Fourier Transform Infrared Studies of Secondary Structure and Orientation of Pulmonary Surfactant SP-C and Its Effect on the Dynamic Surface Properties of Phospholipids†

Belinda Pastrana,‡ Alan J. Mautone,*,§ and Richard Mendelsohn*,‡

Department of Chemistry, Newark College of Arts and Sciences, Rutgers University, 73 Warren Street, Newark, New Jersey 07102, and Departments of Anesthesiology and Physiology, University of Medicine and Dentistry of New Jersey—New Jersey Medical School, 185 South Orange Avenue, Newark, New Jersey 07102

Received June 4, 1991; Revised Manuscript Received August 7, 1991

ABSTRACT: SP-C, a highly hydrophobic, 3.7-kDa protein constituent of lung surfactant, has been isolated from bovine lung lavage, purified, and reconstituted into binary lipid mixtures of 1,2-dipalmitoylphosphatidylcholine (DPPC) and 1,2-dipalmitoylphosphatidylglycerol (DPPG). Fourier transform infrared (FT-IR) spectroscopy has been applied to examine SP-C secondary structure, the average orientation of α-helical segments relative to the bilayer normal in membrane films, and the effect of protein on the thermotropic properties of the phospholipid acyl chains. In addition, dynamic surface measurements were made on phospholipid films at the A/W interface in the presence and absence of SP-C. SP-C (0.5 mol %) was found to possess about 60% α-helical secondary structure in lipid vesicles. Higher levels (1.5 mol %) of SP-C resulted in a slight increase of β-forms, possibly resulting from protein aggregation. The helical segments exhibited an average angle of orientation of about 24° with respect to the bilayer normal, suggesting a trans-bilayer orientation of the peptide. The observation that 70% of the peptide bond hydrogens are hard to exchange in D_2O further reflects the hydrophobic nature of the molecule. SP-C produced little effect on the thermotropic properties of the binary lipid mixture, as measured from acyl chain C–H and C–D stretching frequencies. However, the presence of 1 mol % protein markedly reduced the viscance and increased the elasticity of surface films, suggesting a mechanism by which SP-C facilitates the spreading of phospholipids on an aqueous surface. The possible physiological consequences of these observations are discussed.

Pulmonary surfactant, a mixture of lipids and proteins secreted by type II pneumocytes, possesses as its essential physical characteristic the ability to lower surface tension at the air/alveolar interface to near zero (Scarpelli, 1988). The main lipid in surfactant is 1,2-dipalmitoylphosphatidylcholine (DPPC),[1] with smaller amounts of phosphatidylglycerol (PG), unsaturated PC's, other anionic phospholipids, and cholesterol.

Several surfactant protein classes have been identified. The most abundant is a family (termed SP-A) with a molecular weight range of 28–36K. Ng et al. (1983) have shown this class to be subject to variable posttranslational glycosylation. The gene for human pulmonary SP-A has been isolated and characterized (White et al., 1985). In addition to SP-A, two smaller hydrophobic proteins are also present, namely, SP-B, a 79 amino acid fragment that is the processing product of residues 201–279 from a 42-kDa precursor (Curstedt et al., 1988), and SP-C, a highly hydrophobic moiety (Johansson et al., 1988; Hawgood et al., 1987). The N-terminus of SP-C is heterogeneous, the main form being 35 residues long (Johansson et al., 1988), with S-palmitoylated cysteine residues present in the porcine species (Curstedt et al., 1990). Recently, Persson et al. (1990) have reported the isolation of a collagenous surfactant-associated glycoprotein, SP-D.

The functions of the various protein classes have generally been assumed to be related to surfactant adsorption and spreading at the A/W interface and its reuptake into type II cells. After synthesis, surfactant is secreted into the hypophase where it is believed to exist as tubular myelin. Following poorly understood physical processes, the surfactant lipid adsorbs to the air/water interface.

The spreading of ordered phospholipids such as DPPC at the A/W interface occurs too slowly to model in vivo events (Yu & Possmayer, 1986) or for phospholipids alone to be useful for therapeutic intervention in pathological situations such as respiratory distress syndrome. A possible role for the surfactant proteins is to alter the lipid structure so as to facilitate their spreading and adsorption. SP-B and SP-C (individually and in combination) have indeed been shown to facilitate adsorption at the A/W interface (Hawgood et al., 1987; Suzuki et al., 1986).

To understand the molecular nature of the interaction between the lipid and protein components of surfactant, biophysical approaches are required. To date, the techniques applied have primarily been conventional surface balance determination of monolayer properties such as adsorption rates, γ_{max} and γ_{min}, turbidimetric methods, and calorimetric methods. These approaches provide thermodynamic data about the effect of protein on phospholipid phase behavior or kinetic data about the effect of protein on phospholipid spreading or adsorption, but no direct molecular structural information. Spectroscopic techniques are required for this latter purpose.

† This work was supported by the U.S. Public Health Service through National Institutes of Health Grants GM-29864 (R.M.) and HL-38303 (A.J.M.).

* To whom correspondence should be addressed.

‡ Rutgers University.

§ University of Medicine and Dentistry of New Jersey—New Jersey Medical School.

[1] Abbreviations: FT-IR, Fourier transform infrared; γ, surface tension; A, surface area; E, elasticity; V, viscance; M, viscoelastic index; DPPC, 1,2-dipalmitoylphosphatidylcholine; DPPC-d_{62}, acyl-chain-perdeuterated DPPC; DMPC, 1,2-dimyristoylphosphatidylcholine; DPPG, 1,2-dipalmitoylphosphatidylglycerol; NMR, nuclear magnetic resonance; λ, extension ratio; ATR, attenuated total reflectance; A/W, air/water.

