Depth Profiles of Pulmonary Surfactant Protein B in Phosphatidylcholine Bilayers, Studied by Fluorescence and Electron Spin Resonance Spectroscopy†

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Received June 27, 1997; Revised Manuscript Received April 24, 1998

ABSTRACT: Pulmonary surfactant-associated protein B (SP-B) has been isolated from porcine lungs and reconstituted in bilayers of dipalmitoylphosphatidylcholine (DPPC) or egg yolk phosphatidylcholine (PC) to characterize the extent of insertion of the protein into phospholipid bilayers. The parameters for the interaction of SP-B with DPPC or PC using different reconstitution protocols have been estimated from the changes induced in the fluorescence emission spectrum of the single protein tryptophan. All the different reconstituted SP-B−phospholipid preparations studied had similar K_d values for the binding of the protein to the lipids, on the order of a few micromolar. The depth of penetration of SP-B into phospholipid bilayers has been estimated by the parallax method, which compares the relative efficiencies of quenching of the protein fluorescence by a shallow or a deeper spin-labeled phospholipid probe. SP-B tryptophan was found to be located 10−13 Å from the center of bilayers, which is consistent with a superficial location of SP-B in phosphatidylcholine membranes. Parallax experiments, as well as resonance energy transfer from SP-B tryptophan to an acceptor probe located in the center of the bilayer, indicate that there are significant differences in the extent of insertion of the protein, depending on the method of reconstitution. SP-B reconstituted from lipid/protein mixtures in organic solvents is inserted more deeply in PC or DPPC bilayers than the protein reconstituted by addition to preformed phospholipid vesicles. These differences in the extent of insertion lead to qualitative and quantitative differences in the effect of the protein on the mobility of the phospholipid acyl chains, as studied by spin-label electron spin resonance (ESR) spectroscopy, and could represent different functional stages in the surfactant cycle.

Pulmonary surfactant is essential for facilitating the respiratory mechanics of the mammalian lung. Its biophysical activity is critically dependent on the presence of specific surfactant-associated proteins (*1*−*3*). Surfactant protein - B (SP-B)[1] is a small polypeptide of 79 residues, very hydrophobic in nature, which facilitates the transport of tensoactive surfactant phospholipids through the aqueous lining layer of the alveoli, to adsorb and spread as a phospholipid monomolecular layer at the air−liquid interface (*4*−*6*). SP-B is isolated from alveolar lavage in a disulfide-linked dimeric form. Each monomer has itself three further intramolecular disulfide bridges. Theoretical and experimental studies on the structure of SP-B have suggested that the protein possesses several amphipathic α-helical segments that could be considered important determinants for interaction with lipids (*7*). However, although circular dichroism (*8*, *9*) and infrared studies (*10*) have provided data on the secondary structure of SP-B that support this concept, details on the three-dimensional structure of the protein in solution or in membrane-like environments are still not available.

Qualitative and quantitative features of surfactant lipid−protein interactions of SP-B reconstituted in surfactant phospholipid bilayers or monolayers have been characterized in some detail by different experimental approaches, including differential scanning calorimetry (*8*, *11*), deuterium NMR (*8*), fluorescence (*12*), electron spin resonance (*13*), Fourier transform infrared spectroscopy (*10*, *14*), and Raman spectroscopy (*15*). In most of the cases, the solubility of SP-B in chloroform/methanol mixtures allowed reconstitution of lipid/protein samples by mixing of lipids and proteins in a chloroform solution and preparing either lipid/protein vesicles by hydrating dried films or solvent-spread monolayers. Using these preparations, it has been proposed that SP-B interacts in the polar headgroup region of the bilayer (*8*, *12*, *15*), with the protein helical axis oriented more or less parallel to the surface of the bilayer or monolayer (*5*, *10*).

† This work has been supported in part by FISSS (96/1290) and Comunidad de Madrid (07B/0016/97). A.C. was the recipient of a Short-Term EMBO Fellowship and is now being supported by a grant from Fundacion Ferrer. Collaboration between German and Spanish teams was supported by a Collaborative Research Grant form NATO (CRG 941176).

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[1] Abbreviations: 5-PCSL, 1-acyl-2-[5-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphocholine; 12-PCSL, 1-acyl-2-[12-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphocholine; ACN, acetonitrile; Chl, chloroform; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; ESR, electron spin resonance; MeOH, methanol; PC, egg yolk phosphatidylcholine; RET, resonance energy transfer; SP-B, surfactant protein B; *t*-COPA, *all*-(*E*)-8,10,12,14,16-octadecapentaenoic acid.

S0006-2960(97)01558-4 CCC: $15.00
Published on Web 06/12/1998

A recent study has reported that a peptide from the N-terminal moiety of SP-B, including an α-helical amphipathic segment, is inserted at the polar headgroup region of the bilayer in several surfactant phospholipid mixtures (*16*). Gordon et al. propose a model for the structure and topography of this peptide in the membrane that is consistent with the previously reported data on the interaction of the whole native protein.

We have shown recently that SP-B is able to interact with phosphatidylcholine bilayers in two different modes, depending on the method for reconstituting the lipid/protein samples (*17*). Reconstitution of SP-B/PC samples from mixtures of lipid and protein in organic solvent produced stable lipid/protein bilayers with efficient surface activity and significant effects of the protein on the thermotropic gel-to-liquid crystalline phase transition of the lipid. These samples had structural and functional properties comparable to those prepared by the well-established technique of hydrating lipid/protein films prepared from evaporation of organic solvent mixtures [used, for instance, by Morrow et al. (*8*)]. On the other hand, interaction of SP-B with preformed phospholipid vesicles caused destabilization and aggregation of the bilayers that were associated with a progressive loss of surface activity of the samples after reconstitution.

