Pulmonary surfactant-associated polypeptide SP-C in lipid micelles: CD studies of intact SP-C and NMR secondary structure determination of depalmitoyl-SP-C(1–17)

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Abstract The surfactant-associated polypeptide C (SP-C) is a 35-residue lipopolypeptide which is essential for the function of surfactants used for therapy of infant respiratory distress. Modeling based on the recently determined nuclear magnetic resonance (NMR) structure of native SP-C in an organic solvent showed that SP-C could readily insert into fluid 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine bilayers. The present paper describes further physical-chemical studies of intact SP-C and its N-terminal 17-residue polypeptide fragment, depalmitoyl-SP-C(1–17), in the presence of dodecylphosphocholine micelles. The results obtained provide a link between the NMR solution structure and the behaviour of SP-C in an ordered lipid environment, and thus present new insights for rational design of SP-C analogs for therapeutic purposes.

Key words: NMR; Polypeptide conformation; Lipid-associated polypeptide; Pulmonary surfactant; Surfactant polypeptide-C

1. Introduction

Pulmonary surfactant is a complex mixture of phospholipids, primarily 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), and at least three surfactant-associated polypeptides, SP-A, SP-B and SP-C, of which SP-B and SP-C are pronouncedly hydrophobic. The main function of pulmonary surfactant is to reduce the surface tension at the alveolar air/liquid interface, thereby preventing alveolar collapse at the end of expiration (for reviews see [1,2]). Lack of sufficient amounts of surfactant in premature infants is associated with respiratory distress syndrome (RDS), which is a major cause of neonatal morbidity and mortality. RDS can be effectively treated by airway instillation of exogenous surfactant preparations. Essential components of such preparations are phospholipids, especially DPPC, and small amounts of SP-B and SP-C [3,4].

Endogenous pulmonary surfactant is synthesized in alveolar type II cells and stored intra-cellularly in dense multibilayer structures. These 'lamellar bodies' are excreted into the alveolar liquid subphase, where they are converted into more complex bilayer structures ('tubular myelin'), from which the surface-active monolayers are eventually formed. SP-C is thought to have an important role in facilitating the conversion of subphase bilayer structures into surface-active DPPC monolayers at the air/liquid interface [5–10]. Since large-scale production of natural SP-B and SP-C is difficult, the development of synthetic, possibly non-peptide analogs for therapeutic purposes is of great potential interest [11,12].

The surfactant polypeptide-C (SP-C) is a highly hydrophobic, small protein (Fig. 1), which is difficult not only to prepare in adequate amounts, but also to handle at all stages of an experimental investigation. Recently, we succeeded nonetheless in obtaining a high-quality nuclear magnetic resonance (NMR) structure in a mixed organic solvent [13]. This SP-C structure contains a highly regular α-helix from residues 9–34, whereas the N-terminal octapeptide segment, as well as the C-terminal dipeptide segment are flexibly disordered (Fig. 1). In order to obtain a better foundation for evaluating the physiological significance of the NMR structure in acid chloroform/methanol [13], we now present circular dichroism (CD) and NMR studies of SP-C and of its N-terminal heptadecapeptide, depalmitoyl-SP-C(1–17), in dodecylphosphocholine (DPC) micelles, which mimic more closely the physiological conditions under which the surfactant acts in vivo. Based on the combination of the previously described NMR structure of SP-C [13] with these new experimental measurements, we propose a possible mode of interaction of SP-C with natural lipid bilayers that should help in the future design of artificial compounds with functional properties akin to those of surfactant-associated polypeptides, which they could then possibly replace in exogenous surfactant preparations.

