Binding of Calcium to SP-A, a Surfactant-Associated Protein†

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ABSTRACT: SP-A is a lung-specific pulmonary surfactant-associated protein containing a calcium-dependent carbohydrate recognition domain and collagen-like sequence. The protein is a major component of the extracellular form of surfactant known as tubular myelin. SP-A is thought to influence the surface properties of surfactant lipids and regulate the turnover of extracellular surfactant through interaction with a specific cell-surface receptor. These properties of SP-A are dependent on the presence of calcium. We have estimated calcium binding parameters for SP-A from binding data obtained by equilibrium dialysis and gel permeation chromatography. Our results suggest that each SP-A monomer binds two to three calcium ions in conditions chosen as similar to those found in the alveolar lumen. The binding data are best fit to a model incorporating two calcium binding sites with different affinities. Studies with a fragment of SP-A generated by limited proteolysis suggest the higher affinity site for calcium is located in the noncollagenous carboxy-terminal end of SP-A. This region of SP-A contains a carbohydrate recognition domain homologous to other C-type lectins. The binding of calcium to this region of SP-A causes a conformational change as assessed by a small change in the intrinsic fluorescence spectrum and a marked change in the susceptibility to proteolysis. At physiological calcium concentrations, intact SP-A aggregates in a reversible fashion, a property that may be relevant to the formation of tubular myelin.

Pulmonary surfactant consists of macromolecular complexes of lipids and specific proteins. The lipids of surfactant, particularly dipalmitoylphosphatidylcholine (DPPC), modify the surface forces in the lung, promoting lung stability. Surfactant proteins may be involved both in the transport of DPPC from the pulmonary epithelial type II cell to the surface film at the alveolar air–fluid interface and in the clearance of DPPC from the alveolar space.

The most abundant of the lung-specific surfactant proteins is the glycoprotein surfactant protein A (SP-A) (Possmayer, 1988). The primary structure of SP-A is similar in the human (White et al., 1985; Floros et al., 1986), dog (Benson et al., 1985), rat (Sano et al., 1987), and rabbit (Boggaram et al., 1988). A short NH_2-terminal region of 7–10 amino acids containing an interchain disulfide bridge (Benson et al., 1985) is followed by 72 amino acids in a collagen-like sequence. The remaining COOH-terminal 149 amino acids have short interrupted segments of sequence homology with a number of Ca^{2+}-dependent lectins (Drickamer et al., 1986; Haagsman et al., 1987). The functional form of SP-A is assembled through interactions in the collagen-like domain into a complex oligomer of approximately 18 similar subunits (Voss et al., 1988; King et al., 1989; Haagsman et al., 1989).

The function of SP-A in the alveolar space is not known with certainty, but in vitro studies suggest SP-A has a role in regulating the secretion of DPPC from type II cells (Dobbs et al., 1987; Kuroki et al., 1988; Rice et al., 1987), the formation of the tubular myelin form of surfactant (King & MacBeth, 1981; Efrati et al., 1987; Suzuki et al., 1989), the adsorption of surfactant lipids to an air–fluid interface (King & MacBeth, 1981; Hawgood et al., 1985a, 1987), and the uptake of DPPC into type II cells (Wright et al., 1987). The effect of SP-A in each of these assays is dependent on the presence of calcium.

In this study, we have directly investigated the binding of calcium to purified SP-A. Our results suggest that SP-A has at least one and possibly two specific calcium binding sites and that the binding of calcium by SP-A induces self-association of the intact protein. The relationship between Ca^{2+} binding and protein self-association suggests the interaction between Ca^{2+} and SP-A is one mechanism regulating the major structural transformation of secreted lamellar body contents into the unique tubular myelin form of surfactant. We have also utilized a proteolytic fragment of SP-A, referred to as CRF, which binds to both type II cells (Wright et al., 1989) and carbohydrates in a Ca^{2+}-dependent fashion to investigate conformational changes associated with the binding of Ca^{2+}. The results are consistent with there being more than one mechanism by which calcium regulates the function of SP-A. Parts of this report have been published in a preliminary form (Haagsman et al., 1988).

Materials and Methods

Purification of SP-A. Pulmonary surfactant was isolated from bronchoalveolar lavage of adult dogs and human patients with alveolar proteinosis (Hawgood et al., 1985a). SP-A was purified from the isolated surfactant as described previously

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using sequential butanol and octyl glucopyranoside extractions (Haagsman et al., 1987). Human recombinant SP-A secreted into the medium of Chinese hamster ovary cells transfected with a gene of human SP-A (White et al., 1985) was purified by mannose affinity chromatography (Haagsman et al., 1987). Protein from each source was dialyzed extensively against 5 mM Tris, pH 7.4, after purification. The proteins prepared in this fashion have been previously characterized by us in terms of purity, electrophoretic mobility, charge, size, and amino acid sequence (Hawgood et al., 1985b; Benson et al., 1985; White et al., 1985; Haagsman et al., 1989).

