External Reflection Absorption Infrared Spectroscopy Study of Lung Surfactant Proteins SP-B and SP-C in Phospholipid Monolayers at the Air/Water Interface

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ABSTRACT The interactions of the hydrophobic pulmonary surfactant proteins SP-B and SP-C with 1,2-dipalmitoylphosphatidylcholine in mixed, spread monolayer films have been studied in situ at the air/water interface with the technique of external reflection absorption infrared spectroscopy (IRRAS). SP-C has a mostly α-helical secondary structure both in the pure state and in the presence of lipids, whereas SP-B secondary structure is a mixture of α-helical and disordered forms. When films of SP-B/1,2-dipalmitoylphosphatidylcholine are compressed to surface pressures (π) greater than ~40–43 mN/m, the protein is partially (15–35%) excluded from the surface, as measured by intensity ratios of the peptide bond amide I/lipid C=O stretching vibrations. The extent of exclusion increases as the protein/lipid ratio in the film increases. In contrast, SP-C either remains at the surface at high pressures or leaves accompanied by lipids. The amide I peak of SP-C becomes asymmetric as a result of the formation of intermolecular sheet structures (1615–1630 cm^{-1}) suggestive of peptide aggregation. The power of the IRRAS experiment for determination of film composition and molecular structure, i.e., as a direct test of the squeeze-out hypothesis of pulmonary surfactant function, is evident from this work.

INTRODUCTION

Pulmonary surfactant lines the air/alveolar interface of the mammalian lung. This mixture of lipids and proteins functions in vivo to lower surface tension (γ) to very low values, thereby facilitating the work of breathing and preventing alveolar collapse (Schürch et al., 1976). To function effectively, surfactant must possess two apparently contradictory attributes. First, surfactant must be capable of lowering γ to very low values during expiration. Second, surfactant must easily adsorb to and spread rapidly across the air/water (A/W) interface. 1,2-Dipalmitoylphosphatidylcholine (DPPC), the main lipid component in surfactant, is indeed capable of lowering γ to near zero values during compression. However, this molecule neither adsorbs readily to the A/W interface (Goerke and Clements, 1986) nor spreads sufficiently rapidly to be effective in vivo. Apparently, the fact that DPPC exists in its ordered (gel) state at 37°C is responsible both for its ability to withstand high surface pressures π (equivalent to low surface tension γ) and its spreading inefficiency. In contrast, the unsaturated phospholipid components of surfactant are known to spread relatively rapidly at the A/W interface, but monolayers of these materials collapse at surface pressures well below those required in vivo. To reconcile these apparently contradictory attributes of surfactant (ability to both spread and to sustain high π values) the "squeeze-out hypothesis" was proposed (Watkins, 1968; Clements, 1977; Hildebrand et al., 1979; Hawco et al., 1981a,b; Goerke and Clements, 1986; van Liempd et al., 1987; Egberts et al., 1989; Pastrana-Rios et al., 1994). According to this description of surfactant function, some surface components (unsaturated lipids, proteins, etc.) may be excluded from the monolayer upon compression from near equilibrium (π = 44 mN/m) to high pressures (~70–72 mN/m). Critical testing of this hypothesis is a necessary first step toward understanding the molecular basis of pulmonary surfactant function.

Toward this end, a unique external reflection Fourier transform infrared spectroscopy (IRRAS) experiment capable of monitoring the structure and relative concentrations of both the lipid and protein components of surfactant in situ in monolayers at the A/W interface, under controlled conditions of surface tension, has been designed (Flach et al., 1993; Pastrana-Rios et al., 1994). The feasibility of this spectroscopic approach for studies of phospholipid conformational order was first demonstrated by Dluhy and coworkers (Dluhy 1986; Mitchell and Dluhy, 1988). The current version of the apparatus permits acquisition of IRRAS spectral data from the conformation-sensitive amide I region of protein IR spectra.

The first direct test of the squeeze-out hypothesis evaluated the relative surface concentrations of each component in mixed binary phospholipid films of acyl-chain-perdeuterated DPPC (DPPC-d_{62}) with (in separate experiments) 1,2-dioleoylphosphatidylglycerol (DOPG), 1-palmitoyl-2-oleoylphosphatidylglycerol (POPG), and 1,2-dipalmitoylphosphatidylglycerol (DPPG), under conditions of π ranging from below equilibrium to film collapse. The use of acyl-chain-perdeuterated lipids permitted the separate monitoring of each film component. Under conditions of

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continuous compression, DOPG and POPG were found to be substantially removed (the latter reversibly) from the film, whereas DPPG remained (Pastrana-Rios et al., 1994). Thus, occurrence of the squeeze-out phenomenon at the surface was unambiguously demonstrated. Furthermore, the propensity of disordered acyl chains to be preferentially removed was suggested.

Along with the unsaturated phospholipids, the hydrophobic surfactant proteins SP-B (8.7 kDa) and SP-C (4.2 kDa), which constitute approximately 1% by weight of surfactant, have been shown to enhance the rate of adsorption of phospholipids to the A/W interface (Hawgood et al., 1985, 1987; Suzuki et al., 1986; Yu and Possmayer 1990; Takahashi et al., 1990; Pérez-Gil et al., 1991). SP-C has also been found to enhance the initial rate of relaxation of DPPC/DPPG monolayers upon a fast compression (Pastrana et al., 1991).

The current study extends the testing of the squeeze-out hypothesis by taking advantage of the technical developments in IRRAS instrumentation alluded to above to evaluate the possible occurrence of squeeze-out in mixed monolayer films of DPPC with the surfactant proteins SP-B and SP-C, as well as the secondary structures of these proteins. The results, which constitute a direct molecular evaluation of the structure and conformation of protein at the surface, complement the only other physical studies that have addressed this issue, namely the thermodynamic analysis of π-area (π-A) isotherms reported by Taneva and Keough (1994a–c).

MATERIALS AND METHODS

Materials

DPPC was purchased from Avanti Polar Lipids (Alabaster, AL). Lipid purity was verified by two-dimensional thin layer chromatography. D_2O, 99.9% atom D, was purchased from Sigma Chemical Co. (St. Louis, MO). $CHCl_3$ (ACS grade) and MeOH (ACS grade) were from Fisher Scientific (Pittsburgh, PA). NaCl was certified ACS grade.

Protein isolation

SP-B was isolated from porcine lungs as described in Pérez-Gil et al (1993). The material was analyzed by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (Karas et al (1991). MALDI was performed on a VG TofSpec Spectrometer (Manchester, UK). Typically, protein samples were dissolved in 3:1 $CHCl_3$/MeOH (1:1, v:v) to a final concentration of 0.5 mg/ml. For protein/lipid IRRAS samples, appropriate amounts of SP-B and DPPC were mixed to attain molar ratios of 1:3, 1:12, or 1:32. SP-C was dissolved in $CHCl_3$:MeOH (2:1, v:v) to a final concentration of 0.5 mg/ml. Appropriate amounts of SP-C and DPPC were mixed to attain the desired molar ratios. Protein concentrations were determined by the Bradford (1976) assay.