2. Materials and methods

2.1. Sample preparation

Depalmitoyl-SP-C(1–17), with the sequence LRIPCCPVNLKRLLVVV (see also Figs. 1 and 3), was prepared by chemical synthesis using the *tert*-butyloxycarbonyl method. The peptide was liberated by treatment with hydrogen fluoride, and the protection groups were removed by diethylether extraction. Depalmitoyl-SP-C(1–17) was recovered by extraction with 30% (v/v) acetic acid in H_2O, lyophilized and purified by reverse-phase high-performance liquid chromatography (Vydac C18, 3 × 25 cm, flow 10 ml/min, linear gradient of acetonitrile/0.1% trifluoroacetic acid). Sequence, purity and monomeric state were veri-

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Abbreviations: SP-C, surfactant-associated polypeptide C; depalmitoyl-SP-C(1–17), N-terminal heptadecapeptide fragment of SP-C; DPC, dodecylphosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; TSP, [2,2,3,3-$^2H_4$]-trimethylsilyl-propionate; RDS, respiratory distress syndrome; CD, circular dichroism; NMR, nuclear magnetic resonance; ppm, parts per million; 2QF-COSY, two-dimensional two-quantum filtered correlation spectroscopy; TOCSY, two-dimensional total correlation spectroscopy; NOE, nuclear Overhauser enhancement; NOESY, two-dimensional nuclear Overhauser enhancement spectroscopy.

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fied by amino acid analysis, sequence analysis and mass spectrometry. The micelle-bound form of depalmitoyl-SP-C(1–17) was prepared following established procedures [14], using perdeuterated dodecylphosphocholine ([$^2H_{38}$]DPC) at concentrations above the critical micelle concentration of 1 mM [15] where micelles of defined size but no bilayer structures are formed. 3.2 mg of depalmitoyl-SP-C(1–17) and 42.0 mg of [$^2H_{38}$]DPC were mixed in chloroform/methanol 1:2 (v/v). The mixture was sonicated, dried under a stream of nitrogen, and then dissolved in 500 μl of 50 mM sodium phosphate at pH 3.6 in $H_2O/^2H_2O$, 9:1. The final concentrations of depalmitoyl-SP-C(1–17) and [$^2H_{38}$]DPC were 3.3 mM and 0.2 M, respectively, which corresponds approximately to a 1:1 molar ratio between peptide and micelles [14,16,17]. A solution in ^2H_2O was prepared by twice lyophilizing this sample from ^2H_2O, and redissolving it in 99.96% ^2H_2O.

2.2. NMR experiments

All NMR spectra were recorded at 34°C on Bruker AMX 500 and AMX 600 spectrometers operating at ^1H frequencies of 500 MHz and 600 MHz, respectively. Quadrature detection in the indirectly detected dimension was obtained using the States-TPPI method [18]. The spectra were processed on a Bruker X32 workstation using the program UXNMR. The residual water signal after preirradiation during the relaxation delay was further reduced with the convolution method of Marion et al. [19]. Before Fourier transformation the time domain data were zero-filled and multiplied with shifted sine-bell windows [20]. The spectra were baseline-corrected using third order polynomials. Standard procedures were used to obtain the ^1H resonance assignments: 2QF-COSY [21] (data size 512 × 1024 complex points, t_{1max} = 102 ms, t_{2max} = 205 ms, digital resolution 4.9 Hz in ω_1 and 2.4 Hz in ω_2) and clean-TOCSY [22] (mixing time = 80 ms, data size 400 × 1024 complex points, t_{1max} = 84 ms, t_{2max} = 205 ms, digital resolution 4.7 Hz in ω_1 and 2.4 Hz in ω_2) spectra were recorded at 500 MHz. For the identification of NOEs and the determination of $^3J_{NH\alpha}$ scalar coupling constants [23] (Fig. 3), a NOESY spectrum [24] (mixing time = 100 ms, data size 384 × 1536 complex points, t_{1max} = 67 ms, t_{2max} = 267 ms, digital resolution 5.6 Hz in ω_1 and 1.4 Hz in ω_2) and a soft-NOESY spectrum [25] (mixing time = 100 ms, data size 512 × 1024 complex points, t_{1max} = 80 ms, t_{2max} = 160 ms, digital resolution 6.3 Hz in ω_1 and 3.1 Hz in ω_2) were recorded at 600 MHz. Amide proton exchange rates (Fig. 3) were determined by dissolving the lyophilized depalmitoyl-SP-C(1–17)/[$^2H_{38}$]DPC mixture in ^2H_2O and monitoring the time course of the amide proton signals at 34°C in a series of one-dimensional ^1H NMR spectra at 500 MHz.