A collagenase-resistent fragment of SP-A (CRF) was prepared from SP-A isolated from alveolar proteinosis lavage by digestion with purified bacterial collagenase as previously described (Haagsman et al., 1989). CRF was purified from the digestion mixture by either ion-exchange chromatography (Haagsman et al., 1989) or mannose affinity chromatography (Haagsman et al., 1987) using a binding buffer of 5 mM Tris, 100 mM NaCl, and 25 mM $CaCl_2$ and an eluting buffer containing 2 mM EDTA. Purified CRF was dialyzed extensively against 5 mM Tris and 100 mM NaCl, pH 7.4, before use. The major amino-terminal amino acid of human CRF is the glycine at position 74 in the intact protein (White et al., 1985; Haagsman et al., 1989). Reduced and alkylated SP-A was prepared by incubation of the protein in 50 mM dithiothreitol for 1 h at 37 °C followed by incubation with 100 mM iodoacetamide for 2 h at 4 °C in the dark. Protein concentrations were estimated by the procedure of Lowry (Lowry et al., 1959) using bovine serum albumin as a standard. Electrophoretic analysis of SP-A and CRF was performed according to Laemmli (1970).

Amino Acid Analysis. The amino acid compositions of dog, alveolar proteinosis, and recombinant SP-A were determined after alkaline hydrolysis in 2 M KOH at 110 °C for 22 h. Protein hydrolysates were separated on a Beckman 121M amino acid analyzer using established methods (Hauschka, 1977).

Ion-Dependent Aggregation of SP-A. Aggregation of SP-A as a function of ion concentration and temperature was assessed by recording the change in absorbance of light by the protein sample at a wavelength of 300 nm in a 1-cm pathlength temperature-controlled cuvette in a Gilford response spectrophotometer. The ion concentration was changed by the addition of repeated small volumes of concentrated ion solutions. The SP-A concentration was 100 $\mu g \cdot mL^{-1}$. The buffer in each case was 5 mM Tris adjusted to the stated pH. In the experiments with the divalent cations, the NaCl concentration was 50 mM.

Aggregation of SP-A/Phospholipid Mixtures. Aggregation of the reconstituted lipoproteins was monitored at 400 nm. Vesicles were prepared by hydration of DPPC/PG mixtures (7:3 w/w) at 45 °C in 5 mM Tris buffer, pH 7.4, containing 150 mM NaCl and sonication at three bursts of 30 s at maximal output of an MSE Soniprep 150 sonifier. DPPC and egg PG were from Sigma (St. Louis, MO). SP-A (10 μg) was added to the vesicles (100 μg) in a total volume of 1 mL. The samples were heated to various temperatures for 10 min; then aggregation was initiated by the addition of $CaCl_2$. In a separate series of experiments, SP-A was heated prior to adding the protein to the lipids, and aggregation was then assessed at 37 °C.

Calcium Binding. The binding of calcium to SP-A from alveolar proteinosis lavage, recombinant SP-A, and CRF was determined by equilibrium dialysis. The glassware and dialysis tubing were soaked in EDTA and washed extensively in distilled water before use. SP-A (0.5 mg) was mixed with sonicated vesicles of egg phosphatidylcholine (1 mg) obtained from Avanti Polar Lipids (Birmingham, AL) in a final volume of 0.5 mL of 5 mM Tris, pH 7.4. The sample was then dialyzed for 20 h at room temperature against 250 mL of 5 mM Tris, pH 7.4, 30 mM NaCl, 3 mM $MgCl_2$, and different concentrations of $CaCl_2$ containing 10 μCi of $^{45}CaCl_2$ (3400 $\mu Ci \cdot \mu mol^{-1}$). After dialysis, 5 μL of 10% SDS was added to the bag to solubilize the lipoprotein aggregates and ensure homogeneous sampling of the bag contents. Samples were taken in triplicate for protein and $^{45}Ca^{2+}$ determination. At each calcium concentration, binding was corrected for the binding of calcium to vesicles in the absence of SP-A. The binding data were analyzed by using a nonlinear least-squares program (Systat, Inc.) to derive theoretical curves and binding parameters that best fit the experimental data to the following mathematical models of ligand binding (Murlas et al., 1982):

(1) one class of site: $B = RF/(F + K)$

(2) two classes of sites: $B = R_1F/(F + K_1) + R_2F/(F + K_2)$

where B = the bound $[Ca^{2+}]$, F = the free $[Ca^{2+}]$, R = the binding capacity, and K = the dissociation constant. Molecular masses of 35 000 for intact monomeric SP-A and 25 000 for CRF were used to express the data. The fit of the data to the two models was compared by using the extra sum of squares principle and the F statistic as described by Munson and Rodbard (1980).