IRRAS apparatus

The IRRAS apparatus has been described previously (Flach et al., 1994; Pastrana-Rios et al., 1994). The spectrometer used was a Digilab FTS-40A (Bio-Rad, Cambridge, MA) equipped with an external narrow band HgCdTe detector. IR radiation was directed to the exit port and focused with an angle of incidence of either ~30 or ~42° onto the A/W interface. The teflon trough containing the film was designed with reference and sample wells, connected if needed, to allow accurate matching of subphase heights. Surface pressures were monitored by a platinum Wilhelmy plate connected to a Cahn balance and were controlled as previously described (Boyle and Mautone, 1982; Flach et al., 1993; Pastrana-Rios et al., 1994). The external optics, trough, and detector were enclosed in a plexiglass box and purged with dry air overnight before the experiment.

Cleaning procedures were as previously described (Pastrana-Rios et al., 1994). For IRRAS experiments, 40–60 μl of the desired sample was spread on a 0.15 M NaCl D_2O subphase (pD = 6.6) while the surface area was being increased. To match surface curvature and to retard evaporation, a $CHCl_3$ solution of decanol (3%, v:v) was spread on the reference well when the reflectance-absorbance of the amide I band was less than 1 mAU, as was the case for most SP-B-containing films. A D_2O subphase was used to reduce interference from water vapor absorption and to eliminate the intense liquid H_2O absorption at 1643 cm^{-1}. A 15-min period was allowed for solvent evaporation and film equilibration. To prevent formation of multilayers, initial π values were always less than the equilibrium surface pressure (π_{eq} = ~44 mN/m). Films were rapidly compressed between points at which IR data were collected at a rate of surface area change of 28 or 115 mm^2/s for H_2O and D_2O subphase, respectively. Slow continuous compression was required to hold π constant above the equilibrium spreading pressure. The minimal and maximal aqueous surface areas were 21.6 cm^2 and 83 cm^2, respectively. Subphase volume was 40 cm^3. All experiments were performed at room temperature (~21°C) at a relative humidity of 10–12%, under a dry air purge.

IR data acquisition

Typically, 64 or 512 (D_2O and H_2O subphase, respectively) interferograms were collected, co-added, apodized with a triangular function, and fast Fourier transformed with one level of zero filling to produce spectral data encoded at ~2 cm^{-1} intervals with 4 cm^{-1} spectral resolution. The relatively small number of scans was a limitation imposed by the relaxation properties of the films, to permit the acquisition of several IR spectra along the compression curve for any given sample. Thus, at surface pressures above equilibrium, it was necessary to continually reduce the area (albeit slightly) to hold the pressure constant during IR data acquisition. Interferograms were collected in alternating fashion between sample and reference wells to allow for optimal compensation of water vapor bands. This was achieved via a computer-controlled stepping motor that rotated the trough for alignment of each well. After trough rotation, films were allowed to re-equilibrate for 30 s before data acquisition. The IRRAS spectrum was obtained when the single beam data for the sample and reference wells were ratioed and plotted as reflectance-absorbance ($-\log R/R_o$), where R is the reflectance of the film-covered surface and R_o is the reflectance of the D_2O surface. Under the experimental conditions (unpolarized light, angle of incidence < the Brewster angle) negative-going IRRAS bands are anticipated (Dluhy, 1986; Fina et al., 1991; Gericke et al., 1993).

Data manipulation

Baseline correction and water vapor subtraction were accomplished with software supplied by the instrument manufacturer. Spectra were neither smoothed nor deconvolved before peak area measurements. Frequency and peak area calculations were accomplished with software written at the National Research Council of Canada.

Squeeze-out determination

To determine whether exclusion of the surfactant proteins (SP-B or SP-C) in a protein/DPPC monolayer occurs during compression, the peak area ratios of the protein amide I band/lipid carbonyl band were determined for

each spectrum collected. The proportions of protein to lipid in the film could thus be compared at several points along the compression curve.

RESULTS

Secondary structures of SP-B and SP-C in monolayers at the A/W interface

The IRRAS spectrum of SP-B at the A/W interface is shown over the spectral range of 1520–1780 cm^{-1} in Fig. 1, *A* and *B*. Raw IRRAS data are displayed in Fig. 1 *A* whereas the same data, having been smoothed by using a break point of 0.8, are presented in Fig. 1 *B*. The smoothing tends to minimize interference from the last traces of water vapor. The plotted spectral region contains the peptide bond C=O stretching mode (amide I for proteins in H_2O, termed amide I′ for H→D exchanged peptide bonds) at 1644 cm^{-1} and a full width at half height (FWHH) of 35 cm^{-1}. It is evident, especially from the Fourier-smoothed data of Fig. 1 *B*, that the amide II mode, expected to be between 1525 and 1560 cm^{-1}, is absent within the precision of the measurement, suggesting that the protein has undergone essentially complete H→D exchange within the equilibration/evaporation period (~15 min). The observed amide I′ frequency falls in a frequency range that is appropriate for a random coil secondary structure, possibly overlapped with an H→D exchanged α-helix (Flach et al., 1994; Surewicz et al., 1993). The suggestion that the peak is composed of overlapping contributions from more than one secondary structure is strengthened by comparison with IRRAS data for SP-C (Fig. 1 *A*). The latter shows a sharper amide I mode (FWHH = 25 cm^{-1}) and a frequency, 1652 cm^{-1}, that falls within the range for an α-helical secondary structure. The presence of a residual amide II mode at 1542 cm^{-1} reveals that the peptide bonds in SP-C are more resistant to H→D exchange than in SP-B. The relatively low S/N ratio precludes serious attempts at estimation of the relative contributions of various secondary structures to either protein.

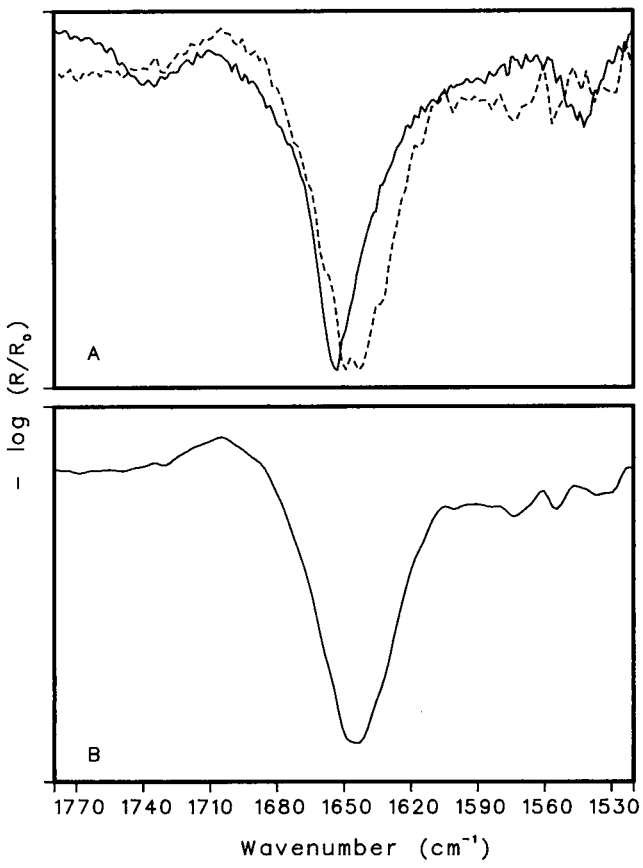

FIGURE 1 IRRAS spectra (1780–1520 cm^{-1}) of hydrophobic surfactant protein as monolayers at the air/D_2O interface. (*A*) Superimposed spectra of: (- - - -) SP-B (collected at π = 22 mN/m), the amide I′ mode (1644 cm^{-1}) has a peak height of ~2 mAU; (—) SP-C (collected at π = ~20 mN/m), the amide I mode (1654 cm^{-1}) has a peak height of 4 mAU. There is a residual amide II mode at 1542 cm^{-1}; (*B*) Fourier-smoothed spectrum of SP-B (collected at π = 22 mN/m) using a break point of 0.8.

