Intermolecular Cross-Links Mediate Aggregation of Phospholipid Vesicles by Pulmonary Surfactant Protein SP-A†

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ABSTRACT: As the most abundant glycoprotein component of pulmonary surfactant, SP-A (M_r = 30000–36000) plays a central role in the organization of phospholipid bilayers in the alveolar air space. SP-A, isolated from lung lavage, exists in oligomeric forms (N = 6, 12, 18, ...), mediated by collagen-like triple helices and intermolecular disulfide bonds. These protein–protein interactions, involving the amino-terminal domain of SP-A, are hypothesized to facilitate the alignment of surfactant lipid bilayers into unique tubular myelin structures. SP-A reorganization of surfactant lipid was assessed in vitro by quantitating the calcium-dependent light scattering properties of lipid vesicle suspensions induced by SP-A. Accelerated aggregation of unilamellar vesicles required SP-A and at least 3 mM free calcium. The initial rate of aggregation was proportional to the concentration of canine SP-A over lipid:protein molar ratios ranging from 200:1 to 5000:1. Digestion with bacterial collagenase or incubation with dithiothreitol (DTT) completely blocked lipid aggregation activity. Both treatments decreased the binding of SP-A to phospholipids. The conditions used in the DTT experiments (10 mM DTT, nondenaturing Tris buffer, 37 °C) resulted in the selective reduction and ^{14}C-alkylation of the intermolecular disulfide bond involving residue ^{9}Cys, whereas the four cysteines found in the noncollagenous domain of SP-A were inefficiently alkylated with [^{14}C]-iodoacetate. HPLC analysis of tryptic SP-A peptides revealed that these four cysteine residues participate in intramolecular disulfide bond formation (^{138}Cys–^{229}Cys and ^{207}Cys–^{221}Cys). Our data demonstrate the importance of the quaternary structure (triple helix and intermolecular disulfide bond) of SP-A for the aggregation of unilamellar phospholipid vesicles.

Recent progress in the clinical use of surfactant replacement therapy for the treatment and prevention of respiratory distress syndrome (RDS) has made knowledge of the biology of lung surfactant increasingly important. Alveolar surface tension increases as air is expired from the lung. The progressive decrease in alveolar radius leads to a continued increase in the interfacial surface tension and, consequently, an increase in pressure toward reducing alveolar volume. By reducing the surface tension along the air–liquid interface, pulmonary surfactant provides the structural stability necessary to prevent collapse of the alveolar air space.

In addition to lung surfactant phospholipids, notably disaturated phosphatidylcholine and phosphatidylglycerol, specific protein components are required for the full biological activity of surfactant. The most abundant surfactant-associated protein has an approximate molecular weight of 30000–36000 and is referred to here as surfactant protein A or SP-A. SP-A has significant surface tension reducing activity when combined with natural surfactant extracts and artificial lipids (King & Macbeth, 1981; Hawgood et al., 1985, 1987). SP-A inhibits the rate of phosphatidylcholine secretion (Dobbs et al., 1987; Rice et al., 1987) and augments the uptake of phospholipids by alveolar type II epithelial cells in vitro (Wright et al., 1987). SP-A may also play an important role in the organization of the surfactant lipid–protein matrix. As surfactant (stored in the type II cell as large lamellar bodies) is secreted into the extracellular space, a structural reorganization takes place, resulting in the formation of tubular myelin structures. Benson et al. (1984) have provided evidence suggesting that this process is dependent on the presence of calcium ions. Chelation of calcium in EDTA-containing buffer caused disruption of tubular myelin structures. Subsequent titration with calcium restored both tubular myelin structure and surface adsorption activity to the surfactant. King et al. (1979, 1983) and Hawgood et al. (1985) demonstrated that SP-A accelerated the calcium-induced aggregation of artificial and surfactant-derived phospholipid vesicles. Calcium-dependent effects on the interaction of hydrophobic surfactant proteins (SP-B plus SP-C) with lipid vesicles have also been reported (Shiffer et al., 1988). More recently, Suzuki et al. (1989) demonstrated that both SP-A and SP-B (M_r = 8000) are necessary for the formation of tubular myelin matrices when added to artificial phospholipid mixtures. These studies suggest that SP-A plays a major role in surfactant lipid organization. Increasing knowledge about SP-A structure now allows us to study these protein–lipid interactions at the level of specific protein subdomains.

We have previously provided evidence that the binding of canine SP-A to multilamellar phospholipid vesicles is facilitated by an internal domain (Ross et al., 1986b) stretching from residues ^{81}Gly to ^{117}Val in the sequence reported by Benson et al. (1985). This region is proposed to contain a classical amphipathic α-helical structure as well as a more a strongly hydrophobic domain. The aggregation of phospholipid vesicles is hypothesized to be mediated by intermolecular cross-linking between SP-A subunits. Two types of cross-links might be involved in SP-A multimer formation. First, the primary sequence of SP-A reveals a collagen-like Gly-X-Y repeating domain which could form a triple-helical structure. King et

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al. (1989) have reported circular dichroism data supporting this hypothesis. Second, a specific cysteine residue located near the amino terminus is involved in intermolecular disulfide bond formation (Ross et al., 1986a). In this study, we have used specific SP-A fragments and disulfide reduction under nondenaturing conditions to demonstrate the requirement for these interactive domains for the aggregation of phospholipid vesicles induced by SP-A.

Materials and Methods

Purification of SP-A. SP-A was purified following saline lavage of intact canine lungs. The isolation of whole surfactant by centrifugation, the extraction of surfactant lipids, and the purification of SP-A by using preparative isoelectric focusing and Cibacron blue CL-6B chromatography have been described previously (Ross et al., 1986a). SP-A was also isolated as described by Haagsman et al. (1987). SP-A purified by either method had similar activity in the phospholipid aggregation assays described below.

