rehydrate the vesicles. Each MLV preparation contained 2.5–3 mg of phospholipid in 1 mL of buffer. To determine the relative amounts of bound and free lipid, samples with the highest lipid/protein ratios (100/1, w/w) were prepared with a trace amount of [$^3$H]-DPPC and centrifuged at $100000g_{av}$ for 16 h through a continuous gradient of 0.25–0.75 M sucrose in the Tes, histidine, and EDTA buffer. Fractions were collected, and the content of [$^3$H]DPPC was determined in a scintillation counter.

*Differential Scanning Calorimetry Measurements.* Each MLV preparation, which was to be run in the Perkin-Elmer DSC-2 instrument, was concentrated by spinning the liposomes at $12200g_{av}$ for 20 min at 4 °C. An aliquot (10 μL) of the concentrated MLV preparation was transferred to an aluminum pan which was sealed hermetically. Two or three pans were prepared from each sample, and each pan was run at least 2 times. Thermograms were performed on a Perkin-Elmer DSC-2 instrument at a scan speed of 5 °C/min over a temperature range of 0–70 °C. The enthalpy of the phase transition was determined from the area under the endotherm using indium as a standard. The base line was determined by eye. The phospholipid concentration was determined by phosphorus analysis (Bartlett, 1959) after dispersing the contents of the pans in 0.5 mL of water. Repeated scans of the same sample in the DSC-2 calorimeter gave nearly identical heat capacity profiles.

*Surface Activity Measurements.* Surface film pressure was measured with a roughened platinum Wilhelmy dipping plate (King & Clements, 1972). A 3-mL Teflon trough (surface area, 3.1 cm$^2$) was placed in a maximally-humidified temperature-controlled chamber at 37 °C. The surface of the subphase buffer (155 mM NaCl, 10 mM Tes, and 0.1 mM EDTA, pH 6.9) was cleaned by aspiration. After a 5-min

equilibration period, MLV (30 μg of phospholipid/mL, final concentration) were carefully injected into the subphase without contaminating the surface, stirring was started, and surface pressure was recorded. Since calcium was not used in the DSC experiments, it was not added to the subphase buffer or samples for the surface activity measurements.

*Circular Dichroism Measurements.* Spectra were recorded on a JASCO J-500 A spectropolarimeter at room temperature. A JASCO DP-500 data processor was used to accumulate and average four spectra for each experiment. Each experiment was performed with a different SP-C preparation. The cell path length was 0.5 mm, and the scan speed was 10 nm/min. Molar ellipticities were expressed in degrees centimeter squared per decimole on the basis of an average molecular weight of 121 per residue. The secondary structure content was predicted using the algorithm of Yang (Yang et al., 1986).

SP-C was prepared for CD analysis at 0.1 mg/mL in 1-butanol (the solvent used to initially solubilize the protein), in 1% SDS in 5 mM Tris, pH 7.4, and in either DPPC or mixtures of DPPC and egg PG (7/3, w/w). The SP-C was reconstituted with the lipids for these experiments at a ratio of 10/1 lipid/protein (w/w) by drying the lipid and protein (both in chloroform/methanol) under nitrogen and then hydrating the lipid/protein mixture in 5 mM Tris, pH 7.4 at 50 °C. The samples were sonicated to clarity prior to recording the spectra at room temperature. Lipid samples without protein were prepared in the same fashion, but the spectra obtained from these samples did not deviate significantly from the base line, so the spectra from the lipid/protein mixtures were used without correction. To assess the effect of palmitoylation on the secondary structure of SP-C, recombinant human SP-C (rhSP-C) was substituted for dog SP-C in some experiments. rhSP-C was expressed as a fusion protein in *Escherichia coli*. The mature protein of 35 amino acids was cleaved from the fusion protein and purified by reverse-phase HPLC. rhSP-C is not palmitoylated (Stults et al., 1991). Similar samples were prepared with SP-B, but in the presence of lipid, turbidity prevented a reliable interpretation of the spectra obtained, and the data were not analyzed further.

## RESULTS

*SP-B Multilamellar Vesicle Studies.* Figure 1A shows the DSC-2 heat capacity profiles of DPPC MLV which contained various amounts of SP-B. The phase transition of pure DPPC MLV was abrupt, as evidenced by the sharpness of the peak. As the DPPC/SP-B ratios were lowered, i.e., as more SP-B was added, the pretransition peak disappeared, the height of the main-phase transition peak at 41.5 °C decreased, and then the main-phase transition peak at 41.5 °C broadened and shifted to 43.5 °C. The DSC-2 scans of DPPG MLV containing various weight ratios of SP-B are shown in Figure 1B. As the DPPG/SP-B ratios were lowered, the pretransition peak disappeared, and the height of the main-phase transition peak at 41.5 °C decreased. However, in this case, at intermediate DPPG/SP-B ratios of 17/1 and 8/1, two partially resolved endotherms were present, and the main-phase transition at 41.5 °C had broadened and shifted to 50–58 °C. At the lowest DPPG/SP-B ratio tested (5/1), only a single broad transition peaking at 58 °C was observed.