The main objective of this work is to characterize these two different forms of SP-B in phosphatidylcholine bilayers. A special focus is on analyzing differences in the extent of insertion of SP-B with phospholipids by using these two methods of reconstitution, because they may be directly relevant to understanding the role of SP-B in surfactant alveolar dynamics.

EXPERIMENTAL PROCEDURES

Materials. Chloroform (Chl), methanol (MeOH), and acetonitrile (ACN) were HPLC-grade solvents from Scharlau (Barcelona, Spain). Sephadex LH-20 and LH-60 chromatography gels were obtained from Pharmacia (Uppsala, Sweden). Dipalmitoylphosphatidylcholine (DPPC) and egg yolk phosphatidylcholine (PC) were from Avanti Polar Lipids (Birmingham, AL). Spin-labeled phosphatidylcholines, with the nitroxide group at different positions of the *sn*-2 acyl chain, were synthesized as described by Marsh and Watts (*18*). The spin-labels were stored at −20 °C in Chl/MeOH (2/1, v/v) solutions at a concentration of 1 mg/mL. The fluorescent fatty acid probe *all*-(*E*)-8,10,12,14,16-octadecapentaenoic acid (*t*-COPA) was a generous gift of Dr. U. Acuña from CSIC (Madrid, Spain) and was stored as an ethanolic solution, saturated with argon, in the dark, at −20 °C. All other reagents and chemicals were from Merck (Darmstadt, Germany).

SP-B Preparation. Surfactant protein SP-B was isolated from minced porcine lungs by an adaptation of the method of Curstedt et al. (*19*), which is described elsewhere (*9*). After isolation, solutions of purified SP-B in Chl/MeOH (2/1, v/v) were stored at −20 °C until they were used. The purity of the protein was routinely checked by SDS−PAGE and quantitated by amino acid analysis.

Reconstitution of SP-B/Phospholipid Samples. First, stock solutions of SP-B were prepared in aqueous mixtures of water-miscible organic solvents (*9*). Two hundred micrograms of SP-B in a Chl/MeOH (2/1, v/v) solution was dried under nitrogen and redissolved in 100 μL of either methanol or acetonitrile/water (60/40, v/v). An aliquot of this solution was used to redetermine the protein concentration. Also, a stock solution of PC or DPPC (50 μg/μL) was prepared in methanol. SP-B was reconstituted in phospholipid bilayers according to two different protocols as described recently (*17*).

In method A, phospholipid vesicles were first formed by injecting the required amount of PC or DPPC (in the region of 5−10 μL of methanolic solution) into 0.7 mL of 50 mM HEPES buffer (pH 7) containing 150 mM NaCl, at a temperature above the phase transition temperature of the phospholipid (25 and 45 °C for PC and DPPC, respectively), with vigorous vortexing. This method has been demonstrated to produce homogeneous uni- or paucilamellar vesicles that are about 100−200 nm in diameter (*17*, *20*). Then, a small volume of SP-B in ACN/water or methanol was injected into the vesicle suspension, also while the mixture was vortexed. All experiments were repeated using both methanolic and acetonitrile/water stock solutions of the protein, three times with each solvent solution, and the results were similar irrespective of the solvent used.

Alternatively, in method B, phospholipid/SP-B vesicles were formed directly by injecting small volumes of organic solutions of protein and phospholipids together into the buffer. Controls were prepared by injecting identical volumes of organic solvent without lipids, without protein, or without both into the buffer.

Intrinsic Fluorescence of SP-B in Phospholipid Bilayers. The parameters governing the association of SP-B with PC or DPPC were determined from the changes in the fluorescence emission spectrum of SP-B upon its interaction with variable amounts of phospholipids. In these experiments, samples containing 10 μg of SP-B were reconstituted with various amounts of PC or DPPC, according to reconstitution methods A and B described above. After reconstitution, the fluorescence emission spectrum of each sample was recorded in a Perkin-Elmer MPF 44-E spectrofluorimeter operating in the ratio mode. Spectra were recorded at 25 and 45 °C for SP-B/PC and SP-B/DPPC samples, respectively, using a scanning speed of 1 nm/s. Samples were in thermostated cells with a 0.2 cm optical path. The slit widths were 7 and 5 nm for the excitation and emission beams, respectively. Spectra were obtained at excitation wavelengths of both 275 and 295 nm. Controls were prepared by injecting the same amounts of phospholipid in organic solution into the buffer, in the absence of SP-B, and were used to correct for the scattering contribution of the lipid vesicles. All the samples had an absorbance at 275 nm of lower than 0.1.

Parallax Fluorescence Quenching Experiments. The depth of penetration of SP-B in phospholipid bilayers was studied by analyzing the relative susceptibility of the SP-B tryptophan fluorescence to quenching by spin probes located at shallow [1-acyl-2-(5-doxylstearoyl)phosphatidylcholine (5-PCSL)], and deeper [1-acyl-2-(12-doxylstearoyl)phosphatidylcholine (12-PCSL)], regions in the bilayers, according to the parallax method proposed by Abrams and London (*21*). Ten micrograms of SP-B was reconstituted in 50 μg of PC or DPPC containing the spin probes in amounts ranging from 0 to 50 mol %. After reconstitution, the fluorescence emission spectra of SP-B were obtained at excitation wavelengths of 275 and 295 nm. The relative fluorescence emission of SP-B at 340 nm in the presence of the quencher,

F/F_0, was analyzed according to the parallax equation (*21*):

$$\ln F/F_0 = -\pi(C/70)(R_0^2 - X^2 - Z^2) \qquad (1)$$

where C is the mole fraction of the spin probe quencher (5-PCSL or 12-PCSL), R_0 is the sphere of critical radius for quenching of tryptophan by the nitroxide group, X is the lateral distance from the fluorophore to the quencher, and Z is the vertical distance from tryptophan to the quencher group. X and R_0 can be considered identical for both quencher probes 5- and 12-PCSL. Therefore, knowing the distance from the nitroxide group to the center of the membrane, we can estimate the distance from the SP-B fluorophore to the center of the bilayer from the simultaneous solution of eq 1 applied to both probes. The distance from the nitroxide group to the center of the bilayer was 12.2 and 5.9 Å for 5- and 12-PCSL, respectively (*22*).

