Secondary structure and biophysical activity of synthetic analogues of the pulmonary surfactant polypeptide SP-C

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Native pulmonary-surfactant-associated lipopolypeptide SP-C, its chemically depalmitoylated form and several synthetic analogues lacking the palmitoylcysteine residues were analysed for secondary structure in phospholipid micelles and for biophysical activity in 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine/phosphatidylglycerol/palmitic acid (68:22:9, by wt.). Compared with the native molecule, with the entire poly-valyl part in a known α -helical conformation, depalmitoylated SP-C was found to be still mainly α -helical, but with an approx. 20% decrease in the helical content. A synthetic hybrid polypeptide where the entire poly-valyl α -helical part of native SP-C had been replaced with the amino acid sequence of a transmembrane helix of bacteriorhodopsin is also predominantly α -helical. In contrast, synthetic SP-C analogues lacking only the palmitoyl groups, by replacement of the palmitoylcysteine residues with cysteine, phenylalanine or serine, or lacking the positively charged amino

acids by replacement with alanine, are considerably less α -helical than both native and depalmitoylated SP-C. The data indicate that the SP-C palmitoyl groups are important for maintenance of the α -helical conformation in parts of the polypeptide, and that the poly-valyl α -helical conformation is not fully formed in synthetic SP-C polypeptides. Furthermore, the helical structure of both native and depalmitoylated SP-C in dodecylphosphocholine micelles is very resistant to thermal denaturation, exhibiting ordered structure at 90 °C. The α -helical content grossly parallels the peptide-induced acceleration of the spreading of phospholipids at an air/water interface and the increase of surface pressure. The data suggest that the α -helical conformation itself, rather than just the covalent structure, is of prime importance for the biological function of synthetic pulmonary-surfactant peptides.

INTRODUCTION

Pulmonary surfactant, synthesized by alveolar type II cells, consists mainly of phospholipids mixed with small amounts of specific proteins. It is stored intracellularly as dense multilayer structures, lamellar bodies, and secreted into the alveolar liquid sub-phase, where it is converted into lattice-like bilayer structures, tubular myelin. Eventually, tubular myelin generates a monolayer of mainly 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) at the air/liquid interface [1,2]. This monolayer decreases surface tension, thereby preventing alveolar collapse at end-expiration. Decreased levels of surfactant in premature babies are associated with respiratory distress syndrome (RDS) [3], a common cause of neonatal mortality [4]. This disease can be treated by airway instillation of isolated surfactant preparations containing phospholipids and 1–2% (by wt.) of the two hydrophobic polypeptides (surfactant proteins) SP-B and SP-C [5]. These polypeptides are important, as they increase the rate of adsorption of surfactant phospholipids to an air/liquid interface [6–10].

The specific importance of the peptides is paralleled by unique structural features. SP-C is an N-terminally truncated 33–35-residue monomer [11,12] which is generated from a 21 kDa precursor, pro SP-C [13–15]. Intact pro SP-C has not been found in alveolar surfactant or lamellar bodies, but a partly processed (16 kDa) form of the precursor has recently been detected in lamellar bodies by immunological means [16]. The middle/C-terminal two-thirds of the airway polypeptide are evolutionarily highly conserved and extremely hydrophobic. Positions 13–28 contain exclusively aliphatic branched-chain residues, predominantly valine [17]. The N-terminal third contains two

conserved basic residues, at positions 11 and 12, and is overall somewhat hydrophilic, except for two palmitoyl groups that are thioester-linked to Cys-5 and Cys-6 [18]. In dog SP-C, Cys-6 (numbering according to the human form) is replaced with Phe [17]. Fourier-transform IR spectroscopy has revealed the overall secondary structure and orientation of native SP-C in phospholipid bilayers [19,20], and recently a high-resolution three-dimensional NMR structure of the molecule has been determined, revealing a well-defined valyl-rich α -helix encompassing positions 9–34 [21], from which it was concluded that the all-hydrophobic part of SP-C exactly spans the hydrocarbon part of a fluid DPPC bilayer. From this detailed structural information it is now feasible to design rationally analogues for structure/function studies of SP-C and for possible use in synthetic surfactant preparations for treatment of RDS. We here provide data which indicate that the functions of SP-C and related synthetic analogues are dependent on the α -helical conformation, and that the valyl-rich α -helix of native SP-C is highly stable, but is not easily adopted in synthetic replicas with amino acid sequences identical with the native polypeptide. However, some of the surface properties of SP-C can be mimicked by an SP-C/bacteriorhodopsin hybrid (SP-C/BR), which completely lacks the poly-valyl segment.

