Pulmonary surfactant protein SP-C causes packing rearrangements of dipalmitoylphosphatidylcholine in spread monolayers

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ABSTRACT The hydrophobic pulmonary surfactant protein SP-C has been isolated from porcine lung surfactant, and it has been incorporated into monolayers of dipalmitoylphosphatidylcholine (DPPC). The monolayers, which contained 1 mol% of a fluorescently-labeled phosphatidylcholine, were observed under various states of compression in an epifluorescence surface balance. SP-C altered the packing arrangements of DPPC in the monolayer, causing the production of many more, smaller condensed lipid domains in its presence than in its absence.

INTRODUCTION

Pulmonary surfactant, material secreted by type II pneumocytes, reduces surface tension at the air–water interface of distal airways and alveoli of lungs, thereby decreasing the work of breathing and the tendency for alveolar collapse at low lung volumes. Experimental data support the assumption that it is a monolayer of surfactant lipid enriched in DPPC which actually effects the surface tension reduction. Surfactant is, however, secreted in the form of bilayer assemblies as lamellar bodies, so that translocation of lipid from bilayers to monolayers must occur in the formation of the monolayer.

The process of adsorption of lipid into the air–water interface from the bilayer form is slow in the absence of the surfactant proteins. The adsorption rate is substantially enhanced in the presence of the hydrophobic surfactant proteins SP-B and SP-C (1, 2) and is further promoted by the protein SP-A which, together with calcium, induces a unique organization called tubular myelin, an assembly thought to be an intermediate between the bilayer and the monolayer forms (3).

Although some studies have investigated the influence of the hydrophobic proteins on adsorption rates, only a few (4, 5) have looked at the way in which the proteins interact with the lipids in order to promote the process of rapid adsorption into the interface or how the proteins behave at the air–water interface.

In this study, the technique of monolayer fluorescence microscopy has been employed to determine how one of the hydrophobic proteins, SP-C, influences assemblies of the principal surfactant lipid, DPPC, at the air–water interface. This technique has been used recently to examine the distribution of lipids and lipid–protein complexes in two-dimensional arrays in various monolayers (e.g., see references 6–10).

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MATERIALS AND METHODS

Materials

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1-palmitoyl-2-{12-[(7-nitro-2-1,3-benzoxadizole-4-yl) amino] dodecanoyl} phosphatidylcholine (NBD-PC) were obtained from Avanti Polar Lipids (Pelham, AL). They were found to be pure by thin layer and gas liquid chromatography and used as received. The subphase used in the monolayer balance was 0.15 M NaCl made in deionized, doubly-distilled water, the second distillation being from dilute potassium permanganate solution.

Pulmonary surfactant was prepared from porcine lungs by a procedure described previously (11). A lipid extract was made and SP-C was obtained from it by chromatography on Lipidex-500 in ethylene chloride-methanol, 1:4 (vol/vol), followed by elution from Sephadex LH-20 in chloroform–methanol 2:1 (vol/vol), and, finally from Sephadex LH-60, in chloroform–methanol 1:1 (vol/vol) containing 5% 0.1 M HCl, in a manner similar to that described by Curstedt et al. (12). The protein, SP-C, gave one band of 5-6 kD on both reducing and nonreducing SDS-PAGE. NH_2-terminal sequence analysis indicated that the protein consisted of ≥85% whole porcine SP-C together with ~10 and 5% of truncated forms of SP-C missing the first one, or the first two NH_2-terminal amino acids, respectively. Fatty acid analysis after of the treatment of the protein with KOH indicated that the protein contained 1.9 mol of palmitic acid per mole of protein. Protein treated with trimethylamine plus dithiothreitol followed by ^{14}C-iodoacetamide incorporated 2.2 mol of iodoacetamide per mole of protein. Proteins treated only with ^{14}C-iodoacetamide for the same time, but in the absence of base and reducing agent, showed almost no incorporation of radioactivity. These results indicate that the SP-C used was dipalmitoylated at the cysteine residues (13).