Preparation of Phospholipid Vesicles. Lipid mixtures consisting of dipalmitoylphosphatidylcholine, egg yolk phosphatidylcholine, dipalmitoylphosphatidylglycerol, and soybean phosphatidylinositol (65:20:7.5:7.5, by weight) were dried under nitrogen from chloroform and hexane stock solutions. The lipid film was resuspended at 10 mg/mL in 40 mM Tris-HCl, pH 7.4, 0.15 M NaCl, and 1 mM Na_2EDTA (Tris/NaCl/EDTA) buffer by sonication on ice for 15 min. Unilamellar vesicles were isolated from the total sonicate by Sepharose CL-6B chromatography and assayed for phospholipid concentration (Chalvardjian & Rudnicki, 1970). The column (l = 30 cm, d = 1.6 cm) was equilibrated in Tris/NaCl/EDTA buffer at a flow rate of 20 mL/h.

Phospholipid Vesicle Aggregation Assay. Aggregation of phospholipid vesicles in suspension was monitored via measurement of absorbance at 400 nm. A Gilford Model 240 spectrophotometer with a 1-cm quartz cuvette was used. The spectrophotometer was blanked against Tris/NaCl/EDTA buffer used in preparation of the lipid vesicles. Phospholipid vesicles (0.15 mg/mL) were added to the cuvette, and the absorbance was monitored for 1 min and plotted by using a chart recorder. Typically, the phospholipid vesicles alone showed insignificant changes in absorbance (less than 0.001 optical density unit/min). Concentrated protein (1–2 mg/mL) and divalent cation (0.3 M) stocks were added in small volumes. The effect of their addition on sample absorbance was recorded and expressed as the initial rate of absorbance change (ΔOD/min), obtained by measuring the initial slope of the recorder tracing immediately following addition of the protein or cation. Net ΔOD/min values were obtained by subtracting out the ΔOD/min value measured before addition of the protein or cation.

In other experiments, lipid vesicle suspensions were measured for absorbance at 400 nm versus a Tris/NaCl/EDTA buffer-only reference blank by using a Gilford Response spectrophotometer. The change in optical density at 400 nm was monitored for 1-min intervals before and after the addition of SP-A and calcium solutions by using the Response Kinetics program. Changes in absorbance (16 measurements/min) were recorded and reported as ΔOD/min ± SD, following a linear least-squares fit analysis. A quadratic least-squares calculation was used to determine whether the data better fit a nonlinear equation over the 1-min interval. In such cases, the Kinetics program provided an initial ΔOD/min estimate. Use of the Response Kinetics program provided more reliable estimates of the rates of absorbance change. However, the absolute values of ΔOD/min obtained were lower than those measured by using the Gilford Model 240 spectrophotometer. While the chart recorder connected to the Model 240 instrument could reveal rapid absorbance changes immediately upon closing of the sample chamber, the Response Kinetics program employed a reference cuvette that first had to be read (lag period 4–5 s) before the initial data point of the test sample could be measured.

At the low protein levels used in these studies (<10 μg/mL), calcium-induced aggregation of the SP-A alone was not observed (initial ΔOD/min < 0.001). In experiments where submillimolar calcium concentrations were required, calcium additions were made in the presence of 0.1 mM EGTA and the final free calcium concentration was calculated (Whitsett & Darovec-Beckerman, 1981).

Phospholipid Binding Assay. Sonicated phospholipid vesicles containing 9 mg/mL DPPC, 1 mg/mL DPPG, and 0.12 μCi/mL [^{14}C]DPPC (100–150 mCi/mmol; New England Nuclear, Boston, MA) were prepared in Tris/NaCl buffer as described above. Lipids (1.25 mg/mL) were mixed with SP-A polypeptides (10 μg/mL) in the presence of 5 mM calcium chloride. The samples were vortexed, chilled on ice for 1 min, and spun at 11000g for 5 min in a MicroCentaur centrifuge (Accurate Chemical and Scientific Corp., Westbury, NY). The pellets were washed with Tris/NaCl/$CaCl_2$ buffer. Where indicated in the figure legends, SP-A aliquots were centrifuged in the absence of added lipids to control for nonspecific precipitation of the protein. Recovery of [^{14}C]phosphatidylcholine was always greater than 90% in the 11000g pellet. SP-A bindng was assessed by SDS–PAGE analysis of the resultant lipid pellet and aqueous fractions. The relative intensities of silver-stained protein bands were estimated by using a Gilford Response spectrophotometer (GelScan) program.

Radioactive ^{14}C-Alkylation of SP-A. Canine SP-A (14 μg) was resuspended in 35 μL of 25 mM Tris-HCl, pH 8.2, and 0.1 mM EGTA buffer. Triplicate samples containing either 0, 1, or 10 mM dithiothreitol were incubated under nitrogen in the dark at 37 °C for 30 min. [^{14}C]Iodoacetic acid (3.5 μL of 0.2 M solution in 1.2 M Tris-HCl buffer, pH 8.2; 1.88 μCi/μL; New England Nuclear) was added to each sample, and the samples were incubated at 37 °C for 15 min. Alkylation of the protein was quenched by addition of 40 μL of 0.1 M DTT to each sample.

To separate the ^{14}C-carboxymethylated protein from free [^{14}C]iodoacetate, each 78.5-μL sample was adjusted to 200 μL with a 1 mg/mL bovine serum albumin stock solution. Double-distilled H_2O (200 μL) was added, and the proteins were precipitated by the addition of 100 μL of cold 100% trichloracetic acid. The samples were chilled on ice for 5 min and microfuged at 13000 rpm for 5 min. The pellets were washed with 500 μL of cold acetone twice and allowed to air-dry.

The samples were resuspended in 100 μL of a buffer containing 0.1 M Tris-HCl, pH 7.3, 0.1 M NaCl, 0.5 mM $CaCl_2$, 0.5 mM $MgSO_4$, and 0.1% NP-40. A 10-μL aliquot was removed and mixed with 10 mL of EcoScint scintillation fluid (National Diagnostics, Manville, NJ). The remaining 90 μL was split in half and incubated at 37 °C for 16 h either in the absence or in the presence of 100 units of bacterial collagenase/mL. The samples were subjected to SDS–polyacrylamide gel electrophoresis (Laemmli, 1970) and transferred to nitrocellulose paper as described by Towbin et al. (1979). The Western blots were exposed to Kodak X-Omat AR film.