EXPERIMENTAL

Peptide synthesis and purification

Peptide synthesis was performed by the t-butyloxycarbonyl method [22] in an Applied Biosystems 430A peptide synthesizer.

Abbreviations used: DPC, dodecylphosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; PA, palmitic acid; PG, phosphatidylglycerol; RDS, respiratory distress syndrome; SP, surfactant protein; TFE, trifluoroethanol.

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The peptides were liberated by treatment with HF, and the protecting groups were removed by repeated diethyl ether extractions.

A modified extraction scheme had to be employed for separation of the polypeptides containing the poly-valyl stretch (Table 1) from the resin. After extraction with chloroform/methanol (1:1, v/v), additional peptide material was recovered by extraction with concentrated formic acid. The latter material was then freeze-dried, resolubilized in small volumes of formic acid and diluted to 10% (v/v) formic acid with propan-2-ol. From this solution, the polypeptides were purified by HPLC on a 4.6 mm × 250 mm reverse-phase C₂ column (Phenomenex, Torrance, CA, U.S.A.); flow 1 ml/min; initial conditions aq. 0.1% trifluoroacetic acid in 40% propan-2-ol; peptides eluted with a linear gradient of 40–70% propan-2-ol. The peptide SP-C/BR (Table 1) was extracted from the resin with 40% (v/v) acetic acid, freeze-dried, and purified by HPLC.

Native SP-C was isolated from pig pulmonary phospholipids by chromatography on Sephadex LH-60 in chloroform/methanol (1:1, v/v) containing 5% 0.1 M HCl [6]. SP-C was depalmitoylated by treatment with trimethylamine and purified by chromatography on Sephadex LH-60 [18].

Protein analysis

Amino acid sequence analysis was carried out on an Applied Biosystems 470A sequencer equipped with an on-line 120A phenylthiohydantoin analyser. For mass spectrometry, the polypeptides were solubilized in chloroform/methanol (2:1, v/v) containing 0.1% trifluoroacetic acid, and 1–5 nmol was analysed in a BioIon ²⁵²Cf plasma desorption time-of-flight instrument [17,18]. Amino acid analysis was performed with an LKB Alpha Plus analyser after hydrolysis in 6 M HCl/0.5% phenol for 24 h at 110 °C in evacuated tubes.

Synthesis of dodecylphosphocholine (DPC)

(a) Dodecyl hydrogen phosphonate triethylammonium salt, **1** (Scheme 1)

Anhydrous imidazole (10.2 g, 150 mmol) obtained by evaporation of added acetonitrile was dissolved in 150 ml of tetrahydrofuran. The solution was kept on ice/water, and PCl₃ (4.3 ml, 50 mmol) in tetrahydrofuran (20 ml) was added slowly, followed by dropwise addition of triethylamine (22.9 ml, 165 mmol) in tetrahydrofuran (50 ml). Dodecanol (2.79 g, 15 mmol) in tetrahydrofuran (200 ml) was then added dropwise

Scheme 1 Synthesis of DPC

during 30 min. The solution was removed from the cooling bath and left stirring at room temperature for 30 min, after which 25 ml of water was added. The solution was evaporated to near dryness and partitioned between triethylammonium bicarbonate (0.5 M) and chloroform/methanol (4:1, v/v). The organic phase was washed with water and evaporated. The residue obtained after evaporation of added acetonitrile was kept under reduced pressure overnight.

The crude material was purified by silica-gel chromatography, by using a stepwise gradient of 5–20% methanol in chloroform to give 3.7 g (70%) pure **1** (Scheme 1).

³¹P NMR [CHCl₃, chemical shift (δ) in p.p.m. relative to an external standard of 2% H₃PO₄ in ²H₂O]: δ = 4.6 (¹J_{PH} = 630 Hz).

