Pulmonary Surfactant Proteins SP-B and SP-C in Spread Monolayers at the Air-Water Interface: II. Monolayers of Pulmonary Surfactant Protein SP-C and Phospholipids

Svetla Taneva* and Kevin M. W. Keough*‡
*Department of Biochemistry and ‡Discipline of Pediatrics, Memorial University of Newfoundland, St. John's, Newfoundland, Canada A1B 3X9

ABSTRACT The interaction of the hydrophobic pulmonary surfactant protein SP-C with dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylglycerol (DPPG) and DPPC:DPPG (7:3, mol:mol) in spread monolayers at the air-water interface has been studied. At low concentrations of SP-C (about 0.5 mol% or 3 weight% protein) the protein-lipid films collapsed at surface pressures of about 70 $mN \cdot m^{-1}$, comparable to those of the lipids alone. At initial protein concentrations higher than 0.8 mol%, or 4 weight%, the isotherms displayed kinks at surface pressures of about 50 $mN \cdot m^{-1}$ in addition to the collapse plateaux at the higher pressures. The presence of less than 6 mol%, or 27 weight%, of SP-C in the protein-lipid monolayers gave a positive deviation from ideal behavior of the mean areas in the films. Analyses of the mean areas in the protein-lipid films as functions of the monolayer composition and surface pressure showed that SP-C, associated with some phospholipid (about 8–10 lipid molecules per molecule of SP-C), was squeezed out from the monolayers at surface pressures of about 55 $mN \cdot m^{-1}$. The results suggest a potential role for SP-C to modify the composition of the monolayer at the air-water interface in the alveoli.

INTRODUCTION

In the first paper of this series we reported on the behavior of monolayers of mixtures of SP-B and phospholipids at the air-water interface. Here we deal with spread monolayers of porcine surfactant protein SP-C and pulmonary phospholipids 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-dipalmitoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (sodium salt) (DPPG). SP-C is a 35-residue polypeptide with an extremely hydrophobic carboxyl-terminal region of 23 residues which undoubtedly plays a role in the interaction with phospholipids (Hawgood, 1989). The reduced hydrophobicity of the amino-terminal moiety is compensated for by two palmitoyl groups covalently bound through thiol esters to cycteines at positions 5 and 6. Their presence suggests the possibility of interaction of the amino-terminal region with phospholipids, also. (Curstedt et al., 1990).

Various experimental techniques have been used to study the interaction of SP-C with lipid bilayers. The ability of SP-C to disturb lipid packing has been investigated using DSC (Shiffer et al., 1993; Simatos et al., 1990). The deuterium-NMR spectra of bilayers of DMPC-d_{54} containing SP-C are also consistent with a perturbation in the lipid packing by the protein (Simatos et al., 1990). Spectroscopic studies (Pastrana et al., 1991; Vandenbussche et al., 1992) showed that SP-C possesses high α-helical content in lipid vesicles (about 60%); the α-helix axis has been suggested to be oriented parallel (Pastrana et al., 1991) or at 24° (Vandenbussche et al., 1992) to the bilayer normal, both indicating a trans-bilayer orientation of the protein. Interaction between SP-C and DPPC has also been detected in spread monolayers at the air-water interface (Oosterlaken-Dijksterhuis et al., 1991). Epifluorescence microscopic study of binary spread monolayers of SP-C and DPPC revealed that SP-C perturbed the packing of lipid, stabilizing the liquid-expanded phase somewhat and reducing the size of condensed domains of the lipid in the mixed phase (liquid expanded-liquid condensed) region during monolayer compression (Perez-Gil et al., 1992).

In this study the surface properties of spread films of SP-C and phospholipids, obtained from compression isotherms, are interpreted in terms of additivity rule of the mean area per "residue" in the mixed films (one amino acid residue of SP-C or one lipid molecule was counted as a "residue"). The partial areas per amino acid residue of SP-C and lipid molecule in the binary monolayers have been used to evaluate compositional changes of the films as a function of surface pressure. Similar analyses are reported for spread monolayers of SP-B plus phospholipids and spread films of an SP-B/SP-C mixture with phospholipids in the accompanying articles.

Received for publication 28 July 1993 and in final form 27 January 1994.

Address reprint requests to Kevin M. W. Keough at the Department of Biochemistry, Room S-4006, Memorial University of Newfoundland, St. John's, Newfoundland A1B 3X9, Canada. Tel.: 709-737-2530; Fax: 709-737-2552; E-mail: kkeough@kean.ucs.mun.ca.

Abbreviations used: All abbreviations and symbols are as defined in the preceding paper.

© 1994 by the Biophysical Society

0006-3495/94/04/1149/09 $2.00

EXPERIMENTAL PROCEDURES

DPPC was purchased from Sigma Chemical Co. (St. Louis, MO) and DPPG from Avanti Polar Lipids Inc. (Pelham, Al). The lipids were determined to be pure by thin-layer chromatography and were used as received.

The isolation of SP-C was described in detail in the preceding paper. Using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (16% gel) followed by silver staining (New England Nuclear Research Products, Boston, MA) SP-C yielded one band at about 5 kDa under nonreducing and reducing conditions. Estimation of the phosphorus content of the protein

(Bartlett, 1959) indicated that it contained less than 0.05 mol of phospholipid per mol of SP-C, which was the detection limit of the lipid determination.

Surface pressure measurements

The isotherms of surface pressure (π) versus mean area per "residue," A_{mean}, where "residue" denotes an amino acid residue of SP-C or a phospholipid molecule, were measured using the trough and surface balance described in the preceding paper. SP-C/phospholipid monolayers were formed by spreading of premixed solutions of the components in chloroform-methanol (3:1). After a 10-min period for evaporation and equilibration, the monolayers were compressed at 12 cm^2/min in 20 steps of approximately 30 s duration. 1 min was allowed between the successive steps. The time required to obtain a complete isotherm was 30 min. 0.15 M NaCl in deionized doubly distilled water with the pH adjusted to 7 immediately before each experiment was used as subphase. The temperature was 22 ± 1°C.