The areas under the main transitions in our DSC-2 thermograms were normalized to the amount of phospholipid analyzed, and an enthalpy of the phase transition ($\Delta H$) was calculated for each. In all cases, the total phospholipid recovered was used for these calculations as we were unable to detect significant free lipid by sucrose density centrifugation

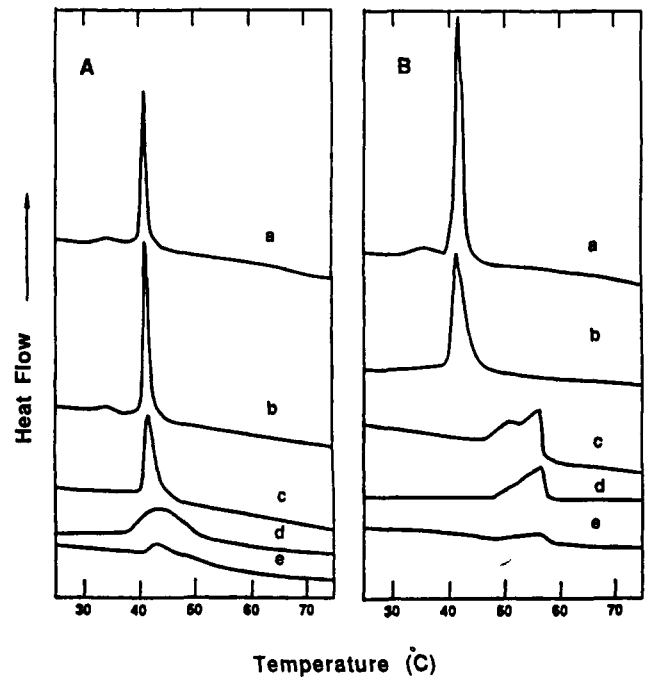


FIGURE 1: Differential scanning calorimetry thermograms. A (left): (a) DPPC; (b) DPPC + SP-B, 50/1; (c) DPPC + SP-B, 17/1; (d) DPPC + SP-B, 8/1; (e) DPPC + SP-B, 5/1. The buffer was 155 mM NaCl, 2 mM L-histidine, 2 mM Tes, and 1 mM EDTA, pH 6.9. All phospholipid/protein ratios were w/w. The heating rate was 5 °C/min. These scans are not normalized for the amount of phospholipid present and are for qualitative comparison only. B (right): (a) DPPG; (b) DPPG + SP-B, 50/1; (c) DPPG + SP-B, 17/1; (d) DPPG + SP-B, 8/1; (e) DPPG + SP-B, 5/1.

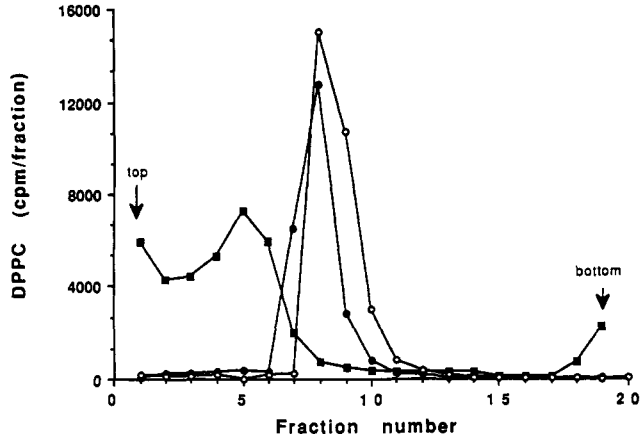


FIGURE 2: Continuous gradient centrifugation of lipid/protein samples. Samples of DPPC (solid squares); DPPC/SP-C, 100/1 (solid circles); and DPPC/SP-B, 100/1 (open circles), were layered onto a continuous sucrose gradient and centrifuged for 16 h at 1000000$g_{av}$. The distribution of lipid in the gradient is shown as cpm of the [$^3$H]DPPC tracer in fractions collected from the gradient. Representative of three separate experiments.

even in the samples with the highest lipid/protein ratios (Figure 2). Table I shows that increasing amounts of SP-B within DPPC or DPPG MLV decreased the $\Delta H$ of the phase transition. Figure 3 shows the phase transition enthalpy, calculated per mole of SP-B, as a function of the lipid/SP-B molar ratio. The interaction of SP-B with DPPC or DPPG MLV reduced the $\Delta H$ values in a linear manner ($r$ = 0.993 and 0.984, respectively) in the range of phospholipid/SP-B ratios studied. By extrapolation of this relationship to the zero enthalpy point, an estimate of the number of lipids removed from the thermal transition (in the temperature range of 0–70 °C) by each SP-B monomer can be obtained. Our