0006-2960/91/0430-10058$02.50/0

The few spectroscopic experiments reported to date on the surfactant components include electron paramagnetic resonance studies (Hook et al., 1984) of surfactant lipid fluidity, FT-IR studies from this laboratory of surfactant melting behavior in vesicles (Mautone et al., 1987), of phospholipid structure in situ at the air/water interface (Dluhy et al., 1989), and of SP-A/phospholipid interaction (Reilly et al., 1989), and recent ^2H NMR studies of Simatos et al. (1990) on the interaction of SP-C with (acyl-chain-perdeuterated) DMPC. In each case, the lipid component was the focus of the study. Little attention has been given to protein conformation, with the exception of a circular dichroism study of SP-A and the resultant suggestion of a collagen-like triple helix as a structural motif (King et al., 1989).

The current work reports three FT-IR experiments probing the interaction between purified SP-C reconstituted with binary mixtures of DPPC (or DPPC-d_{62}) and DPPG. First, the secondary structure of SP-C in aqueous phospholipid dispersions was determined from its amide I vibrations. Second, the orientation of the α-helical segments of SP-C was determined from polarized attenuated total reflectance (ATR) FT-IR of cast films. Finally, the ability of protein to perturb the thermotropic properties of the binary lipid mixture was determined from the temperature dependence of the lipid C–H and C–D stretching modes. In addition, a method to assess dynamic surface properties (Boyle & Mautone, 1982; Mautone et al., 1988) has been used to determine the ability of SP-C to alter the spreading kinetics of the lipid mixture.

Experimental Procedures

Materials. $CHCl_3$ (ACS grade), MeOH (ACS grade), 2-chloroethanol (Eastman-Kodak), and double-distilled H_2O were used throughout. Salts for buffers were of the highest purity commercially available. DPPC-d_{62}, DPPC, and DPPG were all obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). Their purity was checked by differential scanning calorimetry.

Protein Isolation. Calf lung surfactant extract, a fraction obtained from lung lavage and resuspended in saline, was generously supplied by Dr. Bruce Holm, Childrens Hospital, Buffalo, NY. To isolate the hydrophobic fraction, multiple extractions were performed (Bligh & Dyer, 1959). The concentrated organic phase containing SP-C was purified by two successive stages of gel exclusion chromatography, on Sephadex LH-20 and Sephadex LH-60, respectively. The columns were equilibrated with solvent systems of $CHCl_3$/MeOH (2:1, v/v) for the former and $CHCl_3$/MeOH (1:1, v/v) for the latter. Routinely, 1-mL fractions were collected and assayed for protein (Bradford, 1976) and phospholipid content (Chen et al., 1956). Protein concentration was determined only after extensive delipidation, as phospholipid interfered with the Bradford assay. In addition, the latter was used with BSA as a standard and 2-chloroethanol as solvent.

Gel electrophoresis was carried out according to Laemmli (1970), with 20% SDS–PAGE under reducing conditions (8 M urea). Gels were developed by silver staining. One main band corresponding to a molecular weight of 3.7K was observed. Amino acid sequencing of the first 25 amino acid residues of SP-C was performed by the Biotechnology Service Center (Toronto, Ontario, Canada). The samples were analyzed on a Porton gas-phase microsequencer, Model 2090, with on-line PTH analysis.

Reconstitution of SP-C into Lipid Environments. SP-C, codissolved with a binary lipid mixture of DPPC (or its acyl-chain-perdeuterated form DPPC-d_{62})/DPPG (70:30, w/w) in $CHCl_3$/MeOH (2:1, v/v), was either used directly (surface film or polarized ATR FT-IR studies) or dried under a stream of N_2 gas and left in vacuo overnight, to ensure complete solvent removal. For bulk-phase FT-IR studies of protein secondary structure and phospholipid thermotropic processes, isotonic D_2O and H_2O, respectively, were used for rehydration.

Surface Film Studies. For surface film experiments, the lipid and lipid/SP-C mixture were spread directly from $CHCl_3$ solution onto the surface of a buffer which was 150 mM NaCl/20 mM Hepes, pH 7.05. Surface tension (γ)/area data were measured using a vertical film surface balance at 37 °C with a method described previously (Mautone et al., 1988). Briefly, the balance consists of a fine-mesh stainless-steel screen suspended from a movable bar. The screen is moved into and from a hypophase contained in a Teflon beaker, thus decreasing and increasing surface area (A) between A_{min} (34.6 cm^2) and A_{max} (142.6 cm^2), respectively. As the screen is slowly moved from A_{min} to A_{max}, sufficient material from the test preparation is spread from chloroform on the hypophase surface to provide $\approx$1.5× that required to form an equilibrium surface film. The surface is then held at A_{max} for at least 1 min before cycling is begun at 1.5 cycles/min and continued until a stable γ_{max} and γ_{min} are established. At this point, A is held constant at A_{max} until a stable γ is achieved. The surface is then rapidly compressed in <1 s (a step compression) and held at this smaller area while the change in γ (as measured in a Wilhemy-type balance with a platinum dipping plate) with time is sampled at 50 Hz using an A/D converter interfaced to a microcomputer. After a relaxation period of about 3 min, cycling is resumed until stable γ_{max} and γ_{min} are again established. The procedure is repeated for several different levels of compression starting from the same initial A_{max} and γ. Since the relevant γ's for the lung are at and below γ_{equil}, the surface films were compressed to or below the point of γ_{equil}, i.e., $\approx$25 mN/m, the point at which a surface film begins to compress and, ultimately, collapse. The time to γ_{eqil} after a step compression for each preparation was determined in separate experiments by maintaining the relaxation period until γ_{equil} was achieved.