Calcium binding to CRF was determined in a similar fashion both in the presence and in the absence of lipid except that the NaCl concentration was 100 mM. Calcium binding to human recombinant SP-A was also determined by gel permeation chromatography (Hummel & Dreyer, 1962). A Biogel P6DG desalting column (8 mL, 20 cm; Bio-Rad, Richmond, CA) in 5 mM Hepes, pH 7.4, 100 mM NaCl, 3 mM $MgCl_2$, and 0.1% Triton-X-100 was equilibrated with various concentrations of $^{45}CaCl_2$ (1000 $dpm \cdot nmol^{-1}$). Recombinant SP-A (0.6 mg) was equilibrated with the loading buffer containing $^{45}CaCl_2$ for 10 min and then loaded onto the column. Eluted fractions were analyzed for radioactivity and protein content (Bohlen et al., 1973).

Proteolysis of CRF. Proteolysis of CRF, purified by mannose affinity chromatography and extensively dialyzed against 5 mM Tris/100 mM NaCl, pH 7.4, was performed in the presence of either 0.1 mM EDTA or increasing concentrations of either calcium, strontium, or magnesium. Trypsin or chymotrypsin was incubated with CRF at a protein:enzyme ratio of 30:1 (w/w) for 30 min at 37 °C. Digestion was stopped by the addition of SDS sample buffer and boiling for 2 min. The samples were analyzed by electrophoresis on 15% SDS–polyacrylamide gels stained with Coomassie blue. The stained gels were scanned on a Hoeffer GS300 densitometer, and the percent proteolysis was calculated relative to a control sample of undigested CRF run on the same gel.

Intrinsic Fluorescence. The intrinsic fluorescence of SP-A and CRF was measured in 2 mL of 5 mM Tris, pH 7.4, containing 30 mM NaCl and 100 mM NaCl, respectively. The final protein concentrations of SP-A and CRF were 10 and 25 $\mu g \cdot mL^{-1}$, respectively. The samples (buffer blanks and protein samples) were excited at 280 nm using an excitation slit of 0.5 mm. Emission spectra were recorded from 300 to 600 nm using a 0.25-mm slit. The measurements were made on a SPEX Fluorolog fluorescence spectrophotometer (SPEX Industries, Inc., Edison NJ). The sample cuvette was ther-

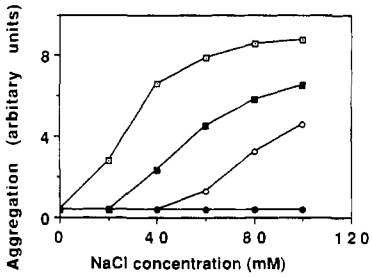

FIGURE 1: NaCl-induced aggregation of human SP-A. Aggregation of human SP-A (100 μg) was studied as a function of NaCl concentration and pH at 25 °C. Sample turbidity was continuously monitored at 300 nm. Increasing amounts of NaCl were added to SP-A adjusted to various pHs in 5 mM Tris-HCl buffers. Open squares, pH 6; closed squares, pH 7; open circles, pH 8; closed circles, pH 9.

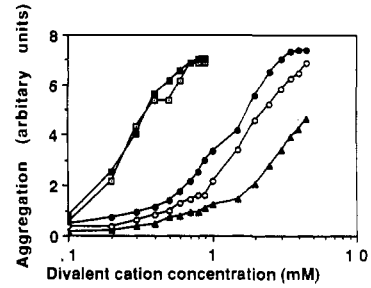

FIGURE 2: Divalent cation dependent self-association of canine SP-A as a function of divalent cation concentration. Self-association of SP-A (50 μg) in 5 mM Tris-HCl, pH 7.0, and 50 mM NaCl (0.5 mL) at 37 °C was monitored by measuring the turbidity at 300 nm. An aliquot of a concentrated solution of the various divalent cations was added to the cuvette every 10 min. Sr^{2+}, closed squares; Ba^{2+}, open squares; Ca^{2+}, closed circles; Mn^{2+}, open circles; Mg^{2+}, closed triangles.

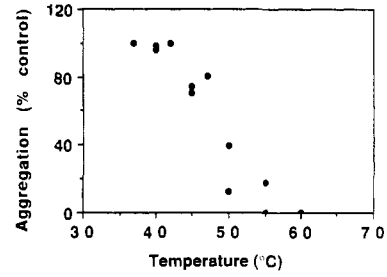

FIGURE 3: Ca^{2+}-induced lipid aggregation by canine SP-A as a function of temperature. Vesicles consisting of DPPC/egg PG (7:3) (100 μg) were mixed with SP-A (10 μg) in 5 mM Tris-HCl, pH 7.4, and 150 mM NaCl in two glass cuvettes in a volume of 1 mL. The mixtures were held for 10 min at different temperatures, and the absorbance was continuously monitored at 400 nm. Subsequently, Ca^{2+} (final concentration 5 mM) was added to one cuvette, and the absorbance was monitored for a further 20 min at the indicated temperature. The aggregation rate was determined at each temperature and expressed as a percentage of the rate observed at 37 °C.

mostatically controlled and continuously stirred. Spectra were recorded first in the presence of 0.1 mM EDTA and repeated 10 min after the addition of $CaCl_2$ or $MgCl_2$ to final concentrations of 1 or 25 mM. The reported spectra were corrected for wavelength-dependent effects.