Methods

The surface balance used to measure epifluorescence of monolayers has been described in detail elsewhere (14). It consists of a teflon trough, having a working area of 148 cm^2 with a variable-speed movable barrier, in which surface tension and epifluorescence from the surface can be simultaneously monitored. Mixtures were formed from a chloroform–methanol (3:1, vol/vol) solution of DPPC that contained NBD-PC and SP-C in desired proportions. Monolayers were formed by spreading aliquots of the chloroform–methanol solution evenly on the surface, and allowing 30 min for evaporation of solvent before compression of the monolayer was started. Data was obtained at 24 ± 1°C. Monolayers were compressed at 0.13 Å^2/molecule/s in 20 steps. At selected surface pressures, a visual recording was made on videotape for 1 min, and then the compression was continued to the next surface

pressure chosen for visual recording. Image analysis was done via the system previously described (14, 15).

Analytical procedures

Phosphorus and fatty acids were measured as described previously (16, 17). SP-C was measured using the fluorescamine assay (18) after validation with quantitative amino acid analysis of selected amino acids (Lys, Ala, Gly) in the SP-C.

RESULTS

Fig. 1 *a* shows surface pressure-area isotherms obtained for various monolayers composed of DPPC plus various amounts of SP-C. The insert shows an isotherm for SP-C under the same conditions. It has substantially greater area per molecule at any given pressure than the lipids. Although small amounts of the probe do not generally substantially affect isotherms of pure DPPC (7, 15, 19, 20), in this case there was a slight effect in one of two isotherms seen with this specific mixture. The liquid expanded to liquid-condensed transition is slightly less distinct than in some isotherms with and without the probe. The liquid to liquid condensed coexistance region of the isotherms was slightly steeper than seen in a previous report (7). This may be due to differences in the probe employed and the method and speed of compression, among other factors. The presence of the protein resulted in expanded films, the degree of expansion being dependent on the amount of SP-C present. This indicated that the protein was incorporated in, or at least associated with, the lipid monolayer.

Fig. 1 *b* shows the isotherm data presented in terms of π vs. average area per "residue". There are 35 amino acid residues in SP-C; DPPC was also considered as a "residue" in these calculations. The insert presents complete compression isotherms up to the maximum surface pressure reached. The high pressures attained are consistent with the behavior of isotherms of DPPC.