Determination of Intramolecular Disulfide Bonds. Canine SP-A (0.8 mg/0.2 mL) was digested with collagenase from

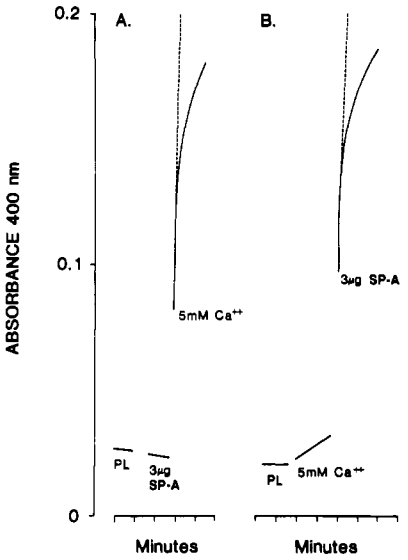

FIGURE 1: Increased light scattering due to aggregation of calcium–phospholipid vesicle SP-A complexes. PL vesicles (65% DPPC, 20% egg PC, 7.5% PG, 7.5% PI by weight) were prepared by sonication and Sepharose CL-6B chromatography in a buffer of 40 mM Tris-HCl, pH 7.4, 0.1 M NaCl, and 1 mM Na_2EDTA (Tris/NaCl/EDTA buffer). The PL vesicles were resuspended at approximately 30 µg/0.3 mL of Tris/NaCl/EDTA buffer in a quartz cuvette. Aliquots of protein and calcium chloride were then added from concentrated stock solutions, and the change in light absorbance at 400 nm was monitored. In (A), 3 µg of SP-A was added first (no OD change), followed by calcium chloride (initial ΔOD/min = 0.456). In (B), 5 mM calcium chloride was added first (ΔOD/min = 0.005), followed by 3 µg of SP-A (initial ΔOD/min = 0.259).

Clostridium histolyticum (Advanced Biofacturers Corp., Lynbrook, NY) at a concentration of 30 units/mL (Ross et al., 1986a). The collagenase-resistant fragment was isolated by dialysis against distilled H_2O by using a Centricon-10 microconcentrator (Amicon Corp., Danvers, MA). The peptide was then lyophilized and digested with 1% (w/w) trypsin-TPCK (Worthington Biochemical Corp., Freehold, NJ) at 37 °C for 1 h. Tryptic fragments were resolved by reverse-phase HPLC using both Beckman C_3 (4.6 mm × 75 mm) and Vydac phenyl (4.6 mm × 25 cm) columns under previously described elution conditions (Ross et al., 1986a). Indicated tryptic peaks were reduced and alkylated by incubation at 37 °C in a buffer of 0.1 M Tris-HCl, pH 8.5, and 6 M guanidine hydrochloride containing 5 mM DTT for 2 h, followed by addition of iodoacetic acid (17 mM, 30 min) and finally excess DTT (40 mM). Reduced alkylated peptides were then rechromatographed on the Vydac phenyl column. The elution program there included a 20-min wash with initial (aqueous) buffer to allow complete removal of salts before beginning the organic solvent (acetonitrile) gradient. Conditions for peptide acid hydrolysis and amino acid analysis were as previously described (Ross et al., 1986a).

Results

SP-A in the presence of supramillimolar calcium is known to cause an increased light scattering of phospholipid vesicle complexes (Hawgood et al., 1985; King et al., 1979, 1983). Our initial experiments sought to determine how the order of addition of SP-A or calcium to the vesicles influenced light scatterings measured by absorbance at 400 nm. Figure 1A shows that addition of SP-A alone causes no significant change in absorbance. Vesicles exposed to 5 mM calcium (Figure 1B), however, show a slow but constant rate of increased absorbance. Subsequent addition of calcium (in Figure 1A) or SP-A (in Figure 1B) results in a dramatic increase in light scattering.

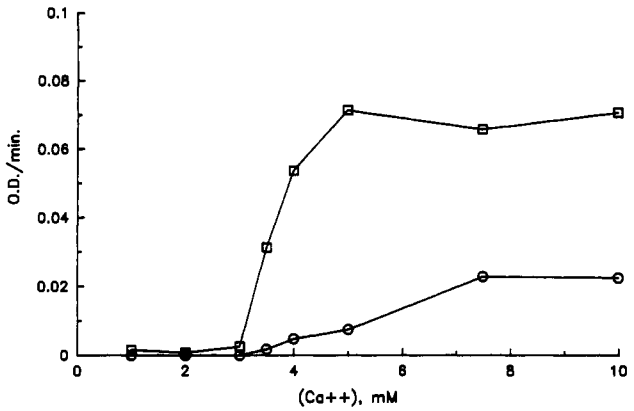

FIGURE 2: Effect of varying calcium levels on rate of PL vesicle aggregation. Sonicated PL vesicles were resuspended at 200 µg/0.3 mL in Tris/NaCl buffer containing 0.1 mM EGTA and incubated either alone (○) or with 3 µg of canine SP-A (□). Calcium chloride was then added to give the indicated final concentrations, and the subsequent absorbance changes were measured. In this experiment, SP-A enhancement of the PL vesicle aggregation rate can first be detected at 3 mM free calcium.

Table I: Dependence of PL Vesicle Aggregation on Both Intact SP-A and Calcium[a]

protein/peptide	cation[b]	rel aggregation activity[c]	*n*
SP-A	Ca^{2+}	100% ± 3.2%	3
	Ca^{2+}	26.0% ± 4.6%	6
albumin	Ca^{2+}	25.1% ± 2.4%	4
SP-A	Mg^{2+}	3.5% ± 2.3%	4
^{118}Gly–^{231}Phe	Ca^{2+}	11.7% ± 5.8%	4

[a] Phospholipid vesicles (72 µg/0.6 mL) were incubated with 5 µg of the indicated proteins. Upon addition of calcium (or magnesium) chloride, the A_{400} changes were recorded and initial ΔOD/min values calculated and averaged (mean ± SD). [b] Present as 4 mM $CaCl_2$ or $MgCl_2$. [c] The rates of absorbance changes are expressed relative to that of SP-A in the presence of 4 mM calcium chloride.