3. Results

DPC-micelles [14,16] solubilize native SP-C, and CD measurements (Fig. 2) indicate that the polypeptide is 80–90% helical in this environment. However, in contrast to other membrane-active polypeptides of similar size, for which NMR structure determinations in DPC micelles were achieved [17,26,27], excessive line broadening for micelle-bound SP-C prevented a determination of the NMR structure. This was true also for the full-length SP-C polypeptide lacking the thioester-linked palmitoyl side chains (Fig. 1), indicating that the observed NMR line broadening cannot be accounted for by possible cross-linking of micelles via the palmitoylcysteinyls.

Unlike full-length SP-C, the micelle-bound form of the synthetic polypeptide comprising the amino acid residues 1–17 of porcine SP-C, depalmitoyl-SP-C(1–17), was amenable to secondary structure determination by NMR. Using standard procedures [28,29], nearly complete sequence-specific ^1H NMR assignments were obtained (Table 1; Fig. 3). Sequential nuclear Overhauser enhancement (NOE) connectivities are missing for Cys^6–Pro^7 and Leu^{13}–Leu^{14} (Fig. 3), because the Cys 6 α-proton resonance is masked by the residual water signal and the chemical shifts of Leu 13 and Leu 14 are nearly degenerate (Table 1). The otherwise consecutive strong d_{NN} connectivities from positions 10–17 and the medium-range NOEs observed in this polypeptide segment (Fig. 3) indicate a helical conformation for this octapeptide segment [28–30]. This is further supported by small values of the $^3J_{NH\alpha}$ spin–spin coupling constants [31] and slowed amide proton exchange rates for residues 13–17 (Fig. 3). After correction for primary structure effects [32] (but not for possible shielding by the micellar environment), the exchange rate constants for residues 14–16 reflect a protection factor of about 10. The NMR data for residues 1–9 of depalmitoyl-SP-C(1–17) (Fig. 3) are compatible with a flexibly disordered conformation of this polypeptide segment [29]. The CD spectrum or depalmitoyl-SP-C(1–17) in DPC micelles (Fig. 2) indicates a helix content of approximately 40%, which coincides well with the extent of helical structure observed by NMR (Fig. 3). Fig. 4A shows a hypothetical arrangement of depalmitoyl-SP-C(1–17) relative to the DPC micelle to which it is bound, which would provide a rationale for the observed conformation of the polypeptide (Fig. 3).

Overall, there are thus several lines of evidence that the

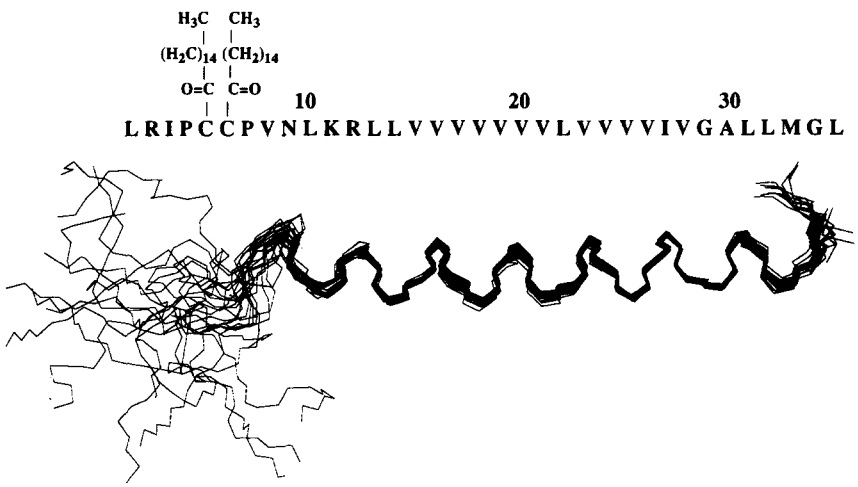

Fig. 1. The upper part shows the primary structure of the porcine lipopolypeptide SP-C [44], with the palmitoylcysteinyl residues in positions 5 and 6 [45]. The lower part shows the NMR solution structure in a mixed solvent of $CDCl_3/CD_3OH$/0.1 M HCl 32:64:5 (v/v) at 10°C [13] represented by a superposition of 20 conformers [29] for optimal fit of the backbone heavy atoms of residues 9–34, where the positions of the C^α atoms of Asn^9 and Gly^{34} align with the corresponding one-letter code in the amino acid sequence.

