Accelerated Publications

Size and Structure of the Hydrophobic Low Molecular Weight Surfactant-Associated Polypeptide†

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ABSTRACT: The most abundant low molecular weight protein of pulmonary surfactant has unusual properties. Its primary structure has now been determined by analysis at the protein level. The highly hydrophobic polypeptide is resistant to cleavage with proteolytic enzymes, but it was possible to generate fragments by limited cleavage with concentrated HCl or with sodium in liquid ammonia. Acid hydrolysis of the peptide required exceptional conditions for release of all residues. The N-terminus is heterogeneous, and in its longest form the primary structure consists of 35 residues. This analysis establishes that the size of the major native hydrophobic surfactant polypeptide is considerably smaller than previously proposed. Biological effects of the polypeptide recombined with phospholipids are confirmed in vitro by using a pulsating bubble system and in vivo by using premature newborn rabbits. The molecule has branched-chain amino acid residues at about two-thirds of all positions and lacks nine types of residue. The middle third is composed entirely of hydrophobic residues, and fragments from this part are sparingly soluble even in organic solvents. The hydrophobic region is preceded by a more hydrophilic, N-terminal segment. Thus, the molecule has two contrasting parts, like a detergent, which may explain its essential role in the pulmonary surfactant system.

Respiratory distress syndrome, a common cause of death in premature infants, is due to deficiency of pulmonary surfactant. This disease is characterized by alveolar collapse, caused by a failure to reduce surface tension to near zero at end expiration. Replacement therapy with organic solvent extracts of natural surfactant has shown excellent effects (Fujiwara et al., 1980; Enhorning et al., 1985; Gitlin et al., 1987; Raju et al., 1987). Essential components of these preparations are hydrophobic low molecular weight proteins, known under designations 3–18 kDa (Phizackerley et al., 1979; Glasser et al., 1987; Hawgood et al., 1987; Possmayer et al., 1987; Curstedt et al., 1987). They facilitate the adsorption of surfactant phospholipids to the air/liquid interface (Suzuki et al., 1986; Takahashi & Fujiwara, 1986; Whitsett et al., 1986; Yu & Possmayer, 1986; Curstedt et al., 1987; Notter et al., 1987). In a previous study, we have separated these proteins from pig surfactant into two fractions of apparently unrelated structure and have shown that addition of either of these fractions to synthetic phospholipid mixtures gives preparations with biophysical properties similar to those of natural surfactant (Curstedt et al., 1987). The structure of the larger type of peptide has been determined by protein analysis (Curstedt et al., 1988). In the present study, we characterize the smaller type of polypeptide, which is the more abundant of the two low molecular weight surfactant-associated proteins. During preparation of this paper, a report on the cDNA structure of a pro form of the corresponding protein from another species appeared (Warr et al., 1987), but the exact size has previously not been determined for the polypeptide from any source. The present analysis proves the structure of the protein itself, establishes the size, and reveals some heterogeneity in size at the N-terminus.

Materials and Methods

The polypeptide, isolated from porcine pulmonary surfactant by Sephadex LH-60 chromatography, was reduced and ^{14}C-carboxymethylated (Curstedt et al., 1987, 1988). Samples solubilized in chloroform/methanol, 1:2 (v/v), were degraded in an Applied Biosystems 470A or a Beckman 890D sequencer. Liberated amino acid derivatives were identified by reverse-phase high-performance liquid chromatography (Curstedt et al., 1988).

CNBr cleavage was performed in 70% formic acid with 0.1 g of CNBr/mL, and limited acid hydrolysis was carried out in 12 M HCl, both at room temperature for 24 h. The CNBr cleavage products were isolated by Sephadex LH-60 chromatography in chloroform/methanol, 1:1 (v/v), containing 5% 0.1 M HCl. The hydrophilic peptides from the limited acid hydrolysis were recovered in the polar phase of the system chloroform/methanol/water, 8:4:3 (by volume), were purified by high-performance liquid chromatography on a C18 column, using a gradient of acetonitrile in 0.1% trifluoroacetic acid, and were then subjected to sequencer analyses. For C-terminal analysis, the intact material was treated with anhydrous hydrazine for 6 h at 110 °C.