Average areas per molecule at π = 10 mN · m^{-1} of 63,

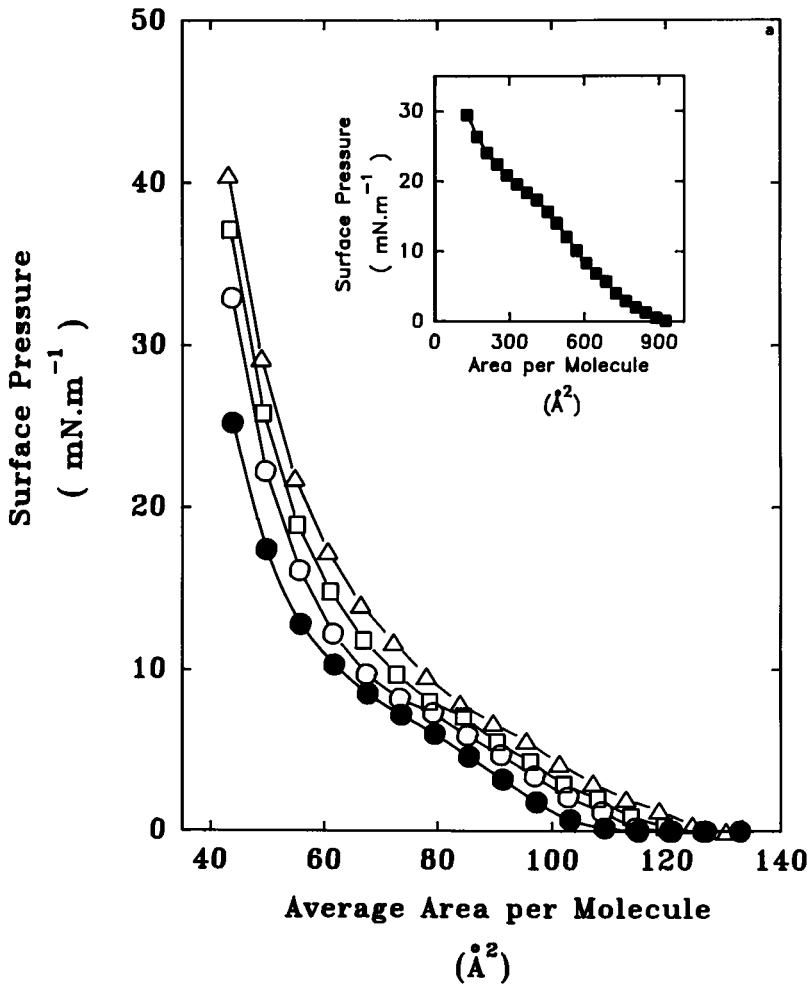

FIGURE 1 (*a*) Isotherms of DPPC plus 1 mol% NBD-PC containing: 0 (● —— ●); 2 (○ —— ○); 6 (□ —— □); and 10 (△ —— △) weight% SP-C. These correspond to 0, 0.36, 1.1, 1.9 mol% SP-C. (*Inset*) π vs. area per molecule for a film of SP-C. (*b*) Data presented as π vs. average area per residue. In this presentation, each DPPC molecule and each amino acid residue has been counted as one residue. (*Inset*) Complete compression and expansion isotherms of DPPC plus 0, 2, 6, and 10 weight% SP-C.

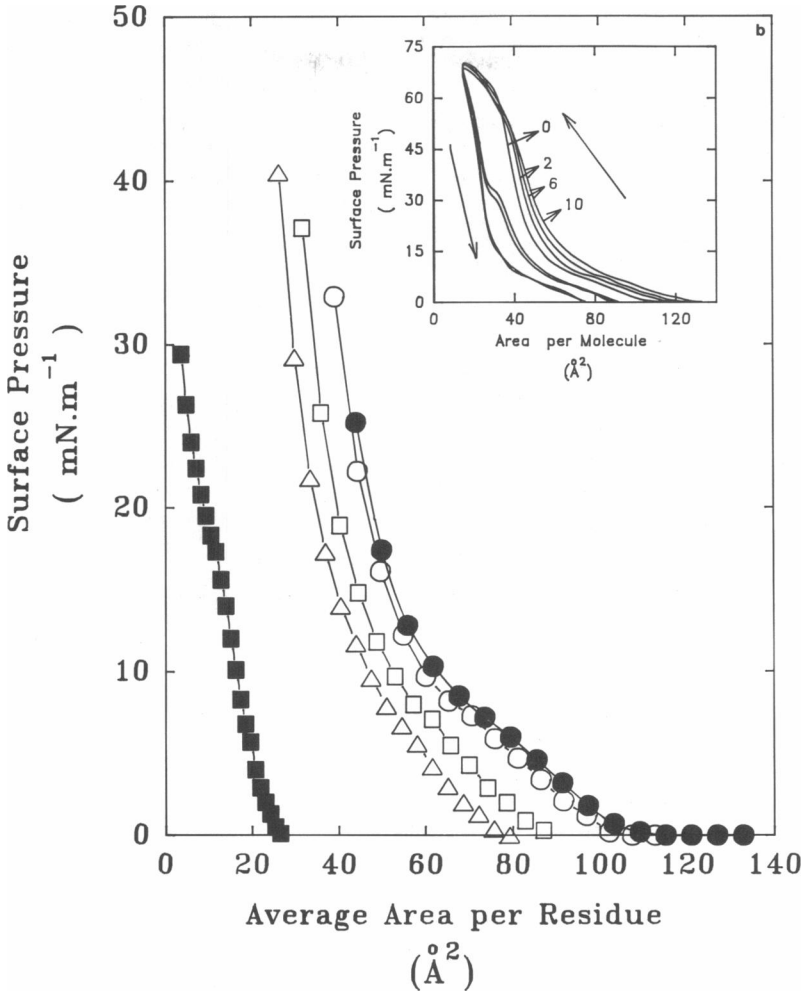

FIGURE 1 (continued)

67, 71, 77, and 530 Å^2 were found for films containing 0, 0.36, 1.1, 1.9, and 100 mol% SP-C, respectively. At π = 20 mN · m^{-1}, the average areas per molecule were 48, 53, 55, 58, and 310 Å^2. The calculated average areas per molecule, based upon ideal mixing, at π = 10 mN · m^{-1}, are 63, 65, 67, 72, and 530 Å^2, and at π = 20 mN · m^{-1} are 48, 49, 51, 53, and 310 Å^2, for 0, 0.36, 1.1, 1.9, and 100% SP-C, respectively. Thus, the protein caused an expansion of the mixed monolayers.

