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Hydrophobic surfactant-associated polypeptides: SP-C is a lipopeptide with two palmitoylated cysteine residues, whereas SP-B lacks covalently linked fatty acyl groups

(thioester/pulmonary surfactant/mass spectrometry)

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ABSTRACT **Pulmonary surfactant contains two hydrophobic polypeptides, SP-B and SP-C, with known amino acid sequences and with truncated subforms lacking the N-terminal residues. Treatment of SP-C with KOH releases fatty acids (palmitic acid to more than 85%) in molar ratios of 1.8–2.0 relative to the polypeptide. Furthermore, plasma-desorption mass spectrometry shows native SP-C of both the intact and truncated types to be monomers with masses about 500 units higher than those expected for the polypeptide chains. After treatment with KOH, trimethylamine, or dithioerythritol, the polypeptide masses are obtained. These results prove that native SP-C is a lipopeptide with two palmitoyl groups covalently linked to the polypeptide chain. The deacylation conditions, the presence of two cysteine residues in the polypeptide, and the absence of other possible attachment sites establish that the palmitoyl groups are thioester-linked to the two adjacent cysteine residues. In contrast, the major form of porcine SP-B is a dimer without fatty acid components. That SP-C is a true lipopeptide with covalently bound palmitoyl groups suggests possibilities for functional interactions. It gives a direct physical link between SP-C and surfactant phospholipid components. Long-chain acylation may constitute a means for association of proteins with membranes and could conceivably modulate the stability and biological activity of surfactant films.**

Surfactant is essential for pulmonary function. It reduces the alveolar surface tension, preventing alveolar collapse, and is a prerequisite for normal respiration (1). The capacity for surfactant synthesis is limited in premature infants, and low amounts of surfactant are associated with neonatal respiratory distress syndrome. This serious disease can be treated effectively by replacement therapy through instillation of surfactant in the airways (2–7).

Natural surfactant is composed of phospholipids and small amounts of specific polypeptides. In particular, two highly hydrophobic, low molecular weight polypeptides named SP-B and SP-C (8) appear to be essential components of surfactant preparations for replacement therapy (2, 3, 5–7). Both are derived from larger precursor polypeptides and have been structurally analyzed at the cDNA (9–16) and protein (17–20) levels. Protein studies are essential for characterization of the mature polypeptides but are complicated by the extreme peptide hydrophobicities. SP-C in particular has essentially no aqueous solubility and is largely inert to conventional hydrolysis and other analytical steps. Furthermore, native preparations are heterogeneous, with the presence of the two polypeptides in different subforms constituting N-terminally truncated molecules (20, 21).

Nevertheless, the primary structures of the polypeptides have been determined (18–20), showing SP-B to be a 79-residue polypeptide with a high content of cysteine residues, and SP-C to be a 35-residue polypeptide with two adjacent cysteine residues in a "palindromic" sequence (-Ile-Pro-Cys-Cys-Pro-Val-). SP-C is of further interest because of the presence of two segments of different apparent polarity, an N-terminal "conventional" region and a middle/C-terminal region with essentially only branched-chain hydrophobic residues (19). A highly limited species variation shows that these properties are strictly conserved (20), but the exact status of the cysteine residues and the type of lipid interaction have been unknown, although we have shown that SP-C fractions contain lipid components that cannot be separated from the polypeptides by chromatography on Sephadex LH-60 (21).

We now show that native, mature SP-C is a monomer with a palmitoyl group thioester-linked to each of the two cysteine residues. The results show that the N-terminal part of SP-C, previously concluded to be hydrophilic, is also hydrophobic. The presence of two palmitoyl groups, if not merely fortuitous, may lead to further understanding of molecular interactions during normal physiology.

MATERIALS AND METHODS

Isolation of SP-B and SP-C. The surfactant-associated polypeptides SP-B and SP-C were isolated from porcine pulmonary phospholipids as described (21). The corresponding human polypeptides were isolated in a similar manner from a lung lobe obtained after a tumor resection. The maximal concentration of phospholipids in the purified polypeptide fractions was determined by phosphorus analysis (22).