When SP-B/lipid films (1:3, 1:12, or 1:32 molar ratios, based on monomer weights for SP-B) are spread from a $CHCl_3$:MeOH (1:1, v:v) solution, the IRRAS data reveal a protein secondary structure unchanged from lipid-free films, as judged by the similarity of the amide I spectral regions. Peptide bond H→D exchange remains complete (data not shown) as judged from the absence of a residual amide II band.

Representative IRRAS data in the 1510–1675 cm^{-1} region for SP-C/DPPC monolayers at several π values are shown in Fig. 2, along with the spectrum of a pure SP-C film. The amide I/II intensity ratio is approximately the same in all spectra, indicating that H→D exchange (about 40% exchanged hydrogens) is unaltered by the presence of the lipid. Of interest is the fact that the amide I contour is significantly altered by lipid. The peak position is lowered from ~1654 to ~1651 cm^{-1} (see Table 1), and there is substantial broadening of the contour on the low frequency side. Difference spectra (not shown) reveal that the broadening arises from the presence of a peak between 1610 and 1635 cm^{-1}.

The origin of the broad feature is explored in Fig. 2 *B* where spectra of SP-C reconstituted in bulk phase D_2O with a 7:3 mole ratio phospholipid mixture of DPPC-d_{62}/DPPG at SP-C/lipid mole ratios of 1:200 and 1:70 are shown and compared with the (inverted) IRRAS spectrum of 1:5 SP-C/DPPC in monolayers. In the bulk phase, protein aggregation is manifest in the 1:70 sample by the appearance of additional spectral intensity (compared with the 1:200 sample) near 1625 cm^{-1} at a band position appropriate for an aggregated intermolecular β-sheet (Pastrana et al., 1991). The amide I region of the IRRAS spectrum (inverted for comparison with bulk phase data) of the 1:5 sample shows even more intensity in this region, suggesting that the SP-C is substantially aggregated in the monolayer in the presence of lipid. The absence of substantial band intensity between 1615 and 1630 cm^{-1} in the IRRAS experiment for mixed

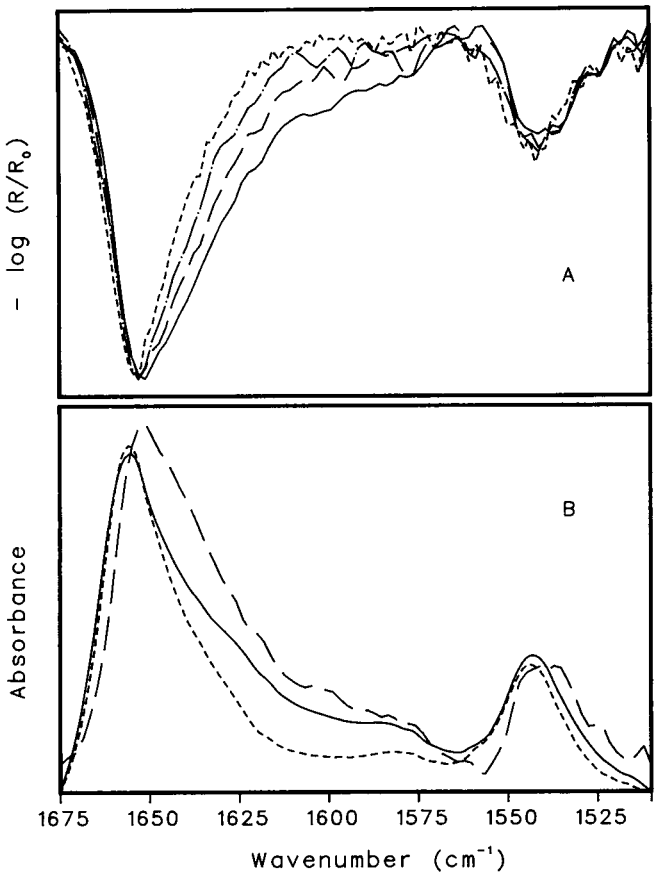

FIGURE 2 Superimposed spectra (1675–1510 cm^{-1}) for the study of SP-C aggregation. (*A*) Reflectance-absorbance spectra of pure SP-C monolayer at π = ~20 mN/m (- - - -) and SP-C:DPPC (1:3, mol:mol) monolayers upon compression at π = 41 mN/m (– · –), at π = 45 mN/m (– – –), and at π = 48 mN/m (——). (*B*) Bulk phase (transmission) IR absorbance spectra superimposed: (- - - -) SP-C:DPPC-d_{62}/DPPG (1:140/60, mol:mol:mol), ——) SP-C:DPPC-d_{62}/DPPG (1:49:21, mol:mol:mol), and an (inverted) IRRAS spectrum of an SP-C/DPPC monolayer (1:5, mol:mol) at π = 53 mN/m (– – –). All spectra on a D_2O subphase.

SP-B/lipid films (e.g., see Fig. 3), suggests that SP-B is not aggregated in the presence of lipid.

Exclusion of SP-B from SP-B/DPPC mixed monolayer films

To maintain the high π values above equilibrium surface pressures, continuous compression had to be applied to the films. Typical spectra (normalized to the lipid C=O intensity) for the amide I band of SP-B along with the DPPC carbonyl modes are shown in Fig. 3, *A–C* for 1:3, 1:12, and 1:32 SP-B/DPPC molar ratios, respectively, at pressures below and above the equilibrium surface pressure for the pure lipid in each instance. Spectral peak positions are constant within the experimental precision either as a function of π or as a function of the protein/lipid ratio. It is noted that there is no additional intensity in the 1610–1635 cm^{-1} spectral region compared with the spectrum of the pure SP-B monolayer, thus indicating that there is no protein

TABLE 1 Lipid carbonyl and amide I frequencies as a function of π for SP-C alone and in SP-C/DPPC monolayers

Film	Ratio (mol:mol)	π (mN/m)	Lipid C=O (cm^{-1})	Amide I (cm^{-1})
SP-C	N/A*	20	N/A	1652
SP-C:DPPC	1:3	37	1735.2	1654
		41	1736.5	1653.1
		45	1734.1	1652.5
		46	1732.5	1651.6
		48	1730.0	1651.6
SP-C:DPPC	1:5	34	1737.4	1654.1
		44	1736.0	1653.2
		49	1730.7	1651.7
		53	1729.9	1650.9

*Not measured.

aggregation in the presence of lipid and that SP-B conformation is not significantly changed in the protein/lipid film upon compression. However, the relative intensity of the amide I band is reduced compared with the lipid C=O stretch in each case at the higher pressure.