¹H NMR [25 °C, C²HCl₃/C²H₃O²H 3:1 (v/v), chemical shift (δ) in p.p.m. relative to an internal standard of tetramethylsilane]: δ = 6.69 (d, ¹J_{PH} = 639 Hz, 1H, H-P); δ = 3.81 (quartet, ³J = 7.1 Hz, 2H, H-C₁); δ = 3.02 (quartet, ³J = 7.4 Hz, 6H, H-C-N); δ = 1.56 (quintet, ³J = 7.1 Hz, 2H, H-C₂); δ = 1.24 (t, ³J = 7.4 Hz, 9H, H-C-C-N); δ = 1.1–1.3 (m, 18H, H-C_{3–11}); δ = 0.80 (t, ³J = 6.8 Hz, 3H, H-C₁₂).

(b) DPC, **2** (Scheme 1)

Dodecyl hydrogen phosphonate triethylammonium salt (**1**, Scheme 1; 1.4 g) and choline acetate (0.7 g, prepared by dissolving choline hydroxide in acetic acid, evaporation of the solvent and of added toluene, washing several times with diethyl ether, and drying under reduced pressure) were dried by evaporation of added pyridine and subsequently dissolved in pyridine (40 ml). Pivaloyl chloride (1.34 ml) was added and the reaction mixture was left stirring for 10 min. Iodine (1.5 g) in pyridine/water (24:1, v/v) (40 ml) was added and the solution was evaporated, dissolved in chloroform/methanol (4:1, v/v), and washed with sodium thiosulphate (10%, aq.), NaHCO₃ (saturated, aq.) and water. The organic phase was evaporated and the residue subjected to silica-gel chromatography with chloroform/methanol (7:3 and 6:4, v/v). The yield of DPC was 1.13 g (81%).

³¹P NMR (25 °C, conditions as above): δ = −3.3 (³J_{PH} = 6.5 Hz).

¹H NMR (40 °C, conditions as above): δ = 4.14 (m, 2H, H-C_{1(choline)}); δ = 3.78 (quartet, ³J = 6.6 Hz, 2H, H-C₁); δ = 3.50 (m, 2H, H-C_{2(choline)}); δ = 3.13 (s, 9H, CH₃-N⁺); δ = 1.54 (quintet, ³J = 6.9 Hz, 2H, H-C₂); δ = 1.1–1.3 (m, 18H, H-C_{3–11}); δ = 0.80 (t, ³J = 6.6 Hz, 3H, H-C₁₂).

CD measurements

Polypeptides (about 50 μg) were dissolved in 150 μl of 20 mM DPC/50 mM sodium phosphate buffer, pH 6.0, and diluted to 10 mM DPC. Due to low solubility even in detergent solutions, the peptide SP-C (AAA) (Table 1) was mixed with DPC in chloroform/methanol (1:1, v/v), the solvents removed and the residue was solubilized in 300 μl of 50 mM sodium phosphate buffer, pH 6.0, giving a final concentration of 10 mM DPC. Experiments were performed in parallel with synthetic decanoyl-glycerophosphocholine (Sigma, St. Louis, MO, U.S.A.) replacing DPC; final concn. 10–24 mM detergent/50 mM sodium phosphate buffer, pH 6.0. For both detergents, the concentrations used are at least 10 times their critical micellar concentration (cf. [23,24]). For analysis in SDS micelles, native SP-C was dissolved in 15 mM SDS/50 mM sodium phosphate buffer, pH 2.5, and for analysis in trifluoroethanol (TFE) it was dissolved in concentrated TFE. After removal of insoluble material by

centrifugation, CD spectra were recorded with a Jasco J-720 spectropolarimeter (Jasco, Japan) at 22 °C, unless otherwise stated. The scan speed was 20 nm/min (184–250 nm), the response time 2 s, and the band width 1 nm. For temperatures above 20 °C, the sample holder was heated by circulating water from a thermostat-controlled water bath, and the values given are those of the circulating water. For melting studies, the temperature was raised by 1.7 °C/min, and the CD value at 222 nm was continuously monitored. In all cases, polypeptide concentrations in the samples were determined by amino acid analysis after the CD measurements, and the mean residue molar ellipticity is expressed in k-degrees $\cdot$ cm² $\cdot$ dmol⁻¹. The contents of α -helical secondary structure given have been estimated from the molar ellipticities at 208 and 222 nm [25].