Fig. 2 shows representations of visual fields seen at a nominal area of 55 Å^2/molecule for DPPC in the presence of increasing amounts of SP-C. The size and shape of the domains of DPPC are influenced by various factors including temperature, surface pressure, and compression rate (15, 20). The conditions used here produced roughly elliptical shapes with only some of the "kidney bean" shapes seen under very slow, near-equilibrium conditions of compression (8, 9, 15). As the amount of protein in the monolayer was increased, the size of the dark condensed domains decreased, and their numbers increased. Fig. 3 shows the numbers of condensed domains as a function of surface pressure. The number of condensed domains was almost independent of π, or of area per molecule of DPPC, at low protein concentrations, and was dependent on these parameters at higher protein concentrations. A film containing 50% by weight protein (15 mol%) was found to contain a very large number of very small condensed domains, but these were not subjected to image analysis because there was difficulty in defining the edges of the condensed domains given the inherent noise in the image recording system.

The average distance between centers of condensed regions is given as a function of π in Fig. 4. Adding protein at 6 or 10 weight%, 1.1 or 1.9 mol%, caused the distances between centers to decrease. The distances between centers were only slightly influenced by the extent of compression, the slopes of all the curves being relatively shallow. The distances between the borders of the dark domains was also influenced by the amount of protein (data not shown), again the maximum effect being seen with 6 weight% SP-C. Not surprisingly, a much

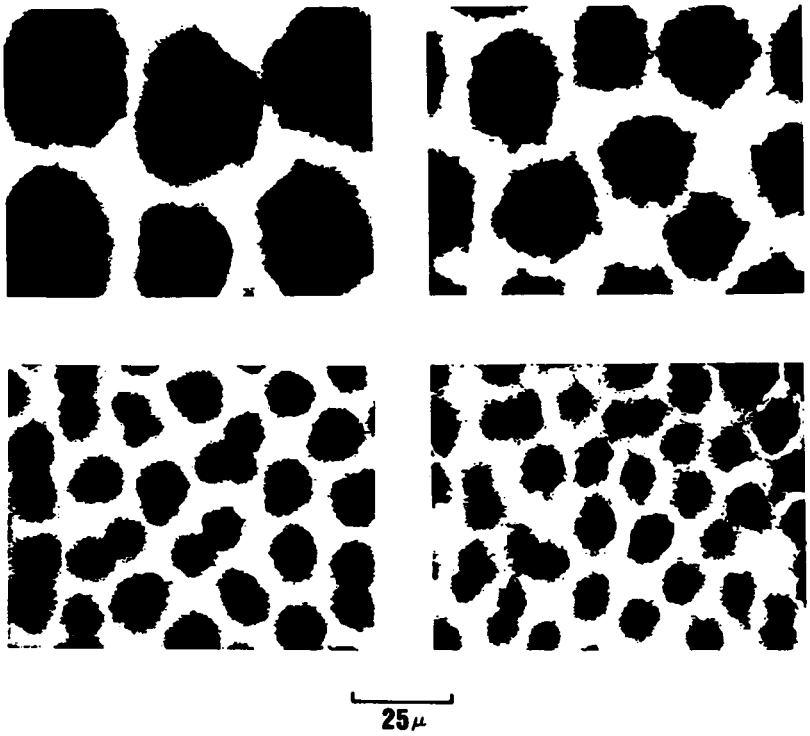

FIGURE 2 Reproduction of typical images from fields containing various amounts of DPPC and SP-C each obtained at 55 Å^2/molecule of DPPC. They represent, from left to right and top to bottom, fields from monolayers containing 0, 2, 6, and 10% by weight SP-C. Dark areas represent condensed domains excluding probe molecules. Light areas are expanded domains containing probe molecules.

stronger influence of the extent of film compression on the distance between borders of condensed domains was seen than on the distances between centers. The distances between borders appeared to converge to a limiting value near 2–3 micrometers.