The initial compositions of the mixed monolayers were determined from the amounts of components spread on the surface and were expressed in terms of the fraction of the amino acid residues of SP-C, X_r (Eq. 1, preceding paper). For SP-C, a molecular weight of 4186 based on the amino acid composition of SP-C plus two palmitates (Curstedt et al., 1990) was used for all calculations. The experimental mean areas per "residue," A_{mean}, in the binary and ternary monolayers of SP-C and phospholipid(s) were determined using Eq. 2 of the preceding paper. The partial areas of a lipid molecule ($\bar{A}_l$), and a protein amino acid residue ($\bar{A}_r$) in the mixed monolayers were determined from the plots $A_{mean}(X_r)$ using the method of intercepts, which was already described in the preceding paper.

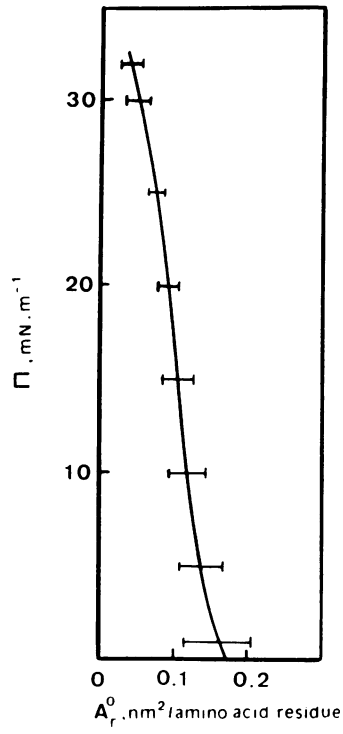

FIGURE 1 Average isotherm of surface pressure versus area per amino acid residue for spread monolayers of SP-C. The bars show means ± SD.

RESULTS AND DISCUSSION

Spread monolayers of SP-C

The average isotherm of surface pressure, π, against the area per amino acid residue, A_r^0, in spread films of SP-C is shown in Fig. 1. The curve represents an average for eight different isolates of SP-C. The surfactant proteins SP-B and SP-C are extremely hydrophobic proteins which makes their delipidation difficult. The extent of delipidation has been reported to influence the functional properties of SP-B and SP-C (Yu and Possmayer, 1988). We also found that the $\pi(A_r^0)$ isotherms for spread monolayers of SP-B and SP-C were influenced by the extent of delipidation of the proteins. Thus a preparation of SP-C containing 1.4 mol of phospholipid per mol of SP-C, which is within the limits of phospholipid remaining in the protein preparations usually reported (Oosterlaken-Dijksterhuis et al., 1991; Williams et al., 1991), yielded an isotherm shifted to higher areas per amino acid residue, e.g., the initial pressure of lift off was at about 0.25 nm^2/amino acid residue, compared to 0.17 nm^2/amino acid residue reported here. The surface pressure-area curves for porcine SP-C measured by us yielded lower areas per amino acid residue than those previously reported (Oosterlaken-Dijksterhuis et al., 1991). The difference has been discussed in detail elsewhere (Pérez-Gil et al., 1992). The limiting area obtained by extrapolation of the $\pi(A_r^0)$ curve to $\pi = 0$ gave 0.14 nm^2/amino acid residue (Fig. 1). This value is consistent with data reported for spread monolayers of predominantly α-helical polymers (Malcolm, 1973; Yamashita, 1971). The inflection of the isotherm for SP-C at minimum compressibility of the protein film (0.018 $m \cdot mN^{-1}$) corresponded to 0.125 nm^2/amino acid residue. This area may be considered to represent the smallest area to which the protein monolayer can be compressed without partial collapse of the film. The $\pi(A_r^0)$ isotherm for the monolayers of SP-C measured with this trough showed a plateau-like region at about 35 $mN \cdot m^{-1}$. Higher surface pressures of about 45 $mN \cdot m^{-1}$ were attained during compression of the protein films when the measurements were performed in a Langmuir trough without separate compartments for surface tension and epifluorescence measurements (see the preceding paper).

The $\pi(A_r^0)$ isotherm for SP-C showed smaller areas per amino acid residue when compared with the curve for SP-B (Fig. 1, preceding paper). The molecular basis of this difference is not known, but it may reflect differences in their hydrophobicities (amino acid composition) and in their secondary structures. Also a role for the two palmitoyl chains covalently bound to the SP-C molecule in the interfacial behavior of the protein has not yet been elucidated.

The conformations of SP-B and SP-C in spread monolayers are not established. Recently, monolayers of SP-B and SP-C collected at 25 $mN \cdot m^{-1}$ have been found to give CD spectra consistent with a high content of α-helix, but the CD spectra have not been qualitatively analyzed (Oosterlaken-Dijksterhuis et al., 1991). More information is available about the secondary structure of the proteins in organic solvent. Since the protein monolayers were formed from organic solvents, and assuming that the conformation of the protein in the spreading solvent is preserved in the monolayer (such behavior having been confirmed for some other polypeptides (Cornell, 1979)), then the $\pi(A_r^0)$ isotherms may be discussed in terms of the secondary molecular structures

found for the proteins in organic solvent. A high content of 52% α-helix was found for bovine SP-C in chloroform: methanol using attenuated total reflection Fourier-transform infrared spectroscopy (Pastrana et al., 1991) and for dog SP-C in butanol using circular dichroism (Shiffer et al., 1993). A content of α-helix of about 45% was also reported for oriented multilayers of porcine SP-B (Vandenbussche et al., 1992). The influence of secondary structure of a polymer in a monolayer on the properties of its $\pi(A_r^0)$ curve (e.g., area per amino acid residue, compressibility, collapse pressure) has been discussed (Malcolm, 1973; Yamashita, 1971). Polymers containing a high percentage of α-helix have been shown to form condensed, incompressible monolayers, whereas polymers which are predominantly in extended β-structures give more expanded and readily compressible films. Also, using computer molecular models of closely packed polypeptide chains lying in the monolayer plane, it has been calculated that the area per amino acid residue in a film of α-helices would be expected to be 0.128 nm^2/amino acid residue compared 0.156 or 0.168 nm^2/amino acid residue in monolayers of β-conformations (Malcolm, 1973). Therefore, the steeper slope of the isotherm and the lower values for the area per amino acid residue for the SP-C monolayer in comparison to that for SP-B are likely consequences of the higher α-helix content of SP-C.