To assess surface viscoelastic behavior (Mautone et al., 1988), an extension ratio (λ) is used, which equals the area to which the surface is compressed (A_e) divided by the initial area from which the compression is begun, A_{max}, or $\lambda = A_e/A_{max}$. The stress on the film, γ, is taken at each time point during the relaxation period. Plots of $\gamma/(\lambda^2 - 1/\lambda)$ vs $1/\lambda$ are constructed for each step compression at several time points during the relaxation period. The slope of the resultant line equals the stress on the surface that is resisting deformation for a given γ at time t during relaxation, or elasticity (E) at time t. The intercept [which is known as the viscance (V) at time t] is the force needed to hold the configuration of material constant for a given γ at time t during relaxation. The sign of V indicates the direction in which the force is applied. In the current work, the surface film was compressed so that the force (viscance) is applied (by convention) in the negative direction. The functions E and V are used to calculate the viscoelastic index for the surface (M), which equals $2E + V$. If M varies with time, the surface is behaving like a fluid; if it does not, the surface is solidlike.

FT-IR Methods. (*i*) *Polarized ATR Studies of Films.* Twenty-microliter aliquots of a 1 mg/mL $CHCl_3$ solution of the desired composition were applied to each face of a hydrophilic germanium 45° ATR crystal (25 mm × 10 mm × 3 mm) from Spectral Systems Inc. (Irvington, NY). The solution was spread with the aid of a Teflon bar which was

rolled across the surface of the crystal until all solvent evaporated. A bluish brown iridescence was then evident on the crystal surface (Brauner et al., 1987). At times, a noniridescent film (streaky in appearance) resulted, apparently caused by aggregation on the germanium surface. Streaky films demonstrated poor orientation upon analysis of FT-IR spectral data.

Interferograms were collected with a Mattson, Inc., Sirius 100 FT-IR spectrometer (Mattson Instruments, Madison, WI) equipped with an HgCdTe detector. The optical setup included a wire grid polarizer. Routinely, 200 scans of the sample and background were separately coadded for both the parallel and perpendicular components, apodized with a triangular function, and Fourier-transformed to provide a resolution of 4 cm^{-1}, with data encoded every 2 cm^{-1}. Data analysis of the amide I contour was accomplished with software from the National Research Council of Canada generously supplied by D. Moffatt. Peak positions for a curve-fitting algorithm were fixed by Fourier self-deconvolution. Standard deviations and goodness-of-fit were used as markers for consistency in the procedure.

(ii) Bulk-Phase Study of SP-C Secondary Structure. Ten milligrams of each of two separate SP-C/lipid reconstitutions (1:70 and 1:200 mol/mol, respectively) was prepared using a lipid mixture of DPPC-d_{62}/DPPG (70:30, w/w) and hydrated with 120 μL of isotonic D_2O. The samples were placed between two CaF_2 windows (25 mm × 2 mm) and sealed with a 12-μm spacer. The assembly was wrapped with "Teflon" tape to prevent dehydration and inserted into a thermostated cell. Temperature was controlled via a Haake Model A80 circulating bath and monitored with a thermocouple positioned in the sample near the point at which the IR radiation was focused.

For these experiments, a Digilab FTS-40 FT-IR spectrometer (Bio-Rad, Cambridge, MA) equipped with a DTGS detector was utilized for data collection. Spectral parameters were similar to those noted above.

(iii) FT-IR Microscopy of Lipid Thermotropic Behavior in Ternary Complexes. Two hundred micrograms of SP-C/DPPC-d_{62}/DPPG (1 mol % protein) or DPPC-d_{62}/DPPG (70:30, w/w) was hydrated in 10 μL of isotonic saline. The samples were placed between two circular CaF_2 windows (6-mm diameter) and sealed with a 12-μm spacer. The assembly was placed in a thermostated cell constructed in this laboratory. Temperature control was achieved with a Haake (Model A80) circulating bath. Temperature accuracy is estimated at ±1 °C.

Spectra were recorded on a Mattson Instruments Sirius 100 spectrophotometer coupled to a Bach–Shearer microscope. The microscope facilitated spectral acquisition from small amounts of material. Spectral parameters were as indicated above.

Spectra were obtained from 0 to 55 °C. The spectrum of solvent matched for temperature and path length was used for background subtraction. Spectra were flattened with a linear base line. The C–H (2700–3000 cm^{-1}) and C–D (2000–2250 cm^{-1}) stretching frequencies were determined with a three-point parabolic routine.

Results

Biochemical Characterization of SP-C. To confirm the identity of the isolated peptide, amino acid sequencing of the first 25 residues was completed. The preparation was found to consist of three closely related peptides with N-terminal heterogeneity in the proportion 56%, 26%, and 18%, as judged from recoveries of their first PTH amino acid residues. These N-termini residues were Leu, Ile, and Phe, respectively. The remainder of the sequences appeared identical. The sequences are

(26%) IPXIPVNIKRLLIVVVVVVLVVVVI

(56%) LIPXIPVNIKRLLIVVVVVLVVVV

(18%) PLIPXIPVNIKRLLIVVVVVVLVVV

The unidentified residue (suggested to be Cys from its breakdown products, but not confirmed as Cys) is marked X. The first 26 residues of the porcine sequence reported by Johansson et al. (1988) are LAIPCCPVNLKRLLVVVVVVLVVVVI which bears a marked similarity to the current bovine sequence. The multiple valine regions are consistent with a highly hydrophobic peptide. The N-terminal heterogeneity presumably arises from nonspecific protease action on the precursor. It is noted that [as in the study of Simatos et al. (1990)] quantitative determination of SP-C proved to be a difficult task. The standard assay of Lowry et al. (1951) failed completely due to the absence of Tyr in the sequence and due to large interference from phospholipids. The Bradford (1976) assay, as modified by Simatos et al. (1990), was found to be more successful. However, phospholipid interference is not completely eliminated. Thus, lipid phosphorus and protein levels were generally determined on the same fractions to test the efficiency of the various separation and purification steps.