Measurement of the initial slope of the aggregation reaction (change in optical density units per minute) allows a comparison of different protein–lipid mixtures. The initial rate of phospholipid aggregation is proportional to the amount of SP-A preincubated with the vesicles. This observation holds for lipid:protein molar ratios ranging from 200:1 to 5000:1; however, the initial aggregation rate plateaus as more SP-A is added (data not shown).

The calcium dependence of the aggregation process is shown in Figure 2. Upon addition of 2 mM calcium, no difference in the rate of aggregation of lipid vesicles alone or vesicles containing SP-A (molar ratio = 1000:1 lipid:protein added) was measured. However, at free calcium levels exceeding 3 mM, SP-A-dependent vesicle aggregation is observed. This effect plateaus in the range of 5–10 mM free calcium. The specificity of the aggregation phenomenon for SP-A and calcium is shown in Table I. At 4 mM calcium, serum albumin has no effect on sample light scattering. Likewise, substitution of magnesium chloride for calcium chloride results in no vesicle aggregation. Table I also shows the results of assays where a defined SP-A fragment was utilized. The C-terminal domain extending from residues ^{118}Gly to ^{231}Phe has been shown to be unable to bind phospholipid (Ross et al., 1986b) and as expected does not induce phospholipid aggregation.

A larger SP-A fragment (^{81}Gly–^{231}Phe) was produced by digestion with bacterial collagenase. Figure 3, top panel, shows the effect of removal of the collagen-like domain of SP-A on the protein's ability to aggregate phospholipid vesicles. When SP-A is incubated with the bacterial collagenase, a progressive

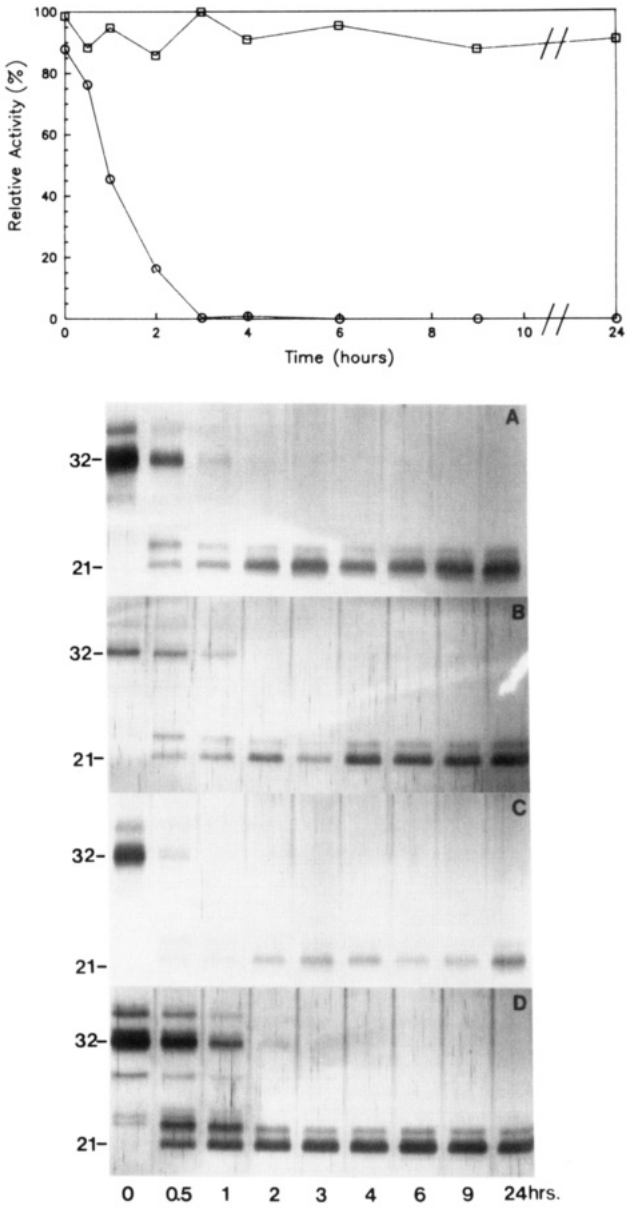

FIGURE 3: (Top) Effect of collagenase digestion on the ability of SP-A to aggregate phospholipid vesicles. Canine SP-A (0.51 mg/mL) was incubated at 37 °C in the absence (□) or presence (○) of 30 units of bacterial collagenase/mL (Ross et al., 1986a). At the indicated times, 5-μL aliquots (2.55 μg of SP-A) were removed and mixed with phospholipid vesicles (72 μg/0.6 mL) in Tris/NaCl/EDTA buffer. After 1 min, calcium chloride was added to 4 mM final concentration and the change in absorbance at 400 nm was measured. Aggregation activity (initial ΔOD/min) is expressed relative to that measured at $t = 0$. The $t = 0$ activity (+collagenase) was 89.2% of the $t = 0$ value measured in the absence of collagenase. The data points represent the average of duplicate measurements and are corrected for the effect of adding 4 mM calcium only to the phospholipid vesicle suspension. (Bottom) Effect of collagense treatment on the binding of SP-A to phospholipid. Canine SP-A (0.51 mg/mL) was incubated at 37 °C in the presence of 30 units of bacterial collagenase/mL. At the indicated times, 2.5-μg SP-A aliquots were removed and assayed for binding to DPPC/DPPG (9:1) vesicles as described under Materials and Methods. In panels A and B, the SP-A samples were centrifuged in the absence of phospholipid. In panels C and D, the samples were mixed with 250 μg of phospholipid sonicate prior to centrifugation. Aliquots of the supernatant (panels A and C) and pelleted (panels B and D) fractions were subjected to SDS–PAGE and silver staining. On the left, the relative M_r of intact SP-A (32 000) and that of the collagenase-resistant fragment (21 000) are indicated.

loss in aggregation activity results. The relative amount of activity at any given time point appears to depend on the level of residual intact SP-A (M_r = 32 000) present in the col-