Although the condensed domains were generally elliptical in shape there was not a great difference between the average lengths of the major and minor axes. The average areas of individual domains were dependent on the amount of SP-C present but appeared to be reaching limiting values (which were dependent on protein concentration) as the monolayers entered the liquid-condensed to solid-condensed transition region (Fig. 5). The lengths of the major and minor axes were similarly dependent on area per molecule of DPPC and surface pressure.

The curves describing the total area occupied by condensed domains as a function of π were essentially superimposable between 0 and 1.1 mol% SP-C (Fig. 6). However, the total amount of condensed domains at any given π was substantially reduced when 1.9 mol% SP-C was present. A value of $\sim$5,500 μ^2 (out of a total field of 7,600 μ^2) or 72% coverage with condensed domains was reached at $\sim\pi$ = 20 mN · m^{-1} for DPPC and DPPC plus the two lower protein concentrations. Higher pressure ($\pi \approx$ 40 mN · m^{-1}) was required to achieve this condensation with 10% protein.

DISCUSSION

The area per residue of SP-C is in the range observed for films of α-helical peptides (21) and of apolipoproteins A-I and A-II (22) and the proteolipid from myelin (23). This suggests that SP-C has substantial α-helical content when it is at the air–water interface. Fourier-transform infrared spectroscopy (24) has indicated that SP-C has $\sim$60% α-helix in bilayers of DPPC-dipalmitoylphosphatidylglycerol (7:3). A similar content of α-helix for SP-C in DPPC has been observed by us (unpublished results) using circular dichroism. Oosterlaken-Dijksterhuis et al. (25) have obtained circular dichroism spectra of monolayers of SP-C at π = 35 mN · m^{-1}, which were consistent with high contents of α-helix.

The 23-residue COOH-terminal portion of SP-C is likely the α-helical part. Based upon the area per residue for isotherms of SP-C films at π = 35 mN · m^{-1}, Oosterlaken-Dijksterhuis et al. (25) have suggested that SP-C has its helical axis oriented parallel to the air–water interface. Our limiting areas per molecule are consistent with the SP-C having α-helix at the interface (21–23, 26). Pastrana et al. (24) find that, in lipid bilayers, the helical part of SP-C is oriented at $\sim$24° with respect to the bilayer normal. The orientation of the α-helix of SP-C when it is in monolayers of lipid plus protein is currently not known.

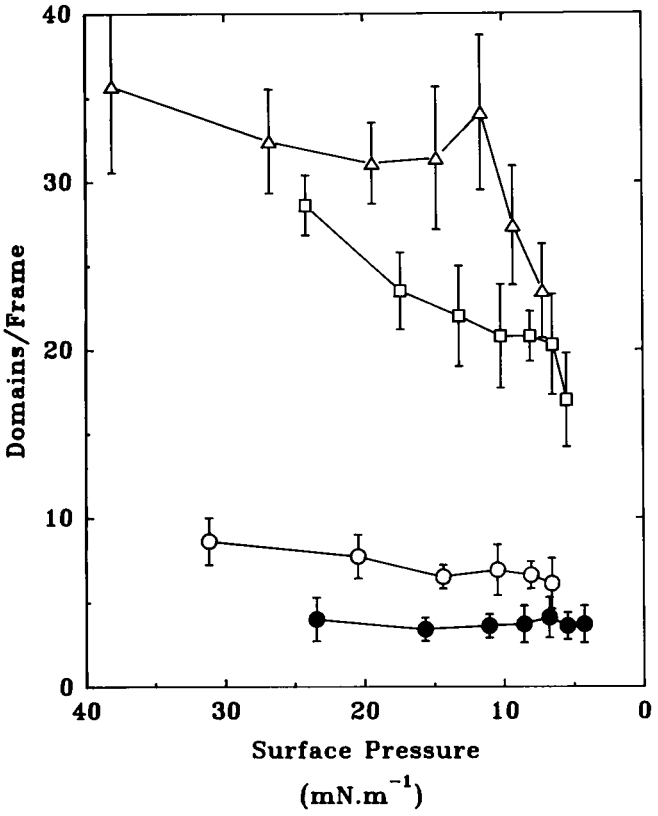

FIGURE 3 Dependence of the number of condensed domains on surface pressure (π) for various protein concentrations: 0 (● —— ●); 2 (○ —— ○); 6 (□ —— □); and 10 (△ —— △) weight%.