Table I: Frequencies, Widths, and Percent of the Band for Components of the Amide I Contour in SP-C/Phospholipid Complexes

sample	SP-C:lipid ratio (mol/mol)	T (°C)	position	width	% area
bulk D_2O	1:70	20	1679	28.0	8.8
			1656	22.8	48.2
			1636	21.3	15.6
			1619	37.8	27.6
bulk D_2O	1:200	22	1678	20.4	6.8
			1656	20.3	59.4
			1637	27.4	33.8
SP-C in $CHCl_3$	0.5% (w/w)	21	1672	24.8	23.8
			1654	18.9	51.9
			1635	19.1	10.9
			1624	28.3	13.4

FT-IR Spectroscopy. The first FT-IR experiment involved determination of SP-C secondary structure in organic ($CHCl_3$/MeOH 1:1) solvents and in reconstituted liposomes with DPPC/DPPG in D_2O suspension. The sample compositions of the liposomes were SP-C/DPPC/DPPG (1:140:60, mol/mol, sample A) and (1:50:20, mol/mol, sample B). The spectrum of SP-C in $CHCl_3$/MeOH solution in the range 1500–1800 cm^{-1} revealed amide I and II vibrations near 1654 and 1545 cm^{-1}, respectively. A sharp feature near 1654 cm^{-1} is assigned to α-helical secondary structure and accounted for 52% of the amide I contour. Additional shoulders seen in this region near 1624, 1635, and 1672 cm^{-1} (Table I) are characteristic of proteins with antiparallel β-sheet structure (Byler & Susi, 1986). However, the high-frequency feature may also be diagnostic of turns (Bandekar & Krimm, 1979). Thus, the assignments to particular secondary structures of the 1672 cm^{-1} feature is uncertain. In any case, the low-frequency motions are characteristic of β-forms.

Spectra of SP-C reconstituted into vesicles of DPPC/DPPG in D_2O (samples A and B) at lipid:protein mole ratios of 200:1 and 70:1 are shown in Figure 1A. The existence of an intense amide II mode at 1540–1550 cm^{-1} in each case, which persists with significant intensity at 50 °C (data not shown), demon-

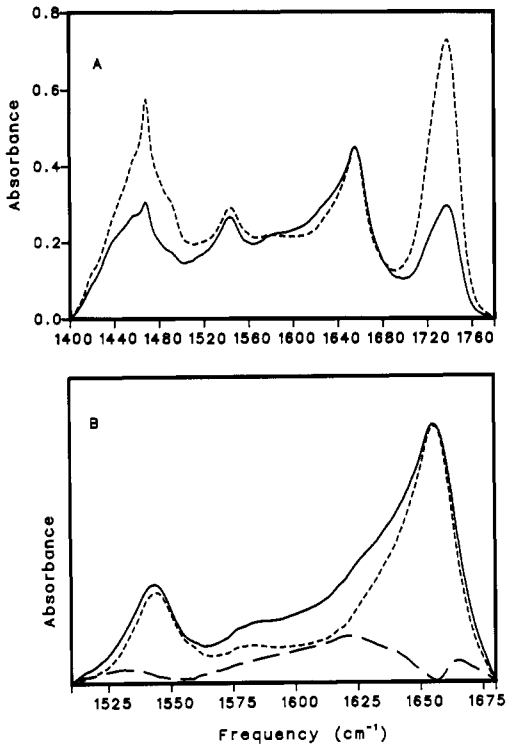

FIGURE 1: (A) Infrared spectra of SP-C reconstituted in bulk-phase D_2O with a 7:3 mole ratio phospholipid mixture of DPPC/DPPG. (---) SP-C/lipid (1:200, mol/mol); (—) SP-C/lipid (1:70, mol/mol). (b) FT-IR evidence for SP-C aggregation in a ternary mixture at T = 20 °C. The spectra shown in Figure 2A have been base-line-flattened and scaled to the peak height of the α-helical amide I band at 1656 cm^{-1}. The spectrum of the 200:1 sample (---) was subtracted from the spectrum of the 70:1 sample (—), and the resultant difference spectrum is displayed. The broad peak in the difference spectrum (--) at 1610–1640 cm^{-1} probably arises from intermolecular β-forms in aggregated SP-C.

strates that the peptide bond hydrogens are not completely exchanged. Exchange would shift the amide II mode to amide II′ at about 1450 cm^{-1}. Lack of exchange suggests that the peptide is sequestered from the aqueous environment and/or that the H-bonds in aqueous environments are located in ordered secondary structures where exchange is slow. A comparison of the relative integrated areas of the amide I to amide II contour in organic solvent, in cast films and in D_2O suspension, suggests that about 70% of the peptide bond hydrogens are not accessible to solvent over the 4-h period of time it takes to prepare a sample, incubate it at the desired temperature (up to 50 °C) in D_2O, and acquire the FT-IR spectrum. It is reasonable (and consistent with the frequency of the residual unexchanged amide II modes) to associate this inaccessible fraction mostly with the α-helical segments.