Reconstitution of surfactant

DPPC, egg phosphatidylglycerol (PG) and palmitic acid (PA), in the proportions 68:22:9, by wt. [26], were dissolved in chloroform/methanol (49:1, v/v), and added to a dried polypeptide (lipid:polypeptide ratio 20:1, w/w). After sonication, the solvent was evaporated under nitrogen and the residue was suspended by gentle ultrasonication (50 W, 48 kHz) in normal saline, giving a lipid concentration of 10 mg/ml.

Measurement of surface activity

Surface properties of the surfactant preparations were measured at 37 °C with a Wilhelmy balance (Biegler, Vienna, Austria) equipped with a Teflon trough (18 cm²), containing a 20 ml hypophase of 150 mM NaCl. A 0.1 ml sample of the surfactant preparation (1 mg of lipid) was added as a droplet on to the hypophase, 4 cm from the platinum plate, and surface pressure was recorded continuously during 1 min. The values presented are representative of three different recordings. Differences in surface pressure were evaluated statistically by analysis of variance and the Newman–Keuls test for between-group comparisons.

RESULTS

Synthesis and purification of polypeptides and DPC

Four SP-C analogues [SP-C (CC), SP-C (FF), SP-C (SS) and SP-C (AAA)], and one analogue (SP-C/BR) where positions 13–35 of SP-C were replaced with the amino acid sequence of positions

Figure 1 Reverse-phase HPLC purification of SP-C (FF)

The peptide (1.5 mg) was injected in formic acid/propan-2-ol (1:9, v/v) and eluted with a linear gradient of 40–70% propan-2-ol in 0.1% trifluoroacetic acid. The arrows show elution positions of SP-C (SS) (first arrow) and SP-C (AAA) (second arrow) under identical conditions.

42–64 of bacteriorhodopsin, with slight modifications (see below), were synthesized (Table 1). For extractions of the SP-C analogues containing poly-valyl segments, chloroform/methanol (1:1, v/v) was used first, yielding about 30% of the peptide material, followed secondly by formic acid, giving a total peptide yield of about 70%. Polypeptides in the formic acid fraction were eluted as a single symmetrical peak after reverse-phase HPLC on a C₂ column (Figure 1). Sequence analysis after HPLC purification showed that more than 90% of the material correspond to the full-length peptide. Plasma-desorption mass spectrometry gave the expected molecular masses, confirming that inadvertent covalent modifications of the polypeptide did not follow from the formic acid treatment. The SP-C/BR polypeptide was quantitatively recovered by extractions with 40% acetic acid.

The synthesis of DPC (Scheme 1) is based on the hydrogen phosphonate approach [27–29], previously applied to the synthesis of glycerophospholipids [30]. This approach is considerably more convenient and efficient than those used previously (cf. [31]). DPC micelles have been well characterized as regards their physico-chemical properties [32] and have been used for extensive structural analysis of lipid-associated polypeptides (e.g. [33]).

Removal of the palmitoyl groups affects the secondary structure of SP-C

Native SP-C solubilized in micellar concentrations of DPC (Figure 2) or decanoylglycerophosphocholine (results not shown) contains 80–90% α -helical structure, in agreement with the helical content of native SP-C in organic solvents as determined by NMR spectroscopy (74%) [21] and in phospholipid bilayers as studied by Fourier-transform IR spectroscopy (60–90%) [19,20]. Similar α -helical contents were also obtained with the peptide solubilized in 15 mM SDS or in TFE (results not shown). This indicates that native SP-C adopts a defined conformation largely independent of the nature of the apolar environment. Thus data from analyses of SP-C and related analogues in lipid micelles are likely to be valid for the polypeptides also in combination with surfactant phospholipids.

Depalmitoylated SP-C is mainly α -helical in DPC micelles,

Table 1 Primary structures of the SP-C analogues analysed

In native pig SP-C, the cysteine residues at positions 5 and 6 are palmitoylated [18] and positions 9–34 form one continuous α -helix [21]. Horizontal lines indicate residues identical with those in the SP-C (CC) line.

