Secondary Structure and Orientation of the Surfactant Protein SP-B in a Lipid Environment. A Fourier Transform Infrared Spectroscopy Study†

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ABSTRACT: Attenuated total reflection Fourier transform infrared spectroscopy was used to investigate the secondary structure of the surfactant protein SP-B. Nearly half of the polypeptide chain is folded in an α-helical conformation. No significant change of the secondary structure content was observed when the protein is associated to a lipid bilayer of dipalmitoylphosphatidylcholine (DPPC)/phosphatidylglycerol (PG) or of dipalmitoylphosphatidylglycerol (DPPG). The parameters related to the $\gamma_w(CH_2)$ vibration of the saturated acyl chains reveal no modification of the conformation or orientation of the lipids in the presence of SP-B. A model of orientation of the protein at the lipid/water interface is proposed. In this model, electrostatic interactions between charged residues of SP-B and polar headgroups of PG, and the presence of small hydrophobic α-helical peptide stretches slightly inside the bilayers, would maintain SP-B at the membrane surface.

During the respiratory cycle, the collapsing tendency of the lung depends largely on the surface tension at the alveolar air/liquid interface. A surface-active material lining the alveoli stabilizes the lung during expiration and prevents atelectasis (Goerke, 1974; van Golde et al., 1988). This pulmonary surfactant is a protein/lipid complex synthesized in the type II pneumocytes and secreted into the alveolar space in the form of multilamellar structures named lamellar bodies. The material is transformed into a lattice-like structure, the tubular myelin (Williams, 1977), which is the precursor of a lipid monolayer at the alveolar air/liquid interface leading to the surface tension decrease (Goerke, 1974).

Extracellular surfactant consists of 85–90% lipids, mainly phospholipids, about 10% proteins, and a small amount carbohydrates. The major component of surfactant phospholipids is dipalmitoylphosphatidylcholine (DPPC).[1] The lipid composition is also characterized by an unusually high percentage of phosphatidylglycerol (PG), up to 10% of the surfactant phospholipids (Shelley et al., 1984; Rooney, 1985). Four proteins, SP-A, SP-B, SP-C, and SP-D, respectively, appear to be closely related to the structure and properties of the pulmonary surfactant (Weaver & Whitsett, 1991).

Two of these proteins, SP-B and SP-C, are water-insoluble and are isolated with the surfactant lipids during extraction with organic solvents (Whitsett et al., 1986; Yu & Possmayer, 1986; Curstedt et al., 1987; Hawgood et al., 1987). They seem to play a crucial role in the rapid adsorption of lipids at the air/liquid interface (Suzuki et al., 1986; Takahashi & Fujiwara, 1986; Curstedt et al., 1987; Hawgood et al., 1987; Oosterlaken-Dijksterhuis et al., 1991a,b) and to be essential components in preparations used for replacement therapy in infants with respiratory distress syndrome (RDS) (Fujiwara et al., 1980; Enhorning et al., 1985; Hallman et al., 1985; Collaborative European Multicenter Study Group, 1988).

Mature SP-C is a 33–35-residue polypeptide, rich in valine and leucine and highly conserved (Warr et al., 1987; Johansson et al., 1988a,b, 1991a). It has one or two Cys residues, stoichiometrically palmitoylated (Curstedt et al., 1990; Johansson et al., 1991a; Stults et al., 1991). SP-B contains more polar and positively charged residues throughout its 79-residue structure (Glasser et al., 1987; Hawgood et al., 1987; Olafson et al., 1987; Curstedt et al., 1988) and is found as a disulfide-linked homodimer in natural surfactant (Curstedt et al., 1990; Johansson et al., 1991b, 1992).

SP-B and SP-C clearly interact with the surfactant phospholipids. A mixture of these proteins induces destabilization and fusion of PG vesicles (Shiffer et al., 1988). The specific interaction of SP-B with PG in model membranes, demonstrated by fluorescence anisotropy measurements, is likely associated to a concomitant ordering of the membrane bilayer surface (Baatz et al., 1990).

The molecular description of the lipid/protein interaction in pulmonary surfactant should contribute to the understanding of its surface-active properties. Infrared spectroscopy has been shown to be well designed to study the structure of proteins and peptides in a lipid environment (Goormaghtigh et al., 1989, 1990, 1991a,b; Martin et al., 1991). Moreover, attenuated total reflection (ATR) infrared spectroscopy gives quantitative information about the orientation of the protein in a lipid matrix (Cabiaux et al., 1989; Goormaghtigh & Ruysschaert, 1990). The structure and the orientation of the SP-C polypeptide in lipid bilayers studied by IR spectroscopy were recently reported (Pastrana et al., 1991; Vandenbussche et

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[1] Abbreviations: SP-A, SP-B, SP-C, and SP-D, surfactant-associated proteins A, B, C, and D, respectively; DPPC, dipalmitoyl-DL-α-phosphatidylcholine; PG, egg L-α-phosphatidylglycerol; DPPG, dipalmitoyl-DL-α-phosphatidylglycerol; ATR, attenuated total reflection; FTIR, Fourier-transform infrared spectroscopy.

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al., 1992). We here describe the secondary structure and a possible orientation of SP-B in a lipid environment.

EXPERIMENTAL PROCEDURES

Materials. Dipalmitoyl-DL-α-phosphatidylcholine (DPPC), dipalmitoyl-DL-α-phosphatidylglycerol (DPPG), and egg L-α-phosphatidyl-DL-glycerol (PG) were purchased from Sigma Chemical Co. (U.S.A.). The homogeneity of both lipids was checked by thin-layer chromatography. The glassware was washed with sulfochromic acid.