Enzymatic cleavages were tested for 4–16 h, with substrate:protease molar ratios of up to 1:1 in 0.1 M ammonium bicarbonate for trypsin and 5% formic acid for pepsin.

Acid hydrolysis of the intact polypeptide was found to require both longer times and higher temperatures (72 h, 150

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Table I: Amino Acid Compositions of the Hydrophilic Peptides Used To Establish the Structure of Porcine Low Molecular Weight Surfactant Polypeptide[a]

	peptide								
	1–6	1–7	1–9	1–13	3–5	7–10	30–32	30–35	34–35
Cys(Cm)	1.4 (2)	1.7 (2)	1.4 (2)	1.8 (2)	0.9 (1)				
Asx			0.9 (1)	0.8 (1)		0.8 (1)			
Pro	1.1 (1)	1.6 (2)	1.9 (2)	2.4 (2)	1.0 (1)	0.9 (1)			
Gly								1.3 (1)	1.1 (1)
Ala							1.0 (1)	1.0 (1)	
Val			0.6 (1)	0.8 (1)		1.0 (1)			
Met								1.1 (1)	
Ile	1.2 (1)	1.1 (1)	1.1 (1)	1.2 (1)	1.0 (1)				
Leu	1.0 (1)	1.1 (1)	1.1 (1)	2.6 (3)		1.1 (1)	2.2 (2)	2.7 (3)	1.0 (1)
Lys				1.2 (1)					
Arg	0.9 (1)	1.0 (1)	1.0 (1)	1.9 (2)					
sum	6	7	9	13	3	4	3	6	2

[a] Peptides are indicated by start and end positions. Values are molar ratios after 24-h hydrolysis with 6 M HCl/0.5% phenol at 110 °C (integers from sequence analysis within parentheses). Total compositions of the hydrophobic peptides 14–27 and 14–30 were not determined since they were recovered incompletely separated from the intact polypeptide.

°C; cf. legend to Table II) than those necessary to obtain complete liberation of residues from most proteins.

A synthetic phospholipid mixture, containing 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol in the weight proportions 11:7:2, was suspended in saline, with or without the purified low molecular weight surfactant polypeptide [protein:phospholipid ratio 1:50 (w/w)]. The properties of the preparations were characterized at 37 °C by using a pulsating bubble system (Enhorning, 1977) (phospholipid concentration 10 mg/mL; pulsation rate 40/min; surface compression 50%) and by experiments with premature, newborn rabbits. These animals were obtained by hysterotomy after 27 days of gestation (term 31 days) and tracheotomized. One group was treated with 2 mL/kg of the preparation containing phospholipids plus protein (phospholipid concentration 80 mg/mL), another group received the same amount of phospholipids only, and the control group consisted of nontreated litter mates. The animals were ventilated for 15 min with 100% oxygen, a peak pressure of 25 cm of H_2O, a frequency of 40/min, and an inspiration:expiration time ratio of 1:1. Tidal volumes were measured with a combined body plethysmograph/pneumotachygraph system (Lachmann et al., 1981).