To quantitatively evaluate the squeeze-out phenomenon in mixed monolayers of SP-B/DPPC, films cast initially at low pressures (π < 44 mN/m) were compressed to high surface pressures (π > 50 mN/m) and IRRAS data acquired at several points along the compression curve. The initial and final π values are summarized in Table 2. The ratios of the integrated band areas of the amide I and lipid carbonyl (~1730 cm^{-1}) vibrations are plotted as a function of π in Fig. 4. This ratio serves as an index of the relative concentrations of SP-B and DPPC at the monolayer surface. The data points represent the mean values for three to five SP-B:DPPC monolayer films at each of the three film compositions studied. Typical precisions were 5% of the measured ratio. The percentages of excluded protein (and precisions of the measurement) were estimated from the average of values at pressures below the onset of squeeze-out compared with the average of values at pressures above the onset. Integration of the spectra for intensity measurements was performed between 1677 and 1588 cm^{-1} for the protein amide I band and between 1705 and 1750 cm^{-1} for the lipid C=O stretch. The reduction in the integrated band intensity ratio, which directly reflects exclusion of protein from the surface (squeeze-out) was observed to commence at π values of ~40 mN/m for the 1:3 (SP-B:DPPC) film and slightly higher for the 1:12 film, i.e., close to the equilibrium surface pressure (π_{eq} = 44 mN/m) for DPPC. π_{max} in each case was 55–60 mN/m, at which point the onset of film collapse was evident. Some exclusion of the protein was noted for each of the three compositions. The extent of squeeze-out appeared to be related to the fraction of SP-B in the film. Values of ~35, ~30, and ~15% exclusion were observed for the 1:3, 1:12, and 1:32 SP-B:DPPC films, respectively.

To address the issue of reversibility of SP-B squeeze-out, films were cycled two to three times. After each cycle, the

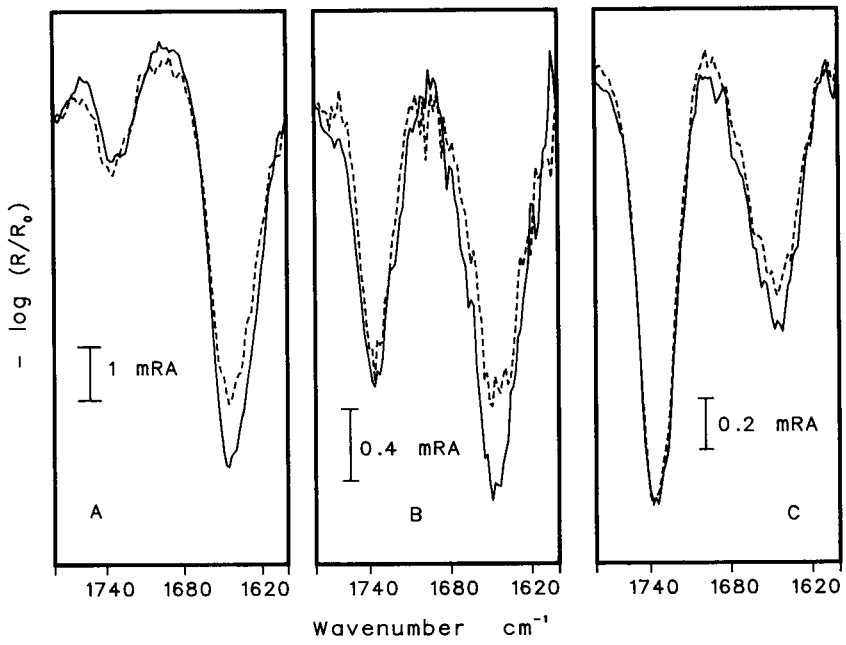

FIGURE 3 Overlaid IRRAS spectra scaled to the lipid carbonyl band of DPPC for SP-B:DPPC monolayers in the region 1780–1600 cm^{-1}. (*A*) SP-B:DPPC (1:3, mol:mol) collected at π = 56 mN/m (- - - -) and π = 36 mN/m (——). (*B*) SP-B:DPPC (1:12, mol:mol) collected at π = 59 mN/m (- - - -) and π = 43 mN/m (——). (*C*) SP-B:DPPC (1:32, mol:mol) collected at π = 56 mN/m (- - - -) and π = 37 mN/m (——).

subsequent surface pressures (at a given surface area) were lower than the previous cycle, thus suggesting that SP-B was not easily readsorbed to the surface, i.e., squeeze-out of SP-B is at least to some extent irreversible under the current conditions of relatively high protein levels. The absence of a signal from the squeezed out protein makes it quite inappropriate for us to comment on possible conformational changes in this fraction of the sample. In addition, determination of the location of the squeezed out material with conventional chemical techniques presents substantial technical difficulties.

A sample of SP-B that had been prepared by a somewhat different protocol, involving the use of trifluoroacetic acetic acid instead of hydrochloric acid during chromotography of the hydrophobic proteins, displayed similar IRRAS characteristics to those described above. Analysis of this SP-B by MALDI mass spectrometry and sodium dodecyl sulfate polyacrylamide gel electrophoresis, however, indicated that it consisted primarily of a dimer with a trace amount of

TABLE 2 Test for exclusion of hydrophobic proteins from DPPC mixed monolayer films the A/W interface as a function of surface pressure

Film	Ratio (mol:mol)	π_i* (mN/m)	π_{max}* (mN/m)	π_1‡ (mN/m)	π_2‡ (mN/m)	Squeeze-out (%§)
SP-B:DPPC	1:32	18	56	37	56	15
	1:12	32	59	37	59	30
	1 :3	31	56	36	56	35
SP-C:DPPC	1 :3	37	48	40	48	0
	1 :5	34	54	44	54	0
	1:10	45	61	47	61	0
	1:20	42	61	43	61	0

*Initial (π_i) and final (π_{max}) surface pressures after film spreading and equilibration.

‡π_1 (π_2) are the first (last) surface pressures at which IR data were collected.

§As determined by the average amide I/lipid carbonyl intensity ratios averaged from data below and above the surface pressure at which the process begins. Averaged (standard deviations) from three to five measurements for each sample. Uncertainties are generally ~5% in absolute value (e.g., 15 ± 5%).