Deacylation of Polypeptides. Three methods were employed. One utilized trimethylamine treatment: SP-C (10–100 nmol) in 2 ml of chloroform/methanol, 1:2 (vol/vol), and 80 μl of 2 M trimethylamine was incubated at 37°C for 4 hr. After chromatography on Sephadex LH-60 (40 × 1.1 cm) in chloroform/methanol, 1:1 (vol/vol), containing 5% 0.1 M HCl, the polypeptide was recovered in the eluate corresponding to the first 60% of the column volume. Addition of 0.02–1% (wt/vol) dithioerythritol in all steps was also tested in order to stabilize the liberated cysteine thiols.

The second method utilized incubation of SP-C (about 10 nmol) at 40°C for 30 min in 1 ml of methanol/water, 98:2 (vol/vol), containing 0.1 M KOH, with heptadecanoic acid as internal standard. After neutralization with 0.1 ml of 1 M HCl, 2 ml of chloroform and 0.65 ml of water were added.

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Polypeptides and fatty acids released were recovered in the organic phase. The latter were methylated with boron trifluoride in methanol (23) and analyzed by capillary gas-liquid chromatography (21) and by gas chromatography–mass spectrometry in a Finnigan 1050 instrument.

The third method involved incubation of SP-C (about 5 nmol) in 1 ml of chloroform/methanol, 1:2 (vol/vol), with 2 μl of 0.5 M dithioerythritol at 37°C for 2 hr under nitrogen.

Chemical Analysis. Limited acid hydrolysis of SP-C was performed in 12 M HCl for 24 hr at ambient temperature (19, 20). Fragments produced were separated according to relative hydrophobicity by a chloroform/methanol/water system (19). Hydrophilic peptides recovered in the aqueous phase were separated by HPLC on Ultropac TSK C_{18} (19).

For reductive alkylation of peptide amino groups, SP-C (25 nmol) was dissolved in 1 ml of chloroform/methanol, 1:2 (vol/vol), adjusted to pH 9.0 with 0.1 M NaOH; 5 μl of 12 M formaldehyde was added, followed after 30 sec by four 20-μl portions of sodium borohydride (50 mg/ml) and 60 sec later by another 100 μl of borohydride solution.

Acid hydrolysis was performed at 110°C for 24 hr. Liberated amino acids were analyzed with a ninhydrin-based Beckman 121M instrument or with a phenylthiocarbamoyl-based HPLC system (24). Methylated and unmethylated lysine residues were separated on the long column of the Beckman analyzer by elution with pH 5.2 buffer. Gas-phase sequencer analysis was performed by degradation in an Applied Biosystems 470A instrument. Liberated amino acids were detected as phenylthiohydantoin derivates by reverse-phase HPLC.

Chemical Synthesis of SP-C. The SP-C polypeptide (porcine form) was synthesized in an ABI model 430A instrument using the *t*-butoxycarbonyl method and subsequent hydrogen fluoride treatment (25). The polypeptide was extracted from the resin with chloroform/methanol, 1:1 (vol/vol), with and without 5% 0.1 M HCl, in a yield of about 30%. After evaporation, the material was solubilized in concentrated formic acid, diluted with chloroform/methanol, 1:1 (vol/vol), and purified on Sephadex LH-60 in the same solvent with 5% formic acid added. The synthetic polypeptide was eluted at a position identical to that for native deacylated porcine SP-C. The quality of the synthetic product was checked by amino acid analysis and sequencer degradation.

Mass Spectrometry. The polypeptide (1–5 nmol) was dissolved in 10 μl of 0.1% trifluoroacetic acid/ethanol, 1:1 (vol/vol), or 10 μl of chloroform/methanol, 1:2 (vol/vol). About 5 μl of the solution was applied on a nitrocellulose-coated sample foil and dried under a stream of nitrogen. The sample was then rinsed with 20 μl of water, dried under a stream of nitrogen, and analyzed by a Bioion 20 ^{252}Cf plasma-desorption time-of-flight mass spectrometer (Uppsala, Sweden). The system was operated at an acceleration voltage of 18 kV. Time-of-flight measurements were made with a resolution of 1 nsec and converted to mass spectra by using the time centroids for H^+ and Al^{3+}. Spectral data were collected over a time period of 1–2 hr and background was subtracted. The data are given as mean ± SD with the number of determinations in parentheses.