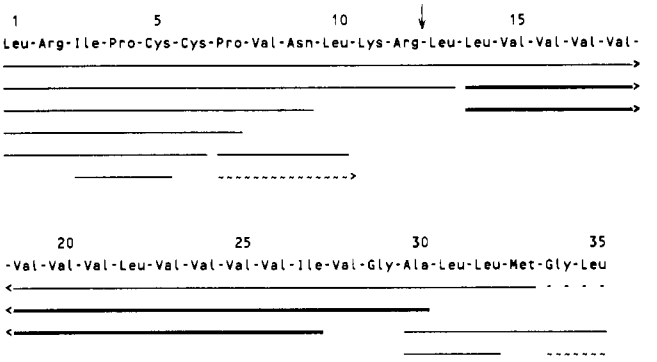

FIGURE 1: Primary structure deduced for the low molecular weight polypeptide from pig surfactant. The longest form is shown (truncated forms start at positions 2 and 3; cf. text). All peptide fragments analyzed are indicated. The top line denotes the intact material analyzed for 33 steps. The remaining straight lines indicate those fragments obtained by limited acid hydrolysis with concentrated HCl and subsequent separation in a chloroform/methanol/water system (cf. text) into peptides recovered from the hydrophilic phase (thin lines) and the lipophilic phase (thick lines). Waved lines denote the peptides obtained by other chemical cleavages (Na in liquid ammonia for the peptide starting at position 7 and CNBr for the one starting at position 34). Positions 21 and 23 were difficult to judge because of the unusual solubility properties, the preceding repetitive Val sequence, and the N-terminal heterogeneity of the starting material. The arrow indicates the transition site from some hydrophilicity to extreme hydrophobicity.

RESULTS

Multiple N-termini were detected by amino acid sequence analysis. Peptide chains start at positions Leu-1, Arg-2, and Ile-3 (Figure 1), in relative amounts of about 70%, 20%, and 10%, respectively. This ragged end, together with the extreme hydrophobicity of the middle region, complicated sequence determination, but an internal Met (position 33, Figure 1) was established by repeated analyses.

Cleavage with CNBr produced two fragments. They were separated by chromatography on Sephadex LH-60 (Figure 2A). As shown, the large fragment (positions 1–33) was detected as a radioactive fraction, while the dipeptide Gly-Leu (positions 34–35) was detected by acid hydrolysis of later fractions (glycine and leucine in equimolar amounts) and by sequence analysis. Attempts to cleave the material with trypsin and pepsin did not produce detectable fragments.

Limited acid hydrolysis (concentrated HCl for 24 h at room temperature) was successful for generation of fragments. The hydrophilic fragments, recovered in the polar phase of a chloroform/methanol/water system were purified by reverse-phase high-performance liquid chromatography (Figure 2B). Analysis of these peptides (Table I, Figure 1) established the structures of the N- and C-terminal regions. The lipophilic phase of the separation system contained intact, uncleaved material and fragments from the extremely hydrophobic middle third of the molecule. After lyophilization, the intact polypeptide was soluble in both chloroform and heptane, while the central fragments required 0.1% SDS for acceptable solubilization.

In an attempt to overcome the N-terminal heterogeneity, intact material was cleaved with metallic sodium in liquid ammonia (Hempel & Jörnvall, 1985) and subsequently extracted with the chloroform/methanol/water system described above. A fragment starting at Pro-7 was obtained, but in a yield of only 20%, and was not separated from the intact material.

The C-terminal end was established from the structures of two fragments (Figure 1) and by recovery of leucine upon hydrazinolysis of the intact polypeptide. Therefore, this surfactant-associated polypeptide has 35 residues in its longest form. Because of the extreme hydrophobicity of the middle fragment, hydrolysis of the whole polypeptide requires many days and elevated temperature for liberation of all