Table I: Effects of SP-B on the Phase Transition Enthalpy of DPPC and DPPG MLV

| condition | phospholipid/ SP-B ratio, w/w | phospholipid/ SP-B ratio, mol/mol | temp of main transition peak, °C[a] | transition enthalpy ($\Delta H$), kcal/mol of lipid[a,b] |
|---|---|---|---|---|
| DPPC | | | 41.5 | 9.1 ± 0.1 (3) |
| DPPC/SP-B | 100/1 | 1353/1 | 42.0 | 8.4 ± 0.4 (3) |
| DPPC/SP-B | 75/1 | 1015/1 | 42.0 | 8.1 ± 0.3 (2) |
| DPPC/SP-B | 60/1 | 812/1 | 42.0 | 8.1 ± 0.1 (3) |
| DPPC/SP-B | 50/1 | 676/1 | 42.0 | 8.5 ± 0.5 (2) |
| DPPC/SP-B | 40/1 | 541/1 | 42.5 | 7.1 ± 0.3 (2) |
| DPPC/SP-B | 17/1 | 230/1 | 42.5 | 6.0 ± 0.7 (2) |
| DPPC/SP-B | 10/1 | 135/1 | 43.5 | 6.5 ± 0.4 (2) |
| DPPC/SP-B | 8/1 | 108/1 | 43.5 | 5.9 (1) |
| DPPC/SP-B | 5/1 | 68/1 | 43.5 | 3.1 ± 1.3 (2) |
| DPPG | | | 41.5 | 8.5 ± 0.1 (6) |
| DPPG/SP-B | 100/1 | 1375/1 | 41.8 | 7.9 ± 0.4 (3) |
| DPPG/SP-B | 75/1 | 1031/1 | 42.0 | 7.2 ± 1.4 (2) |
| DPPG/SP-B | 60/1 | 825/1 | 42.0 | 7.2 (1) |
| DPPG/SP-B | 50/1 | 687/1 | 42.3 | 6.7 ± 0.3 (3) |
| DPPG/SP-B | 40/1 | 550/1 | 42.5 | 6.5 (1) |
| DPPG/SP-B | 17/1 | 234/1 | 55.8 | 5.9 ± 0.1 (2) |
| DPPG/SP-B | 10/1 | 138/1 | 57.5 | 5.0 ± 0.7 (2) |
| DPPG/SP-B | 8/1 | 110/1 | 57.5 | 4.9 ± 0.6 (2) |
| DPPG/SP-B | 5/1 | 69/1 | 57.5 | 1.4 (1) |

[a] Data taken from the heating scan. [b] Values are the mean ± SEM. Numbers of experiments are in parentheses. Buffer = 155 mM NaCl, 2 mM Tes, 2 mM L-histidine, and 1 mM EDTA, pH 6.9.

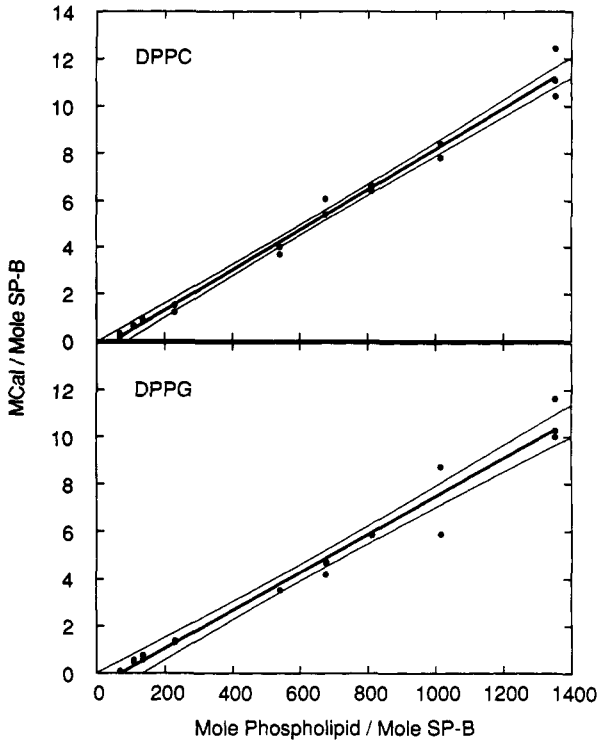


FIGURE 3: Dependence of the phospholipid phase transition enthalpy (megacalories per mole of SP-B) on the phospholipid/SP-B molar ratio. Enthalpies are derived from the heating scans. (Top) DPPC. (Bottom) DPPG. The line and 95% confidence limits of the line that best fit the data were determined by linear least-squares regression.

results are compatible with a model in which approximately 51 (95% confidence levels of 91 to 7) molecules of DPPC and 69 (95% confidence levels of 132 to 0) molecules of DPPG are removed from the thermal transition by each monomer of SP-B.

The slope of this plot can be interpreted as the average transition enthalpy of the lipids which do undergo a phase transition (Lentz, 1988). Values of 8.6 ± 0.2 kcal/mol of DPPC and 8.0 ± 0.4 kcal/mol of DPPG were derived from our results (Figure 3, top and bottom panels, respectively).

