Volume 301, number 2, 165–167 FEBS 10936 April 1992

Human surfactant polypeptide SP-B

Disulfide bridges, C-terminal end, and peptide analysis of the airway form

Jan Johansson[a], Hans Jörnvall[a] and Tore Curstedt[b]

[a]*Department of Chemistry I, Karolinska Institutet, S-104 01 Stockholm, Sweden* and [b]*Department of Clinical Chemistry, Karolinska Institutet at Danderyd Hospital, S-182 88 Danderyd, Sweden*

Received 5 February 1992; revised version received 2 March 1992

Human hydrophobic surfactant polypeptide, SP-B, purified from lung tissue by exclusion chromatography in organic solvents, has been characterized. The polypeptide is 79 residues long, has a C-terminal methionine, and contains seven Cys residues. Native human SP-B lacks free thiol groups. Three intrachain disulfide bridges were defined, linking Cys^{8} to Cys^{77}, Cys^{11} to Cys^{71} and Cys^{35} to Cys^{46}. The remaining Cys^{48} is concluded to link the protein chains into homodimers via an interchain disulfide to its counterpart in a second SP-B polypeptide. These SS bridges are identical to those in the porcine form and confirm a consistant and unique disulfide pattern for SP-B polypeptides in general.

Surfactant polypeptide; SP-B; Disulfide bridge; Structural property

1. INTRODUCTION

Pulmonary surfactant is synthesized and secreted by the alveolar type II cells as a complex of mainly phospholipids and proteins. It is a prerequisite for normal respiration by lowering surface tension, thereby preventing alveolar collapse at end expiration [1]. Low levels of surfactant in premature babies are associated with respiratory distress [2], a common cause of neonatal mortality [3]. This serious disease can now be effectively treated by exogenous instillation of surfactant preparations. Necessary components of such preparations are phospholipids and two hydrophobic polypeptides, SP-B and SP-C [4].

SP-C from several species, including man, is a 33–35 residue polypeptide [5,6] containing one or two (dependent on species) thioester-linked palmitoyl groups [7,8]. Porcine SP-B has been shown to contain 79 residues in its longest form [9], to exist mainly as a 17.4 kDa homodimer and to lack covalently linked fatty acyl groups [7]. One interchain disulfide and three intrachain SS bridges have been determined for porcine SP-B [10]. In contrast, for human SP-B only tentative and conflicting suggestions concerning the C-terminal end (see [11,12]) and the disulfide arrangement exist. Studies of human SP-B at the cDNA level, however, show that the airway polypeptide originates from a much larger 42 kDa precursor [13–15]. This study was undertaken in order to establish the disulfide bridge arrangement and the location of the C-terminal end, and to confirm the overall structure of human SP-B by direct analysis of the airway form.

Correspondence address: J. Johansson, Department of Chemistry I, Karolinska Institutet, S-104 01 Stockholm, Sweden. Fax: (46) 8337462.

2. MATERIALS AND METHODS

2.1. *Protein isolation*

The pulmonary phospholipid fraction was isolated [7,16] from human lung tissue obtained after a tumor resection. SP-B was purified from the phospholipids by Sephadex LH-60 chromatography in chloroform/methanol, 1:1 (v/v), containing 5% 0.1 M HCl [16].

2.2. *Structural analysis*

For detection of free SH groups, native SP-B (25 nmol) was solubilized and denatured in 200 μl 0.4 M Tris–HCl/6 M guanidine–HCl/2 mM EDTA/8 mM SDS, pH 8.1, and treated with 4 μmol [^{14}C]iodoacetate for 4 h at 37°C.

For sequence analysis SP-B was reduced, [^{14}C]carboxymethylated [9] and degraded in an ABI 470A gas-phase sequencer. Liberated phenylthiohydantoin (PTH) derivatives were analyzed by reverse-phase high performance liquid chromatography (RP-HPLC) using a Hewlett-Packard 1090 instrument, or an ABI 120A Analyzer. Under the conditions used diPTH-cystine eluted at the same position as PTH-Tyr. Fragments of SP-B were generated by treatment of about 100 μg [^{14}C]carboxymethylated material with CNBr (0.15 g/ml) in 70% formic acid for 24 h at room temperature. The peptides produced were separated in the two-phase system chloroform/methanol/water, 8:4:3 (by vol). Hydrophilic peptides recovered in the methanol/water phase were further purified by RP-HPLC (Ultropac TSK ODS-120T, 4.6 × 250 mm column, flow 1 ml/min, linear gradient of acetonitrile in 0.1% aqueous trifluoroacetic acid) and subjected to sequence analysis. The chloroform/methanol phase contained uncleaved SP-B. Amino acid compositions were determined with an LKB Alpha Plus or Beckman 121M analyzer after hydrolysis in 6 M HCl/0.5% phenol for 24 h at 110°C.