RESULTS

Porcine SP-C. *Identification of non-amino acid components.* Treatment of SP-C with KOH releases saturated fatty acids, especially palmitic acid (Table 1), indicating that the native molecule is a fatty acyl-polypeptide complex. Stoichiometric determinations show that each SP-C molecule contains up to 1.8–2.0 fatty acyl groups. Due to the low content of phosphorus in the SP-C fraction (Table 1) only a minor fraction (<20%) of the fatty acids may originate from phospholipids. Further, the fatty acid composition of the isolated SP-C fraction and the surfactant phospholipids are quite different, also excluding a phospholipid origin of the fatty acids. Thus, the major form of native SP-C is likely to be a lipopeptide.

Unexpected size. The theoretical molecular weight of the major form of porcine SP-C (19) is 3710. However, the mass spectra show a molecular ion region at m/z 4210 ± 4 (n = 8) (Fig. 1*A*). This indicates that non-amino acid components are covalently linked to the polypeptide. The data also reveal an ion at m/z 4096 ± 6 (n = 5), confirming the presence of a truncated form of porcine SP-C, lacking the N-terminal leucine (Table 2). In two of the analyses, a molecular ion region of low intensity is observed at m/z 3965–3970. These molecules contain only one of the covalently bound non-amino acid components. No other ions are detected within the mass range 2000–10,000 (Fig. 1*A*). Thus, the results clearly show that porcine SP-C is a monomer with truncated forms, but for each form the molecular weight is about 500 units higher than that calculated from the amino acid sequence.

Treatment of SP-C with KOH, trimethylamine, or dithioerythritol removes the extra groups and brings the molecular weight back close to the expected value. The ions at m/z 3734 ± 6 (n = 11) and m/z 3619 ± 7 (n = 6) (Fig. 1*B* and Table 2) fit exactly with the loss of two palmitoyl groups from intact and N-terminally truncated SP-C, respectively. Reduction of SP-C with dithioerythritol for 2 hr gives only a partial deacylation (Fig. 1*C*). The ion at m/z 3968 ± 4 (n = 5) (Table 2) demonstrates the loss of one of the two palmitoyl groups from porcine SP-C. Thus, intact porcine SP-C is a lipopeptide with essentially all molecules containing two covalently bound palmitoyl groups.

Evidence for thioester-bound palmitoyl groups. The covalent linkage between the palmitoyl groups and the polypeptide chain is cleaved with dithioerythritol, indicating that the palmitoyl groups are thioester-linked to cysteine residues.

Table 1. Fatty acid content and composition in various fractions of porcine pulmonary surfactant

		Molar ratios		Fatty acid composition, mol %		
Fraction	Preparation	Fatty acids/ polypeptide	Phosphorus/ polypeptide*	Palmitic acid	Stearic acid	Others
SP-C	1	2.0	0.1	87	9	4
	2	2.0	0.2	87	10	3
	3	1.8	0.1	88	8	4
SP-B	1	0.8	0.3	61	23	16
	2	0.8	0.4	71	18	11
	3	1.2	0.4	75	16	9
Surfactant phospholipids				60	11	29

*The content of phosphorus gives an estimate of the maximum phospholipid value, showing this to be low in SP-C.