RESULTS

Ion-Dependent Aggregation of SP-A. We have previously observed SP-A, extracted from surfactant obtained by bronchoalveolar lavage, is insoluble in physiological saline at room temperature but solubilizes readily in low ionic strength buffers (Haagsman et al., 1987). To better characterize this phenomenon before proceeding with studies with divalent cations, we investigated the aggregation of SP-A as a function of NaCl concentration, pH, and temperature. Figure 1 shows the aggregation of human SP-A at 25 °C as a function of the NaCl concentration and pH. As the pH was lowered, SP-A aggregated at a progressively lower NaCl concentration. At 37 °C, the threshold concentration of NaCl causing aggregation at each pH was approximately 40 mM higher than at 25 °C (data not shown). Minimal aggregation of SP-A was detected at 37 °C at a pH of 7.0 or above in up to 100 mM NaCl. In contrast to human and canine SP-A, obtained from bronchoalveolar lavage, recombinant SP-A at a concentration of 100 $\mu g \cdot mL^{-1}$ did not detectably aggregate in the presence of 100 mM NaCl at pH 7.0 at either 25 or 37 °C.

Calcium induced rapid aggregation of all forms of SP-A at pH 7.0 at both 25 and 37 °C. Aggregation of SP-A in the presence of Ca^{2+} was detectable at concentrations of SP-A as low as 5 $\mu g \cdot mL^{-1}$. The threshold concentration of calcium required to induce aggregation of SP-A at 37 °C was 0.5 mM. The divalent cation sequence for the induction of SP-A aggregation was $Sr^{2+} = Ba^{2+} > Ca^{2+} > Mn^{2+} > Mg^{2+}$ (Figure 2). Ca^{2+}-induced aggregation of SP-A was rapidly and completely reversed by EDTA. Recombinant SP-A was also reversibly aggregated by Ca^{2+} at 37 °C. SP-A that had been boiled for 2 min or reduced at 37 °C no longer aggregated in response to Ca^{2+}. Milder heat treatment at 50 °C for 10 min also significantly decreased the extent of Ca^{2+}-induced aggregation. The rate and extent of Ca^{2+}-dependent SP-A induced aggregation of phospholipid vesicles decreased as the temperature was raised (Figure 3). Similar results were obtained when SP-A was heated for 10 min in the absence of lipid and then cooled to 37 °C before adding to the lipids and assessing aggregation at 37 °C.

Unlike intact SP-A, CRF was soluble in 100 mM NaCl but precipitated in low ionic strength buffers (data not shown). No increase in the turbidity of the CRF sample was observed after the addition of Ca^{2+}. As assessed by gel permeation chromatography (Superose-6, Pharmacia), CRF did not aggregate in the presence of up to 25 mM Ca^{2+}.

Calcium Binding to SP-A. The Ca^{2+} binding properties of human SP-A were determined by equilibrium dialysis. These experiments were performed in the presence of 30 mM NaCl and 3 mM $MgCl_2$ to reduce NaCl-induced aggregation of SP-A and nonspecific binding of divalent cations, respectively. Phosphatidylcholine was included as most if not all SP-A in the lung is lipid-associated. Anionic phospholipids were not used to minimize both the extent of Ca^{2+}-induced lipid aggregation (King & MacBeth, 1981) and the binding of Ca^{2+} to the lipids. As determined from samples containing only lipid, the amount of Ca^{2+} bound to egg PC increased with increasing Ca^{2+} concentrations (data not shown). In 2 mM $CaCl_2$, 1 mol of egg PC bound 0.016 mol of Ca^{2+}, a value similar to that reported for Ca^{2+} binding to POPC under similar conditions (Altenbach & Seelig, 1984). The lipid to monomer SP-A mole ratio used was approximately 100 to 1; therefore, at the higher Ca^{2+} concentrations, the calcium binding to PC accounted for up to 46% of the total binding to the SP-A/PC samples. The total bound Ca^{2+}, at each Ca^{2+} concentration, was corrected for the amount bound to PC before further analysis. The Ca^{2+} bound to SP-A as a function of the free Ca^{2+} concentration is graphically depicted in Figure 4.