For assignment of disulfide bridges, native SP-B was cleaved with pepsin (1:10 enzyme-to-substrate ratio, 24 h incubation at 37°C) in 5% formic acid [10]. After evaporation of the solvents and re-solubil-

ization in 30% acetic acid, the peptides produced were purified by RP-HPLC (conditions as above).

3. RESULTS

3.1. *Amino acid sequence*

Human SP-B is deduced to be a 79-residue polypeptide ending with a methionine (Fig. 1). The C-terminal Met was unambiguously observed in two peptides (C2 and P9, Fig. 1), that were produced independently, by CNBr and pepsin treatment, respectively. The detection of a Met residue at the C-terminus of a CNBr peptide in itself strongly indicates that the methionine already detected was originally the C-terminal residue. The C-terminus established here is not in agreement with suggested structures of human SP-B with 78 residues [11] or 80–81 residues [12] but is supported by previous data from mass spectrometry [7].

All residues of the polypeptide, except at position 28, were unequivocally determined. At this position evidence for a possible Ile/Ala polymorphism was obtained. Analysis of the intact polypeptide clearly showed Ile^{28}, whereas analysis of a fragment starting at position 22 (C3, Fig. 1) identified Ala^{28}. The amino acid sequence deduced for human SP-B is in agreement with the amino acid composition obtained after hydrolysis for 24 h at 110°C, with expected low recoveries of the aliphatic branched-chain residues [5,9]. Total compositions of the fragments produced by CNBr or pepsin are also in agreement with the corresponding amino acid sequences.

The structure of human SP-B with Ala^{28} now established by direct protein analysis is identical with the amino acid sequence of an internal part (positions 201–279) of human pro SP-B, confirming that the biologically active airway polypeptide is a proteolytically derived cleavage product. No N-terminally truncated forms of human SP-B were observed. This is in contrast to the situation with porcine SP-B, where a shorter form was found, lacking the first two residues of the full-length, 79-residue polypeptide [9]. This species difference might indicate that N-terminally truncated forms previously observed for SP-B and SP-C [5,6,8,9] are caused by secondary aminopeptidase-like activities rather than low specificity of the proteolytic enzyme(s) that liberate the active polypeptides.

3.2. *Disulfide bridge arrangement*

No carboxymethylcysteine could be detected by amino acid analysis after iodoacetate treatment of SP-B under denaturing but non-reducing conditions. This shows that in native SP-B all Cys residues are disulfide-linked, since other posttranslational modifications of the Cys residues are excluded as judged by the observed molecular mass of SP-B [7].

Cys-containing peptides isolated by RP-HPLC after cleavage of native SP-B with pepsin (Fig. 2) were identified by amino acid analysis and further characterized by sequence analysis. Equimolar amounts of peptides covering positions 70–74 and 10–12 (P2 and P7, Figs. 1 and 2) eluted in one fraction; 30–37 and 45–47 (P3 and P4) in another; and 1–13 and 70–79 (P10 and P9) in a third. Sequence analysis of the P2+P7 fraction released diPTH-cystine at cycle two, and analysis of the P3+P4 fraction released it at cycle six. This establishes that peptides P2 and P7 are linked via a disulfide bridge, and that P3 and P4 are linked by another disulfide bridge. Sequence analysis of the P10+P9 fraction was expected to release diPTH-cystine at two different positions (8 and 11), but this derivative was not observed at any cycle. The failure to detect these diPTH-cystine residues might be explained by low recoveries from shifts in the disulfide pattern after repeated coupling steps at alkaline pH.