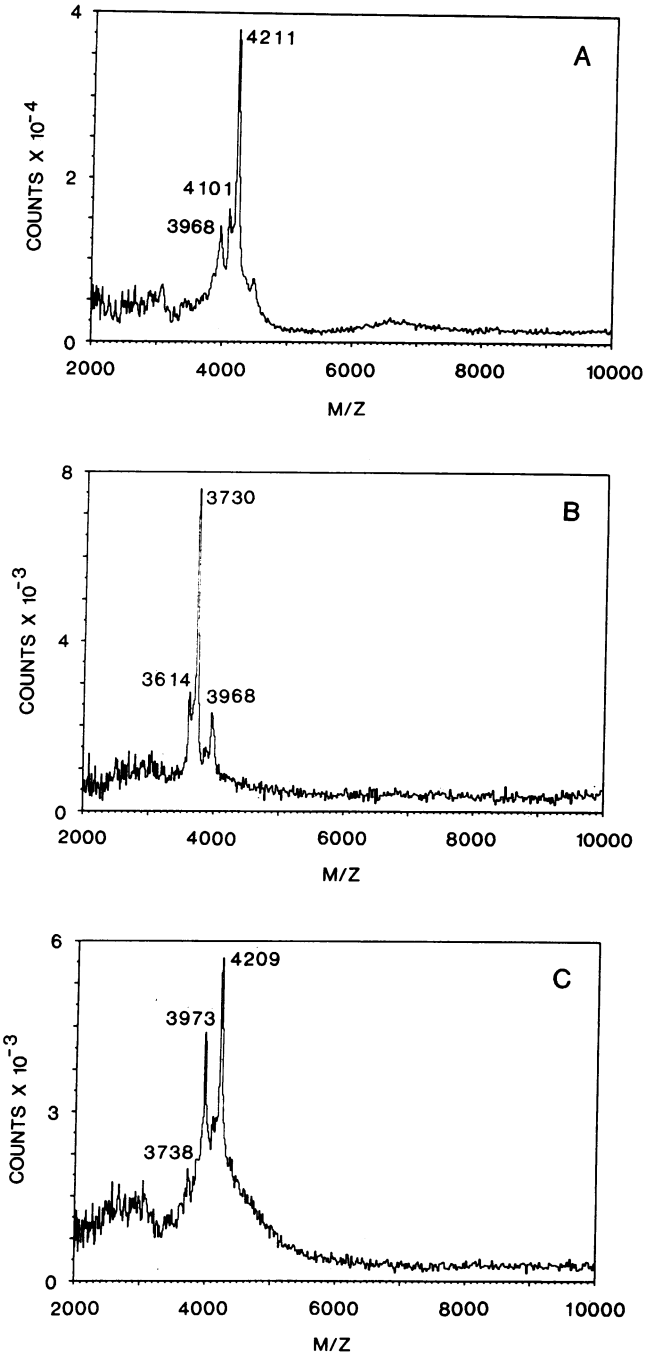

FIG. 1. Plasma-desorption time-of-flight mass spectra of intact (*A*), trimethylamine-treated (*B*), and reduced (*C*) porcine SP-C.

Moreover, the cysteines of intact porcine SP-C cannot be carboxymethylated by direct treatment with iodoacetate, indicating a lack of free thiol groups. In contrast, deacylated SP-C can be carboxymethylated (ion at *m/z* 3852).

For peptides in general, hydroxyl or amino groups can be other sites of acylation. However, such links are not cleaved by dithioerythritol. Further, the whole SP-C peptide lacks side-chain hydroxyl groups (19), and its α-amino group is free as verified by sequencer degradation. Thus, the two palmitoyl groups are concluded to be linked to the thiol groups of the two cysteine residues.

Cation adducts. The ions determined by plasma-desorption mass spectrometry of porcine SP-C are about 25 mass units higher than the molecular weight calculated from the amino acid sequence plus the two palmitoyl groups. A similar discrepancy applies to the deacylated porcine SP-C,

Table 2. Calculated molecular weights (M_r) and calculated and observed ions for various forms of porcine SP-C

SP-C form	Calculated M_r	Calculated $[M+Na]^+$ m/z	Observed m/z
Intact	4186	4209	4210 ± 4
Minus Leu (truncated)	4073	4096	4096 ± 6
Minus Pam	3948	3971	3968 ± 4
Minus 2 Pam	3710	3733	3734 ± 6
Minus Leu and 2 Pam	3597	3620	3619 ± 7

The intact molecule has two palmitoyl (Pam) residues thioester-linked to the cysteine residues. The observed ions are given as mean values ± SD for 5–11 determinations on three different preparations of porcine SP-C.