	10	20	30
SP-C (CC)	L R I P C C P V N L K R L L V V V V V V L V V V V I V G A L L M G L		
SP-C (FF)	— F F —		
SP-C (SS)	— S S —		
SP-C (AAA)	— A — — — — — A A —		
SP-C/BR	— F Y A I T T L V A A I A F T L Y L S L L L G Y

Figure 2 CD spectra of native and depalmitoylated SP-C and of SP-C (CC) in DPC micelles

Spectra were recorded between 184 and 250 nm of native pig SP-C (—), depalmitoylated SP-C (---) and the synthetic polypeptide SP-C (CC) recovered by chloroform/methanol extraction (· · · · ·). All polypeptides were solubilized in 10 mM DPC/50 mM sodium phosphate buffer, pH 6.0, and measured at 22 °C.

exhibiting a pronounced minimum at 208 nm (Figure 2), and a helical content of approx. 70 % is estimated. Thus removal of the palmitoyl groups decreases the helical content by about 20 % when the polypeptide is incorporated into DPC micelles, which is in good agreement with the secondary-structure contents observed for native (92 %) and depalmitoylated (77 %) SP-C in phospholipid bilayers [20].

Synthetic SP-C polypeptides are less helical than depalmitoylated SP-C

Three SP-C analogues containing the poly-valyl sequence, but with different alternatives replacing the palmitoylcysteine residues, were synthesized (Table 1). SP-C (CC) was made to correspond to the native pig molecule minus the palmitoyl groups, i.e. its covalent structure is identical with that of depalmitoylated SP-C. SP-C (FF) corresponds to the molecule with both palmitoylcysteine residues replaced by Phe, and SP-C (SS) to that with both palmitoylcysteine residues replaced with Ser. The Phe analogue was designed from the fact that one of the palmitoylcysteine residues in dog SP-C is replaced by Phe [17]. The Ser substitutions were chosen as hydrophilic alternatives that do not alter the net charge. Surprisingly, CD analyses revealed that all these analogues, irrespective of whether they were recovered by extractions with chloroform/methanol or formic acid (see below), have substantially less α -helical structure than in both native and depalmitoylated SP-C (Figures 2 and 3). No significant differences between the CD spectra of the SP-C (CC) (Figures 2 and 3), SP-C (FF) and SP-C (SS) analogues (spectra not shown), recovered by chloroform/methanol extractions, could be observed. Consequently, it appears that the precise nature of the non-palmitoylcysteine residues at positions 5 and 6 is of minor structural importance. Secondary-structure estimates give about 40 % α -helical content for all these chloroform/methanol-extracted polypeptides.

Apparently, the peptides recovered with formic acid contain even less α -helical structure than those extracted with chloroform/methanol. The spectrum of SP-C (SS) recovered by

Figure 3 CD spectra of SP-C (CC) recovered by chloroform/methanol extraction (—) and of SP-C (SS) (---) and SP-C (AAA) (· · · · ·) recovered by formic acid extractions

Conditions were as described in Figure 2.

formic acid and solubilized in DPC micelles (Figure 3) lacks the minima at 208 and 222 nm, typical of the α -helical conformation [34], but has a broad minimum between 210 and 220 nm, indicative of β -sheet structures [34]. From this we estimate a helical content of less than 20 % for this peptide. The spectra of SP-C (FF) and SP-C (CC), recovered with formic acid, are very similar to that of SP-C (SS) (Figure 3). Together, this indicates that the acid treatment induces the formation of extended polypeptide structures that remain also after removal of the acidic solvent and incorporation into DPC micelles.

To determine whether the positively charged residues of the N-terminal part influence the ability to form α -helical secondary structures, SP-C (AAA) (Table 1) was analysed. SP-C (AAA) was synthesized to correspond to native SP-C without the palmitoyl groups and with all positively charged residues replaced by Ala. CD analysis indicates a different conformation of this peptide mixed with DPC, compared with native SP-C and also compared with the full-length analogues with preserved positive net charge (Figure 3). Apparently, SP-C (AAA) forms mainly, possibly entirely, non- α -helical secondary structures in DPC micelles, since its spectrum lacks the minima at 208 and 222 nm and also the maximum around 195 nm (Figure 3) that are indicative of an α -helical secondary structure [34]. The minimum around 215 nm suggests, however, that it contains β -sheet-like conformations. The CD appearance of SP-C (AAA) was not influenced by the solvents used for extraction of the polypeptide.