The binding parameters estimated by fitting these data to model equations, described under Materials and Methods, for one or two classes of binding sites using a nonlinear least-squares program are given in Table I. The data fit a model incorporating two classes of binding sites for Ca^{2+} on SP-A

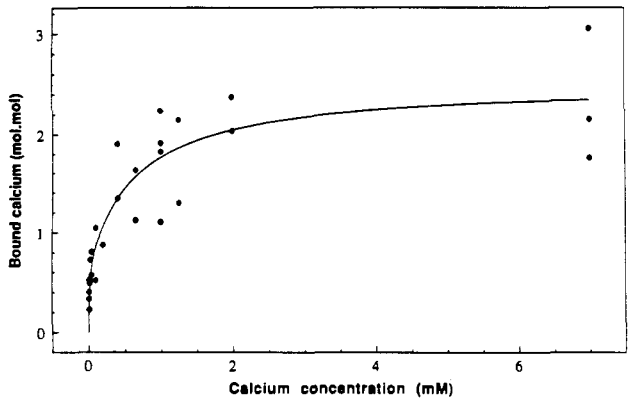

FIGURE 4: Ca^{2+} bound to human SP-A as determined by equilibrium dialysis in the presence of phosphatidylcholine. Binding experiments were performed as described under Materials and Methods. The curve represents the best fit ($r = 0.96$) of the experimental data (27 points) to the equation for a two-site model described in the text.

Table I: Estimate of Ca^{2+} Binding Parameters for Intact SP-A and CRF[a]

	K_1	R_1	K_2	R_2	rms
one site	140 ± 70	2.1 ± 0.2			39
SP-A two sites	11 ± 15	0.7 ± 0.3	690 ± 310	1.8 ± 0.4	35
CRF one site	45 ± 12	0.6 ± 0.1			18

[a] Binding parameters were obtained by fit of the experimental data (corrected for nonspecific binding) to model equations for one and two binding sites (see text for methods). Units for the dissociation constants (K) are μM^{-1} and for the binding capacities (R) are moles of Ca^{2+} per moles of SP-A monomer. The root mean square error (rms) expressed as a percentage represents the average scatter of the data around the estimated best fit (Munson & Rodbard, 1980). For SP-A, the binding data best fit a two-site model ($p = 0.05$–0.1); 27 and 31 individual experimental data points were used for the intact SP-A and CRF estimations, respectively.

slightly better than a model assuming one class of site ($p = 0.05$–0.1). The estimated affinities of the sites were 11 μM^{-1} and 0.7 mM^{-1}, respectively. These estimates should be considered approximate in view of the 35% average scatter of the experimental data around the mathematically derived curve. Within the range of free Ca^{2+} concentrations tested, the binding of Ca^{2+} to SP-A appeared to saturate at 2–3 mol of Ca^{2+} to each mole of protein monomer. Disadvantageous signal to noise ratios in the experimental data precluded experiments in free Ca^{2+} concentrations above 7 mM. The experimental data and mathematically derived binding parameters are graphically represented by using the method of Scatchard (1949) in Figure 5.

As determined by equilibrium dialysis, the amount of Ca^{2+} bound to recombinant SP-A and SP-A from patients with alveolar proteinosis at a free [Ca^{2+}] of 1 mM in the presence of 3 mM $MgCl_2$ was comparable (1.7 and 1.8 mol·mol^{-1}, respectively; average values of three and four determinations). If 3 mM $SrCl_2$ was substituted for $MgCl_2$ in the dialysis buffer, the bound [Ca^{2+}] at 1 mM free Ca^{2+} was reduced to 0.6 mol·mol^{-1} ($n = 2$). The binding of Ca^{2+} to human recombinant SP-A was also determined by an alternate gel filtration method (Hummel & Dreyer, 1962). These experiments were performed at 25 °C in the presence of 100 mM NaCl and 3 mM $MgCl_2$ and in the absence of phosphatidylcholine. Figure 6 shows a representative elution profile of the $^{45}Ca^{2+}$ radioactivity and recombinant SP-A at a free Ca^{2+} concentration of 10 μM and a $MgCl_2$ concentration of 3 mM. Ca^{2+}-induced aggregation of recombinant SP-A at 1 mg·mL^{-1} could be prevented by the addition of Triton X-100 (0.1%) to the binding buffer up to a Ca^{2+} concentration of 0.5 mM. Gel permeation experiments were therefore only performed at Ca^{2+} concentrations up to 0.5 mM. The amount of Ca^{2+} bound to recombinant SP-A at 10, 50, 100, and 500 μM free Ca^{2+} was 0.2, 0.3, 0.6, and 1.7 mol·mol^{-1} SP-A, respectively (average values of four to six determinations at each concentration). These values are in the same range as the values obtained by equilibrium dialysis in the presence of phospholipid.