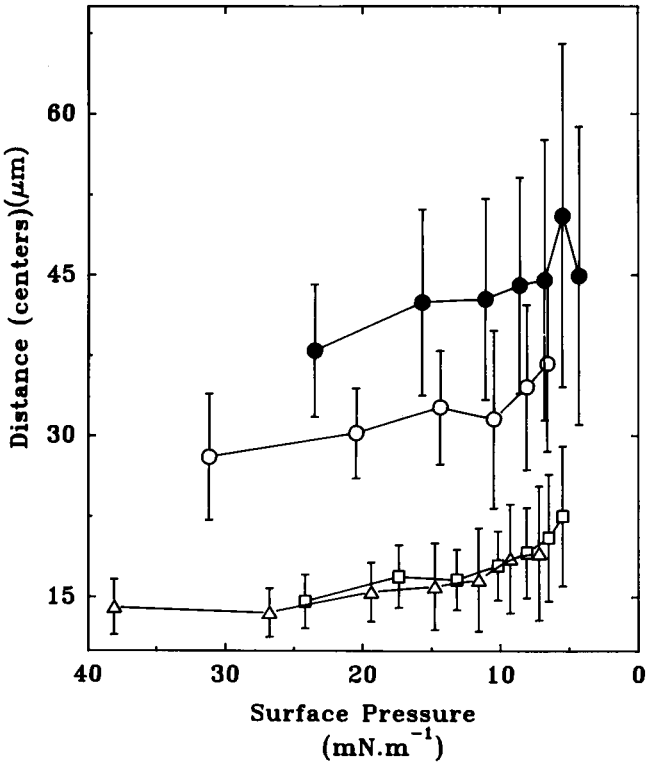

FIGURE 4 Distance between centers of condensed domains (μm) as a function of π and SP-C concentration (symbols as in Fig. 3).

The isotherms we have obtained from SP-C are more condensed (they have lower areas per residue at any surface pressure) than those reported by Oosterlaken-Dijksterhuis et al. (25). We have obtained isotherms similar to the one shown here from three separate preparations of SP-C. When we have examined our protein under conditions employed in reference 25, we found that the isotherm is expanded by only 20–25%, but not as much as seen by the other workers. Even though the amounts of lipid in our preparations of SP-C and the one described in reference 25 are quite low, perhaps there are some differences in lipid content that might help account for the differences in the isotherms. Perhaps their SP-C (25) is more unfolded at lower pressures than ours. The areas per residue we obtain are similar to those obtained by other groups for other proteins, including hydrophobic proteins (e.g., see references 21, 22, 23, 26).

The fluorescence results indicate that SP-C perturbs the packing of DPPC and inhibits formation of condensed phase. Previous work on SP-C in bilayers (5) indicated that SP-C perturbed lipid packing. The average areas per molecule for the protein–lipid films are greater than one predicts given ideal mixing of protein and lipid. This also suggests that SP-C caused perturbation of chain packing of DPPC and, consequently, the expansion of