Purification of Native SP-B. SP-B was isolated from porcine pulmonary tissue (Curstedt et al., 1987). The purity and integrity of the polypeptide were checked by time-of-flight plasma-desorption mass spectrometry. Protein concentration was determined by amino acid analysis.

Insertion of SP-B in a Lipid Matrix. SP-B in chloroform/methanol (1:1 v/v) was added to DPPC/PG (7:3 w/w) in chloroform or to DPPG in chloroform/methanol (1:1 v/v), in a protein/lipid molar ratio of 1:240 or 1:100 for DPPC/PG and 1:240 for DPPG. The organic solvent was evaporated under a stream of N_2. Traces of solvent were completely removed by drying the samples under vacuum overnight. After hydration at 45 °C in 20 mM Tris-HCl and 150 mM NaCl, pH 7.3, at a lipid concentration of 2 mg/mL, the sample was vortexed with small glass beads. The resulting suspension is likely constituted of SP-B associated to multilamellar vesicles. The unbound constituents were eliminated by density gradient centrifugation. Samples in 40% sucrose were placed at the bottom of a continuous sucrose gradient ranging over 2–30% (w/v) and were centrifuged at 100000*g* for 15 h in a Beckman SW 60 rotor at 4 °C. The lipid profile in the gradient was determined with an enzymatic colorimetric test specific for choline (Boehringer Mannheim GmbH) or by measuring the turbidity at 405 nm for DPPG. The fractions containing SP-B associated to the vesicles were pooled and dialyzed against distilled water for 72 h (Microdialyser, System 500, Pierce, U.S.A.).

Attenuated Total Reflection Fourier-Transform Infrared Spectroscopy (ATR-FTIR). ATR-FTIR spectra were recorded at room temperature on a Perkin-Elmer 1720X FTIR spectrophotometer equipped with a liquid nitrogen-cooled mercury cadmium telluride (MCT) detector at a nominal resolution of 4 cm^{-1}, encoded every 1 cm^{-1}. The spectrophotometer was continuously purged with air dried on a silica gel column (5 × 130 cm). The internal reflection element was a germanium plate (50 × 20 × 2 mm, Harrick EJ2121) with an aperture angle of 45°, yielding 25 internal reflections. For each spectrum, 128 scan cycles were averaged; in each cycle, the sample spectra were ratioed against the background spectra of a clean germanium plate, using a shuttle to move the sample or reference into the beam. For polarization experiments, a Perkin-Elmer gold wire grid polarizer was positioned before the sample and reference.

Preparation of SP-B Films and of Oriented Multilayers for ATR-FTIR Spectroscopy. The ATR germanium plates were washed by sonication, rinsed with distilled water, and placed for 5 min in a Plasma Cleaner PDC-23G (Harrick), essential to obtain a clean, hydrophilic germanium surface.

Oriented multilayers were formed by slow evaporation at room temperature under a N_2 stream of the suspension of SP-B associated to lipid vesicles on one side of a germanium plate (Fringeli & Günthard, 1981; Goormaghtigh et al., 1990). The efficiency was checked by measuring the dichroic ratio (see below) for the $\gamma_w(CH_2)$ vibration at 1200 cm^{-1}, and a ratio higher than 3 indicates that the average tilt between the hydrocarbon chains and a normal to the germanium surface is smaller than 25°. Information about oriented multilayers has been described (Asher & Pershan, 1979; Clark et al., 1980; MacNaughtan et al., 1985; Tiede, 1985).

Films of pure SP-B were obtained following the same procedure starting from the protein solution in chloroform/methanol (1:1 v/v).

The ATR plate was sealed in a universal sample holder (Perkin-Elmer 188-0354). The samples were deuterated by flushing with D_2O-saturated N_2 at room temperature until only the readily accessible peptide bonds are exchanged. Importantly, the "random structure" shifts from about 1655 cm^{-1} to about 1640 cm^{-1} upon H/D exchange, allowing the α-helix to be differentiated from the "random structure" (Cortijo et al., 1982; Rothschild et al., 1982). Protons belonging to ordered structures such as α-helices, and especially transmembrane α-helices, are not expected to exchange completely at these conditions (Downer et al., 1986). The fraction of the amide group which remains unexchanged is evaluated by the ratio S_D/S_H, where S_D and S_H are the amide II (1500–1580 cm^{-1}) to amide I (1600–1700 cm^{-1}) area ratios for the deuterated and nondeuterated spectra.

Secondary Structure Estimation of SP-B. The ν(C=O) vibrations of peptide bond groups implicated in different secondary structures occur at wavenumbers between 1600 and 1700 cm^{-1} (Byler & Susi, 1986; Goormaghtigh et al., 1990). These components do not clearly appear in the original amide I infrared spectra. A Fourier self-deconvolution applied on the spectra allows localization of the different components of the amide I′ band (the prime indicates that an H/D exchange has been performed). The self-deconvolution was carried out using a Lorentzian line shape for the deconvolution and a Gaussian line shape for the apodization (Kauppinen et al., 1981). The percentages of the different secondary structures were quantified by a least-squares iterative curve-fitting allowing us to fit Lorentzian line shapes to the amide I′ region of the spectra. A straight base line passing through the ordinates at 1600 and 1700 cm^{-1} was subtracted. A first curve-fitting was realized on spectra deconvoluted with K = 1.8 [K is defined as the ratio of the full width at half-height (FWHH) of the deconvoluting Lorentzian to the FWHH of the Gaussian used for apodization (Kauppinen et al., 1981)]. The number and the position of the bands to be fitted are determined unequivocally by self-deconvolution (Susi & Byler, 1986; Surewicz et al., 1987a–c; Yang et al., 1987). The maxima of the Lorentzian used for the curve-fitting were aligned on the component maxima revealed by the self-deconvolution. Intensities were calculated as two-thirds of the spectrum intensity at the corresponding frequency, and the FWHH was adapted to the extent of deconvolution applied (Goormaghtigh et al., 1990). The position, intensity, and FWHH of each Lorentzian constitute the initial set of input parameters. In order to avoid artifacts from the deconvolution, the secondary structures were evaluated with the undeconvolved spectra, using the initial set of input parameters. During the iterative curve-fitting, none of the input parameters were kept constant. Similar procedures are reported by other authors (Susi & Byler, 1986; Surewicz et al., 1987a–c; Yang et al., 1987; Surewicz & Mantsch, 1988).