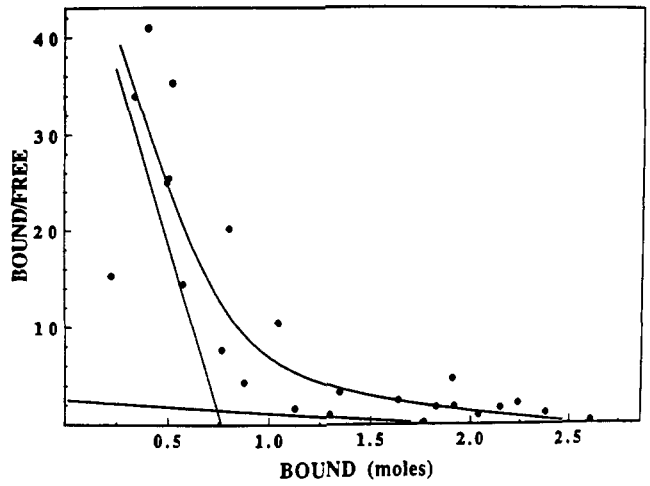

FIGURE 5: Scatchard plot of Ca^{2+} binding to human SP-A as determined by equilibrium dialysis in the presence of phosphatidylcholine. Units for bound are moles of Ca^{2+} per mole of SP-A monomer. Values are corrected for nonspecific binding. Straight lines show individual components corresponding to the high- and low-affinity classes of sites (Table I). The curve represents the best fit of the data to the equation for a two-site model ($r = 0.96$).

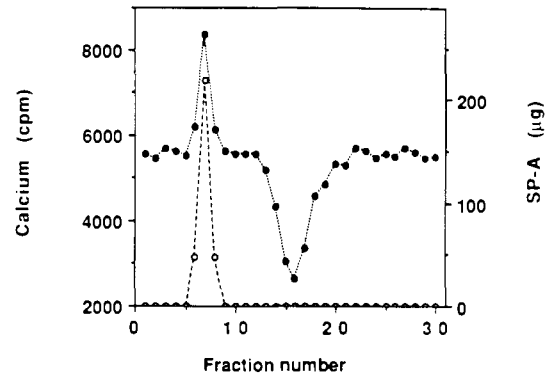

FIGURE 6: Representative elution profile of $^{45}Ca^{2+}$ and human recombinant SP-A from a Bio-Gel P-6DG column in 10 μM free Ca^{2+} and 3 mM Mg^{2+}. The experiment was carried out as described under Materials and Methods. Radioactivity is shown as the solid circles and protein as the open circles.

At 1 mM Ca^{2+}, CRF bound a similar amount of Ca^{2+} in the presence or absence of lipid and in either 30 mM or 100 mM NaCl (data not shown). CRF bound only about half the Ca^{2+} per mole of protein at this Ca^{2+} concentration as the intact protein. The binding of Ca^{2+} to CRF in the absence of lipid as a function of the free Ca^{2+} concentration is shown in Figure 7. These binding data best fit a model for one class of sites for Ca^{2+} on CRF with an affinity of approximately 45 μM^{-1} (Table I).

Alkaline Hydrolysis of SP-A. The amino acid compositions of dog, alveolar proteinosis, and recombinant SP-A after acid hydrolysis have been reported by us (Hawgood, 1985b; Haagsman et al., 1989). Because of a recent report that rat type II cells have an active γ-carboxylase system and carboxylate several proteins including a protein with a similar molecular weight to SP-A (Rannels et al., 1987), we determined the amino acid composition of purified SP-A after alkaline hydrolysis. No significant content of γ-carboxyglutamic acid (less than 1 residue of Gla per 1000 Glu) was

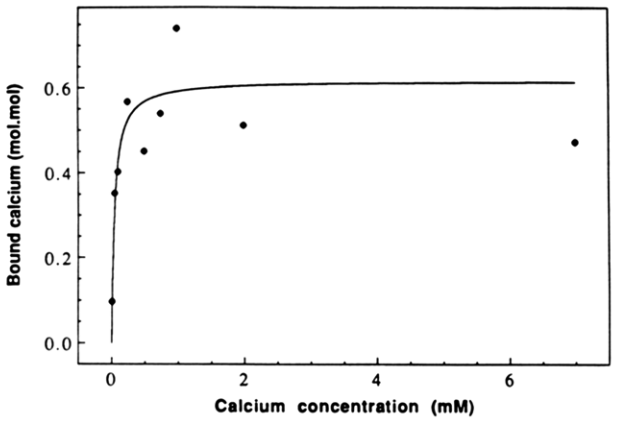

FIGURE 7: Ca^{2+} bound to CRF as determined by equilibrium dialysis in the presence of 100 mM NaCl and 3 mM $MgCl_2$. Each point is the mean of from three to five determinations except the value at 7 mM which represents a single experiment. The curve represents the best fit ($r = 0.88$) of the experimental data (31 points) to the equation for a one-site model described in the text.