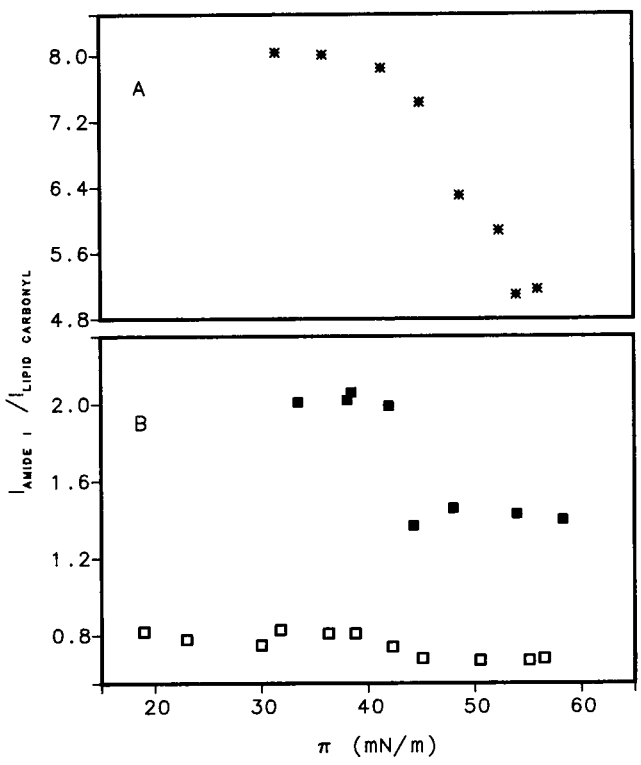

FIGURE 4 Ratio of the integrated areas for the amide I and lipid carbonyl bands as a function of surface pressure upon compression. (*A*) SP-B:DPPC (1:3, mol:mol) monolayer (*). (*B*) SP-B:DPPC (1:12, mol:mol) monolayer (■) and SP-B:DPPC (1:32,mol:mol) monolayer (□). Data points represent the mean of two to five experiments. Mean errors are within 5%.

monomer. The monomer units were smaller by approximately 10–11 amino acid residues than those in the usual SP-B. Amino-terminal analysis showed that the amino portion of this truncated SP-B was normal, so it is suggested that the missing part is near the carboxy terminal. This observation tends to suggest that the last amino acids in the carboxyl-terminal portion of the protein are not required for the partial squeeze-out observed here.

Exclusion of SP-C from SP-C/DPPC mixed monolayer films

IRRAS data for SP-C/DPPC were collected as a function of protein/lipid ratio and surface pressures. The maximal achievable π before film collapse was strongly dependent on the amount of protein in the film. For example, at a protein/lipid mole ratio of 1:20, π_{max} was 61 mN/m, whereas at molar ratios of 1:5 and 1:3, respectively, π_{max} values were reduced to ~54 and 46 mN/m, respectively. The data are summarized in Table 2.

Typical spectra are shown in Fig. 5 for protein/lipid ratios of 1:5 and 1:20 (mol:mol), and the set of amide I/lipid C=O intensity ratios at various π values are shown in Fig. 6. As noted previously (Table 1), the protein amide I bands in Fig. 5 reveal a progressive frequency decrease from 1654.0 cm^{-1} to 1650.9 cm^{-1} as π is increased from 34 to 53 mN/m, along with an increase in the contribution of bands to the low frequency side of the amide I contour as the pressure is increased. Similar frequency shifts are noted for the 1:20 sample. There are additional frequency shifts evident in the lipid C=O band from ~1737 cm^{-1} to 1730 cm^{-1} as π is increased from 34 to 53 mN/m. In contrast to the experimental results for SP-B/DPPC monolayers, an approximately constant amide I/lipid C=O intensity ratio is noted over the range of surface pressures. Taneva and Keough (1994b) found that at this high concentration of SP-C, the monolayer behavior suggested the presence of a collapse phase that included both lipid and protein. If such a phase occurs under the present experimental conditions, and remained at or near the surface, it would be difficult to distinguish from the monolayer itself by the IRRAS approach.

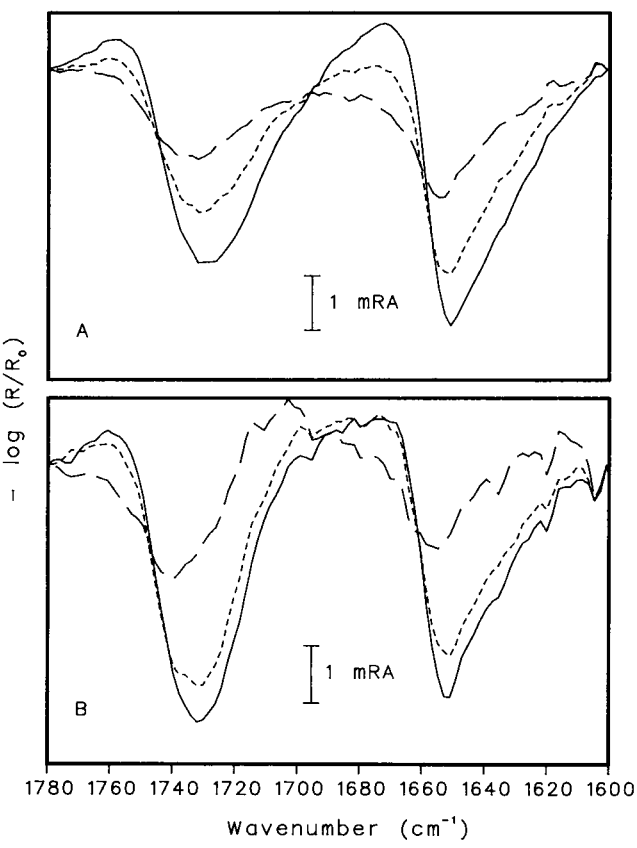

FIGURE 5 Overlaid IRRAS spectra of the lipid carbonyl and amide I bands for SP-C:DPPC monolayers in the spectral region 1760–1610 cm^{-1}. (*A*) For SP-C:DPPC (1:5, mol:mol) collected at π = 34 mN/m (— —), π = 49 mN/m (– – –), and π = 53 mN/m (——). (*B*) For SP-C:DPPC (1:20, mol:mol) collected at π = 45 mN/m (– –), π = 57 mN/m (- - - -), and π = 61 mN/m (——). Vertical bar represents 0.001 reflectance-absorbance unit.

DISCUSSION

SP-B and SP-C conformation at the A/W interface

The first issue addressed in the current study is the conformation of these hydrophobic proteins both in monolayers at the A/W interface and in bulk phases. The secondary structures and extent of H→D exchange of SP-B at the A/W interface are unaltered by its insertion into DPPC monolayers. SP-C exhibits signs of aggregation at the relatively high protein levels required. The α-helical content of the SP-C is greater than that of SP-B, as judged by the greater resistance of the former to H→D exchange and the observation of its characteristic amide I peak in the range 1654–1651 cm^{-1}. As noted previously, the shift of 3 cm^{-1} is caused by the presence of underlying bands due to intermolecular aggregation. SP-B (amide I (I′) mode at 1644 cm^{-1}) reveals an amide I linewidth 30% greater than for SP-C (FWHH of 35 vs 25 cm^{-1}), which suggests overlapping contributions to the amide I contour from additional secondary structures.