It has been shown that intrinsic surface activities of proteins, as determined by surface pressures at the air-water interface, correlate with the product ($\bar{\mu}_H \times F$), where μ_H is the average value of the hydrophobic moment for all helices in the molecule and F is the fraction of α-helix in the protein (Krebs and Phillips, 1983; Krebs and Phillips, 1984; Krebs et al., 1988). Though this analysis was first carried out for adsorbed monolayers of water-soluble proteins, the same approach could be used to approximately estimate the interfacial behavior of spread films of SP-B and SP-C. Values of 0.45 and 0.52 for F were used for SP-B and SP-C as reported (Pastrana et al., 1991; Vandenbussche et al., 1992). Values for μ_H and predicted segments of α-helix, necessary for $\bar{\mu}_H$ calculations, were taken from the study by Takahashi et al. ($\bar{\mu}_H$ = 0.606 kcal/mol helical residue for SP-B and $\bar{\mu}_H$ = 0.036 kcal/mol helical residue for SP-C) (Takahashi et al., 1990). The calculations showed that the product ($\bar{\mu}_H \times F$) for SP-B was 0.27 kcal/mol helical residue, and it was an order of magnitude higher than the value for SP-C (0.02 kcal/mol helical residue), which suggested higher surface activity for the amphipathic SP-B. This result is consistent with the results from surface pressure-area measurements on the spread films of SP-B and SP-C here and in the preceding paper, which showed that at any given area per amino acid residue, SP-B exerted a higher surface pressure than did SP-C. This suggests that a higher fraction of the amino acid residues of SP-B compared to SP-C, resides in the plane of the interface.

The magnitude of ($\bar{\mu}_H \times F$) for SP-B is close to the values calculated for apolipoprotein A-II (0.25 kcal/mol helical residue) and apolipoprotein A-I (0.18 kcal/mol helical residue), whereas the value for SP-C is comparable with the one determined for lysozyme, a water-soluble globular protein (0.07 kcal/helical residue) (Krebs and Phillips, 1983). The $\pi(A_r^0)$ isotherm for spread monolayers of SP-B is consistent with the isotherm for spread monolayer of apolipoprotein A-I (Krebs et al., 1988), whereas the isotherm for SP-C practically superimposes on the curve for the spread film of lysozyme (Mita, 1989). The correlation of the product ($\bar{\mu}_H \times F$) of these proteins of different structure and solubility, spread from different solvents onto different subphases, with the characteristics of the isotherms obtained from compression of their surface films, suggests that this parameter, $\bar{\mu}_H \times F$, could be useful for predicting the properties of spread films of proteins at the air-water interface.

Spread binary monolayers of SP-C and DPPC

The experimental results from surface pressure measurements for SP-C/DPPC monolayers are plotted in Fig. 2, where A_{mean} is the mean area per "residue," ("residue" denotes amino acid residue of SP-C or DPPC molecule). High surface pressures of about 70 $mN \cdot m^{-1}$ were attained in the mixed films of $0 < X_r \leq 0.60$ (corresponding to 4.2 mol% or 20 weight%). At compositions corresponding to $X_r \geq 0.22$ (equivalent to 0.8 mol% or 4.5 weight%) kink points at surface pressure near 50 $mN \cdot m^{-1}$ appeared in the isotherms. They are readily seen as minima in the plots of surface elasticity of the monolayers $E = -(d\pi/d\ln A_{mean})_T$, as a function of surface pressure (Fig. 3). The mean area per "residue" in

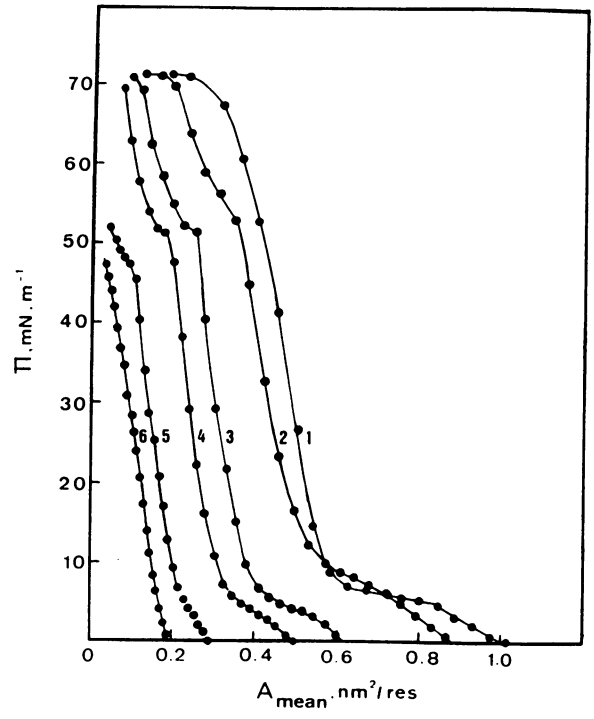

FIGURE 2 Isotherms of surface pressure versus mean area per "residue" for monolayers of SP-C and DPPC of various "residual" fractions of SP-C, X_r: 0.0 (*1*), 0.22 (*2*), 0.48 (*3*), 0.60 (*4*), 0.82 (*5*), and 0.93 (*6*).

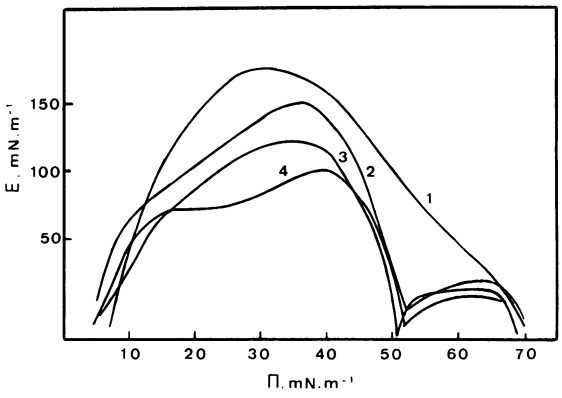

FIGURE 3 Surface elasticity-surface pressure plots for SP-C/DPPC monolayers of various compositions, X_r: 0.0 (*1*), 0.32 (*2*), 0.48 (*3*), and 0.60 (*4*).