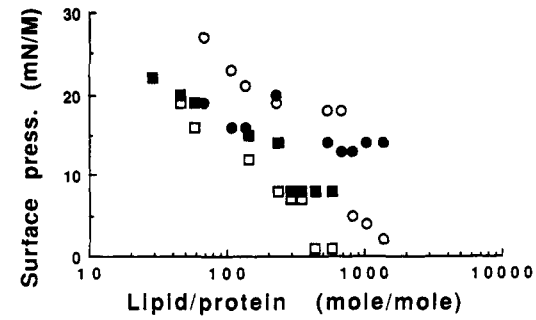


FIGURE 4: Surface activity of phospholipid–protein mixtures. Phospholipid–protein mixtures were placed below the clean surface of a 3-mL subphase buffer consisting of 10 mM Tes, 155 mM NaCl, and 0.1 mM EDTA, pH 6.9, at a final concentration of 30 μg of phospholipid/mL. Stirring was started and the surface pressure continuously measured. The surface pressure 5 min later has been plotted as a function of the phospholipid/protein ratio. Closed circles, DPPG/SP-B; open circles, DPPC/SP-B; closed squares, DPPG/SP-C; open squares, DPPC/SP-C. All values are the mean of two experiments.

These derived molar enthalpies for DPPC and DPPG in the presence of protein are not statistically different from the measured $\Delta H$ values for the respective pure lipids (see Table I; 9.1 ± 0.1 kcal/mol of DPPC and 8.5 ± 0.1 kcal/mol of DPPG), suggesting that the thermodynamic properties of the lipid that participated in the phase transition were minimally affected by the added protein.

As SP-B/phospholipid ratios were raised, the surface activity increased, as represented by the surface pressure reached after 5-min adsorption at 37 °C (Figure 4). At low protein/lipid ratios, DPPG adsorbed faster than DPPC, but at higher ratios, no significant differences were seen. Since calcium was not used in the DSC experiments, it was not added to the subphase buffer for the surface activity measurements. The relatively slow rise in surface pressure in these experiments and those described below with SP-C reflects the absence of calcium, the use of fully saturated phospholipids, and the low lipid concentration at which they were performed.

*SP-C Multilamellar Vesicle Studies.* Figure 5A shows the DSC-2 heat capacity profiles of DPPC MLV which contained various amounts of SP-C. As the DPPC/SP-C ratios were lowered, i.e., as more SP-C was added, the pretransition peak disappeared, the height of the main-phase transition peak at 41.5 °C decreased, and the main-phase transition peak at 41.5 °C broadened and shifted to 46.0 °C. The DSC-2 scans of DPPG MLV containing various weight ratios of SP-C are shown in Figure 5B. As the DPPG/SP-C ratios were lowered, the pretransition peak disappeared, and the height of the main-phase transition peak at 41.5 °C decreased. In addition, at intermediate DPPG/SP-C ratios of 25/1 and 8/1, two partially resolved endotherms were present, and the main-phase transition at 41.5 °C had broadened and shifted to 50–58 °C. At the lowest phospholipid/SP-C ratio tested (5/1), only a single broad transition centered at 54 °C was observed. Table II shows that increasing amounts of SP-C in DPPC and DPPG MLV lowered the phase transition enthalpy value of the phospholipid.

As shown in Figure 6, the phase transition enthalpy calculated per mole of SP-C also varied as a function of the lipid/SP-C molar ratio ($r$ = 0.990 for DPPC and 0.989 for DPPG). Although an inspection of the data suggested a possible break in the linear nature of this relationship around a lipid/protein ratio of about 150/1 with both DPPC and DPPG, an analysis assuming two compartments with different average enthalpies was not statistically better than a single

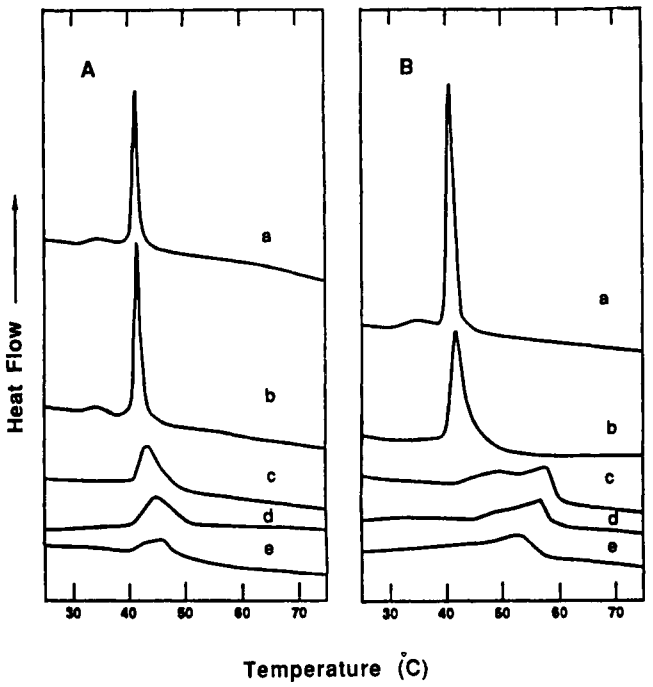


FIGURE 5: Differential scanning calorimetry thermograms. (A) (a) DPPC; (b) DPPC + SP-C, 50/1; (c) DPPC + SP-C, 25/1; (d) DPPC + SP-C, 8/1; (e) DPPC + SP-C, 5/1. (B) (a) DPPG; (b) DPPG + SP-C, 50/1; (c) DPPG + SP-C, 25/1; (d) DPPG + SP-C, 8/1; (e) DPPG + SP-C, 5/1. The conditions are the same as in Figure 1.