to the human intact and deacylated SP-C, and to the synthetic polypeptide (see below). Therefore, the structures were checked for additional modifications, such as hydroxyproline and methyllysine, or simply for misidentification of isoleucine or leucine as valine. However, all these possibilities can be excluded as the cause of the extra mass observed. Thus, amino acid analysis and mass spectrometry of peptide fragments (Table 3) show that SP-C does not contain hydroxyproline or methyllysine. The latter amino acid is also excluded by mass spectrometry of SP-C modified by methylation through reductive alkylation. An ion at *m/z* 3747 and recovery of methyllysine by amino acid analysis then show that this reaction proceeds normally, indicating initially free lysine. Similarly, reduction with sodium borohydride does not change the molecular weight, showing that the initial mass difference between theoretical and experimental values is not due to inadvertent sulfur oxidations. Instead, the extra mass is explained by the strong cationization of the molecules, indicating that the ions detected are the alkali metal (mainly sodium) ion adducts, as in other cases of plasma-desorption time-of-flight mass spectrometry of peptides (26, 27).

Synthetic SP-C polypeptide. This polypeptide has different solubility properties compared to those of native porcine SP-C. It is hardly soluble in organic solvents but can be dissolved in concentrated formic acid and remains largely in solution when chloroform/methanol is added. After purification of the polypeptide on Sephadex LH-60, amino acid analysis and sequencer degradation indicate that more than 90% is full-length material. Mass spectra of this synthetic SP-C polypeptide give a major ion at *m/z* 3733, i.e., 23 mass units higher than the calculated molecular weight, thus again suggesting the presence of sodium ion adducts.

Human SP-C Has the Same Substituents as Porcine SP-C. Human SP-C also has two palmitoyl groups thioester-linked to the cysteine residues of the polypeptide. This structure is established by ions at *m/z* 4204 and *m/z* 4057 (Fig. 2). The two ions, with a difference of 147 mass units, confirm that human SP-C has a truncated form, lacking N-terminal phen-

Table 3. Calculated molecular weights and calculated and observed ions for fragments obtained by limited acid hydrolysis of porcine SP-C

Fragment*	Calculated M_r	Calculated $[M+H]^+$ m/z	Observed m/z
LRIPCC (1–6)	704	705	704
LRIPCCPVNL (1–10)	1127	1128	1127
ALLMGL (30–35)	617	618	618

*Amino acid sequences are given in one-letter code. Residue numbers are in parentheses.

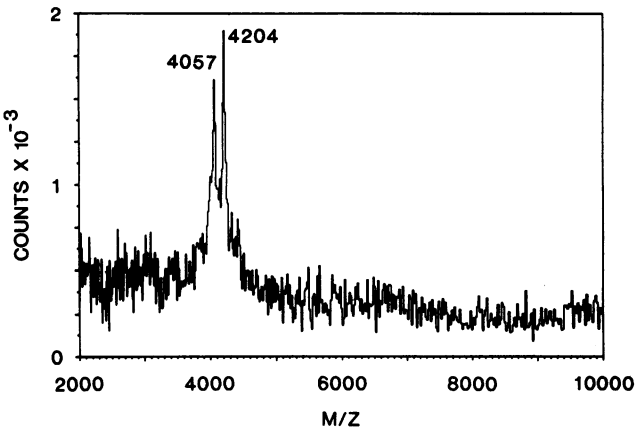

FIG. 2. Plasma-desorption time-of-flight mass spectrum of intact human SP-C.