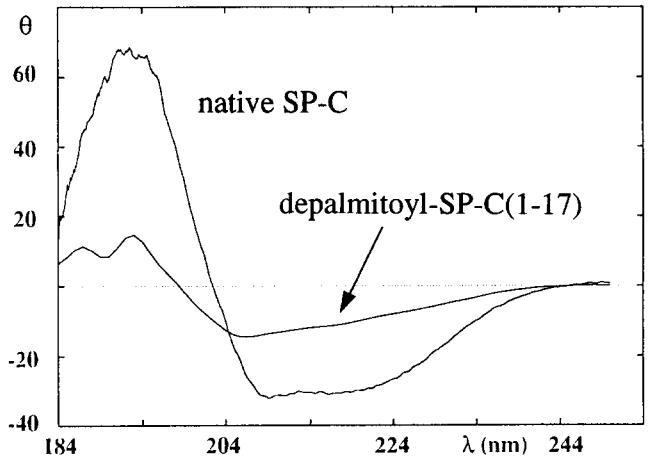

Fig. 2. CD spectra between 184 and 250 nm of native SP-C and depalmitoyl-SP-C(1–17) in 10 mM DPC/50 mM sodium phosphate at pH 6.0. The peptides were solubilized in 150 μl of 20 mM DPC/50 mM sodium phosphate at pH 6.0, and the solution was then diluted with buffer to 10 mM DPC. The CD spectra were recorded with a Jasco J-720 spectropolarimeter at 22°C (scan speed 20 nm/min, one scan per spectrum acquired, concentration of native SP-C = 30 μM and of depalmitoyl-SP-C(1–17) = 60 μM, as determined by amino acid analysis). Θ is in units of 10^4 deg·cm^2·mol^{-1}.

α-helical structure of natural SP-C determined in an organic solvent mixture (Fig. 1) is preserved in phospholipid environments similar to pulmonary surfactant. From our work this includes the CD data of Fig. 2, which indicate a high helix content for SP-C in DPC micelles, and the NMR data of Fig. 3, which show that the onset of helical structure in micelle-bound depalmitoyl-SP-C(1–17) is at a nearly identical sequence location as in native SP-C in solution (Fig. 1). In work by others, Fourier transform infrared spectroscopy of native SP-C in phospholipid bilayers with compositions similar to that of pulmonary surfactant phospholipids showed that the polypeptide is between 60% and 90% α-helical, and that the axis of the SP-C helix is oriented perpendicular to the membrane surface [33,34]. Taken together, these data show that the conformation of SP-C is not significantly affected by the nature of the apolar environment and that, therefore, the α-helical structure determined for native SP-C in an organic solvent (Fig. 1) is also preserved in a phospholipid environment. Similar conclusions resulted from work on isolated transmembrane helices from bacteriorhodopsin, for which similar structures have been observed in organic solvents, detergent micelles and in the intact protein [35].