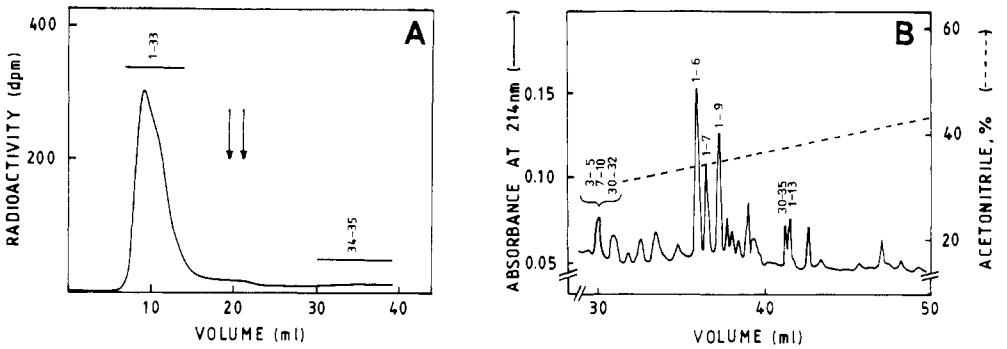

FIGURE 2: Purification of fragments of the low molecular weight surfactant polypeptide. (A) Fragments generated by CNBr cleavage. Separation was on Sephadex LH-60 (1.1 × 40 cm) in chloroform/methanol, 1:1 (v/v), containing 5% 0.1 M HCl (flow 6 mL/h; 2 mL/fraction). The pooled peptide fractions (cf. Results) are indicated by horizontal bars (numbers show positions covered). Arrows indicate elution positions of uncleaved material before (left) and after (right) carboxymethylation (mean of five and eight runs, respectively). As shown, CNBr cleavage (removing two residues) significantly alters the elution to an earlier position, whereas carboxymethylation has only a small effect. (B) Fragments produced by limited acid hydrolysis and recovered in the methanol/water phase of a chloroform/methanol/water system. Separation was by high-performance liquid chromatography on Ultropac C18 (5 μm; 4.6 × 250 mm) with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid (flow 1 mL/min).

Table II: Hydrolysis of the Carboxymethylated Low Molecular Weight Surfactant Polypeptide under Different Conditions[a]

	acid hydrolysis				composition from sequence analysis (Figure 1)
	110 °C		150 °C		
	24 h	168 h	24 h	72 h	
Cys(Cm)	1.8				2
Asx	1.3				1
Pro	2.2				2
Gly	2.1				2
Ala	1.1				1
Val	1.9	3.6	11.6	12.8	13
Met	1.1				1
Ile	1.1	1.4	1.4	1.2	2
Leu	6.0	6.8	7.6	8.0	8
Lys	1.0				1
Arg	2.1				2

[a] Acid hydrolysis was performed in evacuated tubes with 6 M HCl containing 0.5% phenol. Analytical values are molar ratios with all residues indicated only for 24-h hydrolysis at 110 °C. Values for Val, Ile, and Leu increase with time and temperature as shown for these residues (calculated relative to Asx and Ala, which are stable under these conditions). The decreasing yield of Ile at 150 °C is explained by rearrangement during hydrolysis (controls of pure Ile indicated a loss of 40% after 72 h at 150 °C).

branched-chain residues (Table II).

The surface properties of the protein/phospholipid mixture tested in vitro in the pulsating bubble system (Enhorning, 1977) were clearly superior to those of the phospholipids alone. Values (mean ± SEM; $n = 5$) for minimal surface tension, recorded after 5 min of pulsation, were 5 ± 2 and 23 ± 1 mN/m, respectively ($p < 0.01$). Tidal volumes from the experiments with premature, newborn rabbits support these differences. Animals treated with the protein/phospholipid mixture had a median tidal volume of 12 mL/kg body weight (range 2.9–31 mL/kg; $n = 11$) versus 1.5 mL/kg (range 0.9–2.5 mL/kg; $n = 10$) for the controls ($p < 0.001$) and 5.0 mL/kg (range 2.0–11 mL/kg; $n = 10$) for the group receiving phospholipids only ($p < 0.01$).