Table II: Effects of SP-C on the Phase Transition Enthalpy of DPPC and DPPG MLV

| condition | phospholipid/ SP-C ratio, w/w | phospholipid/ SP-C ratio, mol/mol | temp of main transition peak, °C[a] | transition enthalpy ($\Delta H$), kcal/mol of lipid[a,b] |
|---|---|---|---|---|
| DPPC | | | 41.5 | 9.1 ± 0.1 (3) |
| DPPC/SP-C | 100/1 | 580/1 | 41.5 | 8.8 (2) |
| DPPC/SP-C | 75/1 | 435/1 | 42.0 | 9.2 ± 0.6 (2) |
| DPPC/SP-C | 60/1 | 348/1 | 42.0 | 8.7 ± 0.1 (2) |
| DPPC/SP-C | 50/1 | 290/1 | 42.5 | 7.6 ± 0.7 (2) |
| DPPC/SP-C | 40/1 | 232/1 | 42.5 | 7.4 (1) |
| DPPC/SP-C | 25/1 | 145/1 | 43.5 | 5.4 ± 0.1 (2) |
| DPPC/SP-C | 10/1 | 58/1 | 46.0 | 5.0 (1) |
| DPPC/SP-C | 8/1 | 46/1 | 45.5 | 5.5 ± 0.7 (2) |
| DPPC/SP-C | 5/1 | 29/1 | 46.0 | 3.3 (2) |
| DPPG | | | 41.5 | 8.5 ± 0.1 (6) |
| DPPG/SP-C | 100/1 | 590/1 | 42.0 | 6.7 ± 0.3 (3) |
| DPPG/SP-C | 75/1 | 442/1 | 42.0 | 6.9 ± 0.3 (2) |
| DPPG/SP-C | 60/1 | 354/1 | 42.0 | 7.1 ± 0.3 (2) |
| DPPG/SP-C | 50/1 | 295/1 | 42.5 | 5.8 ± 0.1 (3) |
| DPPG/SP-C | 40/1 | 236/1 | 42.5 | 4.7 ± 0.6 (2) |
| DPPG/SP-C | 25/1 | 147/1 | 58.0 | 4.0 (1) |
| DPPG/SP-C | 10/1 | 59/1 | 58.0 | 3.1 ± 0.2 (2) |
| DPPG/SP-C | 8/1 | 47/1 | 57.5 | 3.0 ± 0.4 (2) |
| DPPG/SP-C | 5/1 | 30/1 | 54.0 | 2.4 ± 0.6 (2) |

[a] Data taken from the heating scan. [b] Values are the mean ± SEM. Numbers of experiments are in parentheses. Buffer = 155 mM NaCl, 2 mM Tes, 2 mM L-histidine, and 1 mM EDTA, pH 6.9.

linear analysis assuming one average lipid phase ($p = 0.1$–$0.5$ for the one-compartment model compared to the two-compartment model for both lipids). The slope of the relationship for DPPC (Figure 6, top panel) was 9.4 ± 0.4 kcal/mol of DPPC, a value similar to our measured transition enthalpy value for pure DPPC (see Table II; 9.1 ± 0.1 kcal/mol of DPPC). With DPPG (Figure 6, bottom panel), a value of 7.1 ± 0.3 kcal/mol of DPPG was calculated. This value is less ($p < 0.01$) than the transition enthalpy we measured for pure DPPG (see Table II; 8.5 ± 0.1 kcal/mol of DPPG), suggesting that the lipid free to participate in the phase

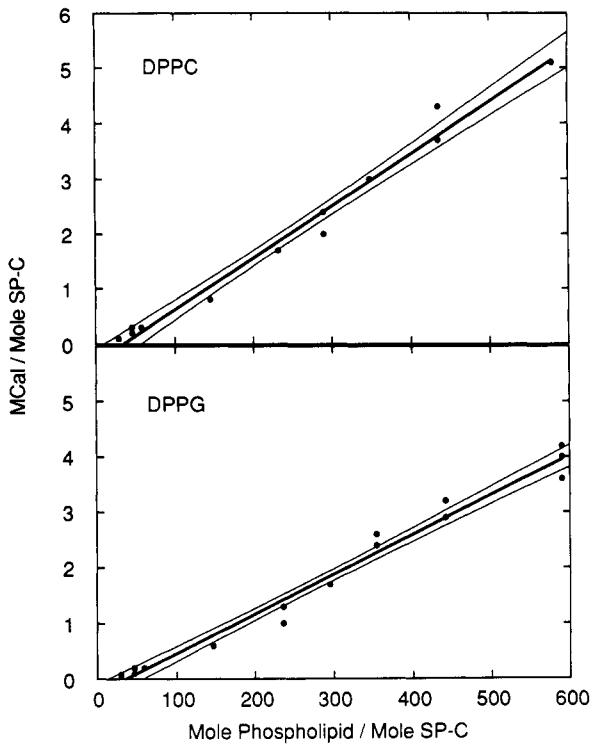


FIGURE 6: Dependence of the phospholipid phase transition enthalpy (megacalories per mole of SP-C) on the phospholipid/SP-C molar ratio. Enthalpies are derived from the heating scans. (Top) DPPC. (Bottom) DPPG. The line and 95% confidence limits of the line that best fit the data were determined by linear least-squares regression.

