Different modes of interaction of pulmonary surfactant protein SP-B in phosphatidylcholine bilayers

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Pulmonary surfactant-associated protein B (SP-B) has been incorporated into vesicles of dipalmitoyl phosphatidylcholine (DPPC) or egg yolk phosphatidylcholine (PC) by two different procedures to characterize the dependence of lipid–protein interactions on the method of reconstitution. In method A the protein was dissolved in a small volume of either methanol or 60 % (v/v) acetonitrile and injected into an aqueous phase containing phospholipid vesicles. In method B the vesicles were prepared by injection of a mixture of phospholipid and SP-B dissolved in methanol or aqueous acetonitrile. Both methods of reconstitution led to the extensive interaction of SP-B with PC bilayers as demonstrated by co-migration during centrifugation, marked protection against proteolysis, change in the fluorescence emission intensity of SP-B, and protection of SP-B tryptophan fluorescence from quenching by acrylamide. SP-B promoted the rapid adsorption of DPPC on an air/liquid interface irrespective of the method of protein reconstitution. However, the interfacial adsorption activity of SP-B reconstituted by method B remained

stable for hours, but that of SP-B prepared by method A decreased with time. Electron microscopy showed that the injection of SP-B into an aqueous phase containing PC or DPPC vesicles (method A) induced a rapid aggregation of vesicles. By contrast, a much longer time was required for detecting vesicle aggregation when the protein was reconstituted by co-injection of SP-B and phospholipids (method B). The presence of 5 % (w/w) SP-B in DPPC bilayers prepared by method B broadened the differential scanning calorimetry thermogram and decreased the enthalpy of the transition. In contrast, the injection of SP-B into preformed DPPC vesicles (method A) did not influence the gel-to-liquid phase transition of DPPC bilayers. Taken together, these results indicate that the mode and extent of interaction of SP-B with surfactant phospholipids depends on the conditions of preparation of lipid/protein samples, and that care should be taken in the interpretation of findings from reconstituted systems on the role of these surfactant proteins in the alveolar space.

INTRODUCTION

Surfactant-associated protein B (SP-B) is a small hydrophobic disulphide-linked homodimeric protein of 79 amino acid residues per monomer that is expressed in the type II alveolar epithelial cells of mammalian lungs [1–3]. It forms part of the complex material called pulmonary surfactant. The main function of surfactant is to lower surface tension at the pulmonary air/water interface, and so decrease the work of breathing and the tendency for alveolar collapse [4,5].

The structural and functional properties of SP-B have been reviewed recently by Johansson and Curstedt [6]. The protein possesses approx. 40 % α -helix, including several amphipathic α -helical motifs [7,8]. Combinations of peptides mimicking these amphipathic domains and surfactant phospholipids can reproduce many of the tensioactive properties of natural surfactant [9]. Studies with ²H-NMR [7], differential scanning calorimetry [10], ESR [11] or fluorescence [12] are consistent with the view that SP-B possibly interacts peripherally with phospholipid bilayers or monolayers. Such lipid–protein interactions have usually been characterized in samples formed by mixing SP-B and phospholipid in chloroform solutions, drying the mixture and hydrating the dry films. In the present study we have analysed two methods of reconstitution of SP-B in phospholipid vesicles based on the solubility of the protein in certain organic solvents [13], and

characterized some features of its interaction with PC bilayers, comparing samples reconstituted by the two different procedures.

EXPERIMENTAL

Materials

Chloroform, methanol and acetonitrile were HPLC-grade solvents from Scharlau (Barcelona, Spain). Sephadex LH-20 and LH-60 chromatography gels were obtained from Pharmacia (Uppsala, Sweden). Dipalmitoyl phosphatidylcholine (DPPC) and egg yolk phosphatidylcholine (PC) were from Avanti Polar Lipids (Birmingham, AL, U.S.A.); radioactive 1,2-di[1-¹⁴C]palmitoyl L-3-phosphatidylcholine (112 Ci/mol) was from Amersham International (Little Chalfont, Bucks., U.K.). Pronase was purchased from Calbiochem (San Diego, CA, U.S.A.), deuterated water from Aldrich (Milwaukee, WI, U.S.A.), and all other reagents and chemicals from Merck (Darmstadt, Germany).