The proportion of particular structures was calculated from the sum of the areas of all the fitted Lorentzian bands having maxima in a particular frequency domain divided by the area of all the Lorentzian bands with maxima between 1689 and 1615 cm^{-1}. The frequency domains for the different secondary structures are as follows: α-helix, 1647.5–1661 cm^{-1}; β-sheet:

1615–1637.5 and 1682–1689 cm^{-1}; "turns", 1661–1682 cm^{-1}; "random", 1637.5–1644.5 cm^{-1}. The frequency limits for each structure were first assigned according to the data determined theoretically (Krimm & Bandekar, 1986) or experimentally (Byler & Susi, 1986). These limits have been slightly adjusted to obtain a good agreement between the proportion of each structure determined by IR-ATR and X-ray crystallographic data (Levitt & Greer, 1977) for a set of purified proteins (Goormaghtigh et al., 1990).

It is important to notice that the same set of initial input parameters was used to evaluate the secondary structure of the isolated SP-B and of the protein associated to lipid vesicles. Prior to deconvolution and curve-fitting, the spectral contribution of lipids, when present, was subtracted.

Orientation Determination. The spectra were recorded with parallel (0°) and perpendicular (90°) polarized incident light, with respect to the ATR plate. Polarization was expressed as the dichroic ratio $R_{atr} = A^{90°}/A^{0°}$. The mean angle between the C=O bond and a normal to the ATR plate surface was then calculated from R_{atr} (Fringeli & Günthard, 1981; Goormaghtigh & Ruysschaert, 1990; Goormaghtigh et al., 1990). In an α-helix, the main transition dipole moment [ν(C=O)] lies closely parallel to the helix axis, while in an antiparallel β-sheet the polarization is opposite, i.e., predominantly perpendicular to the fiber axis (Gremlich et al., 1983). A 27° deviation between the α-helix axis and the C=O dipole moment (Rotschild & Clark, 1979) was taken into account (Goormaghtigh et al., 1990).

Determination of Protein/Lipid Ratios by ATR Spectroscopy. A calibration curve was obtained by recording the nondeuterated spectra of SP-B associated to DPPC/PG (7:3 w/w) vesicles for different protein/lipid mass ratio ranging from 4:100 to 80:100 with 100 μg of lipid in each sample. The area of the lipid [ν(C=O)] band was measured after drawing a straight base line between 1700 and 1780 cm^{-1}. Both limits were slightly adjusted (within 10 cm^{-1}) in order to be located at a frequency corresponding to a minimum of the spectrum. The area of the amide I was evaluated between 1600 and 1700 cm^{-1} using the same procedure. The logarithm of the ν(C=O) amide I/ν(C=O) lipid area ratio was plotted against that of the protein/lipid mass ratio.

RESULTS

The secondary structure of pure SP-B was studied. The protein was deposited on the surface of the germanium plate, from a chloroform/methanol (1:1 v/v) solution. The corresponding spectra from three measurements are illustrated in Figure 1. No significant differences were observed in the amide I shape (1600–1700 cm^{-1}). After hydrogen/deuterium exchange, the spectra are characterized by a maximum at 1654 cm^{-1} and a shoulder near 1629 cm^{-1} associated to α-helical and β-sheet structures, respectively (Figure 2). These structures were quantified from the amide I area occupied by the corresponding Lorentzian bands resulting from the curve-fitting (Figure 2, Table I). The data confirm the presence of a high α-helical content in porcine SP-B. No changes in the secondary structure were observed during the kinetics of deuteration.

SP-B was reconstituted in multilamellar vesicles of DPPC/PG (7:3 w/w) or DPPG at initial protein/lipid molar ratios of 1:240 or 1:100 for DPPC/PG and 1:240 for DPPG (assuming a dimeric protein; $M_r \simeq 17\,400$). The proteoliposomes were separated from unbound material on a continuous sucrose gradient, and the efficiency of reconstitution was evaluated from an ATR calibration curve (Goormaghtigh et al., 1990), specific to SP-B (Figure 3). This procedure gives quick and accurate calculation of the protein/lipid ratio without problematic concentration determinations of small amounts of this hydrophobic protein. The final SP-B/lipid molar ratios in the vesicles isolated on the sucrose gradient were 1:291 and 1:111 for SP-B/DPPC/PG and 1:250 for SP-B/DPPG.