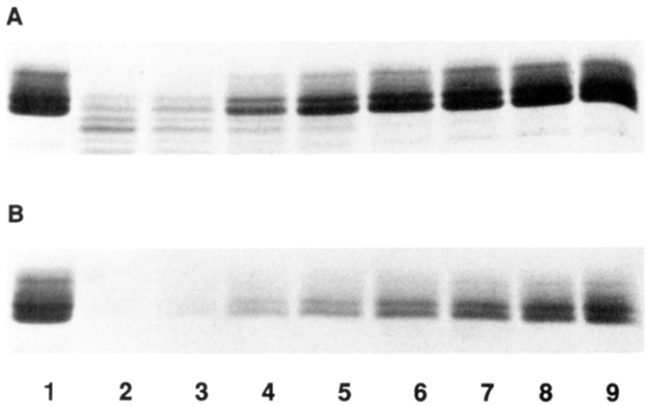

FIGURE 8: Coomassie blue stained gels of CRF digested by trypsin (A) or chymotrypsin (B) in increasing concentrations of $CaCl_2$. Lane 1 contains control CRF with no enzyme. Lanes 2–9 contain CRF with enzyme in 1 mM EDTA and 0.01, 0.05, 0.1, 0.2, 0.4, 0.5, and 1 mM $CaCl_2$, respectively.

detected in dog SP-A or either form of human SP-A after alkaline hydrolysis.

Proteolysis of CRF. The extent of digestion of CRF by both trypsin and chymotrypsin varied as a function of the divalent cation concentration. CRF was almost completely degraded by both enzymes in 0.1 mM EDTA. CRF was also extensively degraded in $MgCl_2$ in concentrations up to 20 mM. Resistance to the proteolysis of CRF by both enzymes was seen as a function of the Ca^{2+} concentration (Figure 8). After exposure to trypsin or chymotrypsin in 1 mM $CaCl_2$, most CRF, 80% and 60%, respectively ($n = 3$), remained undigested. No significant further protection from proteolysis was seen at higher Ca^{2+} concentrations. $SrCl_2$ protected CRF from proteolysis in a similar manner to $CaCl_2$ (data not shown). Bovine serum albumin was digested to a comparable extent in EDTA and 25 mM $CaCl_2$, indicating the protease activity was not Ca^{2+} dependent.

Intrinsic Fluorescence. With excitation of intact SP-A at 280 nm at 37 °C in 30 mM NaCl, there was a small shift in the wavelength of maximum emission from 336 to 332 nm on changing from 0.1 mM EDTA to 1 mM $CaCl_2$. The maximum intensity of emission increased approximately 4% after the addition of Ca^{2+}. Because aggregation of SP-A is likely under these conditions even at the low protein concentration used, further studies were performed with CRF which does not aggregate in Ca^{2+}. All the tyrosine and tryptophan residues in SP-A are located in CRF. At 25 °C in 100 mM NaCl, the addition of $MgCl_2$ to a 1 or 25 mM final concentration caused no change in the wavelength of maximum emission. A small decrease in emission intensity occurred at the higher Mg^{2+} concentration. $CaCl_2$ at a concentration of 1 mM caused a small but consistent shift in the wavelength of maximum emission from 336 nm in EDTA to 334 nm and an increase in emission intensity of 7% (average values of four determinations using two different preparations) (Figure 9). No significant further change was seen when the $CaCl_2$ concentration was increased to 25 mM. Similar changes in the emission spectrum were observed when 1 mM $CaCl_2$ was added to CRF equilibrated with either 25 mM $MgCl_2$ or 0.1 mM EDTA. The spectral changes seen with $CaCl_2$ were completely reversed with an excess of EDTA.

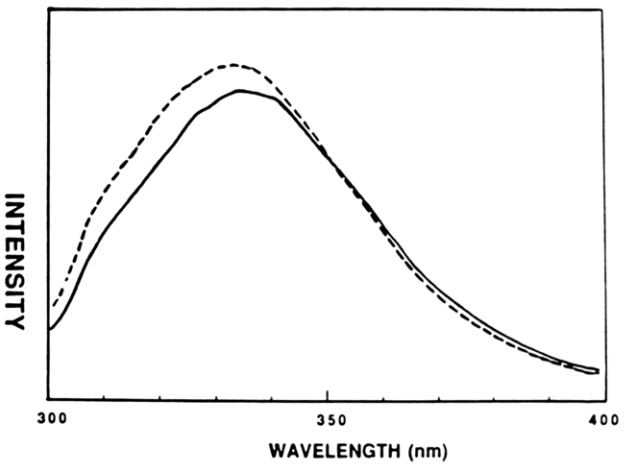

FIGURE 9: Intrinsic fluorescence of CRF at 25 °C in 100 mM NaCl, pH 7.4. The excitation wavelength was 280 nm. Solid line, CRF in 0.1 mM EDTA; dashed line, repeat emission spectrum 10 min after the addition of Ca^{2+} to a final concentration of 1 mM.