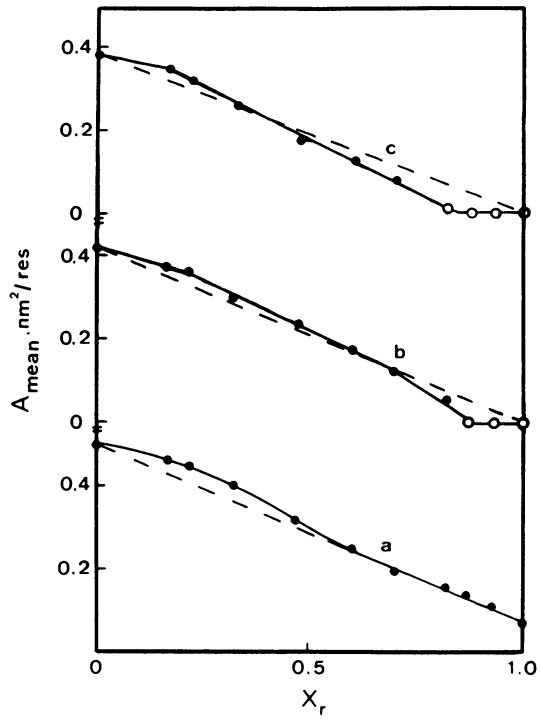

FIGURE 4 Mean area per "residue" in SP-C/DPPC films as a function of their initial composition at different surface pressures: 25 mN·m^{-1} (*a*), 50 mN·m^{-1} (*b*), 55 mN·m^{-1} (*c*). Full circles represent average results of at least two experiments. Open circles represent extrapolated values of A_{mean} at the given surface pressure.

the binary films was determined from the isotherms at selected surface pressures and plotted as a function of monolayer composition, X_r (Fig. 4). At pressures below the collapse pressure of the monolayer of pure SP-C, for monolayers of $X_r < 0.70$ (corresponding to 6.25 mol% or 27 weight%), the mean areas per "residue" have higher values than those calculated for ideal behavior of the two-component monolayers of SP-C and DPPC (*dashed line*). At higher protein concentrations, $X_r \geq 0.70$, the mean areas are additive (Fig. 4 *a*). It is worth noting that a straight-line relationship would occur not only in the case of ideal mixing but also when complete demixing of the components occurs. An interpretation of the apparent additivity of the mean area per "residue" at $X_r \geq 0.70$ can be given for a specific arrangement of SP-C and DPPC in a closely packed two-dimensional layer. The SP-C molecule was treated as a rectangle and DPPC as a circle with cross-sectional area of 0.42 nm^2 (Watkins, 1968). The size of the protein molecule was inferred from the area per amino acid residue corresponding to minimum compressibility of the monolayer (0.125 nm^2/amino acid residue). At this point the protein monolayer was assumed to consist of closely packed α-helices oriented parallel to the air-water interface. While we have made this assumption of all residues in a α-helix for convenience we do not know conformation and orientation of the first 12 residues and the acyl chains. The fact that the palmitates will not associate with the water would confine the first 12 amino acids of SP-C to a relatively compact packing like that seen for α-helices. The assumption for parallel orientation of the α-helices at the air-water interface was based on calculations (Malcolm, 1973) and experimental evidence (Cornell, 1979) for other hydrophobic polypeptides. In this case the area per SP-C molecule would be $35 \times 0.125 = 4.37$ nm^2. To calculate the perimeter of the molecule, and hence the number of phospholipids which could surround the protein in a single adjoining layer, a value of 0.855 nm, which is the separation distance between α-helices lying in a monolayer (Malcolm, 1973), was used for the helix diameter. Assuming that the components were uniformly dispersed in the monolayer, we found that, in a closely packed SP-C/DPPC film, each protein molecule would be surrounded by a shell of 20 lipids. This structure corresponds to a composition $X_r = 0.64$. The expansion in the A_{mean} (X_r) plot for SP-C/DPPC films of $X_r < 0.70$ is consistent with interaction between SP-C and DPPC, hence miscibility of the components. At higher protein concentrations the number of DPPC molecules may be insufficient to provide an individual layer of 20 lipids for each protein, and possibly protein-protein contacts would be present in the surface layer. Strong hydrophobic bonding between the α-helices may result in self-association of SP-C and hence its aggregation in the SP-C/DPPC films of higher protein concentration. Interestingly, it was predicted recently, that in a lipid bilayer with hydrophobic α-helices oriented perpendicular to the plane of the bilayer, the helices would preferentially pack together rather than disperse in the membrane (Wang and Pullman, 1991). Previous experimental data was also consistent with a tendency of SP-C to aggregate in DPPC:PG bilayers (Pastrana et al., 1991; Vandenbussche et al., 1992).

Analysis of A_{mean} (X_r) plots at surface pressures higher than the pressure corresponding to the kink points in the isotherms (Fig. 4 *c*) suggested that some SP-C was still present in the protein-lipid films of initial compositions $X_r \leq 0.30$. For the films of higher protein content, the data was consistent with expulsion of protein-lipid units from the in-

TABLE 1 Calculated composition of SP-C/DPPC monolayers, X_r^{calc}, and excluded phases, X_r^{lost}, as a function of surface pressure

Initial molar ratio (SP-C:lipid)	Initial (X_r)	Surface pressure ($mN \cdot m^{-1}$)									
		Film (X_r^{calc})					Excluded phase (X_r^{lost})				
		25	40	45	50	55	25	40	45	50	55
1:172	0.17	0.17	0.17	0.16	0.18	0.16	-	-	-	-	-
1:124	0.22	0.21	0.24	0.22	0.22	0	-	-	-	-	0.95 1:2*
1:72	0.32	0.33	0.33	0.30	0	0	-	-	-	1.0 1:0*	0.83 1:7*
1:38	0.48	0.50	0.48	0.51	0	0	-	-	-	1:0 1.0*	0.81 1:8*
1:23	0.61	0.60	0.59	0.61	0	0	-	-	-	0.95 1:2*	0.83 1:7*
1:8	0.82	0.83	0.83	0.82	0	0	-	-	-	0.92 1:3*	0.85 1:6*