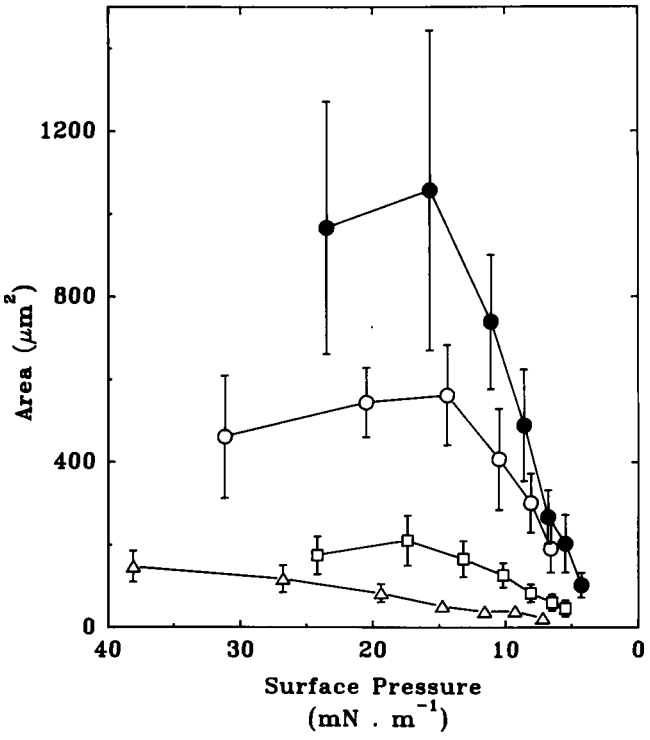

FIGURE 5 Average area of condensed domains (μm^2) as a function of π and SP-C concentration (symbols as in Fig. 3).

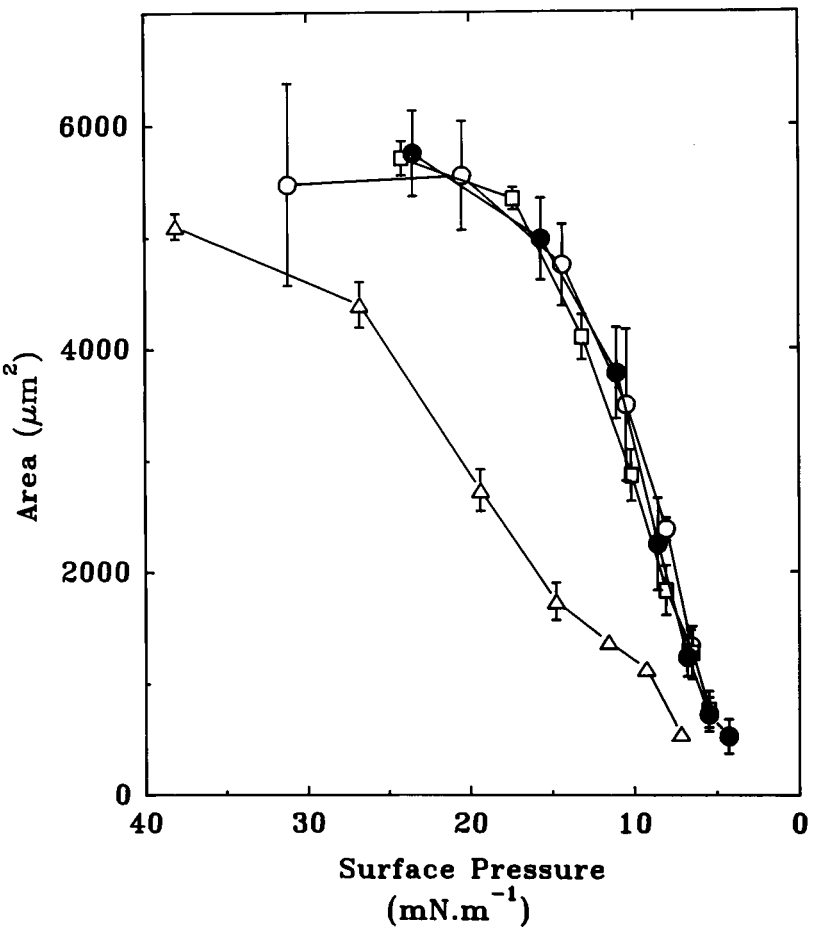

FIGURE 6 Total area of condensed domains (μm^2) as a function of π and SP-C concentration (symbols as in Fig. 3).

the area occupied by a DPPC molecule at any π. SP-C in bilayers of saturated PC perturbs the gel state alignment of the lipids (5). If earlier calorimetric data on bilayers (5) is recalculated to account for the palmitoylated form of SP-C, then about 20 molecules of PC per SP-C molecule could be accounted for as not contributing to the transitional enthalpy, assuming all molecules of DPPC that are not influenced by SP-C "melt" with the enthalpy change of pure lipid. On that basis, we might assume that at least 10 molecules of DPPC are perturbed per SP-C molecule in a monolayer, depending upon the surface pressure.