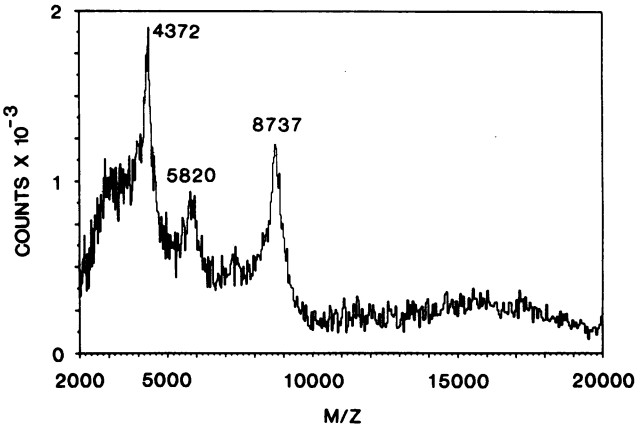

FIG. 3. Plasma-desorption time-of-flight mass spectrum of intact porcine SP-B.

ylalanine (20). No other ions are detected in the mass range 2000–10,000 (Fig. 2).

Deacylation is accomplished by treatment of human SP-C with trimethylamine, giving ions at m/z 3966, 3726, and 3581. The intensity of the ion at m/z 3966 decreases when SP-C is treated for long periods with trimethylamine, clearly showing that two non-amino acid components corresponding to palmitoyl groups are linked to the polypeptide. However, as in the case of porcine SP-C, the ions observed are about 30 mass units higher than the ones calculated from the amino acid sequence and verified by cDNA analysis, indicating that the ions produced by plasma-desorption mass spectrometry are complex, corresponding mainly to sodium/potassium ion adducts.

SP-B Lacks Substituents. Mass spectra of intact porcine SP-B give ions at m/z 8737, 5820, and 4372 (Fig. 3), probably representing ions with two, three, and four charges, respectively. By multiplication of the apparent ion masses with the number of charges, a molecular ion region at m/z 17,400–17,500 is obtained. This ion is similar to the theoretical molecular weight of a homodimer, about 17,400, and excludes linkages of fatty acids or carbohydrates to the molecule. This also proves the C terminus of SP-B to be at position 79, as previously suggested (18). Direct fatty acid analysis of SP-B (Table 1) also supports absence of covalently linked fatty acids in SP-B, as does mass spectrometry of reduced samples, which give ions at about m/z 8740 and 4370, representing ions with one and two charges. The ion at m/z 5820 found in the nonreduced sample is not detected, in agreement with its odd number of charges. Thus, porcine SP-B lacks covalently linked fatty acids and is concluded to be a dimer, although the data do not exclude the presence of monomers. Furthermore, mass spectrometry of nonreduced human SP-B gives weak but clear ions at about m/z 8700 and 4350, showing a lack of fatty acyl groups also in the human form of the polypeptide and hence suggesting SP-B generally not to be modified by any substituents.

DISCUSSION

The results establish that the two adjacent cysteine residues of human and porcine SP-C are both modified by thioester-linked palmitoylation. More-or-less stoichiometric modification is noted for both the full-length and the truncated SP-C forms (19) (Figs. 1 and 2; Table 1).

Thioester bonds are sensitive to cleavage with methylamine (28, 29), hydroxylamine (30), or reduction with mercaptoethanol (31, 32). Treatment with KOH, trimethylamine, or dithioerythritol releases palmitic acid from SP-C. In all cases, molecular ion regions 2 × 240 mass units lower than those of intact SP-C are obtained (Fig. 1). These results show that palmitoyl groups are covalently linked to the polypeptide as thioesters to each of the two cysteine residues in SP-C (Fig. 4) and that deacylation is obtained upon treatment with basic or reducing agents. The only other groups that could be acylated would be the α- or ε-amino groups, since both human and porcine SP-C molecules lack side-chain hydroxyl groups. However, the α- and ε-amino groups are free as shown by sequence analysis and the fact that N-linked acyl groups would require other deacylation conditions.