Resonance Energy Transfer (RET) Experiments. Fluorescence energy transfer from SP-B tryptophan to the fluorescent extrinsic probe *t*-COPA was determined as described by Mateo et al. (*23*). RET was measured in samples composed of 10 μg of SP-B reconstituted in 50 μg of phosphatidylcholine bilayers containing different amounts of *t*-COPA, which was added from a 0.144 μM solution of the probe in methanol, according to either of the two reconstitution methods, A and B. The fluorescence emission spectrum of SP-B/PC/*t*-COPA samples was recorded from 290 to 520 nm, with excitation at 275 nm, immediately after reconstitution.

ESR Experiments. Each sample for the ESR experiments was reconstituted by sequential (method A), or simultaneous (method B), injection of 3 μL of a methanolic solution containing 500 μg of DPPC and the selected spin-labeled probe at 1 mol %, and 100 μg of SP-B in 7 μL of methanol, in 150 μL of 50 mM HEPES buffer (pH 7) containing 150 mM NaCl and 5 mM EDTA at 50 °C under vigorous vortexing. The reconstituted material was then pelleted into capillary tubes for ESR spectroscopy by centrifugation at 3000 rpm in a bench centrifuge. The amounts of lipid and protein in the samples were confirmed by lipid and protein quantitation in the material recovered from the capillaries after recording the ESR spectra. A control sample was prepared by injecting 3 μL of DPPC containing 1 mol % spin probe, plus an additional 7 μL of methanol, into 150 μL of buffer.

ESR spectra were recorded as described previously (*13*), on a Varian E-12 Century Line 9 GHz spectrometer equipped with a nitrogen gas flow temperature regulation system. The sealed capillaries (1 mm outer diameter) were contained within 4 mm quartz ESR tubes containing light silicone oil for thermal stability. The temperature was measured with a fine-wire thermocouple positioned in the silicone oil at the top of the microwave cavity. Spectra were digitized on an IBM personal computer with Labmaster interface. Instrumental settings were 10 mW microwave power, 1.25 G modulation amplitude, 100 kHz modulation frequency, 0.25 s time constant, 4 min scan time, 100 G scan range, and 3245 G center field. Several scans, typically three to five, were accumulated to improve the signal-to-noise ratio.

RESULTS

Intrinsic Fluorescence and Binding of SP-B to PC Bilayers. Porcine SP-B has a single tryptophan residue in its sequence (*24*) which can be used as an intrinsic probe in determining the structure and lipid−protein interactions in its local environment, by using fluorescence spectroscopy. The fluorescence emission spectrum of SP-B has been characterized in aqueous organic solvents (*9*, *25*) and reconstituted in phospholipids (*9*, *17*). In all the environments analyzed, the fluorescence spectra show a principal contribution from tryptophan, with two emission peaks that indicate heterogeneity in the environment of the fluorophore, possibly inequivalence within the SP-B dimer. Figure 1a shows the fluorescence emission spectra of SP-B injected in aqueous buffer in the absence and in the presence of different amounts of egg yolk phosphatidylcholine (PC) vesicles. When the protein was injected in the absence of lipids, the emission spectra showed low fluorescence intensity and emission maxima at 332 and 343 nm. If a fixed amount of SP-B was injected in the presence of increasing amounts of phospholipid, the fluorescence emission spectrum of the protein progressively increased in intensity and the emission maxima shifted to shorter wavelengths, 325 and 338 nm. The reduced fluorescence intensity of SP-B when it is injected in the absence of lipids is probably due partly to some self-aggregation of the hydrophobic protein produced after its transfer from the organic to the aqueous environment. The shorter wavelengths of the emission maxima of SP-B injected in lipids, compared with the spectra of SP-B in their absence, suggest that the interaction of SP-B with PC provides a less polar environment for the tryptophan residue in the protein. The influence of phospholipid on the fluorescence intensity appeared to approach a limit at phospholipid concentrations corresponding to lipid:protein ratios of about 4:1 (w/w) (Figure 1b). The change in the fluorescence emission intensity of SP-B allowed an estimate to be made of the amount of lipid-bound and lipid-free forms of the protein at different lipid:protein ratios. The resulting binding curve was fitted in a classical Scatchard plot (Figure 1c) from which the apparent parameters characterizing the

