Surfactant Protein B: Disulfide Bridges, Structural Properties, and Kringle Similarities†

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ABSTRACT: The disulfide bridges in porcine hydrophobic surfactant protein B (SP-B) were determined. Results show that three intrachain bridges link half-cystine residues 8 and 77, 11 and 71, and 35 and 46, respectively. This gives SP-B an appearance of three loops, a central big loop surrounded by two smaller ones. In the major form of SP-B, the remaining half-cystine, Cys-48, is probably interchain-linked to its counterpart in another molecule, compatible with the existence of dimeric molecules. A minor fraction, with monomeric SP-B but also lacking free thiols, could be due to polypeptides having Cys-57 (instead of Leu in the major form) and hence an additional intrachain bond (Cys-48–Cys-57). Notably, one of the three intrachain bonds common to all SP-B molecules is analogous to one of the disulfide linkages in the kringle structure of complex serine proteases. SP-B and kringles are also similar in size and in positions of half-cystine residues. SP-B and the kringle of coagulation factor XII exhibit 26% residue identity. This structural similarity of SP-B to a binding domain could reflect functional homology, compatible with the notion that SP-B interacts with surfactant anionic phospholipids, which is also in agreement with an SP-B excess of basic residues. Finally, weak similarities between the proform of SP-B and complex serine proteases are also found. This has implications on further possible relationships between kringles, serine proteases, and antiproteases.

Pulmonary surfactant, lowering the alveolar surface tension, is essential for prevention of alveolar collapse at end expiration (van Golde et al., 1988). Reduced surfactant levels are associated with respiratory distress, a common cause of death in premature infants. Surfactant is synthesized by the alveolar type II cells and stored intracellularly as a complex of double-layered membranes, the lamellar bodies, from which it is secreted into the alveoli. The composition of the material is 90–95% lipid, mainly dipalmitoylphosphatidylcholine, and 5–10% protein (van Golde et al., 1988).

Three surfactant-specific proteins have been structurally defined. One protein is a 32-kDa, glycosylated hydrophilic polypeptide (White et al., 1985), named SP-A (Possmayer, 1988), whereas two are water-insoluble polypeptides, SP-B and SP-C, of which SP-C in particular exhibits extremely hydrophobic properties (Johansson et al., 1988a; Curstedt et al., 1990). Presumably, SP-A is important for phospholipid reuptake and inhibition of secretion by the alveolar type II cells (Dobbs et al., 1987; Rice et al., 1987), while SP-B and SP-C are considered essential for transition of double-layered phospholipid structures into the phospholipid monolayer which lines the alveolar air/liquid interface and mediates the lowered surface tension (Takahashi & Fujiwara, 1986; Curstedt et al., 1987; Hawgood et al., 1987).

The two hydrophobic proteins have been purified to homogeneity and structurally analyzed (Curstedt et al., 1987, 1988; Johansson et al., 1988a,b). The results suggest the occurrence of incomplete N-terminal processing (partial truncation), identify the C-terminal ends, and complement analyses at the cDNA level (Glasser et al., 1987, 1988; Hawgood et al., 1987; Jacobs et al., 1987; Warr et al., 1987); the two peptides in the alveoli are derived from larger precursor molecules apparently not secreted, but which generate the extracellular proteins SP-B and SP-C by proteolytic cleavages. The peptide bonds cleaved and their surrounding structures are different in the two precursor molecules.

SP-C, the shorter of the two hydrophobic surfactant proteins (35 amino acid residues in its major, nontruncated form), is modified by thioester linked palmitoyl groups attached to its two Cys residues (Curstedt et al., 1990). This has been proven for two species (human and pig). Another species (dog) initially appeared to lack both these Cys residues (Fisher et al., 1989), but recent data show that canine SP-C has one Cys residue which is also palmitoylated (Johansson et al., 1991).