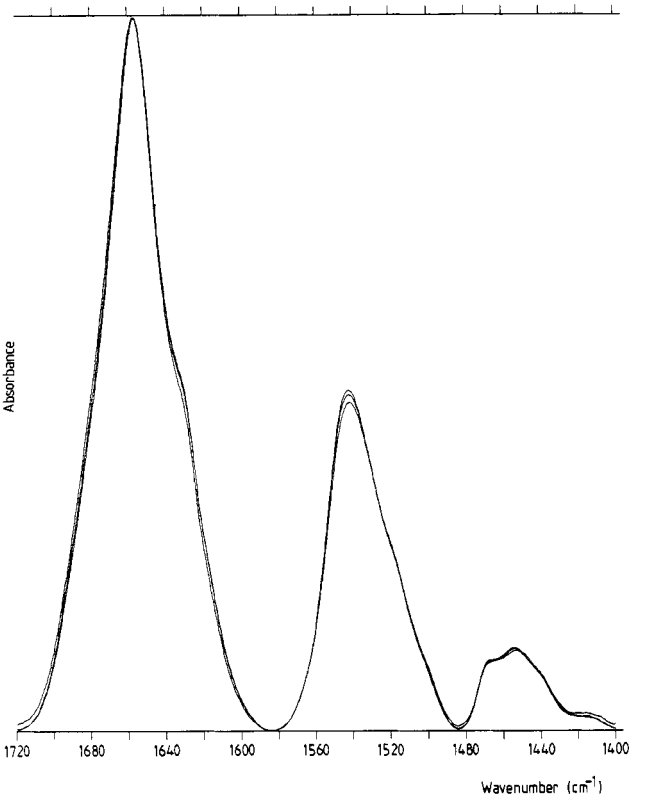

FIGURE 1: ATR-FTIR spectra of three experiments with porcine SP-B. A base-line line was drawn between 1400 and 1720 cm^{-1}. The spectra were rescaled to the same amplitude.

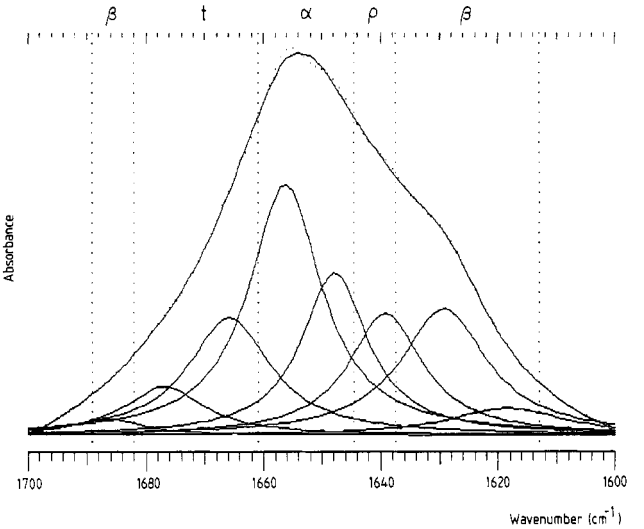

FIGURE 2: Example of curve-fitting for the amide I' band of porcine SP-B deuterated during 6 h. The sum of the Lorentzian line shapes obtained is represented by the dotted spectrum. The vertical dotted lines limit the regions associated to the different secondary structures: α, α-helix; β, β-sheet; t, turns; and ρ, "random".

The shape of the amide I band in the ATR spectra of the two reconstituted samples (Figure 4) was similar to that of the isolated protein (Figure 2), as confirmed by curve-fitting data (Table II). The small discrepancy of 6–8% (Tables I

Table I: Estimation of the Secondary Structure of Porcine SP-B for Different Times of H/D Exchange[a]

deuteration time (h)	α-helix (%)	β-sheet (%)	turns (%)	random (%)
1	44	23	21	12
2	44	22	21	13
3	45	21	21	13
4	44	22	21	13
5	45	22	20	13
6	45	22	20	13

[a] The values represent the mean of the data obtained for three separate experiments (SEM ≤ 1%).

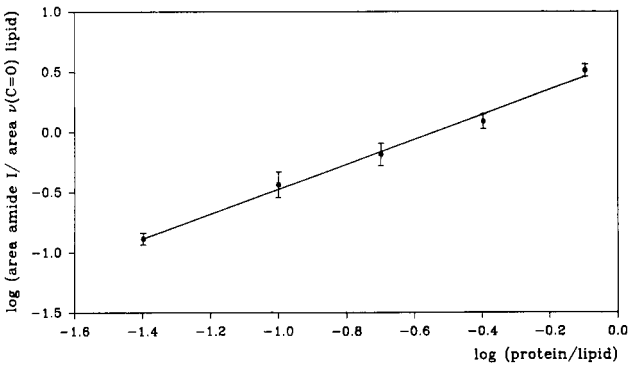

FIGURE 3: Calibration curve for the concentration determination of porcine SP-B associated to DPPC/PG (7:3 w/w) vesicles. Log of the amide I area/lipid ν(C=O) area ratio is plotted as a function of log ([protein]/[lipid]). The values are reported as the mean calculated for three separate determinations.

and II) is not representative of a modification of secondary structure, since the standard deviation of secondary structure estimated by infrared spectroscopy versus X-ray structures is of the order of 8–10% (Goormaghtigh et al., 1990). The secondary structure of SP-B is not influenced significantly by the interactions with the lipid bilayers.

The hydrogen/deuterium exchange of peptide bond protons was evaluated from the evolution of the amide II to amide I area ratios (Figure 5). After 6 h in a D_2O-saturated N_2 stream, about 60% of the polypeptide chain still remained unexchanged. This slow exchange is closely related to the hydrophobic nature of the protein, or to strong hydrogen bonding in ordered structures, like α-helices. The hydrogen/deuterium exchange is slightly more important for SP-B associated to the lipid membrane than for the isolated protein. It appears that when SP-B interacts with the lipid bilayers, the accessibility to the peptide bonds capable of deuterium exchange seems to increase. However, we will avoid associating this small difference to a different folding of the protein after interaction with the membrane.