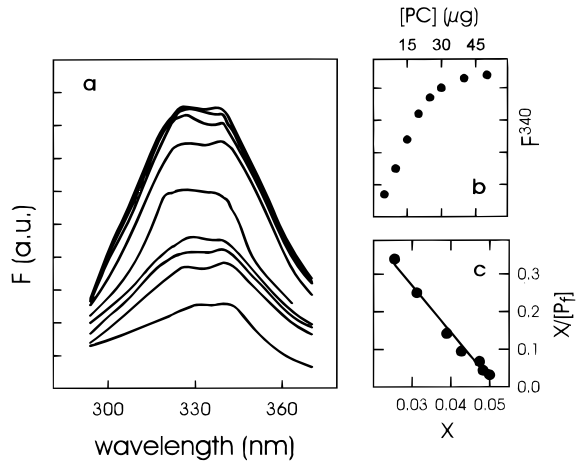

FIGURE 1: Effect of lipid−protein interaction on the fluorescence emission spectrum of SP-B. (a) Fluorescence emission spectra of porcine SP-B (15 μg/mL) injected in buffer 50 mM HEPES (pH 7) containing 150 mM NaCl, in the absence and in the presence of 9, 19, 29, 38, 48, 57, 76, and 95 μM egg yolk phosphatidylcholine (PC) (lower to upper spectra). The excitation wavelength was 275 nm. (b) Dependence of the fluorescence emission intensity of SP-B at 340 nm on the concentration of phospholipid. (c) Scatchard plot of data from the binding isotherm, where X is P_b/L_T, P_b is the concentration of lipid-bound protein at a given phospholipid concentration, L_T, and P_f is the concentration of free protein.

Table 1: Parameters for the Binding of SP-B to Phosphatidylcholine Bilayers

	K_d (μM)[a]	n[a]
PC[b]	4.0 ± 1.0	27 ± 3
PC[c]	2.4 ± 0.7	17 ± 3
DPPC[b]	5.5 ± 1.2	24 ± 3
DPPC[c]	4.5 ± 1.0	15 ± 2

[a] Values are given as means ± SEM ($N = 3$). [b] Injection of SP-B into preformed phospholipid vesicles. [c] Injection of a SP-B/phospholipid organic solvent mixture.

binding of SP-B to PC vesicles could be calculated. The interaction of the protein with the lipid vesicles could be approximated by the equation:

$$P + nL \rightleftarrows P(L)_n$$

where n is the number of lipid molecules with which each protein molecule interacts and K_d is the apparent dissociation constant. A similar approach has been used to estimate the parameters characterizing the lipid−protein interaction with other proteins (*26*−*29*). These parameters were calculated for the interaction of SP-B with both PC and DPPC bilayers by using the two methods of reconstitution described, i.e., sequential (method A) or simultaneous (method B) injection of lipid and protein (Table 1). All preparations gave similar values for K_d, on the order of a few micromolar. The stoichiometry, n, of the lipid−protein interaction was, however, significantly different for the two methods of reconstitution. The number of lipids influenced by a protein molecule was greater when SP-B was added to preformed vesicles than when SP-B was mixed with lipids in organic solution before vesicle formation. This difference can be attributed to a different mode of interaction of SP-B with phospholipid bilayers, depending on the method of reconstitution. The higher stoichiometry of binding of lipid to SP-B in samples prepared by method A compared with those from method B could be a consequence of a different steric accesibility for lipid−protein interaction. SP-B could access, for instance, only the outer leaflet of the membranes when injected into preformed vesicles, whereas it can interact with both sides of the vesicles when they are formed already in the presence of the protein. Thus, the protein in method A would appear to bind to more molecules of lipid for the same number of protein molecules at saturation. Previous studies with electron microscopy and calorimetry showed that the two reconstitution methods used produced lipid−protein complexes with different structural features (*17*).

Parallax Measurements of the SP-B Location. To determine the possible different extents of penetration of SP-B in phospholipid bilayers depending on the reconstitution method, we analyzed the quenching of tryptophan fluorescence in SP-B by nitroxide phospholipid spin probes, using the parallax method first described by Chattopadhyay and London (*30*). The probe having the spin-label at the 5-position of the phospholipid acyl chain had a higher efficiency of quenching SP-B fluorescence than the one with the nitroxide at the 12-acyl carbon (Figure 2). This result is consistent with a relatively superficial location of the SP-B tryptophan in the phospholipid bilayer. The difference in quenching effects of 5- and 12-PCSL was larger with SP-B

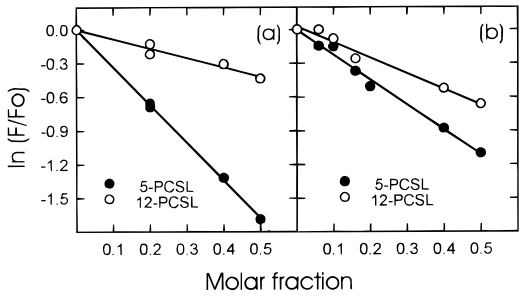

FIGURE 2: Quenching by spin-labeled phospholipids of the fluorescence of SP-B reconstituted in bilayers of phosphatidylcholine. Effect of different molar ratios of spin-labeled PC probes having nitroxide groups either at C5 (5-PCSL) or C12 (12-PCSL) on the fluorescence emission at 340 nm of SP-B reconstituted (a) by injection into preformed PC vesicles or (b) by co-injection of a protein/lipid mixture in organic solvent. The lipid:protein ratio was 5:1 (w/w); excitation and emission wavelengths were 275 and 340 nm, respectively.

Table 2: Distances from the SP-B Tryptophan to the Center of Phosphatidylcholine Bilayers Estimated by the Parallax Method

	distance (Å)[a]		distance (Å)[a]
PC[b]	13.1 ± 0.3	DPPC[b]	11.7 ± 0.3
PC[c]	10.5 ± 0.5	DPPC[c]	9.8 ± 0.2

[a] Values are means ± SEM ($N = 3$). [b] Reconstitution method A, as in Table 1. [c] Reconstitution method B, as in Table 1.

injected in preformed vesicles (Figure 2a) than with SP-B reconstituted by method B (Figure 2b). Table 2 contains the calculated distances of the SP-B tryptophans (one per monomer) from the center of PC or DPPC bilayers. In all the samples, the distances calculated from the SP-B tryptophans to the center of the bilayer are consistent with a peripheral location of the protein in the bilayers. This distance was always slightly greater when the protein was added to preformed vesicles than when lipid and protein were mixed before vesicle formation. These results suggest that the protein approaching from the outside to interact with the lipid penetrates the bilayer to a lesser extent than does the protein mixed with lipid prior to vesicle formation.