* Calculated molar ratio SP-C:lipid of the excluded phase.

terface which resulted in values of A_{mean} below the additive line. The changes in the compositions of the SP-C/DPPC monolayers following increases in the surface pressure were determined using the approach which was applied to the binary monolayers of SP-B and phospholipid (preceding paper). The compositions, X_r^{lost}, of excluded SP-C/DPPC units from monolayers of $X_r \geq 0.22$ were determined in the manner described in the preceding paper. The results, summarized in Table 1, indicate that, for low initial concentrations of SP-C in the SP-C/DPPC films, $X_r \approx 0.17$, (equivalent to 0.5 mol% or 3 weight%), no change in the initial compositions of the monolayers occurred during their compression. In other words, SP-C was not squeezed out from the interface, even at pressures as high as 70 $mN \cdot m^{-1}$. At higher initial concentrations of SP-C, $X_r \geq 0.22$, corresponding to 0.8 mol% or 4 weight%, the protein was excluded from the monolayers at surface pressures $\pi \geq \pi_{kink}$ (about 50 $mN \cdot m^{-1}$). The compositions of the excluded phases from the SP-C/DPPC films, X_r^{lost}, suggest that the exclusion of SP-C was accompanied by removal of phospholipid (about 6–7 mol of lipid per mol of SP-C) at $\pi = 55$ $mN \cdot m^{-1}$. The process of squeeze-out of protein-lipid units resulted in enrichment of the remaining monolayer in the phospholipid component. Comparison with similar data for SP-B/DPPC spread monolayers (Table 2, preceding paper) indicates that the process of exclusion from the protein-lipid monolayers could occur at lower initial protein concentrations for SP-C in comparison to SP-B. At the same time SP-C was retained in the DPPC monolayer at higher surface pressures than SP-B. For example, at 45 $mN \cdot m^{-1}$ no squeeze-out from films containing SP-C was observed in comparison to SP-B/DPPC films where squeeze-out was seen at that pressure. The compositions of the protein-lipid units excluded from the monolayers suggest that a larger amount of DPPC was removed when SP-C was squeezed out than when SP-B was removed (preceding paper). The higher hydrophobicity of amino acids of SP-C compared to those of SP-B (Takahashi et al., 1990) and the two palmitoyl chains of the former molecule likely contribute to a stronger attraction of SP-C than SP-B to phospholipids.

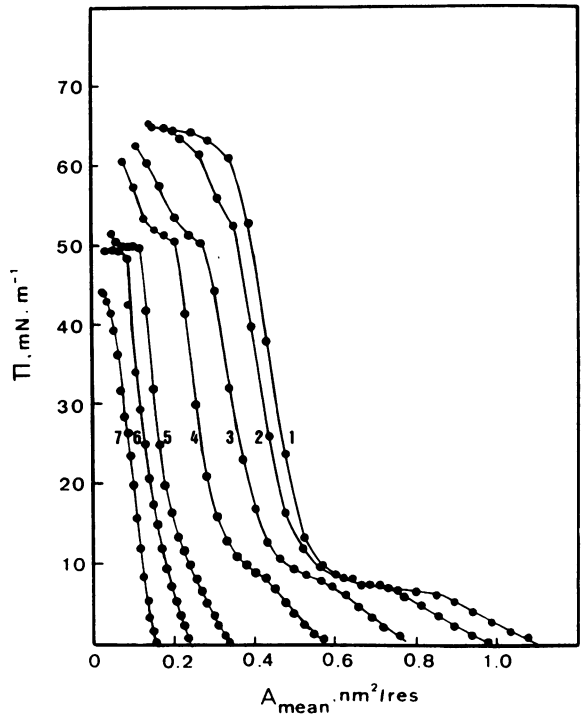

FIGURE 5 Surface pressure-mean area per "residue" curves for SP-C/DPPG monolayers of initial composition X_r: 0.0 (*1*), 0.22 (*2*), 0.39 (*3*), 0.56 (*4*), 0.76 (*5*), 0.86 (*6*), and 0.95 (*7*).

Spread binary monolayers of SP-C and DPPG

The isotherms of surface pressure versus mean area per "residue" for spread monolayers of SP-C and DPPG are shown in Fig. 5. The curves displayed features similar to those of SP-C/DPPC films of comparable compositions. Monolayers of initial protein composition $X_r \leq 0.56$ (equivalent to 3.57 mol% or 17 weight%) collapsed at surface pressures of about 65 $mN \cdot m^{-1}$, typical for the monolayer of DPPG alone. In monolayers of initial composition $X_r \geq 0.22$ a second plateau or kink was detected at pressures of about 50 $mN \cdot m^{-1}$, which

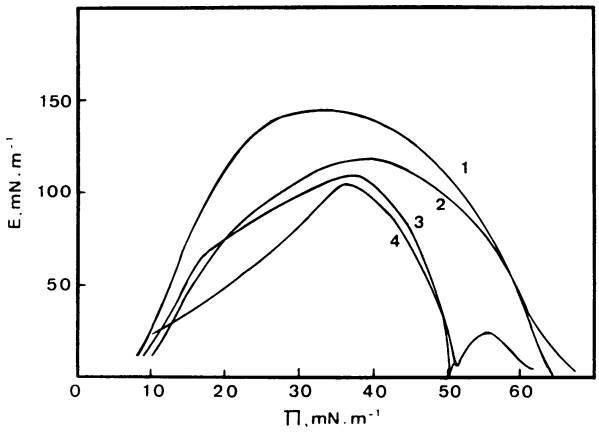

FIGURE 6 Surface elasticity-surface pressure plots for SP-C/DPPG monolayers of different compositions X_r: 0.0 (*1*), 0.15 (*2*), 0.39 (*3*), and 0.76 (*4*).