Table III: Predicted Secondary Structure of Dog SP-C and rhSP-C[a]

| condition | % α helix | % β sheet | % random |
|---|---|---|---|
| butanol (3) ● | 52 ± 2 | 10 ± 8 | 38 ± 7 |
| butanol (3) ○ | 75 ± 8 | 0 ± 0 | 28 ± 8 |
| SDS (2) ● | 56 ± 3 | 1 ± 1 | 43 ± 4 |
| SDS (2) ○ | 71 ± 7 | 4 ± 4 | 27 ± 3 |
| DPPC (3) ● | 46 ± 5 | 31 ± 4 | 23 ± 2 |
| DPPC (3) ○ | 86 ± 7 | 1 ± 1 | 13 ± 7 |
| DPPC/PG (3) ● | 54 ± 4 | 26 ± 5 | 20 ± 1 |
| DPPC/PG (3) ○ | 85 ± 10 | 6 ± 10 | 9 ± 1 |

[a] The secondary structure of dog SP-C (●) and rhSP-C (○) was determined by circular dichroism using the algorithm of Yang (Yang et al., 1986). Spectra of SP-C (0.1 mg/mL) were recorded in butanol/1% SDS, reconstituted with DPPC (1 mg/mL), or with DPPC/"egg" PG (7/3, w/w; 1 mg/mL). Values are mean ± SD (±range for SDS). The numbers of experiments are in parentheses.

transition was affected to some extent by SP-C. At $\Delta H = 0$, the lipid/SP-C molar ratio was 35/1 for DPPC (95% confidence levels of 57/1 to 10/1) and 36/1 for DPPG (95% confidence levels of 58/1 to 13/1), consistent with the notion that SP-C removed a similar number of lipid molecules from the phase transition in both cases.

As SP-C/phospholipid ratios were raised, the surface activity of both phospholipids increased (Figure 4). Again, at low protein/lipid ratios, DPPG adsorbed slightly faster than DPPC, but no differences were observed at the higher ratios.

*Circular Dichroism of SP-C.* The spectra of dog SP-C and rhSP-C in all conditions examined were consistent with a high α-helical content (Table III). The spectra of both proteins were slightly different in the presence of phospholipid compared to those obtained in organic solvent or 1% SDS (Figure 7). Average maximal ellipticities (deg·cm$^2$·dmol$^{-1}$) for dog SP-C were 44 789 at 191 nm in 1-butanol, 45 955 at

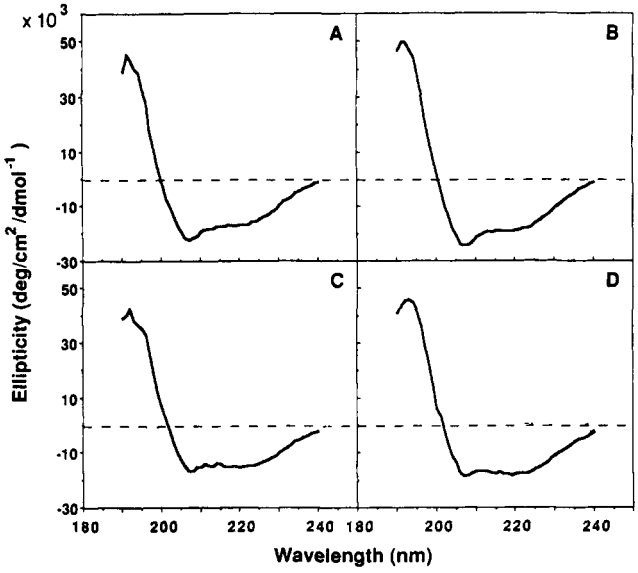


FIGURE 7: Circular dichroism spectra of SP-C. Spectra of canine SP-C (0.1 mg/mL) were recorded in the absence or presence of phospholipids. SP-C was in (A) butanol or (B) 1% SDS in 5 mM Tris-HCl, pH 7.4, or (C) reconstituted with DPPC, at 1 mg/mL, or (D) reconstituted with DPPC/egg PG, 7:3, w/w, at 1 mg/mL. Samples C and D were sonicated to clarity prior to being scanned.

192 nm in 1% SDS, 43 565 at 193 nm in DPPC, and 38 039 at 192 nm in DPPC/PG. Negative extrema (deg·cm$^2$·dmol$^{-1}$) of 22 975, 27 412, 15 163, and 17 407 were observed at 207 nm with SP-C in 1-butanol, 1% SDS, DPPC, and DPPC/PG, respectively. Additional negative extrema were observed at 219 nm with SP-C in DPPC and DPPC/PG, but not in 1-butanol or 1% SDS. Reconstitution of dog SP-C but not rhSP-C with phospholipids appeared to induce increased $\beta$-sheet structure relative to the conformation in butanol or SDS, but the presence of 30% anionic phospholipid did not significantly modify the spectrum obtained in pure DPPC (Table III).