Preparation of SP-B

SP-B was isolated from pig lungs by an adaptation, described elsewhere [13], of the method of Curstedt et al. [14]. The isolated

Abbreviations used: DPPC, dipalmitoyl phosphatidylcholine; PC, egg yolk phosphatidylcholine; SP, surfactant-associated protein.

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protein was stored in chloroform/methanol solution at -20°C . In the absence of reducing agents, SP-B migrated in SDS/polyacrylamide gels as a single band with an apparent molecular mass of 20 kDa, corresponding to the protein dimer. Quantification of SP-B was performed by amino acid analysis.

Reconstitution of SP-B/phospholipid samples

Stock solutions of SP-B (2 mg/ml) were prepared in acetonitrile/water (60:40, v/v) or in methanol [13]. In contrast, stock solutions of phospholipids (50 mg/ml), PC or DPPC, were prepared in methanol.

SP-B was reconstituted in phospholipid bilayers by two methods. In method A, a small volume (2–5 μl) of methanolic phospholipid solution was first injected, with vigorous vortex-mixing, into 0.5–0.7 ml of 50 mM Hepes buffer in 150 mM NaCl, pH 7, at temperatures above the phase transition temperature of the phospholipid (25 and 45°C for PC and DPPC respectively), and then 5–10 μl of SP-B in 60% (v/v) acetonitrile or methanol was injected into the suspension. In method B, the phospholipid (dissolved in methanol) and SP-B (in acetonitrile/water or methanol) were first mixed and then an appropriate volume of the mixture was injected into the buffer under the conditions described above. Controls were prepared by injecting identical volumes of methanol or acetonitrile/water or mixtures of both. A low lipid-to-protein ratio was chosen for spectroscopic experiments to minimize experimental artifacts due to light scattering. It was set to 5% (w/w) protein to lipid when studying biophysical behaviour of lipid/protein systems.

Electron microscopy

Droplets of a suspension of lipid (0.5 mg/ml) with or without protein (5:1, w/w) prepared by method A or B were placed on Formvar-coated grids for 4 min and then stained in a drop of 1% (w/v) phosphotungstic acid for 1 min before observation under a Zeiss EM-902 microscope.

Detection of lipid–protein interaction

Binding of SP-B to phospholipid vesicles was determined by co-floitation of protein and lipid after centrifugation of lipid/protein vesicles in $^2\text{H}_2\text{O}$ buffer [15]. Vesicles of radioactive phospholipids (1,2-di[1- ^{14}C]palmitoyl PC; 55 mCi/mol) were prepared at a concentration of 0.5 mg/ml in the presence or absence of SP-B. Samples of 400 μl of these suspensions were centrifuged in a Beckman Airfuge for 3 h at 4°C and 178 000 *g*. After centrifugation, fractions of 25 μl were recovered starting from the top of the tube. One half of each fraction was subjected to SDS/PAGE, silver-stained and then measured in an Ultrascan 2002 densitometer (LKB, Uppsala, Sweden) to detect and quantify protein. The second half of each fraction was added to vials containing 10 ml of Biogreen scintillation cocktail (Scharlau, Barcelona, Spain) and counted in a Beckman LS 3801 scintillation counter.

Fluorescence of SP-B in PC and DPPC bilayers

Samples containing 10 μg of SP-B were reconstituted with 50 μg of PC or DPPC by methods A and B described above. Fluorescence emission spectra were recorded in a Perkin-Elmer MPF-44E spectrofluorimeter operated in the ratio mode. Spectra were obtained at 25 and 45°C for SP-B/PC and SP-B/DPPC samples respectively in thermostatically controlled cells with a 0.2 cm optical path length, at a scanning speed of 1 nm/s. The slit widths were 7 and 5 nm for the excitation and emission beams

respectively. Spectra were obtained by using two excitation wavelengths, 275 and 295 nm. A control was prepared for each sample that included the same volume of acetonitrile/water or methanol solution without SP-B. The fluorescence emission spectra of these control samples were subtracted from the protein sample spectra.

Quenching experiments

Aliquots (1 μl) of 10 mM acrylamide in water were added to SP-B in 60% (v/v) acetonitrile or to the reconstituted vesicles containing SP-B plus PC or DPPC, and the fluorescence emission spectra were recorded after each addition. Values of fluorescence emission at 325 and 338 nm were corrected for both dilution and the scatter contribution of the lipid vesicles. Corrected data were analysed by the classical Stern–Volmer equation for collisional quenching [16], to calculate the Stern–Volmer dynamic quenching constant, K_{sv} .