Typical results of the fitting process for sample A in the amide I and phospholipid C=O regions are shown in Figure 2 and the data summarized in Table I. The α-helical component (1656 cm^{-1}) comprises 59% of the amide I contour. Assuming, as is often done, that the amide I extinction coefficients are independent of secondary sructure, we may estimate that the fraction of SP-C in α-helix form at a level of 0.5 mol % in the lipid mixture is about 60%. The higher levels of protein in sample B produced significant changes as demonstrated in the difference spectrum (scaled to the peak height at 1656 cm^{-1}) in Figure 1B. A broad band at 1620–1630 cm^{-1} appears in the difference spectrum. The structural origin of this band is increased β-structure, possibly due to aggregation. In addition, the fractional intensity of the helical component decreased to 48%. The results reveal that

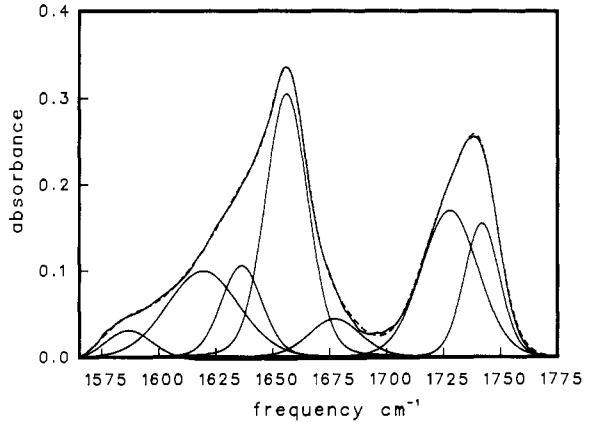

FIGURE 2: Typical result of the curve-fitting algorithm for the amide I and phospholipid C=O regions for bulk-phase D_2O suspensions of SP-C/lipid (1:70, mol/mol). The dominant feature is that of α-helix (1656 cm^{-1}).

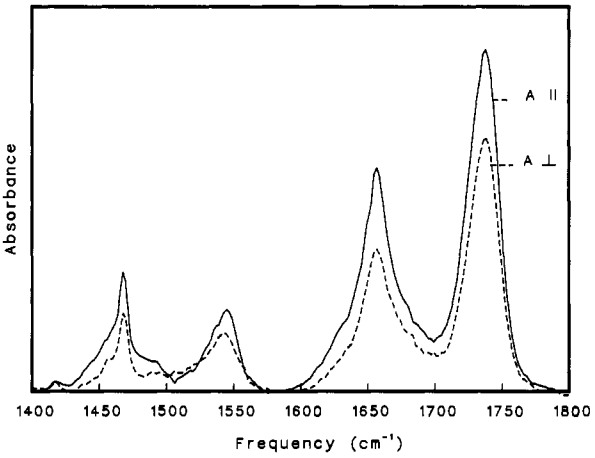

FIGURE 3: Polarized FT-IR ATR overlaid spectra of the parallel and perpendicular components SP-C/DPPC/DPPG at a lipid:protein mole ratio of 170:1 showing the contribution of the 1680 and 1638 cm^{-1} subbands characteristic of β-type secondary structure and the main α-helical feature at 1657 cm^{-1}.

SP-C adopts substantial α-helical secondary structure both in organic solvents and in phospholipid vesicles. Some protein aggregation may take place at higher protein concentrations.

The orientation of the SP-C helical segments in DPPC/DPPG (7:3 mol/mol) films was determined by polarized ATR FT-IR spectroscopy. Typical spectra of iridescent films containing 0.6 mol % SP-C, acquired with light polarized parallel and perpendicular to the plane of incidence, are shown in Figure 3. Curve fitting the contour yielded dichroic ratios for the 1656 cm^{-1} helical component of the amide I contour of 1.98 ± 0.1. As noted under Experimental Procedures, samples which showed a milky, streaky appearance gave much reduced dichroic ratios (1.27 ± 0.03). Analysis of the orientational order of the α-helical regions was carried out as previously described (Brauner et al., 1987). Briefly, the dichroic ratio, R^{atr}, is defined by

$$R^{atr} = \frac{A_\parallel}{A_\perp} = \frac{E_x^2}{E_y^2} + \frac{E_z^2[f\cos^2\alpha + (1/3)(1-f)]}{E_y^2[(1/2)f\sin^2\alpha + (1/3)(1-f)]} \quad (1)$$

where $A_\parallel$ is the band intensity in question under parallel polarized light illumination, $A_\perp$ is the intensity under perpendicular polarized light illumination, and E_x, E_y, and E_z are the electric field amplitudes in the indicated directions. In addition, $f = (1/2)(3\cos^2\theta - 1)$, where θ is the angle the helical axis makes with the normal to the crystal (i.e., normal

to the bilayer in the film) and f itself is the order parameter. α is the angle the transition moment for the amide I mode makes with the helix axis. For the current experiment, the refractive indices of germanium, air, and the film (4, 1.0, and 1.44, respectively) result in relative amplitudes of $E_x = 1.41$, $E_y = 1.46$, and $E_z = 0.728$, as calculated (Harrick, 1967) from the Fresnel equations.

Determination of the orientational order assumes uniaxial symmetry of the distribution of helical segments with respect to the normal to the bilayer (and to the Ge crystal). The analysis requires an estimate of α, the direction of the transition moment of the amide I mode with respect to the helix axis. The value chosen here, 27° (Rothschild & Clark, 1979), leads to calculated values of $f = 0.75$ and $\theta = 24°$. SP-C is therefore well oriented in the iridescent films along a direction parallel to the acyl chains. The orientation of the latter has been determined at about 25 ± 7° (Okamura et al., 1986). Other possible choices for α (Tsuboi, 1962) lead to smaller angles of orientation, while random orientation would produce a measured dichroic ratio (for this experimental protocol) of 1.18, and, according to eq 1, lead to $f = 0$ and $\theta = 54.73°$, the magic angle. The average angle of orientation of SP-C in the streaky films (no iridescence) is near this latter value.