These results give the disulfide arrangement shown in Fig. 1, with intrachain disulfide bridges between Cys^{8}–Cys^{77}, Cys^{11}–Cys^{71} and Cys^{35}–Cys^{46}. No peptide containing Cys^{48} was identified. However, since all Cys res-

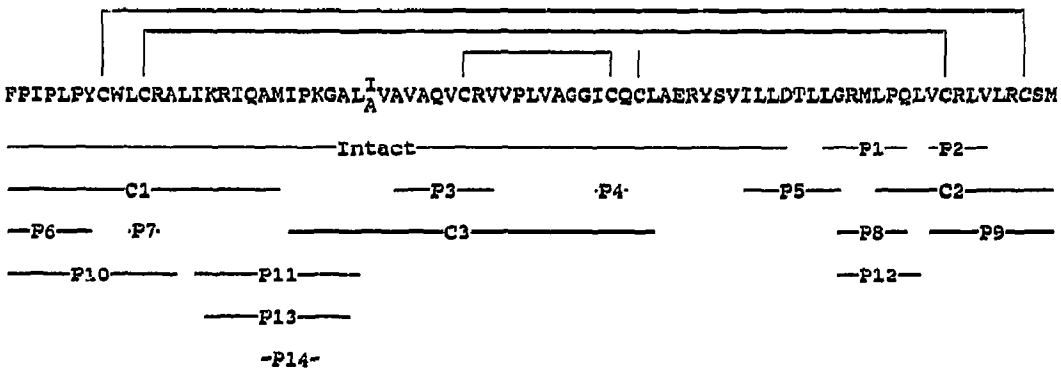

Fig. 1. Amino acid sequence and disulfide bridges of human surfactant polypeptide SP-B. Horizontal lines below the sequence indicate residues identified by sequence analysis. Intact denotes analysis of uncleaved SP-B; P1–P14, analyses of fragments obtained by cleavage with pepsin; C1–C3, analyses of those produced by cleavage with CNBr. At position 28, Ile and Ala were found upon analysis of intact material and C3, respectively. Lines connecting Cys residues represent the disulfide bridges identified. Cys^{48} is indicated by a vertical line to be interchain-linked to another SP-B polypeptide.

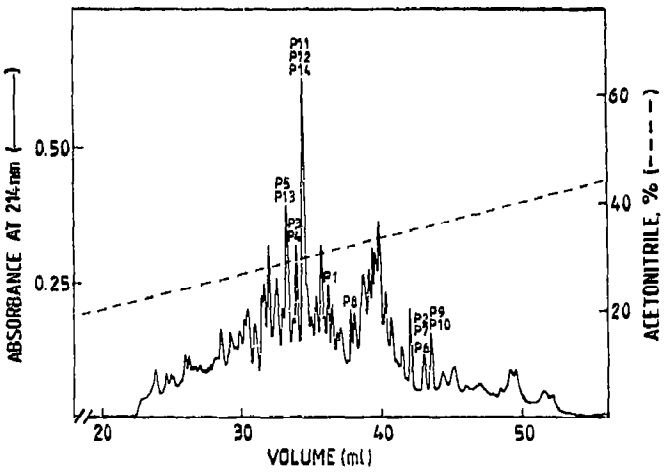

Fig. 2. HPLC separation of peptide fragments of native human SP-B produced by pepsin cleavage. The positions of elution of peptides P1–P14 in Fig. 1 are indicated.

idues are disulfide-linked (see above) the only possible linkage of this Cys is a Cys^{48}–C^{48} interchain disulfide bridge.

4. DISCUSSION

Human surfactant polypeptide, SP-B, like the porcine equivalent [9,10], is a 79-residue polypeptide with three intrachain disulfide bridges. The disulfides are now shown to be identically positioned in human and porcine SP-B, i.e. linking the first half-cystine with the last, the second with the penultimate, and two centrally situated half-cystines with each other (Fig. 1). Thus, disulfide-mediated interactions between distant parts of the SP-B polypeptide appear structurally important, which is supported by a recent finding that reduction of SP-B appears to result in a conformational change of the N-terminal segment [17]. The functional significance of the close interaction between N- and C-terminal parts of SP-B is unclear since synthetic peptides corresponding to smaller parts of SP-B appear to exhibit some biological activity [18]. Furthermore, it has been proposed that SP-B might be involved in binding functions because of its excess of positive charges and an observed similarity between SP-B and the kringle domain [10]. In conclusion, it is possible that intact SP-B contains multiple binding sites for anionic phospholipids, and that the binding sites need not be linked in one polypeptide chain for the lowering of surface tension.