In contrast, SP-B, the longer (79 residues in its nontruncated form) of the two hydrophobic peptides, lacks covalently linked lipids but is present in native preparations largely as a dimer (Curstedt et al., 1990). It is derived from a 40–42-kDa proform (Glasser et al., 1987; Hawgood et al., 1987; Jacobs et al., 1987; Emrie et al., 1989) and, depending on the source, contains eight (Curstedt et al., 1987) or seven (Glasser et al., 1987; Hawgood et al., 1987; Jacobs et al., 1987; Emrie et al., 1989) Cys residues probably involved in disulfide bonds, of which at least one supposedly contributes to the dimerization (Yu et al., 1987).

The aim of this investigation was to ascertain the status and cross-links of the Cys residues in SP-B and to scrutinize the structural features of the molecule and its precursor, in order to define functional characteristics.

Materials and Methods

Porcine SP-B was purified from pulmonary tissue (Curstedt et al., 1987). For carboxymethylation, 100 μg of intact polypeptide in 1 mL of $CHCl_3/CH_3OH$, 1:2 (v/v), was treated with [^{14}C]iodoacetate (30-fold molar excess over Cys residues; 37 °C for 4 h) in the presence of 85 mM trimethylamine.

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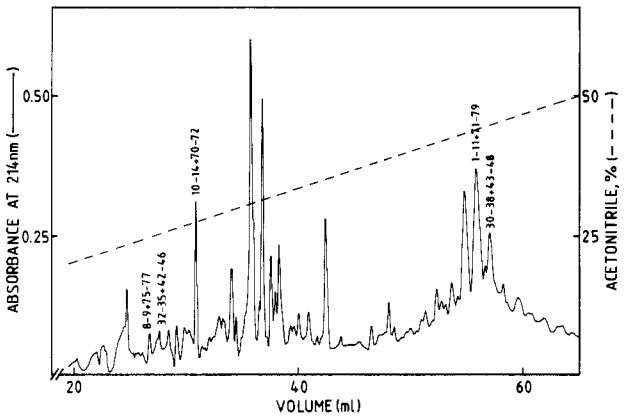

FIGURE 1: HPLC separation of peptic fragments from native porcine SP-B. Peptides recovered in cystine-containing fractions are indicated. Peptides 1–13 and 67–79 were recovered in another batch and eluted at a position corresponding to 42% acetonitrile.

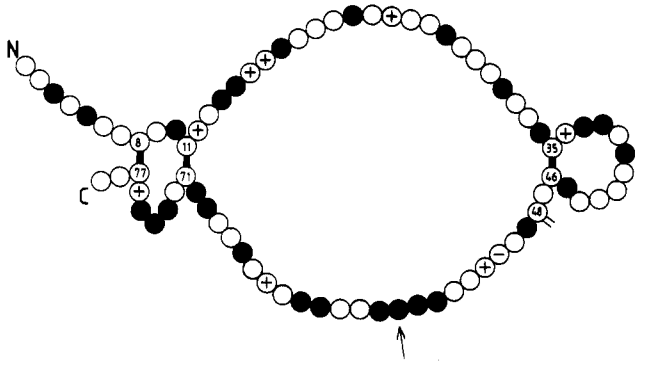

FIGURE 2: Schematic presentation of the disulfide bridges of porcine SP-B. Numbers refer to Cys positions. Cys-48 is supposedly bound to its counterpart in another monomer, thus forming a dimer; alternatively, in a fraction of the molecules, it could be intrachain-linked to position 57 (marked with arrow) where Cys has also been reported (Curstedt et al., 1988). Filled circles denote aliphatic, branched-chain residues and are, like the charged residues, conserved in human (Jacobs et al., 1987), canine (Hawgood et al., 1987), porcine (Curstedt et al., 1988), and rat (Emrie et al., 1989) SP-B.