Resonance Energy Transfer Experiments. Differences in the extent of penetration of SP-B in phospholipid bilayers were also detected in experiments on resonance energy transfer (RET) from SP-B tryptophan to an acceptor fluorescent probe located in the center of the bilayer. The fluorescent fatty acid *all*-(*E*)-8,10,12,14,16-octadecapentaenoic acid (*t*-COPA) can be incorporated into phospholipid bilayers, providing an extrinsic fluorescent probe which is buried deep within the bilayer and away from the lipid−water interface (*23*). The absorption spectrum of *t*-COPA strongly overlaps with the fluorescence emission spectrum of SP-B reconstituted in phospholipid vesicles. The large spectral overlap allows the transfer of excitation energy from the SP-B tryptophan to the fluorescent fatty acid and determination of the distances between the two probes. The values of R_0 for RET of the tryptophan/*t*-COPA couples, which have been estimated to be on the order of 30−34 Å for proteins in phospholipid bilayers (*23*), make this probe useful in determining the depth of penetration of a given protein into the bilayer. Figure 3 shows the fluorescence emission spectra of SP-B reconstituted in bilayers of PC by the two different methods, A and B, in the absence and in

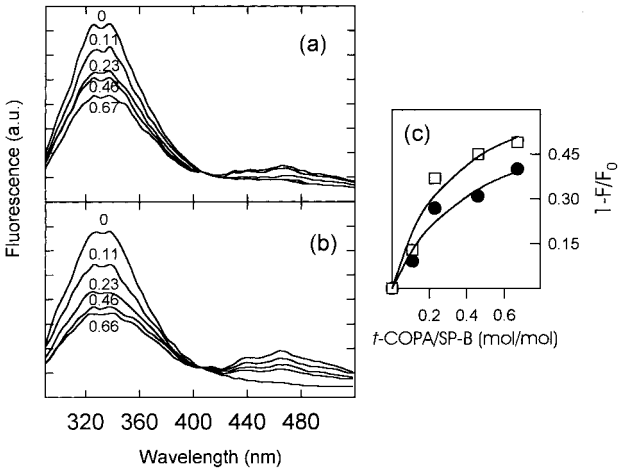

FIGURE 3: Resonance energy transfer (RET) from SP-B to the extrinsic probe *t*-COPA, in bilayers of phosphatidylcholine. Fluorescence emission spectra (excitation wavelength, 275 nm) of SP-B reconstituted in PC bilayers according to method A (a) or B (b), in the absence or in the presence of the fluorescent fatty acid *t*-COPA, at the *t*-COPA:SP-B mole ratios indicated. (c) Dependence of the amount of energy transfer (estimated as $1 - F/F_0$) on the *t*-COPA:SP-B mole ratio, for samples reconstituted by method A (●) or B (□).

the presence of increasing amounts of the acceptor *t*-COPA. For both methods of reconstitution, the fluorescence emission of SP-B was progressively quenched by the presence of *t*-COPA while, simultaneously, the fluorescence of the fatty acid induced by excitation in the SP-B tryptophan band increased (Figure 3a,b). This is an indication of transfer of the excitation energy and therefore a certain proximity between the SP-B tryptophan and the inner core of the bilayer, on the distance scale specified by R_0. The extents of RET at the highest acceptor concentrations in Figure 3c correspond to maximum separations from *t*-COPA that are close to R_0, which is consistent with a superficial location of SP-B on the membrane. Most interestingly, the samples prepared by simultaneous injection of protein and lipid (method B, Figure 3b,c) always showed a significantly higher transfer of energy than did those prepared by addition of SP-B to preformed phospholipid vesicles (method A, Figure 3a,c). Similar differences were found for SP-B reconstituted in DPPC vesicles (data not shown). This result confirms that simultaneous injection of SP-B and phospholipids produced a form of SP-B which is more deeply inserted into the bilayer than is the protein interacting with preformed vesicles.

ESR Spectroscopy Experiments. The implications of these two different forms of insertion of SP-B in phosphatidylcholine bilayers for the lipid−protein interactions have been analyzed by electron spin resonance (ESR) spectroscopy. This technique can give detailed information about the effect of the protein on the thermotropic behavior of the phospholipids, and also positional resolution of the direct motional restriction of the lipid chains upon their interaction with the protein (*13*, *31*). The use of phospholipid probes with nitroxide spin-labels at different positions along the acyl chain in previous studies showed that SP-B increases the order and decreases the mobility of acyl chains in phosphatidylcholine bilayers in the fluid phase, without affecting the gel phase, at least on the time scale of conventional ESR (*13*). In this previous study, SP-B was reconstituted in DPPC

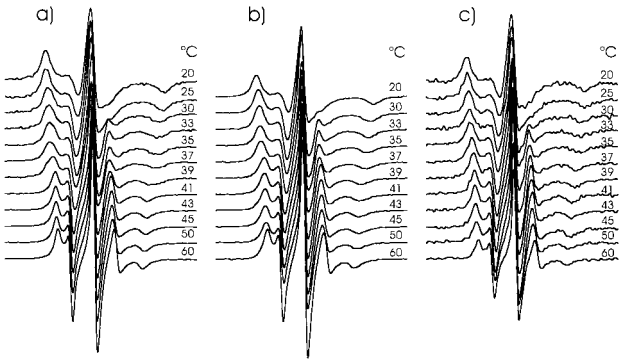

FIGURE 4: Temperature dependence of the ESR spectra from the 5-PCSL spin probe in bilayers of dipalmitoylphosphatidylcholine (DPPC) in the absence (a) and in the presence of 20% (w/w) SP-B reconstituted by addition of the protein to preformed vesicles (b) or by simultaneous protein/lipid injection (c). The total scan width was 100 G.

bilayers by hydrating lipid/protein films obtained after evaporation of SP-B/phospholipid mixtures from organic solvent. The effect of SP-B on the mobility of the acyl chains was stronger for probes located close to the phospholipid headgroups but extended uniformly up to the deepest positions of the chains, close to the center of the bilayer.