To determine the effect of SP-C on the thermotropic properties of DPPC-d_{62}/DPPG mixtures, frequency shifts in the C–H stretching modes of the phospholipid acyl chains were utilized to qualitatively probe acyl chain conformational order. These frequencies increase by 1–4 cm^{-1} during phospholipid phase transitions (Mendelsohn & Mantsch, 1986) and may be observed with a precision of better than 0.05 cm^{-1}. Quantitative relationships between the CH_2 stretching frequency and conformational disorder are not available, although the increase in frequency has been correlated with increased numbers of gauche rotamers (Snyder et al., 1982). Incorporation of acyl-chain-perdeuterated phospholipids as one component of a ternary (two lipids plus protein) mixture permits the simultaneous evaluation of acyl chain conformational order in each lipid component, utilizing the C–D (C–H) stretching modes to monitor the deuterated (proteated) species.

SP-C was reconstituted into vesicles with binary mixtures of DPPC-d_{62}/DPPG (7:3, mol/mol) at a level of 1 mol %. FT-IR melting curves constructed from the symmetric C–H (near 2850 cm^{-1}) and symmetric C–D (near 2100 cm^{-1}) stretching modes from the DPPG and DPPC-d_{62} components, respectively, are shown in panels A and B, respectively, of Figure 4. The lipid mixture undergoes its phase transition at about 40 °C, as judged by the melting profiles for either of the components. The similar thermotropic behavior of each component indicates that the lipids are well mixed; i.e., no phase separation occurs. The introduction of 1 mol % SP-C produces little alteration of the thermotropic properties of either phospholipid. At most, a very slight ordering (close to experimental uncertainty) may be inferred from the data above T_m, while no effect is noted in the phospholipid gel phase.

Surface Viscoelasticity. The change in γ with time after a step compression for DPPC/DPPG and DPPC/DPPG/SP-C at 37 °C is shown in Figure 5. The $t_{1/2}$ to γ_{equil} for the lipids alone is 500 min whereas that for the lipids + SP-C is 9.3 min. γ_{equil} for the lipids is 23.8 mN/m and for the lipids + SP-C 26.6 mN/m. During a step compression to A_{min}, the γ_{min} for the lipids is 4.4 mN/m and, for lipids + SP-C, 7.4 mN/m.

E, V, and M, calculated as described earlier, are plotted against time for the mixtures of DPPC/DPPG and DPPC/DPPG/SP-C in panels A, B, and C, respectively, of Figure 6. Following an initial rapid change, E and V for the lipids

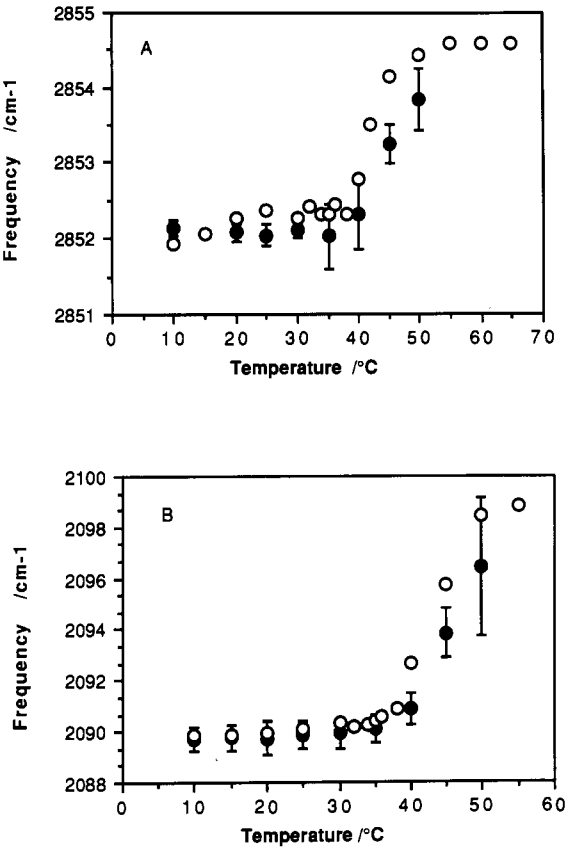

FIGURE 4: (A) Temperature dependence of the CH_2 symmetric stretching mode in (○) DPPC-d_{62}/DPPG (7:3, mol/mol) and (●) SP-C/DPPC-d_{62}/DPPG (1:70:30, mol/mol). (B) Temperature dependence of the CD_2 symmetric stretching mode in (○) DPPC-d_{62}/DPPG (7:3, mol/mol) and (●) SP-C/DPPC-d_{62}/DPPG (1:70:30, mol/mol).

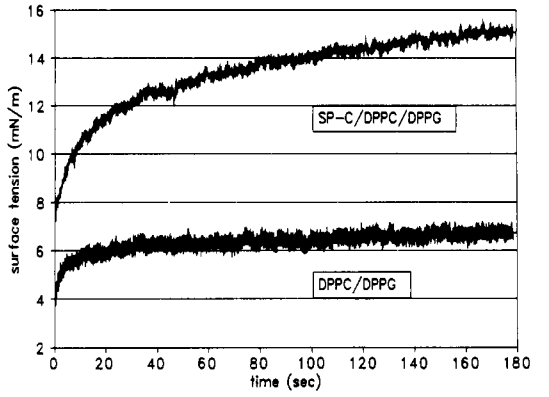

FIGURE 5: Surface tension vs time after a maximal step compression of SP-C/DPPC/DPPG (1:70:30 mol/mol) and DPPC/DPPG (7:3, mol/mol).

are virtually linear after about 10 s into the relaxation period. For the mixture of lipids + SP-C, E and V both increase markedly for about 2 s and then slowly decrease for the next 1–2 min before a plateau is reached. The viscoelastic index for the lipids alone is virtually constant after 5 s, indicating solidlike consistency. In contrast, the viscoelastic index for the mixture of lipids + SP-C continues to change with time, indicative of more fluidlike behavior during most of the relaxation period.