## DISCUSSION

The nature of the interaction of the surfactant proteins SP-B and SP-C with DPPC and DPPG was investigated using differential scanning calorimetry (DSC). In the presence of increasing amounts of either SP-B or SP-C, a progressive broadening of the lipid gel- to liquid-phase transition and a displacement of the center of the transition temperature to a higher temperature occurred. A marked decrease in the enthalpy of the lipid transition was also observed as the amount of either protein relative to either lipid was increased. Evidence for the presence of at least two different phases was obtained with both proteins. We also observed that both proteins improved the surface activity of these phospholipids in a concentration-dependent manner.

SP-B and SP-C had similar qualitative effects on the heat capacity profiles. Both proteins increased the transition temperature of DPPG to a greater extent than DPPC. Also, with DPPG the heat capacity profiles recorded at high protein/lipid ratios were more complex with either protein than those recorded with DPPC at similar ratios. These complex profiles suggest the cationic proteins may form distinct domains with the anionic phospholipid DPPG. Similar conclusions concerning a selective interaction of SP-B with PG have been drawn from fluorescence anisotropy studies (Baatz et al., 1990) and surface activity measurements (Cochrane & Revak, 1991), but no comparable studies with SP-C are available. We have previously reported that a mixture of SP-B and SP-C causes leakage of the contents of liposomes, provided a small amount of anionic lipid is included, again supporting a role for the interaction of the positively charged residues in these proteins with negatively charged phospholipid head groups (Shiffer et al., 1988b).

Each monomer of SP-B influenced the phase behavior of approximately 50 molecules of DPPC and 70 molecules of DPPG in a way that prevented these lipids from participating in the thermal phase transition (see curves e Figure 1). These lipids are commonly thought to lie in a dynamic annular or boundary domain closely associated with the protein (Lentz, 1988). The structure, orientation, or oligomerization of SP-B in lipid membranes is unknown. An analysis of the primary sequence suggests SP-B may fold as an amphiphatic helix, but not necessarily have a transmembrane orientation (Waring et al., 1989; Cochrane & Revak, 1991). Further structural studies will be required before the molecular arrangement of such a large lipid annulus associated with SP-B can be determined.

In contrast, the amino acid sequence of SP-C with an extended hydrophobic sequence of 24 residues (Warr et al., 1987; Waring et al., 1989) and the high content of $\alpha$ helix we report here are both consistent with a single transmembrane helix. Similar conclusions concerning the structure and orientation of SP-C have recently been drawn from the results of studies utilizing attenuated total reflection Fourier-transform infrared spectroscopy (Pastrana et al., 1991; Vandenbussche et al., 1992). Our DSC results suggest that a large number of lipids are removed from the phase transition by SP-C (35/protein monomer) than would be expected if the model of a transmembrane helix with a single boundary layer of protein-perturbed lipids applies. Assuming hexagonal lipid packing, such models predict 12–18 lipids/helix in the boundary layer (Bradrick et al., 1989). In contrast to our results, Simatos et al. (1990) using porcine SP-C and dimyristoylphosphatidylcholine estimated each protein monomer influenced approximately 18 lipid molecules. The different lipids in the two studies may account for these differences, but it is possible that the relatively low sensitivity of the DSC-2 instrument used in our study may have led us to underestimate the transition enthalpy at high protein/lipid ratios, resulting in an overestimation of the size of the boundary layer (Lentz et al., 1983).

An alternate and more interesting explanation for the apparent discrepancy between the experimental and predicted number of lipid molecules influenced by protein relates to the presence of the covalently attached palmitate on the amino-terminal end of SP-C. We noted a different conformation of dog SP-C by CD in the presence of phospholipid that may have reflected a change (random-coil to $\beta$-sheet transition) at the amino-terminal end of the protein as the palmitate interacted with the phospholipid bilayer. Long-chain saturated fatty acids have been reported to increase the $T_m$ and broaden the transition profile of phospholipids (Mabrey & Sturtevant, 1977; Pauls et al., 1983). In contrast, rhSP-C, which lacks the covalently attached palmitate, did not undergo a detectable conformation change in the presence of lipid. We therefore speculate that the acylation of SP-C may affect both the conformation of the amino-terminal end of SP-C and the thermodynamic properties of associated phospholipids. Calorimetry with nonacylated SP-C is being performed to test this hypothesis.

With SP-B and SP-C and either DPPC or DPPG, the molar enthalpies (megacalories per mole of protein) decreased in an

apparently linear fashion with decreasing lipid/protein ratios throughout the entire range of lipid/protein ratios examined. With the possible exception of the SP-C and DPPG mixture, the close agreement between the molar lipid enthalpies obtained from the slopes of these linear relationships (see Results and Figures 3 and 6) and the directly measured values for pure lipid samples (Table I) suggests that the lipid outside of the putative annular layers discussed above was minimally perturbed by the presence of the proteins. According to the model of Lentz (Lentz et al., 1983), the relationship between the molar enthalpy and the lipid/protein ratio should be nonlinear if a second protein-perturbed domain of lipid existed outside of the primary boundary layer. We found no convincing evidence for such a second disordered domain, but due to possible inaccuracies in the calculated transition enthalpies at the highest protein/lipid ratios, we cannot exclude the presence of such domains in our samples. Other models have been proposed to explain changes in lipid transition enthalpy in response to both native and model proteins without invoking the distinct spatially-segregated lipid domains we have inferred from our data (Morrow et al., 1985) [reviewed in McElhaney (1986) and Lentz (1988)]. It is clear that additional studies utilizing techniques such as X-ray diffraction, nuclear magnetic resonance, and electron spin resonance, that examine lipid/protein interactions in different time domains and under conditions that may more closely resemble native surfactant, will be required before reliable molecular models of lung surfactant can be developed.