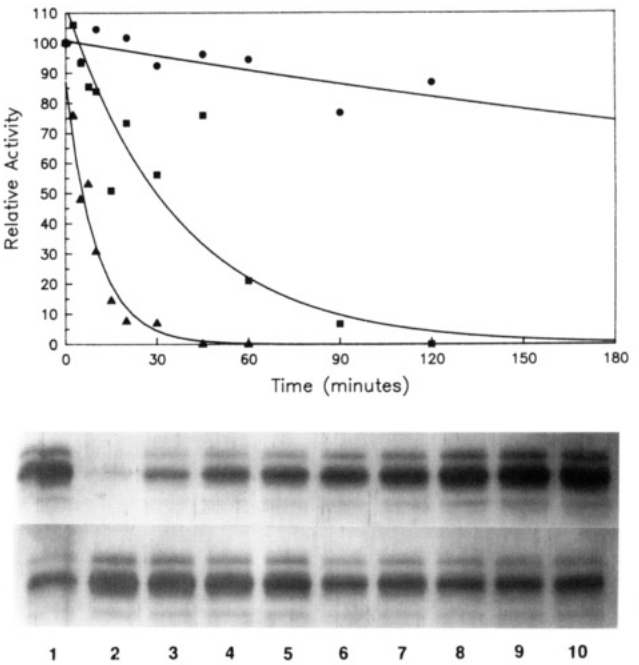

FIGURE 4: (Top) Time-dependent loss of SP-A phospholipid aggregation activity due to disulfide reduction. Canine SP-A (20 μg/mL) in a buffer of 25 mM Tris-HCl, pH 8.2, and 100 μM EGTA was incubated at 37 °C. Samples contained either no reduced DTT (●), 1 mM DTT (■), or 10 mM DTT (▲). At the indicated times, aliquots containing 0.8 μg of SP-A were removed and added to 20 μg of PL vesicles in 0.6 mL of Tris/NaCl/EDTA buffer; DTT was adjusted to a final concentration of 1 mM. After 1 min, calcium chloride was added to 4 mM final concentration and the change in absorbance at 400 nm was measured. Aggregation activity (initial ΔOD/min) is expressed relative to that measured at time zero for each sample. Control experiments show that the presence of 1 mM DTT in the assay did not inhibit the initial activity of unreduced SP-A (relative activity = 100.0% ± 4.9%, $n = 6$). Exponential regression analysis yields half-life estimates of 405 min (no DTT), 26 min (1 mM DTT), and 7.0 min (10 mM DTT), respectively. (Bottom) Effect of preincubation with 10 mM DTT on the binding of SP-A to phospholipid. SP-A was incubated at 37 °C under conditions identical with those used above (plus 10 mM DTT). At varying times, aliquots containing 2.1 μg of SP-A were removed and assayed for phospholipid binding (Materials and Methods). The top row represents SP-A that remained in the sample supernatant, while the bottom row shows SP-A that coprecipitated with the phospholipid. Lane 1, $t = 0$, no lipid present in binding assay; lane 2, $t = 0$, plus lipid; lanes 3–9, t = 5, 10, 15, 20, 30, 45, and 60 min of preincubation with 10 mM DTT, then mixing with lipid; lane 10, $t = 60$ min, no lipid present in binding assay. Densitometric analysis of the bands in lanes 1 and 10 estimates, respectively, that 31% and 35% of the SP-A is nonspecifically pelleted in the absence of phospholipid. The ability of additional SP-A to coprecipitate with lipid is completely lost after 45 min of exposure to 10 mM DTT (lane 8). The progressive loss in the lipid-binding capacity of SP-A proceeds with an approximate half-life of 12.5 min.

lagenase digestion (Figure 3, bottom panel). Aggregation activity is completely lost after 3 h, corresponding with the loss of intact SP-A. Lipid-dependent precipitation of the collagenase-resistant fragment (M_r = 21 000) is observed throughout the digestion period; however, the efficiency of its binding to lipid decreases during continued incubation.

Removal of the collagen-like domain of SP-A by collagenase digestion also results in removal of the cysteine residue involved in intermolecular disulfide bond formation (Ross et al., 1986a; Weaver et al., 1985). We therefore tested the effect of mild reducing conditions on the ability of SP-A to aggregate phospholipids. SP-A was incubated at 37 °C in the absence and presence of 1 mM and 10 mM dithiothreitol. Figure 4, top panel, shows a time- and dose- (DTT-) dependent loss in SP-A activity. Exponential regression of the data yielded estimates of $t_{1/2}$ = 405 min in the absence of DTT, $t_{1/2}$ = 26

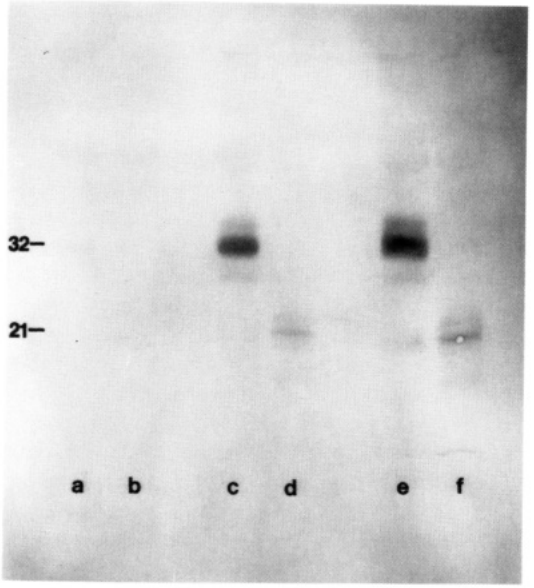

FIGURE 5: Selective reduction of the cysteine residue in the amino-terminal domain of canine SP-A. Triplicate aliquots of canine SP-A were alkylated with [^{14}C]iodoacetate, following 30 min of preincubation with no reducing agent (lanes a and b), 1 mM reduced DTT (lanes c and d), or 10 mM reduced DTT (lanes e and f) (see Materials and Methods). The ^{14}C-alkylated peptides were then divided in half and incubated in the absence (lanes a, c, and e) or presence (lanes b, d, and f) of bacterial collagenase. Following SDS–PAGE and Western blot transfer, the nitrocellulose paper was exposed to X-ray film for 3 days. Bands representing intact SP-A (32 kDa) and the collagenase-resistant domain of SP-A (21 kDa) are seen.

min in the presence of 1 mM DTT, and $t_{1/2}$ = 7.0 min in the presence of 10 mM DTT.