4. Discussion

The length of the entire helix from residues 9–34 of SP-C (Fig. 1) and that of its pronouncedly hydrophobic part from residues 13–28 are about 37 and 23 Å, respectively, which is remarkably close to the experimentally determined overall thickness of a DPPC phospholipid bilayer (37 Å) and its interior acyl-chain part (26 Å) in the liquid–crystalline phase [36]. Thus, in a transmembrane orientation of SP-C in surfactant phospholipids, the rigid hydrophobic helix of residues 13–28 would span the hydrocarbon part of the bilayer (Fig. 4B). This close match of the length of native SP-C with the thickness of a fluid DPPC bilayer and the lesser fit with the larger thickness of 39 Å for the all-hydrophobic part of the same bilayer in the gel phase [37] could be of importance for SP-C function, since mismatches between the length of a transmembrane peptide and the bilayer thickness are known to cause phase separation of peptides and lipids, especially when the peptide is shorter than the bilayer [37]. Consequently, from the size of the SP-C α-helix one would expect that the polypeptide is more favourably accommodated in fluid DPPC bilayers than in gel phase DPPC. This affords a molecular explanation of the recent finding that SP-C is predominantly monomeric in fluid DPPC/dipalmitoylphosphatidylglycerol (DPPG) vesicles, but undergoes self-aggregation in the same lipid mixture below the transition temperature between gel phase and liquid-crystalline phase [38]. Accordingly, the decrease of the phase transition temperature of phospholipid vesicles caused by SP-C [10] may be due to peptide aggregates disrupting the lipid packing in the gel phase.

One observes further that in the trans-membrane orientation of Fig. 4B, the evolutionarily strictly conserved Lys–Arg dipeptide segment in positions 11–12 [39] is suitably located for interacting with the phosphatidyl choline moieties of DPPC. This would also be in agreement with deuterium NMR studies of selectively deuterated DPPC preparations containing variable amounts of SP-C, which provided evidence for direct interactions between the positive charges of SP-C and the lipid headgroups [40]. Furthermore, the palmitoylcysteinyls at positions 5 and 6 are then located near the exterior surface of the DPPC bilayer (Fig. 4B). The exact location of the palmitoyl chains is unknown, but closer inspection of the situation offers only two alternatives: the lipid chains can either fold back and interact with the same bilayer that contains the polypeptide chain to which they are attached, or they can interact with a

Table 1
^{1}H chemical shifts of 3.3 mM depalmitoyl-SP-C(1–17) in 0.2 M [$^2H_{38}$]DPC/50 mM sodium phosphate at pH 3.6 and 34°C

Residue	Chemical shift (ppm)*			
	NH	αH	βH	Others
Leu^1		3.99	1.70	γCH 1.64; δCH$_3$ 0.92, 0.94
Arg^2	8.61	4.47	1.72, 1.79	γCH$_2$ 1.51, 1.57; δCH$_2$ 3.17; εNH 7.33
Ile^3	8.30	4.37	1.88	γCH$_2$ 1.17, 1.56; γCH$_3$ 0.98; δCH$_3$ 0.86
Pro^4		4.48	1.98, 2.31	γCH$_2$ 2.17; δCH$_2$ 3.53, 3.94
Cys^5	8.03	4.41	2.88	
Cys^6	8.70	4.66	2.72, 2.99	
Pro^7		4.54	2.06, 2.36	γCH$_2$ 1.99; δCH$_2$ 3.76, 3.99
Val^8	7.97	4.08	2.05	γCH$_3$ 0.92
Asn^9	8.63	4.69	2.82, 2.89	δNH$_2$ 6.90, 7.81
Leu^{10}	8.16	4.08	1.66	δCH$_3$ 0.84, 0.92
Lys^{11}	8.06	3.86	1.88	γCH$_2$ 1.43, 1.53; δCH$_2$ 1.72; εCH$_2$ 2.94; ζNH$_3^+$ 7.69
Arg^{12}	7.71	4.09	1.82, 1.88	γCH$_2$ 1.71; δCH$_2$ 3.18, 3.23; εNH 7.42
Leu^{13}	7.65	4.09	1.65, 1.81	δCH$_3$ 0.83, 0.91
Leu^{14}	7.59	4.08	1.63, 1.80	δCH$_3$ 0.84, 0.92
Val^{15}	7.25	4.04	2.27	γCH$_3$ 0.98, 1.04
Val^{16}	7.39	4.18	2.28	γCH$_3$ 0.97, 1.02
Val^{17}	7.32	4.17	2.22	γCH$_3$ 0.97

*The chemical shifts are in ppm relative to TSP. For methylene and isopropyl groups two chemical shifts are given only when two separate lines could be identified. Atom groups for which no resonance lines could be identified are not listed.