These results are consistent with reported bulk phase measurements. Attenuated total reflectance and bulk phase Fourier transform IR spectroscopy measurements of SP-C with lipid bilayers reveal the protein to be transmembrane in orientation with 60% α-helical secondary structure. In addition, 70% of the peptide bonds were resistant to H→D exchange (Pastrana et al., 1991; Vandenbussche et al., 1992a,b; Baatz et al., 1992). Similar levels of secondary structure have been observed in solutions of the peptide in organic solvents, as judged by circular dichroism (Pérez-Gil et al., 1993) and proton nuclear magnetic resonance (NMR) spectroscopies (Johansson et al., 1994). The helical content of SP-B is reported to be somewhat lower. For SP-B reconstituted in DPPC bulk phases, its secondary structure con-

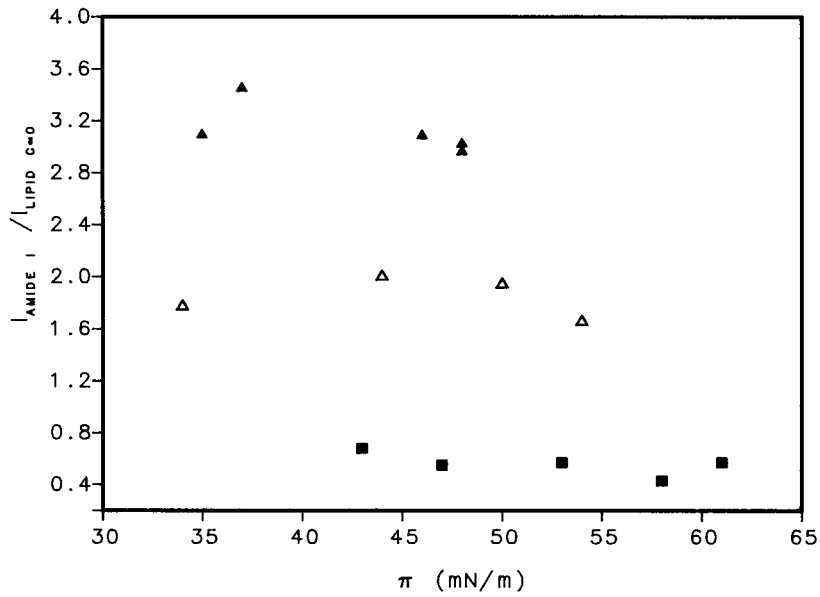

FIGURE 6 Ratio of the integrated band areas for the amide I and lipid carbonyl modes as a function of surface pressure upon compression for SP-C:DPPC (1:3, mol:mol) monolayer (▲), SP-C:DPPC (1:5, mol:mol) monolayer (△), and SP-C:DPPC (1:20, mol:mol) monolayer (■).

stituted 47% α-helical, 40% random coil, 3% β-sheet, and 10% β-turn as determined by circular dichroism (Morrow et al., 1993a).

A difficulty in assigning accurate secondary structure percentages for SP-B may arise from possible confounding effects of exchange on commonly accepted spectra-structure correlations. Shifts as large as 11–12 cm^{-1} to lower wavenumber have been suggested for some α-helical amide I modes upon H→D exchange (Zhang et al., 1992; Flach et al., 1994). If such an effect occurs, it would place the amide I′ frequency of helical segments in a region commonly associated with disordered forms and may preclude the accurate determination of secondary structure. This uncertainty is a general concern about secondary structure determination from IR data of D_2O solutions of proteins. An additional ambiguity may arise in situations where partial exchange occurs.

The extent of H→D exchange for SP-B in mixed SP-B/DPPC monolayers differ from the results obtained in the bulk phase study reported by Vandenbussche et al. (1992a,b) for samples containing PG at various molar ratios (SP-B/DPPC/PG, lipid/protein ratios 1/291 and 1/111). They observed approximately 60% of the polypeptide chain peptide bonds unexchanged after 6 h in a D_2O-staurated N_2 stream. The orientation of SP-B in bulk phase reported to date seems to suggest that SP-B is not a transmembrane (or trans-monolayer) protein. Vandenbussche et al. (1992a,b) studied both multilamellar vesicles and spread films containing SP-B/DPPC/PG by polarized ATR Fourier transform IR spectroscopy and reported the amphipathic domains of SP-B to be associated with the phospholipid head group, with the presence of short helical peptide stretches slightly inside the bilayer. In addition, Baatz et al. (1990) reported fluorescence anisotropy studies suggesting that SP-B was not a transmembrane protein but instead associated with the membrane surface. Morrow et al. (1993a,b) also found results from ^2H NMR studies that were consistent with this orientation for SP-B.

The current results for SP-B conformation in monolayers are not entirely consistent with above experiments. As noted above, complete H→D exchange may produce the observed lower amide I′ frequency (1644 cm^{-1}) compared with the ATR value of 1654 cm^{-1}, without a change in secondary structure. However, this shift in peak maximum is probably too large to be attributed to exchanged α-helix alone and suggests a greater fraction of irregular (unfolded) conformation in the monolayer. The latter interpretation is preferred as it would be consistent with the observation of near complete exchange. Finally, we note that the determination of protein orientation in monolayers at the A/W interface requires the use of polarized incident radiation, which diminishes the incident IR intensity. The resultant loss in S/N ratio, combined with surface relaxation effects at high pressures so that averaging of many scans is not feasible, makes the experiment impractical with the current apparatus design.

Hydrophobic peptide squeeze-out

The IRRAS approach permits direct evaluation of protein squeeze-out upon monolayer compression. SP-B was determined to be partially excluded (15–35%) from SP-B/DPPC monolayers. The only other experiment comparable with the current protocols is the work of Taneva and Keough (1994a), who measured π-A isotherms of spread monolayers of SP-B alone and in varying proportions (SP-B:lipid mole ratios of 1:898 to 1:39) with DPPC and DPPG and 7:3 mixtures of the two lipids. Discontinuities (kinks) in the isotherms at 40–45 mN/m were observed for protein/lipid molar ratios greater than 1:301 and were interpreted as squeeze-out of the SP-B from the surface commencing at

the kink pressure. This result and interpretation are in excellent accord with the onset of squeeze-out (at ~40–43 mN/m) as directly observed in the current IRRAS measurement (Fig. 5). Taneva and Keough (1994a) presented a theoretical analysis of the monolayer composition as a function of π and suggest that the composition of the excluded phase is essentially pure SP-B and that the residual film is composed of essentially pure lipid. The IR measurements show that substantial peptide remains on the surface. Differences in the quantitative results from these experimental approaches may be traced to three factors. First, although both experiments were performed under conditions of film compression, each set of IR measurements required longer measurement times (3–4 h) compared with π-A isotherm construction (30 min). As continuous film compression is required during these experiments, the extent of squeeze-out may be controlled by kinetic factors, as has been observed in studies of exclusion of particular phospholipids from monolayer films (Nag et al., 1993; Pastrana-Rios et al., 1994). It is thus plausible that relaxation processes leading to partial reinsertion of protein would be more pronounced during the longer time taken for the IR data collection. Second, the IRRAS measurements require higher levels of protein than the π-A determinations, simply to detect the amide I modes. Thus, the experiments are not carried out under directly comparable conditions. In fact, the IR data suggest that the extent of squeeze-out depends on the protein concentration in the monolayer. Finally, some of the assumptions (a constant area/residue, no domain formation) necessary for the thermodynamic analysis of π-A curves are untested.