Fragmentation of 1 mg of intact polypeptide was performed with pepsin [1:10 (w/w) enzyme to substrate ratio, 24 h at 37 °C] in 5% formic acid (added as 100% for solubilization and then diluted), and the fragments produced were purified by HPLC (Ultropac TSK ODS-120T, 4.6 × 250 mm column, flow rate 1 mL/min, linear gradient of acetonitrile in 0.1% aqueous trifluoroacetic acid).

For reduction of peptides containing cystine, the dried fractions were dissolved in 1.0 M ammonium bicarbonate, diluted to 0.1 M, and treated with 5–30 mM dithiothreitol for 2–5 h at 37 °C. Peptides present in amounts above the nanomole level were then treated with [^{14}C]iodoacetate and separated by HPLC (conditions as above), while peptides obtained in lower yield were separated by capillary electrophoresis (Beckman P/ACE 2000 with a 75 μm × 50 cm open glass capillary) at 25-kV constant voltage, 25 °C, after sample solubilization in 50 mM phosphate, pH 2.5.

Hydropathy profiles were calculated according to Hopp and Woods (1981).

Results and Discussion

SP-B Disulfide Bridge Arrangement, Structural Properties, and Functional Correlations. (A) Disulfide Bonds. In order to verify the results from mass spectrometry (Curstedt et al., 1990) that SP-B, in contrast to SP-C, is not chemically modified, purified porcine SP-B was treated with [^{14}C]iodoacetate in the presence of trimethylamine. The presence of the amine would release thioester linked acyl groups (Curstedt et al., 1990), but no ^{14}C incorporation into the protein (<0.1 mol/mol of SP-B monomer) was obtained. This was also verified by lack of recovery of (carboxymethyl)cysteine after hydrolysis. We therefore confirm that there is no free thiol group in native SP-B and no thioester linked acyl group.

The purified polypeptide was cleaved with pepsin under conditions of minimal risk for disulfide rearrangement (Schrohenloher & Bennett, 1986). Strong conditions (1:10 enzyme to substrate ratio, 24 h at 37 °C) were needed for significant cleavage, as expected from a tight folding of the disulfide-rich polypeptide. The fragments obtained were separated by HPLC (Figure 1) and analyzed for total composition and amino acid sequence. The results showed that peptides 10–14 and 70–72 eluted in equimolar amounts in one fraction, peptides 1–11 and 71–79 in another, 30–38 and 43–48 in a third, 32–35 and 42–46 in a fourth, 8–9 and 75–77 in a fifth fraction, and 1–13 and 67–79 in a sixth fraction (Figure 1). No evidence for other combinations of these Cys-containing peptides was found. For the first four pairs, the coelution was proven to be derived from disulfide bonding rather than from similar chromatographic properties. Thus, in the first three cases, reduction, ^{14}C-carboxymethylation, and rechromatography by HPLC clearly separated two radioactive peptides. The fourth fraction showed one major peak upon capillary electrophoresis which was separated into two peaks after reduction.

Provided that all the disulfide bonds occur within one SP-B molecule, these results give a disulfide bridge arrangement with Cys-8 linked to Cys-77, Cys-11 to Cys-71, and Cys-35 to Cys-46. This pattern is shown in Figure 2. Thus, the first two half-cystine residues of SP-B are disulfide-linked to the last two, and two Cys residues centrally placed are linked to each other. This results in a gross structure of SP-B with 3 entities, 1 central big loop (composed of 51 residues) surrounded by 2 smaller, but similar-sized (11 and 12 residues) structures.