Temperature-stability of micelle-bound native and depalmitoylated SP-C

To characterize further the stability of the SP-C α -helix, with and without palmitoyl groups present, the temperature effects on native and depalmitoylated SP-C in DPC micelles were analysed. A gradual increase in the temperature from 20 to 90 °C gives a continuous, small, increase in the molar ellipticity at 222 nm for both polypeptides, but no sigmoidal increase indicative of co-operative melting is observed in this temperature range (results

Figure 4 Temperature effects on the secondary structure of depalmitoylated SP-C

CD spectra of depalmitoylated SP-C in 10 mM DPC/50 mM sodium phosphate buffer, pH 6.0 at 20 °C (—) and at 90 °C (·····).

not shown). Figure 4 shows the spectra of depalmitoylated SP-C in DPC micelles at 20 °C and at 90 °C. A moderate decrease in α -helical content can be observed at the higher temperature, but a significant amount of ordered secondary structure remains also at 90 °C, corresponding to approx. 45% α -helical structure, and the spectrum remains virtually constant for at least 1 h at this temperature. The presence of the SP-C palmitoyl groups does not significantly influence the thermal stability in detergent solutions, since a native SP-C/DPC mixture exhibits nearly identical spectral changes. The temperature effects observed are partly reversible, since cooling the mixtures to 20 °C gives spectra similar to, although not identical with, those obtained before heating.

The SP-C/bacteriorhodopsin hybrid adopts the expected α -helical secondary structure

The SP-C/BR hybrid (Table 1) was designed to replace the transmembrane poly-valyl α -helix of native SP-C [21] with an unrelated amino acid sequence of a known transmembrane α -helix. For these reasons, the transmembrane α -helix B of bacteriorhodopsin was chosen, corresponding to positions 42–64 of that intact protein (see [35]), with the only exceptions that Pro-50 was replaced with Ala, in order not to interfere with the helical backbone hydrogen bonding, and that Met-56 and Met-60 were replaced with Leu to simplify synthesis. SP-C/BR, as predicted, forms a mainly α -helical structure in DPC micelles, and the overall secondary structure is similar to that of native SP-C (Figure 5). SP-C/BR contains about 80% α -helix. This is in agreement with the α -helical content of the NMR structures of bacteriorhodopsin positions 34–65 determined in organic solvents and SDS micelles (about 70%) [36]. This shows that the poly-valyl part of SP-C and the bacteriorhodopsin segment 42–64 differ significantly in their ability to form a stable α -helical conformation in isolated synthetic peptides, although both segments occur as transmembrane α -helices *in vivo*.

The helical conformation of SP-C/BR in DPC micelles is also resistant to thermal denaturation, exhibiting CD spectra at 20 °C and 90 °C that are not significantly different from those of SP-C at these temperatures (Figure 4).

Figure 5 CD spectrum of SP-C/BR in 10 mM DPC/50 mM sodium phosphate buffer, pH 6.0, at 22 °C

Figure 6 Plots of surface pressure versus time with native pig SP-C (■—■), SP-C (CC) recovered with chloroform/methanol (●—●), SP-C (CC) initially recovered with chloroform/methanol and later treated with formic acid (○—○), SP-C (SS) (○—○), SP-C (FF) (▲—▲), SP-C (AAA) (□—□) or SP-C/BR (△—△) mixed with DPPC/PG/PA (68:22:9, by wt.) compared with phospholipids alone (■—■)

The SP-C (SS), SP-C (FF) and SP-C (AAA) peptides were recovered with formic acid (see the text for details). All values are representative of three separate measurements. At 6 and 10 s, native SP-C > SP-C (AAA), SP-C (SS), SP-C (FF), SP-C (CC, formic acid) ($P < 0.05$). At 20 s, as at 6 and 10 s, plus SP-C (CC) and SP-C/BR > SP-C (SS), SP-C (FF) ($P < 0.05$). At 30 and 60 s, native SP-C, SP-C (CC), SP-C/BR > SP-C (AAA), SP-C (SS), SP-C (FF), SP-C (CC, formic acid) ($P < 0.01$).