Figure 4 shows the ESR spectra at different temperatures of a phosphatidylcholine spin probe (5-PCSL), labeled in the *sn*-2 chain close to the lipid headgroup, in bilayers of DPPC in the absence and in the presence of 20% (w/w) SP-B reconstituted by either method A or B. The decrease in the outer hyperfine splitting in the ESR spectra from the pure lipid reflects the gel-to-liquid crystalline phase transition of DPPC bilayers, indicating a sharp increase in acyl chain mobility at around 40 °C (Figure 4a). The presence of SP-B which was mixed with the lipid before the formation of bilayers (reconstitution method B) caused a considerable broadening in the transition of the lipid, resulting in a continuous and progressive change of the spectral splittings, instead of the abrupt shift observed with the pure lipid (Figure 4c). Such behavior was already described in samples prepared by mixing SP-B and DPPC in chloroform solutions, drying the solvent, and hydrating the dry lipid/protein films (*13*). On the other hand, the interaction of the same amount of SP-B with preformed DPPC vesicles (method A) had a much smaller effect on the ESR spectra of the 5-PCSL spin probe (Figure 4b), which additionally showed a sharp increase in the outer hyperfine splitting at temperatures around the phase transition of the lipid. The outer hyperfine splittings of the 5-PCSL spectra from DPPC bilayers alone and DPPC bilayers containing 20% (w/w) SP-B, reconstituted either by co-injection with the lipid or by addition to preformed vesicles, are plotted against temperature in Figure 5. The phase transition profile of pure DPPC bilayers prepared by organic solvent injection is comparable to that previously published for bilayers prepared by solvent removal and subsequent hydration (*13*). This indicates that any perturbations by the small volume of solvent present in the injection samples are relatively minor, once the solvent is diluted out. The addition of SP-B to DPPC bilayers by either reconstitution method caused a similar decrease in the total amplitude of the transition of the lipid, by increasing the splittings of the ESR spectra in the fluid phase without very significant effects in the gel phase. The protein did not cause

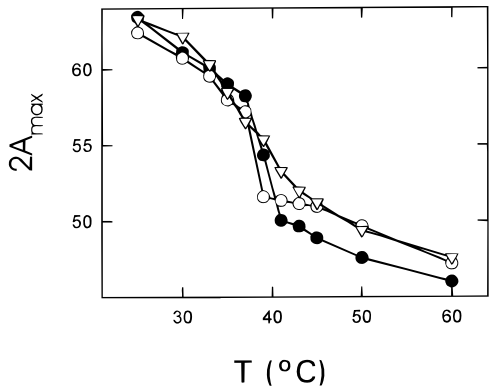

FIGURE 5: Temperature dependence of the outer hyperfine splitting, $2A_{max}$, of the 5-PCSL phosphatidylcholine spin-label in DPPC bilayers in the absence (●) and in the presence of 20% (w/w) SP-B reconstituted by method A (○) or method B (▽).

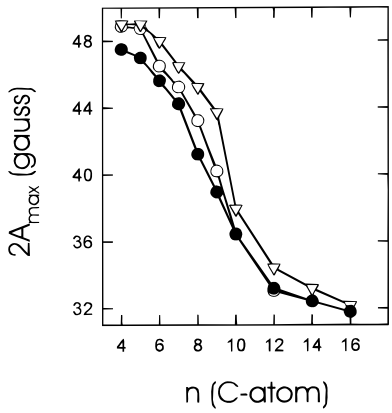

FIGURE 6: Outer hyperfine splittings ($2A_{max}$) at 45 °C as a function of nitroxide position, *n*, in the *sn*-2 chain for the *n*-PCSL positional isomers in DPPC dispersions, in the absence (●) and in the presence of 20% (w/w) SP-B reconstituted by addition of the protein to preformed vesicles (method A) (○) or simultaneous protein/lipid injection (method B) (▽).

significant shifts of the phase transition temperature in either sample in comparison with the behavior of the lipid in the absence of protein. However, it is clearly seen in the figure that the temperature profile of the splittings from samples reconstituted by method B shows a broadened phase transition, while the samples prepared by method A have a cooperative chain-melting behavior similar to that of the pure lipid.