We also tested the effect of DTT on the ability of SP-A to bind phospholipids. Figure 4, bottom panel, shows that, over the course of a 1-h exposure to 10 mM DTT, there is a progressive loss in the amount of SP-A coprecipitated with phospholipid. The loss in the ability of SP-A to bind lipids following preincubation with DTT (Figure 4, bottom panel) proceeds with $t_{1/2}$ = 12.5 min compared to $t_{1/2}$ = 7.0 min for the DTT-induced loss in lipid aggregation activity in Figure 4, top panel.

Experiments were then carried out to identify which specific disulfide bonds were actually reduced under the nondenaturing Tris/EGTA buffer conditions used in Figure 4. We hypothesized that the cysteine residue (^{9}Cys) near the amino terminus and adjacent to the start of the collagenous domain of SP-A would be most readily reduced, having previously shown this cysteine residue to be involved in intermolecular disulfide bond formation (Ross et al., 1986a). The other cysteine residues are located in the C-terminal, noncollagenous domain of SP-A, specifically at positions 138, 207, 221, and 229 in the canine SP-A sequence reported by Benson et al. (1985).

SP-A was incubated under conditions identical with those in Figure 4, top panel. After 30 min, reduced sulfhydryl groups were alkylated via incubation with an excess of [^{14}C]iodoacetic acid. Figure 5, lanes a, c, and e, show that ^{14}C-alkylation of SP-A was stimulated by DTT in a dose-dependent manner. The relative incorporation of ^{14}C label (mole per mole of SP-A, based on M_r = 26000) was estimated by scintillation counting to be 0.007 in the absence of DTT, 0.83 in the presence of 1 mM DTT, and 1.10 in the presence of 10 mM DTT. Collagenase digestion was then used to monitor the incorporation of ^{14}C label into the amino-terminal (collagenase-sensitive) versus carboxy-terminal (collagenase-resistant) domain of the protein. Figure 5, lanes d and f, show only faint labeling of the 21-kDa C-terminal polypeptide. Densitometric analysis of the SP-A bands shown in Figure 5 estimates that the labeling intensity in the 21-kDa peptide (lane f) is only 20% of the total label incorporated into the 35-kDa protein (lane e). Complete labeling of all the SP-A cysteines would have resulted in a corresponding ratio of 0.8. Under nondenaturing conditions, the ^{9}Cys residue in the amino-terminal domain is selectively reduced by dithiothreitol.

Table II: Amino Acid Analysis of SP-A Tryptic Fragments[a]

	A_1	A_2	B_1	B_2	B_3
CM-Cys	0.65	0.75	0.96	1.32	2.24
Asx	0.41	4.78	3.12	4.19	5.24
Thr		0.27	1.04	1.04	1.22
Ser		3.52	0.62	0.80	0.46
Glx	1.40	6.85	4.36	6.23	6.79
Pro		1.72		0.30	
Gly	0.58	4.32	2.74	2.58	2.71
Ala	1.30	5.85	0.38	1.45	0.68
Val	0.35	1.89	1.02	1.37	1.33
Met		0.75	0.14	0.46	1.03
Ile	1.09	4.22	0.18	0.91	0.12
Leu	1.29	1.22	0.30	1.13	1.47
Tyr		0.14	1.02	1.70	2.34
Phe	1.0[b]	1.74	0.14		
His					
Lys		1.0[b]	2.0[b]	2.22	2.0[b]
Trp					
Arg	0.27	0.12	0.20	1.0[b]	1.53
matching peptide[c]	^{226}Leu–^{231}Phe	^{121}Val–^{162}Lys	^{203}Gly–^{219}Lys	^{203}Gly–^{225}Arg	*d*
cysteine	^{229}Cys	^{138}Cys	^{207}Cys	^{207}Cys, ^{221}Cys	^{207}Cys, ^{221}Cys

[a] Peaks correspond to those labeled in Figure 6. Values given are moles of amino acid per mole of peptide. [b] The picomole values of these amino acids were used to calculate the molar amount of the other amino acids per mole of peptide. [c] Derived from the cDNA sequence published by Benson et al. (1985). [d] The composition is most consistent with coelution of peptides ^{203}Gly–^{225}Arg and ^{220}Asn–^{225}Arg, recovered here in a molar ratio of 2:1.

The inability to ^{14}C-alkylate SP-A in the absence of DTT (Figure 5, lane a) supports the conclusion that all the cysteine residues in the canine SP-A sequence are present in the oxidized disulfide bond state. The four C-terminal-domain cysteines are involved in intramolecular disulfides (Ross et al., 1986a). We sought to identify the pairings of the two disulfide bonds hypothesized to be present in the noncollagenous domain of canine SP-A. SP-A was digested first with collagenase and then trypsin. Tryptic peptides were resolved by reverse-phase HPLC, collected, and then reduced and alkylated in the presence of 6 M guanidine hydrochloride. Figure 6 shows that rechromatography of two samples on the reverse-phase phenyl column yields multiple peaks. Amino acid analysis (Table II) of these peaks is consistent with their resulting from the reduction of disulfides between residues ^{138}Cys and ^{229}Cys (Figure 6, panels a and b) and between residues ^{207}Cys and ^{221}Cys (Figure 6, panels c and d).