This result leaves only one Cys residue unaccounted for in intramolecular disulfide bonding, Cys-48, which in all likelihood is intermolecularly disulfide-linked to its counterpart in a second SP-B polypeptide chain, explaining the native occurrence of dimers. Alternatively, in some molecules (with an additional Cys residue at position 57, cf. below), Cys-48

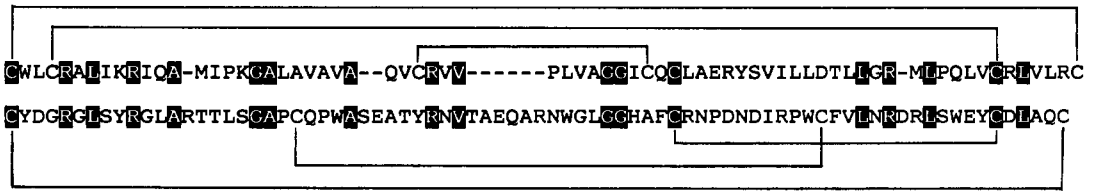

FIGURE 3: Alignment of human SP-B (Jacobs et al., 1987; top) and the kringle (bottom) of human coagulation factor XII (McMullen & Fujikawa, 1985). Positions 8–77 of SP-B, encompassing the first and last Cys residues, are shown. Identical residues have been marked and gaps introduced to maximize the identities. Disulfide bridges are given, as now determined for porcine SP-B (Figure 2) and as known for the kringles (Magnusson et al., 1975).

may be involved in a fourth intramolecular disulfide bridge, perhaps explaining the presence of monomers.

Cys-57, previously detected (Curstedt et al., 1988), could not be confirmed now. Instead, a peptide covering positions 54–58 with leucine at position 57 was found. This suggests a possible polymorphism, as indicated also for other positions of the SP-B molecule, i.e., a tentative Met/Arg polymorphism at position 64 in the porcine form (Curstedt et al., 1988) and an Ala (Jacobs et al., 1987)/Arg (Glasser et al., 1987)/Ile (Johansson et al., unpublished observation) polymorphism at position 28 in the human form. Similarly, the native molecule has been reported to exist both in dimeric form, from gel electrophoresis (Yu et al., 1987) and mass spectrometry (Curstedt et al., 1990), and in monomeric form, also from gel electrophoresis (Whitsett et al., 1986).

The apparent Cys/Leu polymorphism at position 57 in porcine SP-B could conceivably constitute an explanation to the heterogeneity in monomer/dimer status, if the minor fraction having Cys-57 would have an intrachain Cys-48–Cys-57 disulfide, yielding monomers, and the major fraction having Leu-57, instead a Cys-48–Cys-48 interchain link, yielding a dimer. However, no fragment with Cys-48–Cys-57-linked residues was observed in this investigation, and all cDNA analyses show Leu-57 (Glasser et al., 1987; Jacobs et al., 1987; Hawgood et al., 1987; Emrie et al., 1989). Consequently, the major form of SP-B definitely has seven half-cystines in total, six intra-chain-linked and one interchain-linked, all now defined, and the corresponding molecule is a dimer. Additional forms, monomers, and heterogeneities, may occur but are not fully established.

(*B*) *Structural Similarities with Kringles.* The SP-B structure (Figure 2) has some gross features in common with the kringle structures (Magnusson et al., 1975) present in complex serine proteases and in fibronectin and haptoglobin (Patthy et al., 1984). Thus, SP-B and kringles both possess a disulfide bond between their first and last Cys residues, are similar in size, and also exhibit distant residue similarities.

An alignment of primary structures (Figure 3) shows positions 8–77 of SP-B to have 26% residue identity with the kringle of coagulation factor XII (McMullen & Fujikawa, 1985). Furthermore, although SP-B and the kringle only have one of the disulfide bridges in common (the "external" one), four of the six Cys residues in the kringle (Figure 3) are separated by no more than two positions from the Cys residues in SP-B (or five if Cys-57 of SP-B is considered). The two internal disulfide bridges characteristic of kringles (Magnusson et al., 1975) are not present in SP-B. Nevertheless, those Cys positions in the tertiary structure of the first kringle of prothrombin (Park & Tulinsky, 1986) are positioned in such a manner that they would be adjacent also in the disulfide bonding present in SP-B. Consequently, sizes, patterns, and actual residue identities all suggest the possibility that kringles and SP-B disulfide domains are structurally related.