To explore further the extent of perturbation in the mobility of the phospholipid acyl chains induced by SP-B, we have analyzed the effect of the protein on the ESR spectra of phosphatidylcholine spin probes having the nitroxide group attached at different positions of the *sn*-2 acyl chain, in DPPC bilayers. Figure 6 shows the chain flexibility gradient of DPPC bilayers in the fluid phase, in the absence and in the presence of 20% (w/w) SP-B obtained by plotting the outer hyperfine splittings of the *n*-PCSL spin-label positional isomers versus the chain position, *n*, bearing the spin-label. The presence of SP-B which was mixed with the lipid before reconstitution of bilayers (method B) caused a perturbation of the chain flexibility profile similar to that previously described for reconstitution from a dry mixed lipid/protein film (*13*). In these samples, the protein caused the largest decrease in mobility in the acyl chain segments close to the phospholipid headgroups, but the perturbation extended more

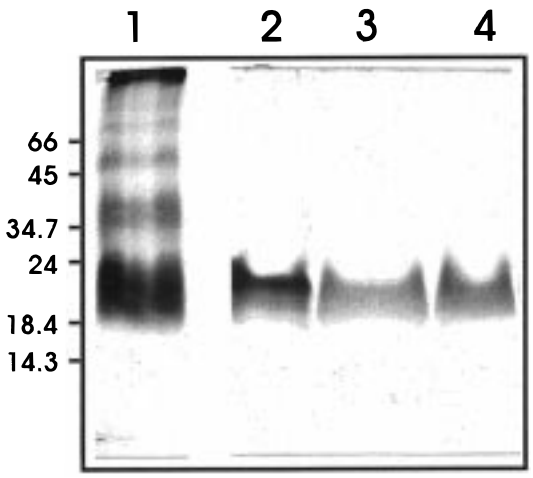

FIGURE 7: SDS−PAGE of lipid-free and lipid-reconstituted SP-B under conditions of limited solubilization: lane 1, aggregated SP-B prepared by transfer of a 1.9 mg/mL solution of protein to aqueous solution; and lanes 2−4, 5 μg of SP-B injected into aqueous buffer in the absence (lane 2) and in the presence of 50 μg of egg yolk PC reconstituted by method A (lane 3) or B (lane 4). The sample buffer contained only 0.5% SDS, and the samples were not boiled. The separating gel was polymerized with 12% acrylamide. Molecular mass markers (in kilodaltons) are indicated to the left of the gel.

or less uniformly to the center of the bilayer. On the other hand, the effect of SP-B added externally to the bilayers (method A) was qualitatively different. In these samples, SP-B caused a decrease in the mobility of probes close to the polar headgroups that was similar to that observed for samples prepared by method B. For probes having the spin reporter group at positions deeper than C5, the effect of externally added SP-B was always smaller than that of SP-B reconstituted by method B. Furthermore, when the protein was added to preformed DPPC vesicles, it showed no effect on the mobility of probes located at positions deeper than C9 of the *sn*-2 acyl chain. These features demonstrate that the interaction of SP-B with preformed PC vesicles induces a much more superficial perturbation of the chain mobility than that of the protein reconstituted from lipid and protein mixed in organic solvent.

To check if differences in the mode of lipid−protein interaction of SP-B reconstituted by the two methods could arise from a different extent of protein aggregation prior to the interaction with vesicles, we performed SDS−PAGE on samples reconstituted by methods A and B under conditions of limited solubilization (Figure 7). Treatment of lipid/protein samples with only 0.5% SDS, without heating at 100 °C, still showed a similar oligomerization state for SP-B (90% dimeric, in nonreducing electrophoresis gels) irrespective of the method used (A or B; lanes 3 and 4 in the gel of Figure 7) for reconstitution of the samples. Under the same electrophoresis conditions, aggregated protein samples in the gels showed significant bands with mobilities corresponding to higher SP-B oligomerization forms having 4−6-mers and more (lane 1). Interestingly, SP-B injected in the absence of lipids at low protein concentrations, such as the sample used for the fluorescence spectrum of Figure 1, was also mainly dimeric (lane 2), excluding nonspecific protein−protein interactions as possible artifacts altering the lipid−protein interaction processes studied here.

Table 3: Parameters for the Binding of Some Membrane-Interacting Proteins to Phospholipid Bilayers

	lipid[a]	parameters	method	reference
SP-B	PC, DPPC	$n = 15-27$ $K_d = 2-5\ \mu M$	fluorescence	this work
cardiotoxin	PS, PI, PA	$n = 7.8$ $K_d = 0.2\ \mu M$	fluorescence	*26*
cardiotoxin	cardiolipin	$n = 3.8$ $K_d = 50$ nM	fluorescence	*27*
melittin	DEPC	$n = 25$ $K_d = 1.5\ \mu M$	fluorescence	*28*
apo A-IV	PC	$K_d = 1.36\ \mu M$	fluorescence	*29*
myelin basic protein	POPC/POPG	$n = 17$ $K_d = 0.48\ \mu M$	ESR	*45*
α-sarcin	DMPG	$n = 40$ $K_d = 60$ nM	filtration	*46*

[a] POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; DEPC, 1,2-dielaidoyl-*sn*-glycero-3-phosphocholine; PS, 1,2-diacyl-*sn*-glycero-3-phosphoserine; PI, 1,2-diacyl-*sn*-glycero-3-phosphoinositol; PA, 1,2-diacyl-*sn*-glycero-3-phosphoric acid; DMPG, 1,2-dimyristoyl-*sn*-glycero-3--phosphoglycerol.

DISCUSSION

The studies on the lipid−protein interactions of SP-B available to date are not completely conclusive with regard to the extent of perturbation induced by the protein in surfactant phospholipids. The nature and extent of this perturbation are probably important for several of the effects induced by SP-B in the properties of the phospholipids, such as the fusogenicity of SP-B-containing bilayers (*32*, *33*), bilayer−monolayer transitions (*5*), or exclusion of the protein from the monolayer upon compression (*34*, *35*). Several studies suggest that the perturbation induced by SP-B in phospholipid bilayers is very limited, only affecting the headgroup region of the membrane (*8*, *10*, *12*). Other studies, however, suggest that the effect of SP-B extends to deeper regions of the phospholipid acyl chains, beyond the headgroup region of the bilayer (*11*, *13*, *36*).