For the SP-C/DPPC system, some apparent differences exist between the IRRAS data and the analysis of the π-A curves. The latter show kink points in the isotherms near 50 mN/m, interpreted as arising from the onset of SP-C removal from the interface. At SP-C/DPPC lipid levels of 1:23 and 1:8, the quantitative analyses suggest that substantial levels of lipid accompany the removal of protein. That is, the composition of the excluded phase ranges from an SP-C/lipid molar ratio of 1:1 to 1:7.

The current IRRAS spectra reveal that SP-C aggregates in the presence of lipid when the protein concentrations are high. In the presence of a very high protein to lipid ratio, protein-protein contacts or protein aggregation are inevitable because insufficient lipid is present to surround each protein molecule separately. At intermediate protein concentrations, aggregation is driven by unequal protein-protein, lipid-lipid, and lipid-protein interaction energies. Protein associations at intermediate concentrations would not be unexpected given the physical constraints in these systems and the difference in structures of the protein and lipids. As SP-C is generally not incorporated into condensed domains upon compression of spread monolayers (Pérez-Gil et al., 1992), some aggregation of SP-C would be expected at high compression even when its nominal concentration is low.

The IRRAS technique requires the use of fairly high protein-to-lipid ratios to obtain a sufficient signal-to-noise ratio in the spectra. Thus, only monolayers with relatively high concentrations of SP-C could be investigated (25–68 wt % or $X_r = 0.64–0.92$, where X_r is the "mole fraction" of amino acid residues as defined by Taneva and Keough, 1994b). At such high levels of SP-C, Taneva and Keough (1994b) found behavior that suggested that any excluded phases of the SP-C-lipid films at high pressures were likely "collapse" phases with the removal of the SP-C and DPPC into three-dimensional structures, these possibly residing at or near the surface. The IRRAS data are in good agreement with this interpretation. Taneva and Keough (1994b) suggested that "selective" exclusion of SP-C accompanied by some simultaneous removal of DPPC occurred during compression of films containing SP-C in the amounts of X_r of 0.22–0.48 above the pressure of ~50 $mN.m^{-1}$. Unfortunately, the sensitivity of the IRRAS technique did not allow for observation of SP-C/DPPC films in this concentration range, but the co-removal into a separate surface phase of lipids and SP-C may not have been easily differentiated from SP-C plus lipids in the monolayer in any case.

General comments concerning the squeeze-out phenomenon

In earlier IRRAS studies of lipid squeeze-out, the process was shown to be (for POPG/DPPC mixtures) substantially reversible. In contrast, the squeeze-out of SP-B may have a significant irreversible component. This suggests that the types of structures and the location of the excluded component in these two instances may differ. One possible structure is the formation of micelles or vesicles in the aqueous phase. These must reside at least 1–2 μm below the film surface at high surface pressures to remain undetected by IRRAS techniques. For the SP-B-containing films this is a reasonable possibility, as a substantial fraction of the squeezed out material does not readsorb to the surface upon re-expansion during the time scale of the IRRAS measurement (~10–30 min). Micelle or vesicle formation is less probable for the squeezed out lipid observed in our previous investigation, as these would have to travel a relatively large distance compared with their size to readsorb to the surface, while remaining undetected in the IRRAS experiment. The reversibility of the exclusion process may depend substantially on the operating parameters of the experiment. Taneva and Keough (1994d) have found some evidence from conventional surface pressure measurements of DPPC films containing small amounts of SP-B that selectively excluded protein may return to the surface or at least facilitate the re-spreading of lipids in the surface after collapse. This property depends on factors such as initial spreading pressures and pressures achieved on recompression and the extent of collapse of the films during compression.

The current measurements begin to define the possible molecular events that may occur during film compression. It

is evident from our earlier report (Pastrana-Rios et al., 1994) that unsaturated and/or disordered lipid components may be easily excluded. The current data reveal that SP-B may be excluded, whereas SP-C apparently remains at the surface, in an aggregated state, possibly as part of a collapsed phase. The squeeze-out process occurs to an extent that depends on compression rate, as does the reversibility of the process. The physical location and state of the excluded material remain obscure, as these can only be inferred and not easily measured. It would be of some interest to examine three component systems (two lipids plus protein) for possible synergistic effects on squeeze-out and reversibility. The number of concentration variables (lipid molar ratios, lipid/protein ratios, binary mixture controls, etc.) is such that the experiment is not quite yet practical with the current apparatus design. In addition, experiments at higher temperatures (e.g., 37°C) would be of much interest. However, the increased vapor pressure of water is expected to create interference from HDO and H_2O rotation-vibration bands that would be difficult to overcome.

Finally, although the possible relevance of these experiments to the in vivo mechanism of surfactant function remains to be evaluated, IRRAS of monolayer films is demonstrated to be a powerful and unique approach for evaluation of the relative concentrations and conformations of particular film constituents.

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REFERENCES

Baatz, J. E., B. Elledge, and J. A. Whitsett. 1990. Surfactant protein SP-B induces ordering at the surface of model membrane bilayers. *Biochemistry*. 29:6714–6720.

Baatz, J. E., K. L. Smyth, J. A. Whitsett, C. Baxter, and D. R. Absolom. 1992. Structure and functions of a dimeric form of surfactant protein SP-C: a Fourier transform infrared and surfactometry study. *Chem. Phys. Lipids*. 63:91–104.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.

Boyle, J. III, and A. J. Mautone. 1982. A new surface balance for dynamic surface tension studies. *Colloids Surfaces*. 2:77–86.

Clements, J. A. 1977. Functions of the alveolar lining. *Am. Rev. Respir. Dis.* 115:67–71.

Dluhy, R. A. 1986. Quantitative external reflection infrared spectroscopic analysis of insoluble monolayers spread at the air-water interface. *J. Phys. Chem.* 90:1373–1379.

Egberts, J., H. Sloot, and A. Mazure. 1989. Minimal surface tension, squeeze-out and transition temperatures of binary mixtures of dipalmitoylphosphatidylcholine and unsaturated phospholipids. *Biochim. Biophys. Acta*. 1002:109–113.

Flach, C. R., J. W. Brauner, and R. Mendelsohn. 1993. Coupled external reflectance FT-IR/miniaturized surface film apparatus for biophysical studies. *Appl. Spectrosc.* 47:982–985.

Fina, L. J., and Y.-S. Tung. 1991. Molecular orientation of monolayers on liquid substrates: optical model and FT-IR methods. *Appl. Spectrosc.* 45:986–992.

Flach, C. R., J. W. Brauner, J. W. Taylor, R. C. Baldwin, and R. Mendelsohn. 1994. External reflection FT-IR of peptide monolayer films in situ at the air/water interface: experimental design, spectra-structure correlations, and effects of hydrogen-deuterium exchange. *Biophys. J.* 67: 402–410.