(*C*) *Functional Conclusions.* The exact functions of the kringles are unknown, but they have been reported to be involved in binding to different molecules, such as phospholipids (Gitel et al., 1973; Magnusson et al., 1975), L-lysine (Lerch et al., 1980), and other proteins (Patthy et al., 1984). Therefore, a similarity in function between kringles and SP-B domains appears possible. Thus, the SP-B dimer is expected to bind negative molecules, like anionic phospholipids, which is fully compatible with its native surfactant action. The kringle similarity may indicate a noncovalent lipoprotein nature of SP-B, which, together with the covalent association of lipids to SP-C (Curstedt et al., 1990), could explain the complex

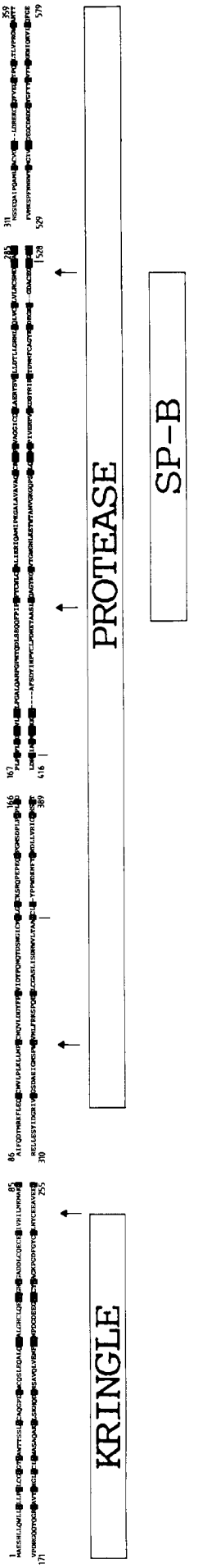

FIGURE 4: Alignment of human pro-SP-B (Jacobs et al., 1987) and human prothrombin (Degen et al., 1983). Long gaps have been omitted as shown by the positional numbers of the first and last residues of the segments compared (upper line for pro-SP-B and lower line for prothrombin). The catalytically essential His, Asp, and Ser residues in the serine protease are marked with vertical bars. Arrows mark exon borders in the prothrombin gene (Degen et al., 1983; Rogers, 1985). The positions of the second kringle and the protease part of prothrombin, as well as the SP-B part of pro-SP-B, are indicated. The activation segment is between the kringle and the protease part.

FIGURE 5: Hydropathy profiles of prothrombin positions 272–522 (solid) and the second prothrombin kringle (dashed) repeated 3 times. Each kringle repeat is underlined, and the dots in the third repeat indicate two gaps (one and four residues, respectively). Positive and negative values denote hydrophilicity and hydrophobicity, respectively, calculated according to Hopp and Woods (1981).

relationships and molecular mechanisms of the surfactant preparations. SP-B has a strong positive net charge at physiological pH (Figure 2): eight basic residues are conserved between the human, porcine, canine, and rat proteins, versus only one acidic counterpart. This pattern appears significant in the interaction between SP-B and the anionic phospholipids. Although the nature of the interactions is unknown, basic proteins have been shown to be essential for the passage of negatively charged macromolecules through phospholipid membranes (Jay & Gilbert, 1987).

The only negatively charged residue conserved is adjacent to a positive charge also conserved (Figure 2). Furthermore, these opposite charges are close to Cys-48 which is suggested to participate in the disulfide interlink between the monomers (above). It is therefore possible that charge interactions may contribute also to the dimerization of SP-B.

In spite of its many charged residues, SP-B is insoluble in water. This is likely to be related to the large fraction (34%) of conserved aliphatic branched-chain residues (Figure 2). Both the charged residues and the hydrophobic residues are distributed evenly in the primary structure of SP-B, in contrast to the situation for the structure of the shorter SP-C molecule (Johansson et al., 1988a).