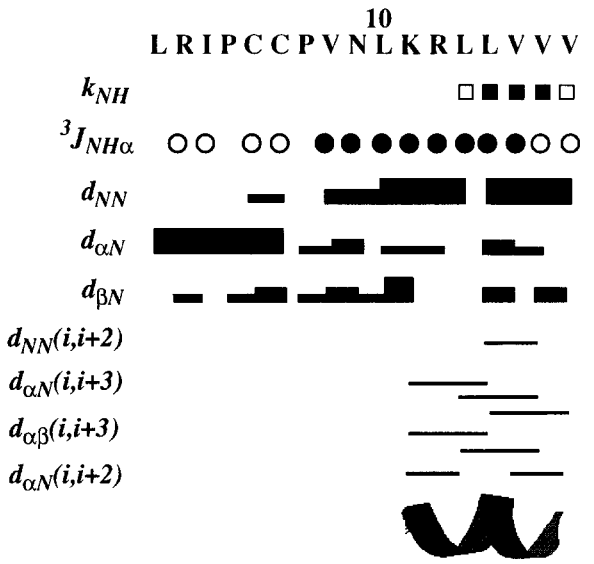

Fig. 3. Survey of experimental NMR data for depalmitoyl-SP-C(1–17) bound to [$^2H_{38}$]DPC micelles. In the row k_{NH}, filled and open squares identify, respectively, the residues with amide proton exchange rate constants $k_{NH} < 1.0 \cdot 10^{-2}$ min^{-1}, and $1.0 \cdot 10^{-2}$ min^{-1} < $k_{NH} < 1.0 \cdot 10^{-1}$ min^{-1} at p^{2}H 3.6 and 34°C. In the row $^3J_{NH\alpha}$, filled and open circles denote the residues with $^3J_{NH\alpha} < 6.0$ Hz, and 6.0 Hz < $^3J_{NH\alpha} < 8.0$ Hz, respectively. The sequential NOE connectivities d_{NN}, $d_{\alpha N}$ and $d_{\beta N}$ are presented by horizontal bars, where the thickness of the bars reflects the intensities of the NOEs. The medium-range NOEs $d_{NN}(i,i+2)$, $d_{\alpha N}(i,i+3)$, $d_{\alpha\beta}(i,i+3)$ and $d_{\alpha N}(i,i+2)$ are represented by lines starting and ending at the positions of the residues connected. At the bottom the location of α-helical structure determined by the NMR data is indicated.

second, neighbouring bilayer. The possible effects of such interactions on the structure and dynamics of the N-terminal octapeptide segment cannot be evaluated from the present structural data, which were obtained either in an organic solvent in the absence of lipids (Fig. 1, [13]) or using depalmitoyl-SP-C(1–17) (Fig. 3). The palmitoylcysteinyls and the basic residues 11 and 12 are both located in the N-terminal half of SP-C, while the C-terminal half of the sequence contains exclusively residues with non-polar side chains (Fig. 1). The N-terminal dodecapeptide segment with the basic residues and the palmitoyl 'side chains' is thus likely to anchor the SP-C molecules on one side of the DPPC bilayer in the sense that lateral movements of the lipopolypeptide are restricted, while the C-terminal part contains no groups that would tend to interact specifically with the head groups. It is tempting to speculate that the herewith implicated mobility gradient across the lipid bilayer is linked to the observed capacity of SP-C to accelerate the transformation of lipid bilayers to lipid mono-layers, and it correlates with the finding that addition of SP-C increases the ordering of the bilayer surface but tends to disrupt the interior acyl chain packing [7].