Discussion

Both the surfactant polypeptide now analyzed and the one in the other fraction discussed above [with 79 residues (Curstedt et al., 1988)] require organic solvents for solubilization. However, their amino acid sequences are unrelated and the structure now established is unique. It lacks acidic residues, aromatic residues, and residues containing hydroxyl groups. Its central region has only Val, Ile, and Leu and

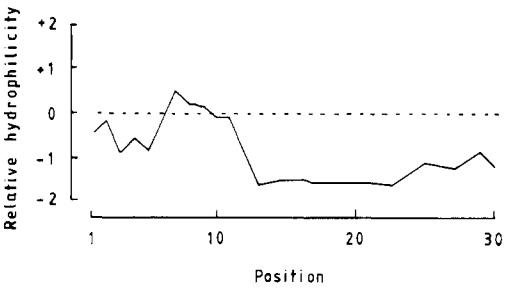

FIGURE 3: Hydropathy profile of the surfactant-associated low molecular weight polypeptide. Values are calculated for six-residue segments as in Hopp and Woods (1981) and plotted against the starting position of each segment.

includes a stretch of seven consecutive Val residues.

The amino acid sequence of the middle part, especially positions 21–28, was difficult to establish. Peptides from that part were generated only by limited acid hydrolysis (Figure 1) and were recovered as mixtures. Moreover, the multiple N-terminal starting points and the seven consecutive valine residues complicated the analyses, especially at positions 21 and 23. However, the final structure now determined for the pig polypeptide is in excellent agreement with the total composition (Table II). Furthermore, it is strictly homologous to a segment of the precursor of the human protein, the structure of which was deduced from the nucleotide sequence of a cDNA clone (Warr et al., 1987). N-Terminal sequences have been reported for human (Whitsett et al., 1987) and bovine (Phelps et al., 1987; Yu et al., 1987) low molecular weight surfactant-associated proteins and are similar to the N-terminal part of the structure now determined. Differences could reflect species variations, difficulties in interpretation arising from the unusual properties of the polypeptide, or preparation heterogeneities.

Together with surfactant phospholipids, this polypeptide generates a film that reduces surface tension to low values during surface compression. The essential physiological properties of the polypeptide were confirmed in experiments on artificially ventilated immature, newborn rabbits and by in vitro bubble experiments. These properties may be explained by the hydropathy pattern of the surfactant 35-residue polypeptide (Figure 3). The C-terminus now established for the native peptide exactly coincides with the end of the hydrophobic segment defined from the precursor molecule studied at the cDNA level (Warr et al., 1987). The structural arrangement of the native polypeptide resembles that of a de-

tergent, consisting of two parts with opposite properties, an N-terminal hydrophilic "handle" with three residues positively charged at physiological pH and two juxtapositioned half-cystine residues, adjacent to a part with extraordinary hydrophobicity. Presumably, these properties account for the vital surfactant activity of the polypeptide in the lung.

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Uptake of Cesium Ions by Human Erythrocytes and Perfused Rat Heart: A Cesium-133 NMR Study

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ABSTRACT: Cesium-133 NMR studies have been carried out on suspended human erythrocytes and on perfused rat hearts in media containing CsCl. The resulting spectra exhibit two sharp resonances, arising from intra- and extracellular Cs^+, separated in chemical shift by 1.0–1.4 ppm. Thus, intra- and extracellular resonances are easily resolved without the addition of paramagnetic shift reagents required to resolve resonances of the other alkali metal ions. Spin–lattice relaxation times in all cases are monoexponential and significantly shorter (3–4 times) for the intracellular component. When corrections are made for the pulse repetition rate, the total intensity of the intracellular and extracellular Cs^+ resonances in erythrocytes is conserved, implying total observability of the intracellular pool. The uptake of Cs^+ by erythrocytes occurs at approximately one-third the reported rate for K^+ and was reduced by a factor of 2 upon addition of ouabain to the sample. These results indicate that ^{133}Cs NMR is a promising tool for studying the distribution and transport of cesium ions in biological systems and, in some cases such as uptake by cellular Na,K-ATPase, for analysis of K^+ ion metabolism.

Interest in the biochemistry and physiology of cesium ions derives primarily from three areas: (1) applications related to elucidating the general properties of alkali metal ion transport and enzyme activation (Eisenman & Krasne, 1975), (2) toxicologic problems related to the uptake and passage through food chains of radioactive Cs^+ produced in fission