The organization and the conformation of the lipid molecules were studied in the SP-B/lipid complex. The dichroic ratios of the $\gamma_w(CH_2)$ vibration at 1200 cm^{-1} are 4.0 ± 0.2 for a film of pure DPPC/PG and 4.2 ± 0.2 and 3.5 ± 0.5 for the SP-B/DPPC/PG complex with a protein/lipid molar ratio of 1:291 and 1:111, respectively. The dichroic ratios are 2.3 ± 0.1 for a film of pure DPPG and 2.9 ± 0.2 for the SP-B/DPPG complex. From these values, the corresponding mean angles between the axis of the hydrocarbon chains and a normal to the ATR plate surface were calculated for an order parameter equal to 1. The DPPC/PG acyl chains make a maximum tilt of 20° with respect to the normal to the germanium surface whereas angles of 19° and 22° are obtained for the SP-B/DPPC/PG complex with protein/lipid molar

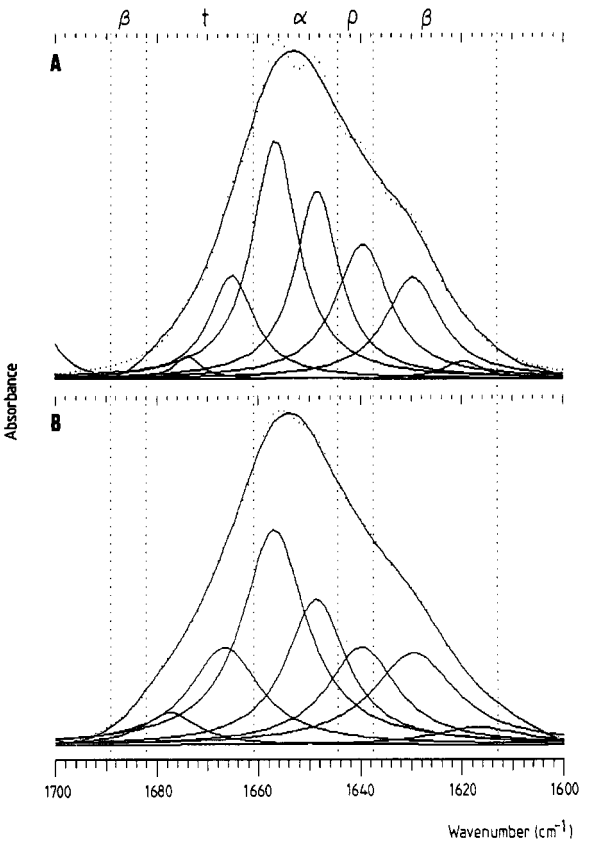

FIGURE 4: Curve-fitting for the amide I′ band of porcine SP-B associated to DPPC/PG (7:3 w/w) vesicles deuterated during 6 h. (A) SP-B/lipid (1:291 mol/mol); (B) SP-B/lipid (1:111 mol/mol). The initial set of parameters of the Lorentzian bands used for the least-squares curve-fitting are the same as in Figure 2. The sum of the Lorentzian line shapes obtained is represented by the dotted spectrum. The vertical dotted lines limit the regions associated to the different secondary structures.

Table II: Estimation of the Secondary Structure of Porcine SP-B Associated to Lipid Vesicles[a]

deuteration time (h)	α-helix (%)			β-sheet (%)			turns (%)			random (%)		
	A	B	C	A	B	C	A	B	C	A	B	C
1	52	46	42	17	20	22	15	20	24	16	14	12
2	52	48	43	17	20	22	15	17	23	17	15	11
3	52	48	43	17	19	22	14	17	23	17	15	12
4	52	48	44	15	20	22	14	16	22	19	16	11
5	50	48	44	17	20	22	14	16	22	19	16	11

[a] (A) SP-B/DPPC/PG (1:291 mol/mol); (B) SP-B/DPPC/PG (1:111 mol/mol); (C) SP-B/DPPG (1:250 mol/mol). The data are representative of two separate experiments (SEM ≤ 2%).

ratios of 1:291 and 1:111, respectively. Angles of 29° and 25° are found for the acyl chain orientation in the film of DPPG and of the SP-B/DPPG complex, respectively. The association of SP-B with the lipid bilayers does not disturb the mean orientation of the DPPC or DPPG molecules.

The dichroic spectra 90°–0° (Figure 6) provide another way to confirm the formation of oriented multilayers. As the transition dipole moment of the $\gamma_w(CH_2)$ peak at 1200 cm^{-1} is parallel to the all-trans DPPC hydrocarbon chains, a strong 90° polarization of this absorption band and, therefore, a positive peak in the dichroic spectra 90°–0° reveal an orientation of the hydrocarbon chains of DPPC nearly normal to the ATR element surface. This orientation is confirmed in the dichroic spectra by the negative peak observed near 1468 cm^{-1} corresponding to the $\delta(CH_2)$ peak, whose transition dipole moment is perpendicular to the axis of the lipid acyl

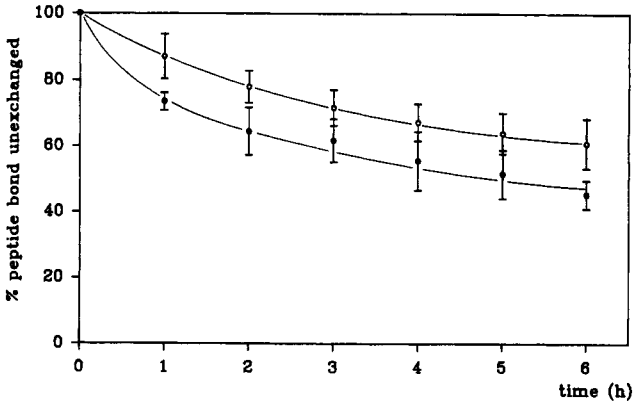

FIGURE 5: Evaluation of the proportion of peptide bonds unexchanged during hydrogen/deuterium exchange. (○) Pure SP-B polypeptide (n = 3); (●) SP-B/DPPC/PG complex (n = 2).

chains (Figure 6). The same result was obtained for the SP-B/DPPG complex (data not shown).