Several technical factors in our experimental design may have influenced the results we obtained and their relevance to the properties of native lung surfactant. First, we chose to use MLV as a model system because much of the natural surfactant is in a multilamellar form and because the phase transition of phospholipids in a multilamellar suspension is sharper than it is for small sonicated unilamellar vesicles (Epand & Surewicz, 1984). We felt we were more likely to achieve homogeneous reconstitution of the lipids and hydrophobic proteins by this method. To assess this, SP-B- and SP-C-containing samples with the highest lipid/protein ratios (100/1, w/w) were analyzed by sucrose density gradient centrifugation. By this method, these samples were homogeneous with a narrow range of density that overlapped minimally with the free lipid. We did not determine the morphology or actual stoichiometry of the lipid–protein complexes studied and recognize that microheterogeneity in the structure and composition of our samples may have influenced our results (McElhaney, 1986). The morphology of DPPC/PG mixtures reconstituted with surfactant proteins by a variety of methods has been recently studied (Suzuki et al., 1989; Williams et al., 1990). In certain conditions, both SP-B and SP-C can form small disklike structures from vesicles of mixed lipid (Williams et al., 1990), but no information is available on the morphology of complexes reconstituted using pure DPPC or DPPG.

Second, any impurities in the protein preparations could have influenced our results, particularly at higher protein/lipid ratios. The protein purification procedures we used have been well characterized (Curstedt et al., 1987; Warr et al., 1987). The chromatographic procedures allow the purification of SP-B and SP-C without overlap detectable by SDS–PAGE or microsequence analysis. The use of acidified solvents minimizes lipid contamination of the protein preparations, but small amounts of fatty acid were detectable in both SP-B and SP-C by GLC after acid hydrolysis and methanolysis of the samples. By this analysis, the lipid contamination of our preparations did not exceed 2 mol of fatty acid/mol of SP-B or SP-C. The origin of these fatty acids is uncertain, but it is known that canine SP-C is covalently palmitoylated at cysteine position 5 (Stults et al., 1991). Palmitate was the major fatty acid detected in both proteins, but small amounts of stearate and oleate were also detected. Similar results have been reported for porcine SP-B and SP-C (Curstedt et al., 1990). Therefore, even at the highest protein/lipid ratio examined, the fatty acid impurity would not have exceeded 3 mol % of the total lipid.

Finally, single pure lipids were chosen for this study, rather than more complex mixtures which would have more closely resembled the composition of natural lung surfactant. This was done in order to simplify the nature of the thermal transition and therefore allow a less ambiguous assessment of the effect of the proteins. For the same reason, calcium was omitted from our samples although the effects of this ion on surfactant structure and function are well recognized (Benson et al., 1984; Hawgood et al., 1985; Suzuki et al., 1989; Williams et al., 1990) and will ultimately have to be taken into account. DPPC was studied because it is both the major phospholipid of surfactant and it is thought to be primarily responsible for lowering surface tension at the air/fluid interface in the alveoli during the normal breathing cycle. DPPG was also selected for study because there is an unusually large amount of PG present in lung surfactant (i.e., 8–10 mol %) (King & Clements, 1972), and we were interested to see whether SP-B and SP-C, as positively charged proteins, would have different effects on a phospholipid with a negatively charged head group. Since DPPC and DPPG both have a sharp main-phase transition at 41–42 °C, we were able to study a temperature range that included 37 °C, the temperature at which surface activity was examined. The potential influence of acyl chain length on the interaction with SP-C is suggested by the fact that SP-C caused a small decrease in the peak transition temperature of DMPC (Simatos et al., 1990) but clearly increased the peak transition temperature of DPPC in our studies. SP-C reduced the transition enthalpy of both lipid species in a dose-dependent manner. Studies with more complex lipid mixtures in the presence of calcium are in progress using a high-sensitivity calorimeter. The proteins induce extremely complex thermograms under these conditions (data not shown), but the basic observations of a protein-induced shift to higher transition temperatures and a reduction in transition enthalpies are maintained.

It is difficult to directly relate the thermodynamic properties of the surfactant lipid/protein complexes to their surface activity. We found that the rate of adsorption of lipid to an air/fluid interface at 37 °C, as reflected by the rising surface pressure, is increased as more SP-B or SP-C is combined with the lipid. As our calorimetry results suggested, with increasing protein more lipid would be in protein-influenced domains (either boundary or secondary domains). The number of lipid molecules at domain boundaries where lipid packing is most variable would also increase in the presence of more protein. Such rearrangements may facilitate lipid adsorption, but the precise mechanisms involved in this step, so critical to the physiological function of native and therapeutic lung surfactants, remain to be clarified.

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