Gericke, A., A. V. Michailov, and H. Hühnerfuss. 1993. Polarized external infrared reflection-absorption spectrometry at the air/water interface: comparison of experimental and theoretical results for different angles of incidence. *Vib. Spectrosc.* 4:335–348.

Goerke, J., and J. A. Clements. 1986. Alveolar surface tension and lung surfactant. *In* Handbook of Physiology: The Respiratory System III. P. T. Mackelm and J. Mead, editors. Washington, DC, American Physiology Society. 247–261.

Hawco, M. W., K. P. Coolbear, P. J. Davis, and K. M. W. Keough. 1981a. Exclusion of fluid during compression of monolayers of mixtures of dipalmitoylphosphatidylcholine with some other phosphatidylcholines. *Biochim. Biophys. Acta*. 646:185–187.

Hawco, M. W., P. J. Davis, and K. M. W. Keough. 1981b. Lipid fluidity in lung surfactant: monolayers of saturated and unsaturated lecithins. *J. Appl. Physiol.* 51:509–515.

Hawgood, S., B. J. Benson, and R. L. Hamilton, Jr. 1985. Effects of surfactant-associated protein and calcium ions on the structure and surface activity of lung surfactant lipids. *Biochemistry*. 24:184–190.

Hawgood, S., B. J. Benson, J. Schilling, D. Damm, J. A. Clements, and R. T. White. 1987. Nucleotide and amino acid sequences of pulmonary surfactant protein SP-18 and evidence for cooperation between SP-18 and SP-28–36 in surfactant lipid adsorption. *Proc. Natl. Acad. Sci. USA*. 84:66–70.

Hildebrand, J. N., J. Goerke, and J. A. Clements. 1979. Pulmonary surface film stability and composition. *J. Appl. Physiol.* 47:604–611.

Johansson, J., T. Szyperski, T. Curstedt, and K. Wüthrich. 1994. The NMR structure of the pulmonary surfactant-associated polypeptide SP-C in an apolar solvent contains a valyl-rich α-helix. *Biochemistry*. 33: 6015–6023.

Karas, M., R. C. Beavis, F. Hillenkamp, and B. T. Chait. 1991. Matrix-assisted laser desorption/ionization mass spectrometry of biopolymers. *Anal. Chem.* 63:1193A-1203A.

Mitchell, M. L., and R. A. Dluhy. 1988. In situ FT-IR investigation of phospholipid monolayer phase transitions at the air-water interface. *J. Am. Chem. Soc.* 110:712–718.

Morrow, M. R., J. Perez-Gil, G. Simatos, C. Boland, J. Stewart, D. Absolom, V. Sarin, and K. M. W. Keough. 1993a. Pulmonary surfactant-associated protein SP-B has little effect on acyl chains in dipalmitoylphosphatidylcholine dispersions. *Biochemistry*. 32:4397–4402.

Morrow, M. R., S. Taneva, G. A. Simatos, L. A. Allwood, and K. M. W. Keough. 1993b. ^{2}H NMR studies of the effect of pulmonary surfactant SP-C on the 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine headgroup: a model for transbilayer peptides in surfactant and biological membranes. *Biochemistry*. 32:11338–11344.

Nag, K., and K. M. W. Keough. 1993. Epifluorescence microscopic studies of monolayers containing mixtures of dioleoyl- and dipalmitoylphophatidylcholines. *Biophys. J.* 65:1019–1026.

Pastrana, B., A. J. 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.

Pastrana-Rios, B., C. R. Flach, J. W. Brauner, A. J. Mautone, and R. Mendelsohn. 1994. A direct test of the "squeeze-out" hypothesis of lung surfactant function: external reflection FT-IR at the air/water interface. *Biochemistry*. 33:5121–5127.

Pérez-Gil, J., A. Cruz, and C. Casals. 1993. Solubility of hydrophobic surfactant proteins in organic solvent/water mixtures: structural studies on SP-B and SP-C in aqueous organic solvents and lipids. *Biochim. Biophys. Acta*. 1168:261–270.

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.* 63:197–204.

Pérez-Gil, J., J. Tucker, G. Simatos, and K. M. W. Keough. 1991. Interfacial adsorption of simple lipid mixtures combined with hydrophobic surfactant protein from pig lung. *Biochem. Cell Biol.* 70:332–338.

Schürch, S., J. Goerke, and J. A. Clements. 1976. Direct determination of surface tension in the lung. *Proc. Natl. Acad. Sci. USA.* 73:4698–4702.

Surewicz, W. K., H. H. Mantsch, and D. Chapman. 1993. Determination of protein secondary structure by Fourier transform infrared spectroscopy: a critical assessment. *Biochemistry.* 32:7720–7726.

Suzuki, Y., T. Curstedt, G. Grossmann, T. Kobayashi, R. Nilsson, K. Nohara, and B. Robertson. 1986. The role of the low-molecular weight ($\leq$15,000 daltons) apoproteins of pulmonary surfactant. *Eur. J. Respir. Dis.* 69:335–345.

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

Taneva, S., and K. M. W. Keough. 1994a. Pulmonary surfactant proteins SP-B and SP-C in spread monolayers at the air-water interface. I. Monolayers of pulmonary surfactant protein SP-B and phospholipids. *Biophys. J.* 66:1137–1148.

Taneva, S., and K. M. W. Keough. 1994b. 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. *Biophys. J.* 66:1149–1157.

Taneva, S., and K. M. W. Keough. 1994c. Pulmonary surfactant proteins SP-B and SP-C in spread monolayers at the air-water interface. III. Proteins SP-B plus SP-C with phospholipids in spread monolayers. *Biophys. J.* 66:1158–1166.

Taneva, S., and K. M. W. Keough. 1994d. Dynamic surface properties of pulmonary surfactant protein SP-B and SP-C and their mixture with dipalmitoylphosphatidylcholine. *Biochemistry.* 33:14660–14670.

van Liempd, J. P. J. G., A. A. H. Boonman, R. A. Demel, P. M. C. Gieles, and T. C. M. Gorree. 1987. Nonselective squeeze-out of dioleoylphosphatidylcholine and dioleoylphosphatidylglycerol from binary mixed monolayers with dipalmitoylphosphatidylcholine. *Biochim. Biophys. Acta.* 897:495–501.

Vandenbussche, G., A. Clerx, M. Clerx, T. Curstedt, J. Johansson, H. Jornvall, and J. Ruysschaert. 1992a. Secondary structure and orientation of the surfactant protein SP-B in a lipid environment: a Fourier transform infrared spectroscopy study. *Biochemistry.* 31:9169–9176.

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

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

Yu, S. H., and F. Possmayer. 1990. Role of bovine pulmonary surfactant-associated proteins in the surface-active property of phospholipid mixtures. *Biochim. Biophys. Acta.* 1046:233–241.

Zhang, Y., R. N. A. H. Lewis, R. S. Hodges, and R. N. McElhaney. 1992. FT-IR spectroscopic studies of the conformation of amide hydrogen exchange of a peptide model of the hydrophobic transmembrane α-helices of membrane proteins. *Biochemistry.* 31:11572–11578.