Proteolysis experiments

Samples consisting of 5 μg of SP-B were digested with pronase at 0.5 mg/ml, in the absence or presence of 25 μg of phospholipids that had been added in accordance with method A or method B. The digestion was performed in 100 μl of 20 mM Tris/HCl buffer (pH 8.0)/10 mM CaCl_2 at 50°C for 30 min. The digestion was stopped by boiling the samples for 15 min at 100°C . After the reaction had been stopped, 25 μg of phospholipid was added to the control, where SP-B was digested with Pronase in the absence of lipids. Samples were analysed by SDS/PAGE followed by silver staining of the gels and densitometry in an Ultrascan 2002 densitometer (LKB).

Differential scanning calorimetry

High-sensitivity differential scanning calorimetric scans of dispersions of DPPC and DPPC containing 5% (w/w) SP-B, reconstituted by both methods, were performed in a Microcal MC-2 calorimeter (Amherst, MA, U.S.A.) as described previously [17]. DPPC (1 mg), with or without 50 μg of SP-B, was reconstituted in 1.5 ml of 50 mM Hepes buffer (pH 7)/150 mM NaCl. A 1.2 ml aliquot of each dispersion was loaded in the calorimeter sample cell and run against buffer in the reference cell. Calorimetric scans were performed at a rate of $30^{\circ}\text{C}/\text{h}$. Each sample yielded reproducible thermograms over three heating cycles from 20 to 60°C .

Surface activity

Interfacial adsorption experiments with SP-B/DPPC samples were performed at room temperature as previously described [18]. DPPC (600 μg) was dispersed in 0.45 ml of 50 mM Hepes buffer (pH 7)/150 mM NaCl, in the presence or the absence of 5% (w/w) SP-B, prepared by method A or method B. At zero time, 100 μl samples of these suspensions were injected into a Teflon beaker containing 5 ml of a hypophase consisting of 5 mM Mes (pH 6.9)/150 mM NaCl/5 mM CaCl_2 , which was stirred continuously. Interfacial adsorption of the samples was followed by measuring the changes in surface pressure with a platinum dipping plate suspended from a strain gauge.

RESULTS

The injection of a very small volume of PC or DPPC in methanol into a much larger volume of aqueous buffer, with vigorous vortex-mixing, produced relatively homogeneous suspensions of

Figure 1 Electron microscopic images of PC (upper panels) and DPPC (lower panels) vesicles after negative staining with phosphotungstic acid, in the absence and in the presence of SP-B

Lipid/protein samples were reconstituted by method A (a) or method B (b). Scaled bars represent 100 nm.

vesicles, ranging mostly from 100 to 200 nm in diameter as observed by electron microscopy (Figure 1). Similar phospholipid vesicles have been produced by other authors by an analogous solvent injection method [19]. The injection of PC/SP-B or DPPC/SP-B mixtures into the buffer (method B) yielded vesicles with similar sizes and morphologies to those found for lipids alone (Figure 1b). In contrast, the injection of SP-B into previously formed lipid vesicles of either PC or DPPC (method A) led to preparations with extensive vesicle aggregation for both lipids (Figure 1a). The different structures of lipid/SP-B samples prepared by the two methods are detected at short times after

Figure 2 Adsorption kinetics isotherms of dispersions of DPPC (●) and DPPC plus 5% (w/w) SP-B reconstituted by method A (▽) or method B (▼)

Lipids (130 μ g) were injected in a total volume of 5 ml of 0.15 M NaCl in 50 mM Hepes buffer (pH 7)/5 mM CaCl_2 . Π , surface pressure.

Figure 3 Stability of interfacial adsorption activity of dispersions of DPPC plus 5% (w/w) SP-B prepared by method A (a) or method B (b) with time

After reconstitution, the samples were preincubated at 37 °C for 20 (○), 75 (□), 135 (▽), 275 (◇) or 345 (△) min in (a), and for 30 (○), 135 (▽), 235 (△) or 350 (□) min in (b) before injection into the adsorption beaker under the conditions described in the legend to Figure 2.

reconstitution. However, after 3–4 h all samples showed extensive aggregation as judged by a pronounced turbidity, regardless of the method used for reconstitution.