Further information can be gained from the dichroic spectra. In the gel state, the hydrocarbon chain in the α-position of DPPC or DPPG is all-trans from the ester group down to the methyl group. This conformation allows a resonance to occur between the ester group and the methylene groups of the chain, giving rise to the so-called $\gamma_w(CH_2)$ progression between 1200 and 1350 cm^{-1}. The number of peaks composing the progression is equal to $n/2$ in an all-trans acyl chain, where n is the number of methylenes in the chain (Meiklejohn et al., 1957). Deviation of the hydrocarbon chain conformation from all-trans produces modifications in the wagging progression (Fringeli & Günthard, 1981). Figure 6 shows that the number of bands in the progression is conserved for the SP-B/DPPC/PG vesicles (peaks at 1200, 1221, 1246, 1266, 1286, 1309, and 1330 cm^{-1}) and that no modification of the shape or intensity of these bands is observed. No modification of the $\gamma_w(CH_2)$ progression was observed for the SP-B/DPPG complex (data not shown). As a conformational modification of the acyl chains would be associated to a perturbation of this progression, our data indicate the absence of conformational change in the DPPC or DPPG molecules upon interaction with the SP-B protein.

Interaction of SP-B with the phospholipid headgroup is suggested by the analysis of the bands associated to PO_2^- stretching between 1050 and 1250 cm^{-1} (Fringeli & Günthard, 1981; Casal & Mantsch, 1984). Upon association of SP-B with DPPG, the symmetric PO_2^- stretching peak shifts from 1094 to 1110 cm^{-1} and is broadened (spectra not shown). As the $\gamma_w(CH_2)$ progression is superimposed to the antisymmetric PO_2^- stretching (around 1225 cm^{-1}), a modification of the latter is difficult to visualize.

Finally, the curve-fitting applied to the polarized spectra in the amide I′ region allows the calculation of the dichroic ratios for the α-helical structure of SP-B associated to the liposomes (Table III). From this, it is possible to calculate a corresponding angle between the long axis of the α-helix and a normal to the germanium plate. Splitting of the α-helix domain into two resolved bands, α_1 and α_2, during the curve-fitting step is also noted. Both components can be associated to specific orientations of an α-helix population (Goormaghtigh et al., 1991a), but for SP-B the splitting probably arises from the procedure since the dichroic ratios are similar for both components (cf. below) (Goormagtigh et al., 1990). The dichroic ratio will be discussed in the next section.

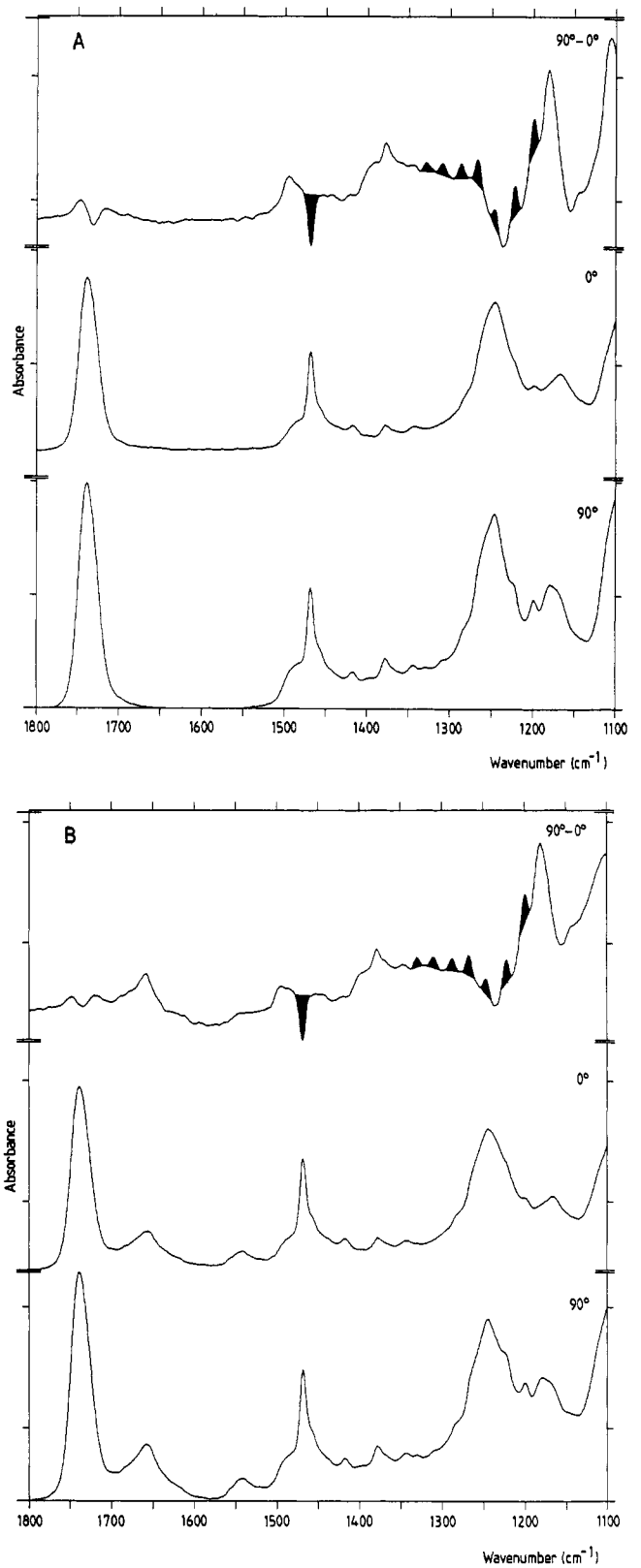

FIGURE 6: Dichroic spectra of (A) DPPC/PG mixture (7:3 w/w) and (B) SP-B/lipid complex (1:291 mol/mol). The dichroic spectrum obtained by subtracting the 90°–0° spectra is plotted on the top of each figure, expanded 4-fold in the ordinate direction.