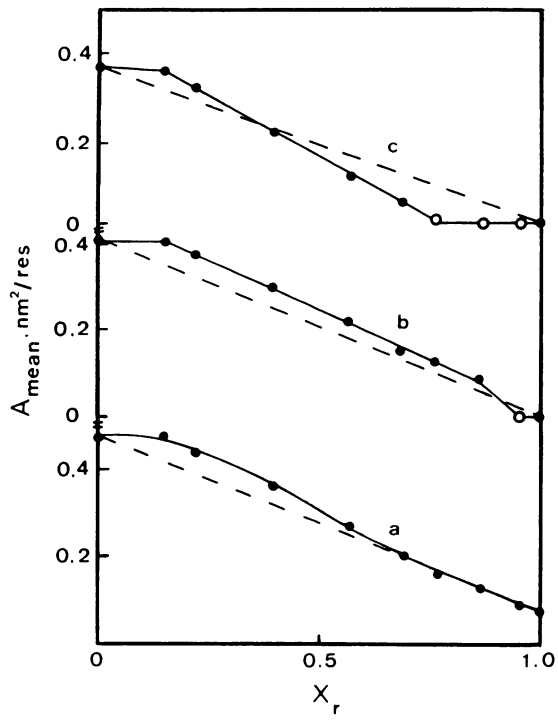

FIGURE 7 Mean area per "residue" versus monolayer composition for SP-C/DPPG mixtures at surface pressure of 25 mN·m^{-1} (*a*), 50 mN·m^{-1} (*b*), 55 mN·m^{-1} (*c*).

are seen as minima in the curves of surface elasticity of the monolayers versus surface pressure (Fig. 6). When the mean areas per "residue" in the SP-C/DPPG films were plotted against the initial monolayer composition at low surface pressures, positive deviations from ideal behavior were observed for $X_r < 0.70$ (Fig. 7 *a*). At higher protein levels, the mean areas per "residue" were additive. At surface pressures above the kink points in the isotherms, an expansion effect of SP-C was seen only in SP-C/DPPG films of $X_r < 0.30$ (Fig. 7 *c*). The mean areas in the SP-C/DPPC films showed similar dependence on the composition and pressure (Fig. 4). This similarity suggests that the expansion seen in the films of the basic SP-C (porcine SP-C has three side chains which are positively charged at physiological pH (Curstedt et al., 1990)) with either DPPC or DPPG are likely due predominantly to hydrophobic interactions between the protein and phospholipids.

Similar to the case of SP-C/DPPC films, analyses of the compositions of the SP-C/DPPG films as a function of surface pressure were carried out. The results, summarized in Table 2, indicated that no change in the initial composition of SP-C/DPPG films of $X_r \leq 0.15$ (0.5 mol% or 3 weight%) occurred during their compression. In films of higher protein concentration, exclusion of protein-lipid units (about 10 lipids per SP-C molecule) was observed at surface pressures of about 55 mN·m^{-1}. This result, when compared to the compositions of the excluded phases from SP-C/DPPC films (Table 1), suggests that SP-C removed a slightly larger amount of lipid from the binary SP-C/DPPG monolayers than from the SP-C/DPPC films of similar initial composition. The surface pressure required for squeeze-out was slightly higher for SP-C/DPPG than for SP-C/DPPC films. These observations are consistent with the idea that there are slightly stronger interactions in the SP-C/DPPG than SP-C/DPPC films, which may be accounted for by electrostatic attraction between SP-C and DPPG.

Ternary spread monolayers of SP-C with a mixture of DPPC and DPPG

The isotherms of surface pressure versus mean area per "residue" in the ternary films of SP-C with a mixture of DPPC:DPPG (7:3, mol:mol) are shown in Fig. 8. Monolayers of composition $X_r < 0.57$ (corresponding to 3.57 mol% or 17 weight%) collapsed at about 70 mN·m^{-1}, corresponding to the collapse pressure of the binary DPPC:DPPG (7:3, mol:mol) film. For films of compositions $0.26 \leq X_r \leq 0.73$ ($1 \leq$ mol% SP-C ≤ 7.14, or $5 \leq$ weight% protein ≤ 30) kink points in the isotherms were observed at surface pressure of about 50 mN·m^{-1}. The plots of the elasticities of the films as a function of the surface pressure (Fig. 9) showed minima at $\pi \approx 50$ mN·m^{-1}, similar to the E(π) plots for the binary films of SP-C plus phospholipid. The expansions in the mean areas per "residue" in the ternary films had values of similar magnitude to those of SP-C/DPPC and SP-C/DPPG films of comparable compositions and surface pressures. Changes in the compositions of the SP-C/(DPPC:DPPG) films following their compression were determined using the same approach as used for the binary SP-C/phospholipid monolayers. The lipid partial molar ("residual") area, $\bar{A}_l$, and apparent area, A'_l, were determined assuming that there was no difference in the behavior of DPPC and DPPG in the ternary films. The calculated compositions for some ternary films as a function of surface pressure are listed in Table 3. The compositions,

TABLE 2 Calculated composition of SP-C/DPPG monolayers, X_r^{calc}, and excluded phases, X_r^{lost}, as a function of surface pressure

Initial molar ratio (SP-C:lipid)	Initial (X_r)	Surface pressure ($mN \cdot m^{-1}$)									
		Film (X_r^{calc})					Excluded phase (X_r^{lost})				
		25	40	45	50	55	25	40	45	50	55
1:197	0.15	0.15	0.16	0.15	0.15	0.16	-	-	-	-	-
1:125	0.22	0.22	0.22	0.20	0.23	0	-	-	-	-	0.76 1:11*
1:54	0.39	0.38	0.40	0.40	0.35	0	-	-	-	-	0.76 1:11*
1:27	0.56	0.55	0.56	0.54	0.53	0	-	-	-	-	0.76 1:11*
1:11	0.76	0.76	0.77	0.76	0	0	-	-	-	0.85 1:6*	0.77 1:10*

* Calculated molar ratio SP-C:lipid of the excluded phase.