The α -helical content of the SP-C analogues influences the biophysical activity

The pure lipid mixture DPPC/PG/PA (68:22:9, by wt.) has a low surface activity. Addition of one of the synthetic peptides (Table 1) to this mixture accelerates the spreading at the air/liquid interface and increases the surface pressure of the film. As seen in Figure 6, the effects of the peptides on these parameters vary considerably. Native SP-C, SP-C/BR and SP-C (CC) recovered by chloroform/methanol, all containing α -helical secondary structures (Figures 2 and 5), significantly increase the spreading rate compared with pure lipid mixtures (Figure 6), and the surface pressures achieved after 1 min (about 45 mN/m) are comparable with those obtained under the same conditions with pig surfactant preparations containing phospholipids, SP-B and

SP-C [37]. SP-C (SS) and SP-C (FF) recovered with formic acid and SP-C (AAA), all of which contain little or no α -helical conformations (Figure 3), mixed with the same lipids show rapid spreading, but the surface pressure reaches a significantly lower level, only about 30 mN/m ($P < 0.01$ versus native SP-C at 20–60 s; Figure 6). At the time points 6 and 10 s statistically significant differences in surface pressure were found for native SP-C versus SP-C (AAA), SP-C (SS), SP-C (FF) and SP-C (CC) treated with formic acid as described below ($P < 0.05$), and for the phospholipid mixture versus all other preparations ($P < 0.01$). (Further statistical analysis is given in the legend to Figure 6.) In a control experiment, SP-C (CC) initially recovered by chloroform/methanol was solubilized in conc. formic acid, incubated for 24 h at room temperature, the solvent was evaporated, and the peptide reconstituted with the lipid mixture. After such acid treatment SP-C (CC) contains very little, if any, α -helical structure exhibiting a CD spectrum very similar to that of SP-C (SS) in Figure 3, and shows spreading kinetics nearly identical with those of the peptides initially recovered by formic acid extractions (Figure 6). This confirms the importance of an α -helical conformation for the spreading properties. The surface properties of all non-helical peptides are clearly inferior to those of surfactant preparations from natural sources, suggesting that such polypeptides may not be appropriate constituents of synthetic surfactant substitutes.

DISCUSSION

We have investigated the secondary structures of native and depalmitoylated SP-C as well as five 35-residue analogues in micelles of synthetic DPC. In addition, the biophysical activities of the peptides combined with DPPC/PG/PA were studied. By this, the overall secondary structures and spreading kinetics of SP-C and related analogues in phospholipid environments can be rapidly evaluated by using only small amounts of protein, and this method appears suitable for screening of structure–function relationships of synthetic analogues. The α -helical contents now estimated from CD data seem to be about 10% above those determined from available atomic-resolution structures of native SP-C [21] and of bacteriorhodopsin transmembrane segment B [36]. Slight overestimation of α -helical contents of lipid-associated polypeptides by CD spectroscopy has been encountered [38], but this is here considered to be of minor importance, since the relative helical contents of similar peptides are considered.

In agreement with observations from IR spectroscopy of native and depalmitoylated SP-C in phospholipid bilayers [20], the thioester-linked palmitoyl chains at positions 5 and 6 are now found to affect the polypeptide structure (Figure 2). Their removal causes an approx. 20% loss of the α -helical structure. Assuming that this loss occurs entirely in the N-terminal part, where the palmitoylcysteine residues are located, the helix would start near position 15 in depalmitoylated SP-C, instead of at position 9 as in the native molecule [21], leaving essentially only the poly-aliphatic part of the peptide chain in an α -helical conformation. This decrease in α -helical structure may be brought about via loss of direct palmitoyl/peptide contacts, for instance hydrogen bonds between palmitoyl carbonyl oxygen atoms and peptide amide protons in the first turn of the helix. Alternatively, loss of palmitoyl/phospholipid interactions may indirectly make SP-C less firmly anchored in the phospholipid environment, and thereby affect the secondary structure. The fact that no palmitoyl/peptide nuclear Overhauser effects (NOEs) could be observed in the NMR study of native SP-C in organic solvents [21] favours the view that the palmitoyl groups mainly influence the α -helical structure via indirect effects. Additional