The visual characteristics of the monolayer can be consistent with the assumption that SP-C perturbs lipid packing and inhibits lipid condensation. In the monolayers, the number of dark areas (condensed lipid) increased, and the size of the individual condensed domains decreased, with increasing concentration of SP-C. If SP-C perturbed lipid packing it would inhibit the growth of large condensed lipid domains. At the same time, increasing the surface pressure would exert a packing force on molecules of DPPC that were remote from SP-C, causing them to form condensed areas. As the SP-C concentration increased, the available nonperturbed lipid would be confined to more, but smaller, interstitial spaces between the perturbed or expanded regions that were in contact with protein. This interpretation could also account for the fact that the dependency of the average area of the condensed domains on surface pressure is smaller in the presence of greater amounts of proteins than it is at lower protein concentrations (Fig. 5).

This interpretation is also consistent with the interesting property of the monolayers seen in Fig. 3. In the absence of protein, the number of dark condensed domains was independent of π, but in the presence of SP-C, the number of condensed domains increased with increasing π. This could happen if, when π was increased, more SP-C molecules were forced into contact with what would otherwise be the condensed phase. Such additional SP-C-lipid interaction would cause more condensed lipids to enter the fluid phase, and force the remaining condensed lipid into more, smaller condensed regions (Fig. 3).

The data in Fig. 6 show an interesting property of the total dark area in each visual field (the percentage of the total area that is covered by condensed lipid). Up to 1.1 mol% protein, the total condensed areas are equivalent at any given π. This data and the observation that con-

densed domains are smaller when SP-C is present than in its absence, indicate that SP-C reduces the cooperativity of the liquid-expanded to liquid-condensed phase transition. At 1.9 mol% SP-C, substantially less total condensed area existed at any given π, although all the curves appeared to be approaching the same limit at higher π. Thus, while SP-C causes a redistribution of lipid domains in the monolayer, it seems to do so in two different ways, depending on protein concentration. Up to a certain SP-C concentration (c) ($1.1 < c < 1.9$ mol%) one type of protein–lipid packing occurs. Above that limit ($c \leq 1.9$ mol%) a different type of protein–lipid interaction is encountered, as evidenced by the reduced amount of condensed phase at any given π. Since this limit is in the range of concentration that has been estimated for hydrophobic proteins in surfactant (e.g., 1, 3, 27, 28), it could be important in surfactant dynamics. This observation suggests that it could be important to determine as precisely as possible the amounts of hydrophobic proteins, SP-B and SP-C, that are present in surfactant.

The isotherms in the insert in Fig. 1 *b* indicate that the presence of SP-C does not prevent the monolayers from attaining very high surface pressures. Monolayers of SP-C alone did not attain such high surface pressures, as was also found by Oosterlaken-Dijksterhuis et al. (25). Perhaps SP-C is somehow removed from the monolayer at high surface pressure. This may not be the case under all conditions, especially if PG is present and the compression is rapid and large (24). There were some small changes in the isotherms at $50 < \pi < 60$ mN · m^{-1}, which might suggest some rearrangement in the lipids and proteins. At this point, however, the balance is not totally leak free (14). This fact, the possibility that the probe might be self quenching at these very high pressures, and the visual texture of the densely packed state prevent reliable quantitative analyses of the high pressure regions currently. In mammals, although obviously not in many ectotherms living at lower temperatures, the pulmonary surfactant is at 37°C. Technical difficulties have prevented us from analyzing the films at this temperature at this time. Since the phase transition of DPPC is at 41°C, however, the fundamental nature of the lipid–protein interactions are likely not different at 24 and 37°C. Future experiments will be directed at establishing more information about the distribution of the protein in the lipid matrix, and its effect on the characteristics of more complex lipid systems particularly those including acidic lipids.

We thank Drs. Mendelsohn and Oosterlaken-Dijksterhuis for providing us with preprints of their work, and Lorne Taylor for help with the graphics.

This work was supported by the Medical Research Council of Canada (to Kevin M. W. Keough) and a NATO fellowship to Jesus Perez-Gil. Kaushik Nag thanks Memorial University of Newfoundland for the award of a Graduate Fellowship.

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