Discussion

The secondary structure and orientation of SP-C as well as the molecular nature of its interaction with phospholipids are

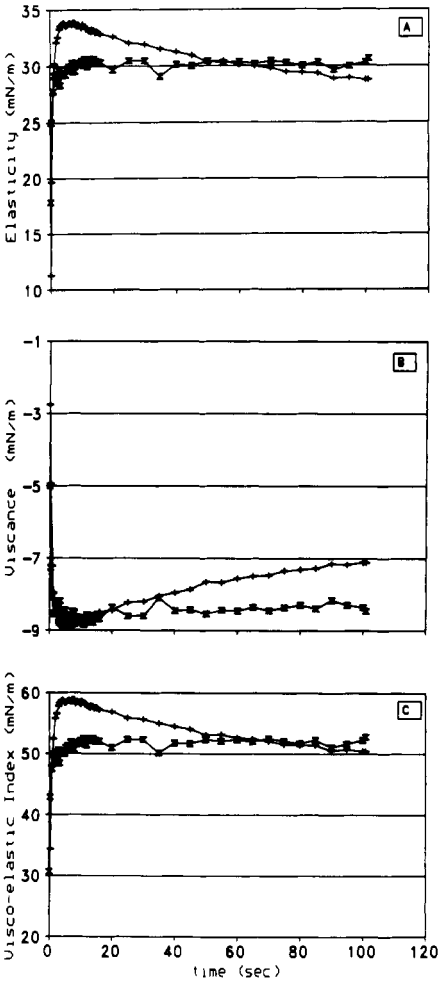

FIGURE 6: Dynamic surface study of the effect of SP-C on the spreading of surface films. (A) Elasticity vs time; (B) viscance vs time; (C) viscoelastic index vs time as indicated for (+) SP-C/DPPC/DPPG (1:68:30, mol/mol) and DPPC/DPPG (×) (7:3, mol/mol). See text for a detailed definition of each quantity.

revealed from the current IR experiments. The protein has 50–60% α-helical content, appropriate for a highly membrane-active species. Its ability to interact with phospholipids and to penetrate into the hydrophobic regions is demonstrated in two ways. First, the lack of exchange (D_2O, 50 °C, 4 h) of 70% of the peptide bond hydrogens, as measured by the persistence of the amide II mode in D_2O environments, reveals the sequestering of SP-C from the aqueous medium. Second, the orientation of helical segments along a direction that parallels the phospholipid acyl chains is evident from the ATR data. The relationship between these structural findings and the function of SP-C is addressed through the surface chemistry measurements.

Both dynamic surface measurements (current work) and surface adsorption experiments (Hawgood et al., 1987; Suzuki et al., 1986) reveal that SP-C can enhance the ability of ordered phospholipids to spread at the A/W interface. It has been suggested that certain components of the alveolar surfactant system are "squeezed out" of the surface during compression to A_{min} (Goerke & Clements, 1986). The γ-time data (Figure 6) show that addition of SP-C to the lipid mixture decreases the $t_{1/2}$ 50 times (≈9–10 min in the presence of protein to ~500 minutes in its absence). This fact, coupled with differences in the γ_{min} between the two preparations, suggests that SP-C is not "squeezed out" but remains in the surface upon compression. This result meshes nicely with the ATR-derived model of transbilayer incorporation for this predominantly α-helical peptide. In addition, the strongly hydrophobic nature of SP-C, as revealed from sequence data, renders it unlikely that this protein would squeeze out of its lipid environment without substantial energy input.

Under nonequilibrium conditions, the configuration of the surface is altered and E becomes time-dependent. The surface is therefore viscoelastic with γ changing as a function of time. The extent and type of this variation are indicated by M. The time dependence of M (Figure 6C) shows that the lipids exhibit solidlike behavior ~10 s into the relaxation period whereas the lipid/SP-C mixture exhibits more fluid characteristics. The effect of SP-C on dynamic γ_{min} and surface viscoelastic parameters suggests that when SP-C packs within the lipid (surface) matrix the configuration is extremely stable even with large compressions.

If the orientation of SP-C in lipid bilayers as revealed from the ATR experiments is assumed to also occur in surface films at the A/W interface, then SP-C may function to prevent the phospholipids from packing as tightly together as the lipids alone in phospholipid gel phases. At the A/W interface, this poorer packing would tend to increase γ_{min} and would lead to more fluid (dynamic) behavior of the resultant system. A positive, nonzero, E value is thought (Goerke & Clements, 1986; Notter, 1984) to be necessary in vivo to provide stability to terminal air units by providing energy to replenish a perturbed surface film in response to the local decrease in surface concentration of surfactant. In addition, a stable, low γ is required for alveolar stability. The time scale and magnitude of the increase in γ for the lipid/SP-C mixture are greater than those needed to maintain low γ in alveoli (see Figures 5 and 6). Thus, to maintain low surface tension (especially in the presence of SP-C), the surface must be constantly compressed or γ will increase to its equilibrium level. It is difficult to reconcile this finding with the currently widely held belief that a continuous monomolecular film exists at the alveolar/air interface.