DISCUSSION

The secondary structure of porcine SP-B, free or associated to a lipid matrix, was deduced from the corresponding Fourier-transform infrared spectra. Nearly half of porcine SP-B (45%) adopts an α-helical structure. The β-sheet components account

Table III: Dichroic Ratios of the α-Helical Components of the SP-B/Lipid Complex[a]

	dichroic ratio		
	SP-B/DPPC/PG (1:291 mol/mol)	SP-B/DPPC/PG (1:111 mol/mol)	SP-B/DPPG (1:250 mol/mol)
α_1 component (1657 cm^{-1})	1.57	1.79	1.57
α_2 component (1649 cm^{-1})	1.61	1.66	1.55

[a] These data are representative of two separate experiments (SEM ≤ 0.1).

for about 22%. This result confirms the presence of a high content of α-helical structure in SP-B revealed by circular dichroism of a pure protein monolayer collected at the air/water interface (Oosterlaken-Dijksterhuis et al., 1991b). The secondary structure of SP-B is conserved when the protein is associated to a DPPC/PG (7:3 w/w) or a DPPG matrix. Multilamellar liposomes were chosen because their bilayer organization strongly resembles the structure of the lamellar bodies.

The α-helical content estimated by IR spectroscopy for SP-B is consistent with the presence of potential amphipathic helix segments [cf. Glasser et al. (1987), Takahashi et al. (1990), and Bruni et al. (1991)]. Two synthetic peptides covering residues 1–25 and 49–66, respectively, could approximately fit an amphipathic model with a high proportion of α-helical structure in the interfacial environment of detergent micelles or in a lipid environment (Bruni et al., 1991; Fan et al., 1991).

The absence of secondary structure modification upon association of the protein with the lipid bilayers suggests that SP-B does not possess important membrane domains or that these anchoring domains adopt an α-helical structure already in the isolated protein. Indeed, the insertion of a membrane or a transmembrane protein into a lipid core is generally accompanied by a concomitant increase of its α-helical content (Martin et al., 1991). Thus an increase of about 15% of the α-helical content was demonstrated for porcine SP-C inserted in DPPC/PG vesicles (Vandenbussche et al., 1992). This structural modification was, to a lesser extent, also observed for calf SP-C associated to DPPC/DPPG liposomes (Pastrana et al., 1991).

The secondary structure of SP-B was not influenced by the protein/lipid ratio. Although this ratio is higher than the physiological concentration, this result brings arguments in favor of a reconstituted model composed of SP-B associated or inserted in the lipid matrix instead of SP-B aggregated at the membrane surface. In the latter case, the proportion of β-sheet structure would be dependent on protein concentration. Furthermore, it seems unlikely that aggregates of protein remain associated to the lipid vesicles after the gradient density separation. For a high SP-C/lipid ratio, a broad band in the β-structure domain of the IR spectrum, likely associated to protein aggregation, was reported (Pastrana et al., 1991).

The protein does not affect the organization and orientation of the lipid molecules in the membrane. In the case of the DPPC/PG bilayer, this conclusion cannot be extended to the whole lipid matrix as the parameters presented are only valid for all-trans chains. This restriction prevents the measurement of the PG component properties. Alternatively, our result could be explained by the presence of a lateral phase separation induced by SP-B. In this case, the insertion of the protein in a fluid phase of phosphatidylglycerol would not strongly interfere with the DPPC hydrocarbon chains. The presence of small segregated domains of PG molecules is plausible, and different studies bring arguments to this hypothesis. Raman spectroscopy of a synthetic peptide representing residues 59–81 of human SP-B showed that bilayer stability of DPPC/PG liposomes increased (Vincent et al., 1991). An ordering of the surface of DPPC/DPPG membrane with SP-B was also reported by fluorescence anisotropy (Baatz et al., 1990). Interaction of the positively charged residues along SP-B and multiple PG polar headgroups would reduce the lipid lateral diffusion, leading to patches enriched in PG molecules. However, we have demonstrated that the organization of the DPPG hydrocarbon chains was not affected upon association with SP-B. Moreover, no alteration of the lipid ordering in the hydrophobic domain of DPPC/DPPG liposomes was detected by fluorescence anisotropy in the presence of SP-B (Baatz et al., 1990), suggesting that the protein is positioned close to the lipid/water interface. Effectively, it is improbable from the complex structure of SP-B with its three intramolecular disulfide bonds (Johansson et al., 1991b, 1992) that the protein can be deeply embedded in the lipid matrix without induction of important perturbations of the hydrocarbon chain order.