SP-B promotes the interfacial adsorption of phospholipids on the air/liquid interface [20,21]. Our DPPC/SP-B samples, prepared by either method, also underwent rapid adsorption on the air/liquid interface compared with the control samples in the absence of SP-B (Figure 2). The effect of SP-B on the adsorption of DPPC was similar to that seen in suspensions reconstituted by the ‘classical’ method of mixing lipid and protein in chloroform solution and drying, then hydrating the dry films [7]. The main difference between the surface activity of the two preparations proved to be in the duration of their tensioactive properties. Samples of DPPC/SP-B prepared by method B continued to have similar interfacial adsorption activity when measured at various times up to 5 h after reconstitution (Figure 3b). In contrast, the adsorption activity of samples prepared by method A decreased significantly after 2 h following reconstitution, and it was very low after 5 h (Figure 3a).

Centrifugation in deuterated buffer of DPPC/SP-B samples prepared by method A or B showed that a substantial part of the protein remained in the top part of the tube, associated with the lipid vesicles (Figure 4). The fact that both lipid and protein profiles overlapped in the top fractions suggested that they existed in the form of lipid/protein complexes.

Quantification of vesicle-bound protein in samples reconstituted by both methods, and incubated for different times up to 7 h, revealed that irrespective of the method of reconstitution SP-B was stably bound to bilayers. Therefore the loss of ability for interfacial adsorption of lipid/protein samples prepared by method A does not seem to be due to a decrease in lipid-bound protein.

Each SP-B monomer has a single tryptophan residue that can be used as an intrinsic fluorescence probe to study the effect of lipid–protein interactions on its local environment. The fluorescence emission spectrum of SP-B after its interaction with preformed PC vesicles (reconstitution method A) is shown in Figure 5(a). It was similar to that previously reported for SP-B reconstituted in lipid protein mixtures made in the ‘classical’ manner [13]. The spectrum is dominated by contributions from tryptophan, with two emission peaks at 325 and 338 nm that are

Figure 4 Detection of SP-B binding to DPPC phospholipid vesicles

Protein (30 μ g) and lipid (200 μ g) were reconstituted in 400 μ l of deuterated 50 mM Hepes buffer, pH 7, containing 150 mM NaCl, by method A (a) or method B (b). After centrifugation at 178000 g , fractions were obtained starting from the top of the tube and quantified for lipid and protein. The percentage of lipid (○) and protein (●) relative to the total amount is plotted against the fraction number. In (c) two control experiments are shown, in which only vesicles or only protein were run.

Figure 5 Fluorescence emission spectrum of SP-B reconstituted in vesicles of PC (left panel) or DPPC (right panel)

SP-B (10 μ g) was reconstituted in 50 μ g of lipid by method A (traces a) or method B (traces b). Traces c are the spectra of 10 μ g of SP-B injected into 0.7 ml of buffer in the absence of lipids. The excitation wavelength was 275 nm. Abbreviation: a.u., arbitrary units.

due to heterogeneity in the environment of the fluorophore. The spectrum of SP-B in vesicles made by method B was similar to that of the protein reconstituted by method A. When the same amount of SP-B was injected into the aqueous buffer in the

Table 1 Stern–Volmer constant (K_{sv}) of quenching by acrylamide of fluorescence emission of SP-B in organic solvents and reconstituted in phospholipid vesicles

Environment	K_{sv} (M^{-1})
Acetonitrile	11.7 ± 0.6
PC (method A)	1.4 ± 0.1
PC (method B)	1.7 ± 0.1
DPPC (method A)	1.3 ± 0.2
DPPC (method B)	1.9 ± 0.2

Figure 6 Susceptibility of phospholipid-interacted SP-B to digestion by Pronase

SP-B (5 μ g) were digested with Pronase 0.5 mg/ml in the absence (lane 2) and in the presence of 25 μ g of DPPC reconstituted by method A (lane 3) or method B (lane 4). A control (lane 1) was run with 5 μ g of SP-B without Pronase. DPPC (25 μ g) was added to samples loaded in lanes 1 and 2 to obtain a comparable silver staining signal in all the lanes.

absence of phospholipid vesicles the fluorescence emission spectrum had a decreased intensity, probably due to self-aggregation of the protein. Figure 5(b) shows that fluorescence emission spectra of SP-B reconstituted in vesicles of DPPC by the two methods are very similar to those of the protein in PC bilayers.