Human SP-B is concluded to exist as a homodimer because of a Cys^{48}–Cys^{48} interchain SS bridge, compatible with the molecular mass, with the absence of a detectable free thiol group, and the fact that all other Cys residues are occupied in disulfide bonding. The failure to detect a fragment containing Cys^{48} might be explained by the hydrophobicity of the surrounding region, rendering peptic cleavage or fragment isolation difficult. The only heterogeneity now detected for human SP-B is an apparent Ile/Ala polymorphism at position 28. Therefore, the proposed coexistence of thiol-free dimeric and monomeric porcine SP-B due to a Cys/Leu polymorphism at position 57 [10] does not seem to be a general phenomenon and the major form of native SP-B is concluded to be homodimeric.

In summary, the identical disulfide patterns established for human and porcine SP-B by direct analyses of the polypeptides confirm a consistent and unique structure for this pulmonary surfactant molecule.

Acknowledgements: This study was supported by the Swedish Cancer Society (project 1806), Oscar II Jubileumsfonds, Axel Tielman Fund, and the Swedish Society for Medical Research.

REFERENCES

[1] van Golde, L.M.G., Batenburg, J.J. and Robertson, B. (1988) Physiol. Rev. 68, 374–455.
[2] Avery, M.E. and Mead, J. (1959) Am. J. Dis. Child. 97, 517–523.
[3] Farrell, P.M. and Avery, M.E. (1975) Am. Rev. Respir. Dis. 111, 657–688.
[4] Collaborative European Multicenter Study Group (1988) Pediatrics 82, 683–691.
[5] Johansson, J., Curstedt, T., Robertson, B. and Jörnvall, H. (1988) Biochemistry 27, 3544–3547.
[6] Johansson, J., Jörnvall, J., Eklund, A., Christensen, N., Robertson, B. and Curstedt, T. (1988) FEBS Lett. 232, 61–64.
[7] Curstedt, T., Johansson, J., Persson, P., Eklund, A., Robertson, B., Löwenadler, B. and Jörnvall, H. (1990) Proc. Natl. Acad. Sci. USA 87, 2985–2989.
[8] Johansson, J., Persson, P., Löwenadler, B., Robertson, B., Jörnvall, H. and Curstedt, T. (1991) FEBS Lett. 281, 119–122.
[9] Curstedt, T., Johansson, J., Barros-Söderling, J., Robertson, B., Nilsson, G., Westberg, M. and Jörnvall, H. (1988) Eur. J. Biochem. 172, 521–525.
[10] Johansson, J., Curstedt, T. and Jörnvall, H. (1991) Biochemistry 30, 6917–6921.
[11] Sarin, V.K., Gupta, S., Leung, T.K., Taylor, V.E., Ohning, B.L., Whitsett, J.A. and Fox, J.L. (1990) Proc. Natl. Acad. Sci. USA 87, 2633–2637.
[12] Revak, S.D., Merritt, T.A., Hallman, M., Heldt, G., La Polla, R.J., Hoey, K., Houghten, R.A. and Cochrane, C.G. (1991) Pediatr. Res. 29, 460–465.
[13] Jacobs, K.A., Phelps, D.S., Steinbrink, R., Fisch, J., Kriz, R., Mitsock, L., Dougherty, J.P., Taeusch, H.W. and Floros, J. (1987) J. Biol. Chem. 262, 9808–9811.
[14] Glasser, S.W., Korfhagen, T.R., Weaver, T., Pilot-Matias, T., Fox, J.L. and Whitsett, J.A. (1987) Proc. Natl. Acad. Sci. USA 84, 4007–4011.
[15] Hawgood, S., Benson, B.J., Schilling, J., Damm, D., Clements, J.A. and White, R.T. (1987) Proc. Natl. Acad. Sci. USA 84, 66–70.
[16] Curstedt, T., Jörnvall, H., Robertson, B., Bergman, T. and Berggren, P. (1987) Eur. J. Biochem. 168, 255–262.
[17] Fan, B.R., Bruni, R., Taeusch, H.W., Findlay, T. and Waring, A.J. (1991) FEBS Lett. 282, 220–224.
[18] Cochrane, C.G. and Revak, S.D. (1991) Science 254, 566–568.