factors may, however, also be of importance, since synthetic SP-C molecules without palmitoyl chains are less α -helical than is depalmitoylated SP-C (Figure 2) and can also adopt entirely different, non-helical, conformations after acid treatment (Figure 3). It cannot be ruled out that the trimethylamine treatment used for depalmitoylation as such induces non-helical conformations and thereby contributes to the observed loss of α -helical structure. Literature data further indicate a complex nature of the SP-C conformation. A dimeric form of SP-C that lacks palmitoyl groups and exists predominantly in β -sheet conformations has been reported to be present in surfactant purified from natural sources [39]. On the other hand, the helical content of palmitoylated and non-palmitoylated recombinant human SP-C, in an interfacial model system, is apparently not significantly different [40], and non-palmitoylated recombinant SP-C has even been reported to exhibit a higher helical content (about 70%) than native dog SP-C (about 50%) [41]. These apparent discrepancies cannot be explained at present, but it seems likely that even small variations in experimental conditions may cause significant effects, since, for example, native SP-C dissolved in acidified organic solvents exhibits a time-dependent conversion into a non-helical structure [21]. The significance of protein palmitoylation in general is also not fully understood and may vary depending on the protein modified, since, in the case of mammalian rhodopsin, which like SP-C contains two juxtaposed palmitoylcysteine residues [42], depalmitoylation does not significantly affect the secondary structure but decreases the thermal stability [43].

The present data indicate that the poly-valyl α -helix of SP-C exhibits several distinct properties which are unrelated to the presence or absence of the covalently linked palmitoyl side chains. The α -helix of native SP-C and that of depalmitoylated SP-C, known and assumed, respectively, to be located mainly in the poly-valyl part, are both highly insensitive to thermal unfolding in DPC micelles (Figure 4), and remain to a large extent also after prolonged incubation at 90 °C. The close packing of the valyl γ -methyl groups in the native α -helical conformation [21] contributes to this stability by limiting the side-chain rotational freedom and the opening of the polypeptide backbone structure [43a]. Additional factors, for example hydrophobic interactions between the helix surface and surrounding lipid acyl-chains, must also be of importance, as SP-C/BR, which lacks the poly-valyl part, is thermostable in DPC micelles. The rigidity of the SP-C α -helix may be a prerequisite for the ability to affect phospholipid organization in pulmonary surfactant. The folding into this α -helical conformation is, however, apparently not fully achieved under conditions *in vitro* now used, since the synthetic polypeptide SP-C (CC) exhibits significantly lower content of α -helical secondary structure than does depalmitoylated SP-C (Figure 2), although they are covalently identical. Whether the low α -helical content of SP-C (CC) is caused by the sole existence of a peptide population with a shorter α -helical part or by coexistence of helical and non-helical peptides cannot at present be resolved. Notably, however, the α -helical content of SP-C (CC) observed in chloroform/methanol (about 40%; Figure 2) is similar to the α -helical content of a synthetic 15-residue poly-valyl segment in aq. 98% methanol (about 50%) [44], reinforcing the idea that a stable poly-valyl α -helix as observed in native SP-C [21] is not easily adopted by synthetic poly-valyl-containing peptides. The problems of forming an α -helix in synthetic SP-C is in all likelihood caused by the many consecutive valine residues, since valine residues are under-represented in helical structures [45]. Together, these circumstances make it tempting to hypothesize that, once formed, the native SP-C poly-valyl α -helix is highly stable, but

that the SP-C precursor and/or other factors present during folding of SP-C *in vivo* are necessary for complete formation of this structure.

A consequence of the problems encountered in forming an α -helix *de novo* with synthetic SP-C polypeptides containing the long stretch of consecutive β -carbon branched residues (Figures 2 and 3), and the apparent significant concomitant decrease in effect on phospholipid spreading of non-helical SP-C analogues (Figure 6), is that synthesis of the correctly folded biologically active SP-C by chemical means appears not to be fully feasible. To investigate the effects on structure and function by replacements of the entire poly-valyl segment with an α -helix containing a completely unrelated amino acid sequence, we synthesized the SP-C/bacteriorhodopsin hybrid (Table 1) and compared it with the poly-valyl SP-C analogues. The hybrid polypeptide adopts the secondary structure expected (Figure 5), and its effects resemble those of the native SP-C on phospholipid spreading and surface pressure in a model system *in vitro* (Figure 6). These results are promising for the design of peptide analogues for use in synthetic surfactant preparations, as they indicate that primarily the secondary structure determines the surface properties of such analogues. This also raises important questions concerning the general structure–function relationships of α -helical peptides in surfactant preparations.

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