Table 1 presents the calculated Stern–Volmer constants (K_{sv}) for the quenching by acrylamide of tryptophan fluorescence of SP-B in aqueous organic solution or reconstituted in lipid vesicles. SP-B reconstituted in phospholipid vesicles by either method had a significantly lower accessibility of tryptophan to the quencher in comparison with SP-B in aqueous acetonitrile. There were no significant differences between the K_{sv} values for the protein reconstituted in the two phospholipids, PC and DPPC. However, the interaction of SP-B with preformed vesicles of PC or DPPC (method A) produced a slightly greater protection of SP-B fluorophore from quenching by acrylamide (lower K_{sv}) than that observed for samples reconstituted by SP-B/phospholipid co-injection. These minor differences might originate from a different extent of insertion of the SP-B molecule into the bilayers, or from a decrease in the accessibility of SP-B tryptophan because of the rapid vesicle aggregation induced by SP-B when it was injected into preformed vesicles.

To assess the extent of interaction of SP-B in phospholipid bilayers we performed proteolysis of the lipid/protein complexes. SP-B was resistant to proteolysis by several proteases checked, except for Pronase, which is a mixture of several proteases from *Streptomyces griseus*. Figure 6 shows the SDS/PAGE analysis of the products of digestion of SP-B by Pronase. In the absence of

Figure 7 Differential scanning calorimetry thermograms of DPPC vesicles in the absence and in the presence of 5% (w/w) SP-B, reconstituted by method A (a) or method B (b)

lipids, incubation with Pronase produced almost total digestion of the protein (lane 2). As shown in lane 1, when SP-B was incubated in the same buffer without lipid and Pronase it was fully recoverable as the initial protein. The reconstitution of SP-B in DPPC vesicles produced a marked protection against proteolysis (lanes 3 and 4). A similar protective effect was induced by interaction with vesicles made of PC (results not shown). In both lipids, method A resulted in more protection against proteolysis than method B. This might be because of the greater initial aggregation of vesicles in method A.

The lipid-protein interaction of SP-B has been also analysed by running high-sensitivity differential calorimetric scans of DPPC in the absence and the presence of 5% (w/w) SP-B (Figure 7). The scanning calorimetry thermogram of vesicles of DPPC prepared by the methanol injection method had similar features to the typical thermograms of DPPC multilamellar vesicles [22,23]. It showed a main, sharp transition peak at 41.9 °C with a pretransition peak at 34.7 °C. The enthalpy change associated to the gel-to-liquid phase transition, calculated from the area under the main peak, was 8.03 kcal/mol (33.6 kJ/mol), which is in the range of the enthalpy change previously found for the transition of DPPC multilamellar vesicles. This result demonstrated that the small volume of solvent used to reconstitute the lipid or lipid/protein samples was not significantly perturbing the phospholipid thermotropic behaviour. Addition of 5% SP-B to preformed DPPC vesicles did not substantially affect the gel-to-liquid phase transition of DPPC bilayers, which also showed a sharp main transition at 41.7 °C with an associated enthalpy change of 7.99 kcal/mol (33.4 kJ/mol). In contrast, bilayers of DPPC formed in the presence of 5% SP-B by method B showed a broadened main

transition peak at 40.7 °C. The enthalpy change was 7.03 kcal/mol (29.4 kJ/mol). This result is similar to the effect of SP-B on multilamellar vesicles of DPPC reported previously [7,10].

DISCUSSION

Most of the studies so far published about the structure and function of SP-B were performed with samples where SP-B was reconstituted in the 'classical' way, by mixing protein and lipids in chloroform solutions, drying the mixtures and hydrating the dry films in the desired aqueous buffer [7–12,21]. Samples reconstituted by this method possess good activity in promoting a rapid adsorption of phospholipids on the air/liquid interface [7,21,24]. However, various reports suggest that the extent and stability of adsorption activity are dependent on the conditions of SP-B/phospholipid reconstitution. Parameters such as temperature, choice of organic solvent, rehydration, the procedure used to prepare the aqueous protein/lipid suspensions or the time of incubation of samples after reconstitution seem to influence the biophysical properties of the suspensions. In contrast it has been proposed that SP-B possesses membrane fusogenic activity, because either the injection of a methanolic solution of SP-B [25,26] or the addition of phospholipid vesicles containing the protein [27] causes lipid mixing in suspensions of phospholipid vesicles. Because protein orientation in the lipid could be influenced by conditions of formulation it was desirable to analyse possible consequences of reconstitution methods for features of SP-B.