Discussion

Pulmonary surfactant associated protein with average M_r = 35000 (SP-A) is the most abundant glycoprotein component bound to surfactant lipids. SP-A lowers the surface tension of aqueous dispersions containing either artificial (King & Macbeth, 1981; Hawgood et al., 1987; Ross et al., 1986b) or surfactant-derived lipids (Hawgood et al., 1985). A second function of SP-A involves the regulation of extracellular surfactant lipid pools. Addition of purified SP-A to cultured type II epithelial cells in vitro decreases the rate of [^{3}H]-phosphatidylcholine secretion by these cells (Dobbs et al., 1987; Rice et al., 1987). Wright et al. (1987) have also reported that SP-A stimulates the reuptake of extracellular lipids by type II cells. SP-A also is thought to play a role in the organization of surfactant lipids into a unique tubular myelin-like

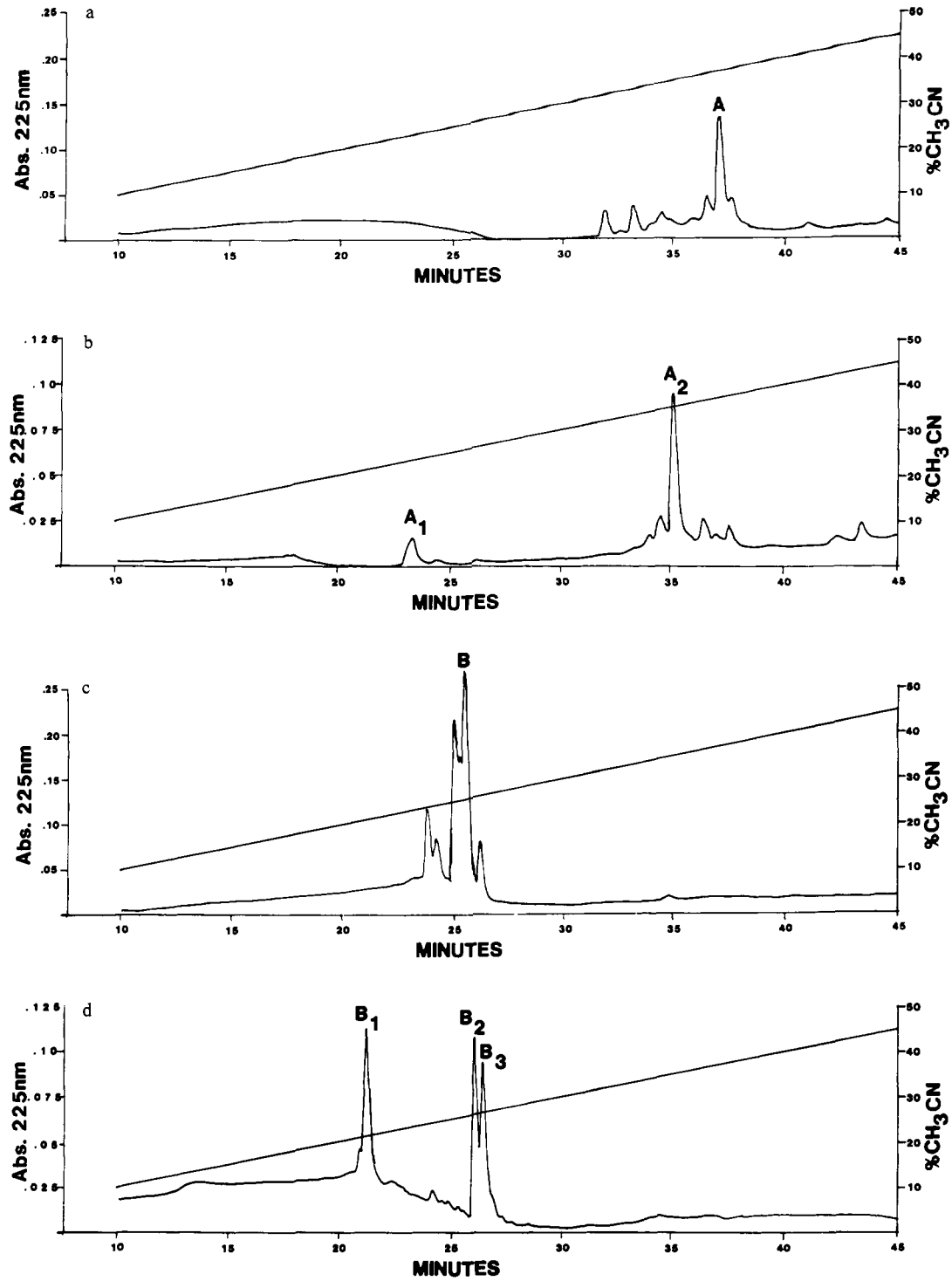

FIGURE 6: Reverse-phase HPLC analysis of two SP-A tryptic peaks before and after reductive alkylation of disulfide bonds. Canine SP-A was sequentially digested with collagenase and trypsin (Materials and Methods). The tryptic peptides were then chromatographed on Beckman C_3 and Vydac phenyl reverse-phase columns (Ross et al., 1986a). Phenyl column elution of SP-A tryptic peptide A is shown before (panel a) and after (panel b) reductive alkylation, which results in the separation of peptides A_1 and A_2. Similarly, peptide B eluted from the Vydac phenyl column (panel c) yields peptides B_1, B_2, and B_3 (panel d) following reductive alkylation. The amino acid compositions of these peptides are given in Table II.

structure. Suzuki et al. (1989) have recently demonstrated tubular myelin formation upon addition of SP-A *and* SP-B to lipid membranes. Reconstitution of SP-A with surfactant lipids has been shown to produce large lipid aggregates in the precence of calcium ions (Hawgood et al., 1985). Similarly, reconstitution of SP-A with artificial lipids also leads to a calcium-dependent reorganization (i.e., aggregation) (King & Macbeth, 1979; King et al., 1983). In the present study, we investigated the specific SP-A domains that are involved in the binding and aggregation of lipid. The structural subdomains of SP-A have recently been revealed by analysis of purified mammalian SP-A and analysis of cDNA and genomic clones predicting the primary sequence of the protein. Those domains of canine SP-A most relevant to this study include (1) an amino-terminal collagen-like domain, sensitive to bacterial collagenase (Ross et al., 1986a) and proposed to be the basis of triple-helical SP-A interactions, (2) a specific cysteine residue, near the N-terminal start of the collagen-like domain, which is involved in intermolecular disulfide bonding between SP-A monomers (Ross et al., 1986a), (3) a potential

lipid-binding domain (Ross et al., 1986b), consisting of hydrophobic residues and a potential amphipathic α-helical structure, and (4) a proposed calcium-binding domain, most likely located in the more hydrophilic C-terminal region of SP-A. In combination, these structural features of SP-A are hypothesized to enable the protein to bind phospholipid vesicles and enhance aggregation via protein–protein intermolecular cross-links.