The amino acid sequence around the palmitoylation sites is notable (Fig. 4). The two cysteine residues are adjacent and situated in a largely palindromic sequence, -Ile-Pro-Cys-Cys-Pro-Val-. The flanking proline residues might be of special importance in imposing rigid constraints on rotations of the peptide backbone. Adjacent cysteine residues are palmitoylated in other proteins (33), but a palindromic sequence appears not to be a prerequisite for palmitoylation in these other cases. The sequence requirements for palmitoylation are unknown except that basic residues often are situated nearby (34), which applies also to SP-C, which has conserved basic residues at positions 11 and 12 (20). Interestingly, canine SP-C fundamentally differs by having both cysteine residues replaced by phenylalanine (16). The greater hydrophobicity of phenylalanine compared to cysteine might compensate for the loss of the palmitoyl residues. Alternatively,

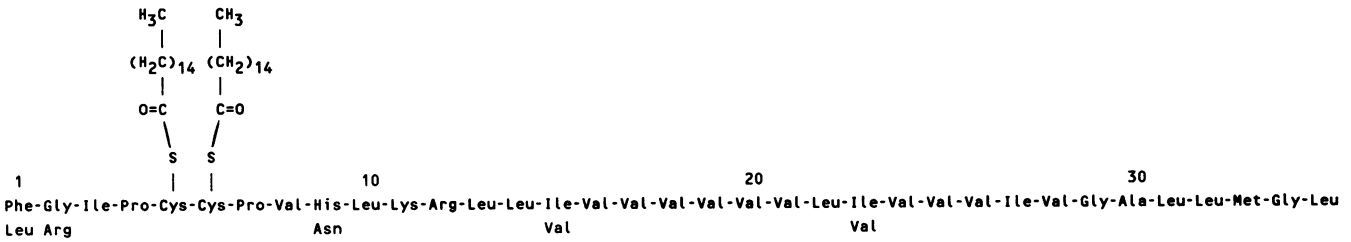

FIG. 4. Structure of intact SP-C. The amino acid sequence of human SP-C is shown, with residues that differ in the porcine molecule indicated below the human sequence. The sulfurs implicit in the Cys symbols are shown in order to emphasize the thioester nature of the linkages.

two adjacent serine residues in the canine molecules (16) could perhaps be acylated.

The covalent modification of native SP-C by long-chain fatty acids shows the presence of a direct physical link between the lipid and polypeptide components known to be necessary constituents of SP-C preparations (21). Different types of acylation/alkylation are encountered in many proteins. Apart from the frequent α-amino acetylation of polypeptides (35), three entirely different modifications by long-chain hydrocarbons are known: palmitoylation, myristoylation, and farnesylation (36–38). Myristoylation is associated with the α-amino group, and farnesylation with a C-terminal cysteine residue, while palmitoylation affects internal positions as found in an increasing number of proteins. In common, the three types of long-chain acylation/alkylation appear important for localization of the water-soluble protein in close vicinity of lipid membranes, although at least myristoylated proteins in some cases also remain cytosolic (34). In the case of SP-C the true lipopeptide nature may have less implication for its solubility properties, since the SP-C polypeptide itself is highly lipophilic (19). However, the two palmitoyl groups in the N-terminal part of SP-C will increase the hydrophobicity of the segment previously calculated to be unexpectedly hydrophilic (19). Hence, native SP-C may not have two highly different ends as previously suspected. Instead, both ends now appear to be hydrophobic, with corresponding functional implications for the whole SP-C molecule. Palmitoylation perhaps also occurs in the precursor, ensuring its membrane association prior to proteolytic processing to SP-C.

The chemical modifications of native SP-C also provide a potential mechanism by which the biological activity of SP-C or its precursor may be regulated by acylation/deacylation. Internal thioesters at important segments of other proteins affect the biological activity, as in the case of the hinge-region thioester of α_2-macroglobulin (28, 39). However, the present data suggest complete stoichiometry of modification and hence do not support the presence of different molecules. Furthermore, palmitoylated cysteine residues in other proteins appear to represent structural components, perhaps even fortuitous modifications, rather than functional entities. Nevertheless, in those cases the structures are not palindromic, lack the proline residues, and frequently do not affect adjacent cysteine residues.

In conclusion, the present results establish a link between the two major constituents of surfactant preparations, suggest stoichiometric modifications in native SP-C, and document the different natures of SP-C (lipopeptide) and SP-B (not a true lipopeptide). These and previous results with SP-C demonstrate the value of direct analysis. Although DNA analyses rapidly give polypeptide chain structures, it was the direct studies that established the position of the C terminus and the chemical modification of the cysteine residues.

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