Here we have analysed two different methods (A and B) of SP-B reconstitution. Both methods lead to the interaction of SP-B with the phospholipid vesicles, as demonstrated by (1) hydrodynamic co-migration of protein and lipids in deuterated buffer, (2) protection of SP-B against digestion by Pronase, (3) an increase in the fluorescence emission spectrum of SP-B, and (4) a decrease in the susceptibility of SP-B tryptophan fluorescence to quenching by acrylamide that was similar to that observed in other membrane-interacting proteins (Table 2).

The extent of interaction of SP-B with phospholipid vesicles differs significantly depending on the method of reconstitution. SP-B reconstituted in phospholipid vesicles by method B, i.e. co-injection of SP-B and phospholipid into an aqueous phase, interacts to a somewhat greater extent with PC bilayers than SP-B injected into preformed phospholipid vesicles (method A). This conclusion is supported by the stronger effect of the protein on the thermotropic behaviour of the DPPC bilayers.

The typical SP-B/phospholipid samples reconstituted by hydrating dry films formed from lipid/protein mixtures made in organic solutions (typically, chloroform/methanol mixtures) probably produce similar structures to those formed by method B. The observation that DPPC/SP-B samples prepared by method B produced differential scanning calorimetry thermograms similar to those previously reported for the effect of SP-B on DPPC multilamellar vesicles [7,10] would be consistent with this assumption.

The instantaneous and extensive aggregation of lipid vesicles caused by the addition of SP-B (method A) suggests that there is, at least initially, a difference in SP-B orientation with respect to the lipid in method A in comparison with method B. A similar location could be responsible for the lipid-mixing activity reported for SP-B [25,26]. This rapid aggregation process cannot be the cause of the progressive loss of adsorption capacity. First, samples made by method A initially show good surface activity although they are extensively aggregated. Secondly, samples made by method B continue to show a high surface activity at

Table 2 Effect of phospholipid/protein interaction on the Stern–Volmer constant (K_{SV}) of dynamic quenching by acrylamide of the fluorescence emission of some membrane-interacting proteins

Protein	Phospholipid	K_{SV} (M^{-1})		Reference
		Without lipid	With lipid	
Pig SP-B	PC	11.7	1.4–1.7	This study
	DPPC	11.7	1.3–1.9	This study
Pig SP-A	DPPC	9.3	1.0	[31]
Cardiolipin II	Cardiolipin	12.5	3.3	[32]
PhoE signal peptide	PC	4.1	1.6	[33]
	PG	4.1	0.7	[33]
Colicin A thermolytic peptide	DMPG	1.07	0.76	[34]
Tachyplesin	PG	8.7	< 0.5	[35]

longer times even though they have become aggregated over those times. Thirdly, in other cases aggregation has been associated with enhanced biophysical activity [28,29]. Therefore we conclude that the introduction of SP-B by method A causes a different type of lipid–protein interaction from that of method B. This raises the question of whether or not the aggregation process and aggregated forms are the same in each method, but we do not yet have sufficient information to answer that question.

The possible functional implications of these different SP-B/phospholipid structures for the actions of surfactant *in vivo* might be important. A reason to consider that the samples reconstituted by co-injection of SP-B and phospholipids (method B) are more similar to the arrangement of SP-B in the surfactant bilayers *in vivo* would be that these structures have a more stable surface activity than samples made by method A. However, it can be argued that the process of exposing hydrophobic domains of SP-B to preformed vesicles as is done in method A could be analogous to the interaction of SP-B with lipids in the surfactant cycle *in vivo*. SP-B is synthesized as a large precursor of 381 amino acid residues, the fundamental role of which has been suggested to keep the mature sequence of the protein away from the lipid environment until the precise moment at which the protein has to participate in surfactant assembly [30]. The processing of pro-SP-B to generate the mature form of the protein could be comparable, in biophysical terms, to exposure of the hydrophobic, mature SP-B from organic solvents to the lipid bilayers in aqueous suspensions. In that case, the transformations detected in the lipid structures made by method A might be more representative of real surfactant dynamics.

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