The aggregation of artificial unilamellar vesicles is dependent on the amount of SP-A added (data not shown), as well as the presence of calcium ion at $[Ca^{2+}]_{free}$ greater than 3 mM (Figure 2). Hawgood et al. (1985) have previously demonstrated that progressive calcium titration of surfactant liposome SP-A mixtures results in an aggregation threshold at 0.5 mM calcium. Differences in our assay system from that of Hawgood et al. (1985) may account for these varying calcium sensitivities. Our experiments were performed exclusively with artificial phospholipid vesicles. Hawgood et al. (1985) studied SP-A effects on liposomes extracted from canine surfactant, which would be expected to contain the organic solvent soluble hydrophobic surfactant proteins SP-B and SP-C (Whitsett et al., 1986a,b; Phizackerly et al., 1979; Suzuki et al., 1982). In addition, Hawgood et al. (1985) reported their aggregation event by using turbidity measurements taken 4 min after titration of each aliquot of calcium. We have chosen here to focus on the initial aggregation event as measured by the initial absorbance changes induced at the time of protein or calcium addition.

Testing of several modified SP-A structures (Table I) has yielded the following results: (1) The C-terminal domain of canine SP-A (residues ^{117}Val–^{231}Phe) does not induce phospholipid vesicle aggregation. This result was expected, as this peptide fails to associate with lipid (Ross et al., 1986b). (2) The noncollagenous domain of SP-A (extending from residues ^{81}Gly to ^{231}Phe) also does not accelerate phospholipid aggregation (Figure 3). This fragment contains the most hydrophobic SP-A sequences and has been shown previously to bind phospholipids (Ross et al., 1986b). Lipid-dependent precipitation of the noncollagenous fragment (M_r = 21 000) is observed throughout the 24-h collagenase digestion period (Figure 3, bottom panel), while lipid aggregation activity is destroyed by 3 h (Figure 3, top panel), the time when intact (M_r = 32 000) SP-A is no longer detectable. This suggests that the N-terminal disulfide and collagen-like domains involved in protein–protein interaction are critical for the aggregation of neighboring lipid vesicles.

The importance of intact intermolecular disulfide bonds in phospholipid aggregation is supported by the observation that incubation of SP-A in the presence of dithiothreitol under nondenaturing conditions leads to a progressive loss of aggregation activity (Figure 4, top panel). Under nondenaturing conditions, selective, though not exclusive, reduction of the amino-terminal ^{9}Cys residue occurs, as demonstrated in Figure 5. Disulfide reduction, by 10 mM dithiothreitol, leads to subsequent [^{14}C]iodoacetate labeling, which is very sensitive to collagenase digestion. The [^{14}C]iodoacetate labeling (Figure 5) and DTT inactivation experiments (Figure 4) support the conclusion that the ability of SP-A to aggregate phospholipid vesicles is destroyed under conditions where the ^{9}Cys residue is preferentially alkylated; that is, where intermolecular SP-A disulfide bonds are reduced. Following incubation with DTT, the ability of SP-A to bind to phospholipids is decreased (Figure 4, bottom panel). This result is similar to the situation where the noncollagenous domain of SP-A no longer forms multimeric structures (Ross et al., 1986a) yet still binds to phospholipids (Ross et al., 1986b), though with lower affinity. It is possible that reduction of the disulfide bond at residue ^{9}Cys also destabilizes the neighboring collagen-like triple helix. Intermolecular disulfide bonds in the carboxy-terminal junction region of procollagen types I, II, and III precede the formation and lend stability to collagen triple helices (Bachinger et al., 1980, 1981). In the presence of DTT, therefore, unwinding of the SP-A triple helix could result in a quaternary structure similar to that obtained by collagenase digestion (i.e., a monomeric species is produced). This is consistent with a model wherein the intermolecular structure of SP-A would enhance the cooperative binding and organization of neighboring membrane structures. Our experiments with DTT can be summarized as follows: preincubation of canine SP-A with DTT, under nondenaturing conditions, selectively reduces the intermolecular disulfide bond formed between residue ^{9}Cys of two SP-A chains. Reduction of this disulfide bond results in a progressive loss in SP-A's ability to aggregate phospholipid vesicles due, in part, to a reduced affinity for the lipids themselves.

The cysteine residues located in the C-terminal, noncollagenous domain of canine SP-A exist as intramolecular disulfide bonds (Figure 6, Table II). The pairings of ^{138}Cys–^{221}Cys and ^{207}Cys–^{221}Cys in canine SP-A are similar to the disulfide bonding structures described for the carbohydrate recognition domains of lectins isolated from sea urchin [*Anthocidaris crassispina* (Giga et al., 1987)], tunicate [*Polyandrocarpa misakiensis* (Suzuki et al., 1990)], and acorn barnacle [*Megabalanus rosa* (Muramoto et al., 1986)] species. SP-A and these invertebrate lectins belong to a family of calcium-dependent (C-type) lectins described and reviewed by Drickamer et al. (1986, 1988).

By sequence homology to this family of calcium-dependent lectins, it is inferred that a calcium-binding domain of SP-A resides in this C-terminal region. Recent work by Haagsman et al. (1990) demonstrated the decreased lipid aggregation activity of N-glycosidase F treated SP-A, suggesting the importance of lectin-like interactions among SP-A molecules in this aggregation process. Efrati and Hawgood (1987) have reported that terminal sialic acid residues present on the asparagine-linked carbohydrates of SP-A are not critical for the binding and aggregation of surfactant-derived unilamellar liposomes. Taken together, these two studies infer that SP-A–SP-A interactions would involve lectin binding to more proximal sugars present on the asparagine-linked oligosaccharide of a neighboring SP-A chain. In the present study, we have demonstrated that this noncollagenous domain alone, however, is not sufficient to induce the calcium-dependent acceleration of lipid vesicle aggregation (Table I; Figure 3, top panel). Our data support the conclusion that the macromolecular aggregation of lipids by SP-A, though possibly initiated by calcium binding in the C-terminal domain, requires the integrity of the N-terminal collagen and intermolecular disulfide structures.

Registry No. Ca, 7440-70-2; Cys, 52-90-4.

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