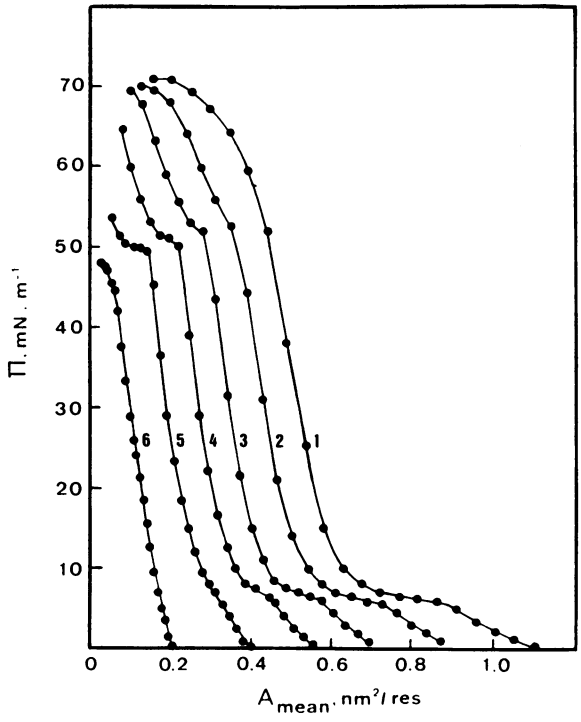

FIGURE 8 Surface pressure versus mean area per "residue" curves for ternary films of SP-C and DPPC:DPPG (7:3, mol:mol) of initial compositions X_r: 0.0 (*1*), 0.26 (*2*), 0.41 (*3*), 0.57 (*4*), 0.73 (*5*), and 0.92 (*6*).

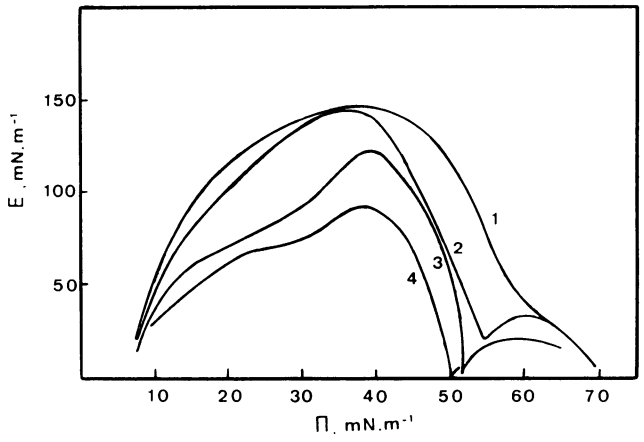

FIGURE 9 Surface elasticity-surface pressure plots for SP-C/(DPPC: DPPG) films of initial composition X_r: 0.0 (*1*), 0.26 (*2*), 0.41 (*3*), and 0.73 (*4*).

X_r^{lost}, of the excluded protein-lipid units from the ternary films are shown in the same table. The data are consistent with squeeze-out of protein-lipid complexes (about 10 lipid molecules per molecule of SP-C) at $\pi \geq 55$ $mN \cdot m^{-1}$ from films of initial composition $X_r \geq 0.26$. The method used to estimate the compositions of the excluded phase did not distinguished between the two phospholipids in the ternary mixtures and no conclusion could be drawn about selective squeeze-out of a SP-C/DPPC or SP-C/DPPG complex from the SP-C/-(DPPC:DPPG) films when they were compressed to high surface pressures.

Comparison of the data for monolayers of SP-B/(DPPC: DPPG) (Table 4 in the preceding paper) and SP-C/(DPPC: DPPG) (Table 3) shows that at $\pi = 55$ $mN \cdot m^{-1}$ a larger amount of phospholipid was squeezed out from monolayers containing SP-C compared to that eliminated from those containing SP-B. This conclusion also could be drawn from a comparison of the $E(\pi)$ plots for the ternary SP-B/(DPPC: DPPG) and SP-C/(DPPC:DPPG) films (Fig. 13 of the preceding paper and Fig. 9 in this paper). For example, at $\pi \geq 55$ $mN \cdot m^{-1}$, the SP-B/(DPPC:DPPG) film of composition $X_r = 0.42$ (*curve 3*, Fig. 13, preceding paper) displayed elasticity values, comparable to those seen in the monolayer of DPPC:DPPG without protein. This was possibly because only a small amount of phospholipid was removed with SP-B during compression of the films, and therefore the remaining phospholipid monolayer displayed high elasticity. At the same surface pressure, the SP-C/(DPPC:DPPG) monolayer of the same initial concentration, $X_r = 0.41$ (*curve 3* in Fig. 9), showed lower elasticity than did the monolayer of DPPC:DPPG without protein. This observation is consistent

TABLE 3 Calculated composition of SP-C/(DPPC:DPPG) monolayers, X_r^{calc}, and excluded phases, X_r^{lost}, as a function of surface pressure

Initial molar ratio (SP-C:lipid)	Initial (X_r)	Surface pressure ($mN \cdot m^{-1}$)									
		Film (X_r^{calc})					Excluded phase (X_r^{lost})				
		25	40	45	50	55	25	40	45	50	55
1:231	0.13	0.13	0.13	0.13	0.13	0.13	-	-	-	-	-
1:99	0.26	0.25	0.27	0.27	0.25	0	-	-	-	-	0.79 1:9*
1:50	0.41	0.41	0.40	0.40	0.39	0	-	-	-	-	0.78 1:10*
1:27	0.57	0.57	0.55	0.54	0.55	0	-	-	-	-	0.78 1:10*
1:13	0.73	0.74	0.72	0.72	0	0	-	-	-	0.87 1:5*	0.79 1:9*

* Calculated molar ratio SP-C:lipid of the excluded phase.

with the removal of a larger amount of phospholipid during compression of the SP-C/(DPPC:DPPG) films compared to SP-B/(DPPC:DPPG) ones. In this case the depletion in the surface concentration of the phospholipid in the remaining monolayer resulted in lower values of the elasticities compared to the ones for SP-B/(DPPC:DPPG) films (preceding paper).