The above description is based on the ability of SP-C to alter the packing of lipid gel phases. Yet this highly hydrophobic species, though clearly penetrating the lipid bilayer, seems to change the conformational properties of the phospholipid acyl chains very little, as monitored both by FT-IR in the current work and by ^2H NMR (Simatos et al., 1990). To reconcile the dynamic surface measurements with constant gel-phase phospholipid conformational order (as seen from the C–H stretching frequencies in the IR), an excellent match must exist between the shape of the protein surface and the lipid acyl chains. SP-C-induced changes in the elasticity, viscance, and viscoelastic index in the early stages of relaxation after a step compression all indicate that the peptide decreases the viscous drag while increasing the surface elasticity of the film. The step compression may produce a deviation from the ideal matching and a rapid return to equilibrium, during which the protein–lipid interface behaves in springlike fashion.

ACKNOWLEDGMENTS

We thank Professor Kevin Keough for discussions concerning the merits and lack thereof of the various protein assays for SP-C.

Registry No. DPPC, 2644-64-6; DPPG, 4537-77-3.

REFERENCES

Bandekar, J., & Krimm, S. (1979) *Proc. Natl. Acad. Sci. U.S.A. 77*, 774–777.

Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. 37*, 911–917.

Boyle, J. B., III, & Mautone, A. J. (1982) *Colloids Surf. 4*, 77–85.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.
Brauner, J., Mendelsohn, R., & Prendergast, F. G. (1987) *Biochemistry 26*, 8151–8158.
Byler, D. M., & Susi, H. (1986) *Biopolymers 25*, 469–487.
Chen, P. S., Toribara, T. Y., & Warner, H. (1956) *Anal. Chem. 28*, 1756–1758.
Curstedt, T., Johansson, J., Barros-Soderling, J., Robertson, B., Nilsson, G., Westberg, M., & Jornvall, H. (1988) *Eur. J. Biochem. 172*, 521–525.
Curstedt, T., Johansson, J., Persson, P., Eklund, A., Robertson, B., Lowenadler, B., & Jornvall, H. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 2985–2989.
Dluhy, R. A., Reilly, K. E., Hunt, R. D., Mitchell, M. L., Mautone, A. J., & Mendelsohn, R. (1989) *Biophys. J. 56*, 1173–1181.
Fraser, (1953) *J. Chem. Phys. 21*, 1511–1515.
Goerke, J., & Clements, J. A. (1986) *Handbook of Physiology—The Respiratory System III* (Macklem, P. T., & Mead, J., Eds.) pp 247–261, American Physiological Society, Washington, D.C.
Harrick, N. J. (1967) in *Internal Reflectance Spectroscopy*, Interscience, New York.
Hawgood, S., Benson, B. J., Schilling, J., Damm, D., Clements, J. A., & White, R. T. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 66–70.
Hook, G., Spalding, J. W., Ortner, M. J., Tombropoulos, E. G., & Chignell, C. F. (1984) *Biochem. J. 223*, 533–542.
Johansson, J., Curstedt, T., Robertson, B., & Jornvall, H. (1988) *Biochemistry 27*, 3544–3547.
King, R. J., Simon, D., & Horowitz, P. M. (1989) *Biochim. Biophys. Acta 1001*, 294–301.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Mautone, A. J., Reilly, K. E., & Mendelsohn, R. (1987) *Biochim. Biophys. Acta 896*, 1–10.
Mautone, A. J., Ritter, A., & Boyle, J. (1988) *Colloids Surf. 300*, 223–241.
Mendelsohn, R., & Mantsch, H. H. (1986) in *Progress in Protein–Lipid Interactions 2* (Watts, A., & De Pont, J. J. H. H. M., Eds.) Chapter 4, Elsevier Science Publishers, Amsterdam, The Netherlands.
Ng, V. L., Herndon, V. L., Mendelson, C. R., & Snyder, J. M. (1983) *Biochim. Biophys. Acta 754*, 218–236.
Notter, R. H. (1984) *Surface Chemistry of Pulmonary Surfactant: the Role of Individual Components in Pulmonary Surfactants* (Robertson, B., Van Golde, L. M. G., & Batenburg, J. J., Eds.) Elsevier, Amsterdam.
Okamura, E., Umemura, J., & Takenaka, T. (1986) *Biochim. Biophys. Acta 856*, 68–75.
Persson, A., Chang, D., & Crouch, E. (1990) *J. Biol. Chem. 265*, 5755–5760.
Reilly, K. E., Mautone, A. J., & Mendelsohn, R. (1989) *Biochemistry 28*, 7368–7373.
Rothschild, K. J., & Clark, N. A. (1979) *Biophys. J. 25*, 473–488.
Scarpelli, E. M. (1988) *Surfactants and the Lining of the Lung*, Johns Hopkins University Press, p 34, Baltimore, MD.
Simatos, G. A., Forward, K. B., Morrow, M. R., & Keough, K. M. W. (1990) *Biochemistry 29*, 5807–5814.
Snyder, R. G., Strauss, H. L., & Elliger, C. A. (1982) *J. Phys. Chem. 86*, 5145–5150.
Suzuki, Y., Curstedt, T., Grossmann, G., Kobayashi, T., & Nilsson, R. (1986) *Eur. J. Respir. Dis. 69*, 335–345.
Tsuboi, M. (1962) *J. Polym. Sci. 59*, 139–153.
White, R. T., Damm, D., Miller, J., Spratt, K., Schilling, J., Hawgood, S., Benson, B., & Cordell, B. (1985) *Nature 317*, 361–363.
Yu, S. H., & Possmayer, F. (1986) *Biochem. J. 236*, 85–87.