A qualitative analysis of the dichroic spectrum (Figure 6) reveals a positive peak both at 1654 cm^{-1} in the α-helix domain and at 1200 cm^{-1} associated to the $\gamma_w(CH_2)$ of the lipids, showing that both vibrations have their dipole moments mainly oriented in a close parallel direction. Evaluation of the mean angle between the long axis of the α-helix structure and a normal to the germanium plate is obtained from the dichroic ratios (Table III). Assuming a single α-helix containing 35–40 residues, the maximum tilt between the α-helical long axis and a normal to the ATR element surface would be 32°, assuming a parameter of order equal to 1, with the α-helix deeply inserted in the bilayers. The primary sequence of successive basic and hydrophobic regions and the complex kringle tertiary structure (Johansson et al., 1991b) make the presence of a single α-helix not realistic. A more realistic model of the orientation of SP-B with respect to the lipid interface would be that a fraction of the helices lie parallel to the membrane surface and another fraction is slightly embedded in the bilayers, parallel to the acyl chains. These two situations represent the orientation of α-helices commonly found in biological membranes.

The splitting of the α-helical domain into two components in the curve-fitting analysis arises from an arbitrary cut in a complex α-helix contribution to amide I′ (Goormaghtigh et al., 1990), whose exact frequency depends on the length of the helix or on the position of the amino acid residues in this structure (in the middle or at the end of the helix) (Chirgadze et al., 1976; Nevskaya & Chirgadze, 1976). If more than one helical segment is present in the protein, we assume that they all contribute to the two components resolved by the curve-fitting procedure.

The fraction of helices parallel to the acyl chains and to the membrane surface can be computed from the dichroic ratio data on the basis of the two equations (Goormaghtigh & Ruysschaert, 1990):

$$A = (A^{90°} + 2A^{0°})/3 \quad (1)$$

$$R = A^{90°}/A^{0°} \quad (2)$$

where A, $A^{0°}$, and $A^{90°}$ are the absorbances measured from the spectrum and the two orthogonal polarized spectra and R is the dichroic ratio. From eqs 1 and 2 we calculate that

$$A^{0°} = 3A/(R + 2) \quad (3)$$

$$A^{90°} = 3AR/(R + 2) \quad (4)$$

In our SP-B/lipid systems, we have found that the maximum tilt between the lipid hydrocarbon chains and a normal to the ATR plate was around 20°. An α-helix parallel to the acyl chains would have a dichroic ratio of 1.977 [see Goormaghtigh and Ruysschaert (1990)]. Conversely, an α-helix parallel to the membrane would have a dichroic ratio of 0.980. The $A^{0°}$ and $A^{90°}$ components for both orientations are calculated by injecting the respective R values in eqs 3 and 4. The dichroic ratio is described by the following equation resulting from a linear combination of the two orientations:

$$R_{total} = \frac{A^{90°}_{total}}{A^{0°}_{total}} = \frac{XA_1^{90°} + YA_2^{90°}}{XA_1^{0°} + YA_2^{0°}} \quad (5)$$

where X and Y are the fractions of α-helices parallel to the acyl chains and parallel to the membrane surface, respectively, and the indices 1 and 2 are associated to the absorbance of the orthogonal polarized spectra for orientations parallel to the acyl chains and parallel to the lipid/water interface, respectively. By plotting the curve describe by eq 5, we found that the dichroic ratios obtained for the different α-helical components (Table III) indicate that 60–80% of the α-helices are parallel to the acyl chains. This proportion could represent several stretches of hydrophobic residues slightly anchored near the membrane interface and short enough not to disturb the acyl chain organization. The existence of these hydrophobic stretches was postulated from studies of the interaction of a lipid matrix with simplified peptides containing hydrophilic and hydrophobic residues mimicking the SP-B polypeptide chains (Cochrane & Revak, 1991; Vincent et al., 1991). The other part of the α-helix content would be associated to charged residues and oriented parallel to the lipid/water interface. This orientation would fit the amphipathic helix model (Glasser et al., 1987; Takahashi et al., 1990; Bruni et al., 1991). Argument for electrostatic interactions between the charged residues of SP-B and the phospholipid head group comes from the spectral changes of phosphate vibrations in the SP-B/DPPG complex. The symmetric and antisymmetric PO_2^- stretching is sensitive to dehydration of the phosphate group, which is characterized particularly by a shift to higher frequencies of the $\nu_{as}(PO_2^-)$ (Fringeli & Günthard, 1981; Dluhy et al., 1983; Casal et al., 1987). In the SP-B/DPPG complex, we measured a shfit of the $\nu_s(PO_2^-)$ to higher wavenumbers and a broadening of this band. This modification, very likely, denotes an interaction of the surfactant protein with lipid polar heads at the membrane surface. The combination of electrostatic and hydrophobic interactions would enable a stable association of the protein at the surface of the lipid membrane.

Regarding the dichroic spectrum (Figure 6), a positive deviation of the amide I′ band should be theoretically accompanied with a negative deviation of the amide II′ band (localized near 1545 cm^{-1}). The dipole moments of the amide I and II bands are usually considered as perpendicular to each other, as the first is mainly due to C=O stretching (80% of the potential energy of the vibration) and the second arises mainly from a N–H bending. In fact, the situation is more complex as the amide II is made of $\simeq$60% δ(N–H), $\simeq$40% ν(C–N), and some ν(N–H). An uncertainty in the angle between the dipole moment and the C=O direction results from the complexity of this absorption band. The abnormal comportment observed for the amide II′ band in this study can therefore not be interpreted as was already observed for other proteins (Rothschild & Clark, 1979; Nabedryk et al., 1988; Goormaghtigh et al., 1991a).

In summary, this paper shows that native SP-B adopts mainly an α-helical structure. This structure is not influenced by the interaction of the protein with a lipid membrane, and unlike the surfactant-associated protein SP-C (Pastrana et al., 1991; Vandenbussche et al., 1992), the presence of a membrane-spanning segment is not necessary to maintain SP-B at the surface of the model membrane.

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