SUMMARY

In the three monolayer systems composed of SP-C plus phospholipid(s) (SP-C/DPPC, SP-C/DPPG, and SP-C/(DPPC:DPPG), at surface pressures below the collapse points of the components, an expansion effect in the mean film areas was detected for protein concentrations $X_r < 0.70$, equivalent to 6 mol% or 27 weight%. At higher concentrations of SP-C in the films, additivity of the mean areas was observed, consistent with aggregation of the hydrophobic protein in the phospholipid monolayers. Low amounts of SP-C, $X_r \leq 0.17$, corresponding to 0.5 mol% or 3 weight%, did not appear to be excluded from the phospholipid monolayers up to high surface pressures (60 – 70 $mN \cdot m^{-1}$). At higher initial concentrations of SP-C in the monolayers, complexes of protein and lipid were squeezed-out when the films were compressed to $\pi > 50$ $mN \cdot m^{-1}$. The pressure required to achieve exclusion of material from SP-C/lipid monolayers was higher than that for SP-B/lipid complexes. SP-C showed a higher efficiency for removing phospholipid from the protein-phospholipid monolayers than did SP-B.

This work was supported by the Medical Research Council of Canada and, in part, by the Heart and Stroke Foundation of Canada and Newfoundland and Labrador.

REFERENCES

Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* 234:466–468.

Cornell, D. 1979. Circular dichroism of polypeptide monolayers. *J. Colloid Interface Sci.* 70:167–180.

Curstedt, T., J. Johansson, P. Persson, A. Eklund, B. Robertson, B. Lowenadler, and H. Jörnvall. 1990. Hydrophobic surfactant-associated polypeptides: SP-C is a lipopeptide with two palmitoylated cysteine residues, whereas SP-B lacks covalently linked acyl groups. *Proc. Natl. Acad. Sci. USA.* 87:2985–2989.

Hawgood, S. 1989. Pulmonary surfactant apoproteins: a review of protein and genomic structure. *Am. J. Physiol.* 257:L13–L22.

Krebs, K., J. Ibdah, and M. Phillips. 1988. A comparison of the surface activities of human apolipoprotein A-I and A-II at the air-water interface. *Biochim. Biophys. Acta.* 959:229–237.

Krebs, K., and M. Phillips. 1983. The helical hydrophobic moments and surface activities of serum apolipoproteins. *Biochim. Biophys. Acta.* 754: 227–230.

Krebs, K., and M. Phillips. 1984. The contribution of α-helices to the surface activities of proteins. *FEBS Lett.* 175:263–266.

Malcolm, B. 1973. The structure and properties of monolayers of synthetic polypeptides at the air-water interface. *Prog. Surface Membr. Sci.* 7: 183–229.

Mita, T. 1989. Thermotropic behavior of proteins and acylated proteins in monolayers. *Bull. Chem. Soc. Jpn.* 62:2299–2306.

Oosterlaken-Dijksterhuis, M., H. Haagsman, L. van Golde, and R. Demel. 1991. Characterization of lipid insertion into monomolecular layers mediated by lung surfactant proteins SP-B and SP-C. *Biochemistry.* 30:10965–10971.

Pastrana, B., A. Mautone, and R. Mendelsohn. 1991. Fourier transform infrared studies of secondary structure and orientation of pulmonary surfactant SP-C and its effect on the dynamic surface properties of phospholipids. *Biochemistry.* 30:10058–10064.

Pérez-Gil, J., K. Nag, S. Taneva, and K. M. W. Keough. 1992. Pulmonary surfactant protein SP-C causes packing rearrangements of dipalmitoylphosphatidylcholine in spread monolayers. *Biophys. J.* 33:197–204.

Shiffer, K., S. Hawgood, H. Haagsman, B. Benson, J. Clements, and J. Goerke. 1993. Lung surfactant proteins, SP-B and SP-C, alter the thermodynamic properties of phospholipid membranes: A differential calorimetry study. *Biochemistry.* 32:590–597.

Simatos, G. A., K. B. Forward, M. R. Morrow, and K. M. W. Keough. 1990. Interaction between perdeuterated dimyristoylphos-phatidylcholine and low molecular weight pulmonary surfactant protein SP-C. *Biochemistry.* 29:5807–5814.

Takahashi, A., A. Waring, J. Amirkhanian, B. Fan, and H. Taeusch. 1990. Structure-function relationships of bovine pulmonary surfactant proteins: SP-B and SP-C. *Biochim. Biophys. Acta.* 1044:43–49.

Vandenbussche, G., A. Clercx, T. Curstedt, J. Johansson, H. Jörnvall, and J.-M. Ruysschaert. 1992. Structure and orientation of the surfactant-associated protein C in a lipid bilayer. *Eur. J. Biochem.* 203: 201–209.

Vandenbussche, G., A. Clercx, M. Clercx, T. Curstedt, J. Johansson, H. Jörnvall, and J.-M. Ruysschaert. 1992. Secondary structure and orientation of the surfactant protein SP-B in a lipid environment. A

Fourier transform infrared spectroscopy study. *Biochemistry*. 31: 9169–9176.

Wang, J., and A. Pullman. 1991. Do helices in membranes prefer to form bundles or stay dispersed in the lipid phase? *Biochim. Biophys. Acta*. 1070:493–496.

Watkins, J. 1968. The surface properties of pure phospholipids in relation to those of lung extracts. *Biochim. Biophys. Acta*. 152:293–306.

Williams, M., S. Hawgood, and R. Hamilton. 1991. Changes in lipid structure produced by surfactant proteins SP-A, SP-B and SP-C. *Am. J. Respir. Cell. Mol. Biol.* 5:41–50.

Yamashita, T. 1971. Conformation of synthetic polypeptide monolayers. *Nature (Lond.)*. 231:445–446.

Yu, S.-H., and F. Possmayer. 1988. Comparative studies on the biophysical activities of the low-molecular-weight hydrophobic proteins purified from bovine pulmonary surfactant. *Biochim. Biophys. Acta*. 961: 337–350.