In contrast to the near-perfect fit with the dimensions of a fluid DPPC bilayer, the solution structure of SP-C (Fig. 4B) could hardly be stably incorporated into the surface-active DPPC monolayer at the air/liquid interface, since the width of such monolayers corresponds to only about half of the length of the helical part of SP-C in the conformation of Fig. 1, and the poly-valyl α-helix is an outstandingly rigid structure that could not readily kink or even fold back on itself. This implies that the principal influence of SP-C on phospholipid adsorption rates should occur rather by destabilization of bilayer structures in the alveolar liquid subphase than by direct interactions with the monolayer structures. This would also be in agreement with a recent study [41] which suggests that SP-C may act by enhancing the respreading of collapsed DPPC structures that have been squeezed out of the compressed monolayer to the interphase during expansion of the alveolar surface. Our conclusion may also be correlated with the fact that in a model system, SP-C installed in a monolayer is less effective than SP-B in accelerating adsorption of phospholipids from the alveolar subphase [8]. Furthermore, in the same model system the presence or absence of the palmitoyl groups in positions 5 and 6

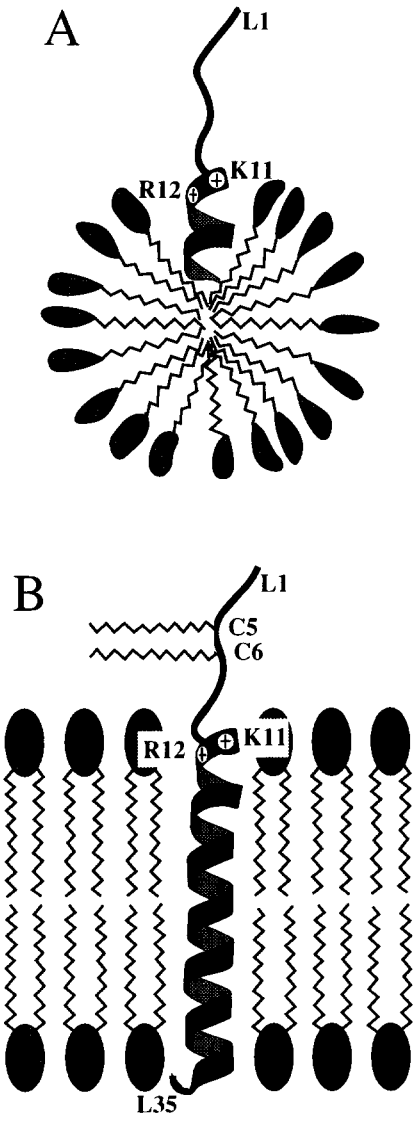

Fig. 4. Schematic representations of depalmitoyl-SP-C(1–17) and SP-C in lipid environments (see text). (A) Depalmitoyl-SP-C(1–17) in DPC micelles. The locations of the N-terminal residue and the positively charged residues 11 and 12 are indicated. (B) Native SP-C in a DPPC bilayer, assuming that the α-helical conformation observed in an organic solvent [13] is preserved in this environment. The N-terminus, the palmitoylcysteinyl residues in positions 5 and 6, the positively charged residues in positions 11 and 12, and the C-terminus are indicated. In both drawings, which were prepared using the program MOLSCRIPT [46], the shaded ovals and the wavy lines represent, respectively, the polar head groups and the fatty acyl chains of the lipid molecules.

(Fig. 1) apparently does not correlate with the ability of the peptide to induce phospholipid insertion into the monolayer [42]. Due to the fundamental differences between the monolayer model system and the *trans*-bilayer orientation of SP-C now deduced from the structure determination in solution [13] (Fig. 4B), further work is needed to investigate the functional implications of the palmitoyl groups when SP-C interacts with the phospholipid bilayers.

The indications from the present study on structure–function correlations of SP-C should provide a lead for rational design of SP-C analogs with grossly preserved molecular parameters and hopefully also functional properties, but with covalent structures that would make them easier to synthesize and purify than the natural product. In addition to the biomedical interest, further studies of the SP-C system also bear on questions of stability and functionality of biologically active polypeptide conformations. It remains to be seen whether formation of a long α-helical stretch by residues with β-branched aliphatic side chains (Fig. 1), which has previously also been reported for model peptides with poly-valyl blocks [43], is simply an extreme adaptation to a hydrophobic environment, or is more specifically related with biological function. Further investigations of structure and function of SP-C analogs might also shed some light on this fundamental question.

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