Proform of SP-B: Comparisons with Complex Serine Proteases. The facts that the mature SP-B domain has some resemblances to kringles and that both SP-B and SP-C are proteolytically derived from larger precursors motivated comparisons of pro-SP-B with members of the serine protease family. The best matches are shown in Figure 4, attempting an alignment of human pro-SP-B (positions 1–359; Jacobs et al., 1987) with positions 171–579 of human prothrombin [(Degen et al., 1983) including the protease part (Magnusson et al., 1975)]. Although not unambiguous in gap segments, this alignment shows two features of possible interest:

(1) Overall residue identity is low (15%), but the functionally important Asp, Ser, and His residues of the protease may have matches in the pro-SP-B molecule. Residues around the Asp and Ser even exhibit further positional identities (Figure 4).

(2) The mature SP-B corresponds to an exon in many of the serine protease genes (Rogers, 1985), suggesting proteolytic cleavage of pro-SP-B to have occurred at positions corresponding to those of the protease exon borders. Likewise, the two exon borders flanking SP-B in the human pro-SP-B gene (Pilot-Matias et al., 1989) are close by, nine and five residues, respectively, outside the positions corresponding to the protease exon borders.

If pro-SP-B and complex serine proteases are related as aligned in Figure 4, the SP-B part would correspond both to a kringle (Figure 3) and to the serine protease part (Figure 4). In that case, one would expect a relationship also between kringles and serine protease domains. Therefore, the hydropathy of prothrombin positions 272–522 (i.e., from the activation segment to the position corresponding to the end of SP-B, Figure 4) is compared (Figure 5) with that of a three times repeated second kringle of prothrombin. These segments exhibit no significant residue identity, but hydrophilicity patterns appear similar. This may be compatible with similar overall conformations and is of interest for evaluations by other means.

Finally, positions 43–53 of SP-B have been proposed to be homologous to serine protease inhibitors (Emrie et al., 1989). Thus, SP-B may show similarities both to serine proteases (Figure 4) and to their inhibitors. Consequently, as suggested (Skern et al., 1990), proteases and antiproteases could be structurally related.

A protease/antiprotease activity of pro-SP-B could be of functional importance, as both SP-B and SP-C are cleavage products. Although fascinating, a hydrolase activity is however unlikely since rat pro-SP-B (Emrie et al., 1989) has Ala at the "active site" Ser position, indicating that a homology, if present, is not accompanied by identical functional roles. On the other hand, if the structural similarity between SP-B and antiproteases (Emrie et al., 1989) reflects an antiproteolytic function of SP-B, the specificity rules (Laskowski & Kato, 1980) would fit with inhibition of activities on Gln–Xxx bonds, like the Gln_{200}–Phe_{201} (Figure 4) bond which must be cleaved to produce SP-B from pro-SP-B. Another activity of interest would be an antiesterase function, because of the thioester bonds in SP-C (Curstedt et al., 1990).

Different Functions for SP-B and SP-C. Irrespective of the protease/pro-SP-B relationships, if any, the present results clearly demonstrate that SP-B and SP-C carry through separate functional roles. These two molecules differ in size, primary structure, distribution of charged and hydrophobic residues, Cys status (disulfide bridges in SP-B, thioester palmitoylation in SP-C), and essentially all properties except a lipoprotein nature. These facts suggest that the two hydrophobic proteins of the surfactant system have different functions and that both proteins may be needed for optimal lowering of surface tension.

Added in Proof

During the final preparation of this article Patthy (1991) reported that pro-SP-B, like prothrombin (cf. Figure 5), appears to exhibit an internal triplication and that the repeated unit may be homologous to units, also repeated, in prosaposin and sulfated glycoprotein 1. To what extent the prosaposin/glycoprotein 1 and protease/antiprotease similarities to SP-B support each other is presently difficult to judge.

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