The work described here presents the first quantitative data available on the extent of insertion of SP-B into phosphatidylcholine bilayers. The distances calculated from the SP-B tryptophan to the center of the bilayers were consistent, in all the preparations assayed, with a peripheral location of the protein when it interacts with membranes. This feature had previously only been inferred indirectly from structural predictions for the protein (*7*). The binding parameters estimated in this work for the interaction of SP-B with egg PC or DPPC bilayers lie in the same region as those determined for the interaction of other proteins with lipids (Table 3). All the proteins in this table possess amphipathic structural motifs that are responsible, as for SP-B, for their ability to insert into lipid bilayers.

Several lines of evidence presented in this paper suggest that SP-B reconstituted from lipid/protein mixtures in organic solvents inserts more deeply in PC bilayers than does the protein added to preformed vesicles. The quenching effect of spin-labeled lipids on the fluorescence of SP-B is consistent with a slightly more superficial location of the SP-B tryptophan in protein reconstituted by method A compared with that reconstituted by method B. Data from Table 2 on the estimated distance of the SP-B tryptophan from the center of the bilayer must be taken with some caution. Extension of the parallax analysis to the polar region of the membrane (*37*) probably would be necessary to ensure precise determination of slight differences in the degree of insertion into the headgroup zone. Significantly, however, similar qualitative differences in insertion were also observed in resonance energy transfer experiments from the SP-B tryptophan to *t*-COPA.

Differences in the extent of insertion of SP-B into PC bilayers also lead to qualitative and quantitative differences in the effect of the protein on the mobility of the phospholipid acyl chains, as registered by spin-label ESR. When SP-B interacts with preformed vesicles, its effect in the bilayer is much more superficial. A correlation, therefore, can be established between the estimated distance from the protein to the bilayer core and the extent of perturbation induced by SP-B in the segmental motion of the acyl chains. Several authors have pointed out that aromatic side chains in membrane proteins have some intrinsic preference for being located in regions close to the phospholipid headgroups (*38*). Then, the slight differences detected in the insertion of SP-B from the fluorescence experiments could reflect a relatively superficial location of the region of SP-B where the tryptophan is located, in all the samples. Larger differences in the insertion of other regions of SP-B could be responsible for the significant differences found in the effect of the protein on the mobility of the phospholipid acyl chains, depending on the method of reconstitution of SP-B/phospholipid samples. Deuterium NMR studies did not detect significant perturbation induced by SP-B in the phospholipid acyl chains of DPPC/SP-B bilayers prepared by the "classical" method of hydrating dry lipid/protein films (*8*, *39*). The reason for this can be traced to the long time scale of the ^{2}H NMR technique relative to that of spin-label ESR (and fluorescence), a result that is well-known in the study of lipid−protein interactions (see ref *31*). It might be also argued that the differential behavior of SP-B in restricting the mobility of the acyl chains could be, at least partially, also caused by the formation in some cases of discoidal SP-B/PC particles, such as those described by Williams et al. (*40*). In these structures, some of the protein molecules could be located in the edges of the disks, laterally closing and shielding the hydrophobic core of the bilayers, and therefore interact with the deeper regions of at least some of the acyl chains. A similar model has been proposed recently to explain certain perturbations induced by SP-B in dipalmitoylphosphatidylglycerol (DPPG) or DPPC/DPPG bilayers, detected by ^{2}H NMR (*39*). However, electron microscopic analysis of our samples (*17*) excludes the existence of significant amounts of such structures.

Taking together both the effect of lipid−protein interaction on the location of SP-B fluorophores in the bilayer and the extent of perturbation induced by SP-B on the mobility of the phospholipid acyl chains, we can conclud that reconstitution of SP-B/phospholipid samples by lipid/protein coinjection, as well as by the classical method, simulates insertion of SP-B into bilayers, while addition of SP-B to preformed vesicles only produces protein association with the bilayers, without a real membrane penetration.

The conformation of SP-B in samples prepared by sequential injection, analyzed from its circular dichroism and fluorescence spectrum, its sensitivity of SP-B tryptophan

fluorescence to quenching by acrylamide, and its susceptibility of the inserted protein to proteolysis (*17*), is similar to that of the protein reconstituted by simultaneous lipid/protein injection. Furthermore, there are no differences in the apparent extent of aggregation of SP-B when comparing lipid/protein samples prepared by the two methods. All these features seem to exclude the possibility that a shallower penetration of SP-B in phospholipid bilayers could be related to the occurrence of protein−protein interactions originating from the transient exposure of the protein to the aqueous environment.

It is possible to speculate on the possible significance of the different forms of interaction of SP-B with lipids for the physiological role of this protein in the surfactant cycle. Either of the two different forms of interaction of SP-B described here could have physiological relevance. The more deeply inserted form of SP-B has greater and more stable activity in promoting adsorption of surfactant phospholipids to the air−liquid interface (*17*), a property which is essential for the biophysical function of surfactant. On the other hand, the large structural transformations induced by SP-B when it is injected into dispersions of preformed phospholipid bilayers, including processes of vesicle aggregation (*17*, *32*) or fusion (*32*, *41*), have been related to the essential participation of SP-B in the formation of tubular myelin, a specific surfactant structure which has been linked to optimal tensoactive properties (*40*, *42*). It is even possible that SP-B undergoes a dynamic behavior with a different location, and extent of insertion and lipid−protein interactions in surfactant bilayers or monolayers, at different stages of the surfactant cycle. It has been reported, for instance, that SP-B is squeezed out from the air−liquid interface at a certain surface pressure during compression, without being accompanied by lipid molecules (*34*, *35*). Such protein "de-insertion" could lead to a more peripheral location of the protein in the monolayer, such as the superficial structures described here, which might well be more accessible to recycling (*43*, *44*) or to other processes in surfactant dynamics.

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BI971558V