Discussion

In the experiments described here, we have shown SP-A binds Ca^{2+} in the presence and absence of phospholipids and in the presence of competing concentrations of other divalent cations. The binding of Ca^{2+} induces self-association of the protein and a conformational change in the fragment of SP-A containing binding sites for carbohydrates and type II cells. The free Ca^{2+} concentration in the fluid in the alveolar space of adult animals is approximately 1.5 mM at a pH of 6.9 (Nielson & Lewis, 1988). Our results suggest that under these conditions the binding sites for Ca^{2+} on SP-A would be fully occupied with two to three Ca^{2+} ions on each subunit of the SP-A oligomer.

SP-A Calcium Binding Parameters. The conditions in which our binding studies were conducted were selected after an investigation of the solution properties of purified delipidated SP-A. Temperature, pH, and ionic strength were found to influence the state of SP-A aggregation in the range of protein concentrations tested (5–100 $\mu g \cdot mL^{-1}$). In low ionic strength buffers at a physiological pH, purified SP-A is soluble in a presumably native octadecameric form (Voss et al., 1988; King et al., 1989; Haagsman et al., 1989). Aggregation of this form of SP-A occurs as the temperature and pH are lowered and the ionic strength is raised. The solvent conditions for our binding studies were a compromise selected to minimize aggregation of SP-A and to avoid nonspecific cation binding. Although our determinations of the binding parameters using SP-A from different sources (native and recombinant) and by different experimental approaches (equilibrium dialysis and gel permeation) in different solvent conditions (30–100 mM NaCl) were similar, we cannot be certain that the affinity and

capacity of SP-A for Ca^{2+} in the alveolar space are the same as the values we estimated. The majority of the binding studies with intact SP-A were performed in the presence of phosphatidylcholine as most SP-A appears to be lipid-associated in vivo. We did not, however, determine the extent of SP-A and lipid association or the morphology of the lipoprotein complexes in this study and therefore cannot exclude the possibility that there may be differences between the Ca^{2+} binding properties of lipid-associated and free SP-A or that lipoprotein aggregation may influence the amount of Ca^{2+} bound.

At present, no other quantitative binding data for SP-A are available for comparison with our results, but the Ca^{2+} binding characteristics of the partially homologous asialoglycoprotein receptor have been investigated (Andersen et al., 1982; Loeb & Drickamer, 1988). Both SP-A and the asialoglycoprotein receptor share partial sequence homology with a number of Ca^{2+}-dependent lectins (Drickamer et al., 1986) and might therefore be expected to share similar Ca^{2+} binding properties. When fully saturated, the asialoglycoprotein receptor binds two to three Ca^{2+} ions per chain with an affinity of 0.35 mM (Andersen et al., 1982). Allowing for differences in the analytical technique, these results are similar to our estimates of the Ca^{2+} binding parameters for SP-A.

The actual residues involved in the binding of Ca^{2+} are unknown, but SP-A has an abundance of carboxyl groups scattered throughout the sequence that could potentially participate in Ca^{2+} binding. The properties of SP-A that are dependent on Ca^{2+} are lost after heating or reducing the protein, suggesting Ca^{2+} binding may be dependent on an intact tertiary structure. We have not, however, determined directly if the binding sites for Ca^{2+} are dependent on correct protein folding. Type II cells, a major site of SP-A synthesis (Williams, 1977; Phelps & Floros, 1988), have γ-carboxylase activity (Rannels et al., 1987), but we were unable to detect any γ-carboxyglutamic acid residues in our samples of human, canine, or recombinant SP-A. This result is consistent with the recent report of Wallin (Wallin et al., 1988).

Implications for the Regulation of Surfactant Structure. SP-A induces a rapid Ca^{2+}-dependent aggregation of phospholipid vesicles prepared from surfactant extracts or simple phospholipid mixtures (King & MacBeth, 1981; Hawgood, 1985a). Our earlier results suggested that the surfactant lipid membrane aggregation induced by SP-A was followed by rearrangement of the vesicular membrane structure into tubular myelin-like forms (Efrati et al., 1987). Recent reconstitution studies have more clearly established the roles of SP-A and Ca^{2+} in the formation of tubular myelin (Suzuki et al., 1989). Our results here suggest the lipoprotein aggregation induced by Ca^{2+}, at least the rapid reversible component, is most likely mediated by Ca^{2+}-induced association of protein molecules on different vesicles. There is an excellent linear correlation ($r = 0.996$) between the extent of vesicle aggregation induced by SP-A at each Ca^{2+} concentration and the extent of aggregation of SP-A in the absence of lipid at the same Ca^{2+} concentration (Figure 10). The order of potency of divalent cations for the induction of both protein and lipoprotein aggregation is also identical (Efrati et al., 1987). The rank order of the divalent cations in the aggregation assay suggests a specificity in the binding site that is similar to other Ca^{2+} binding proteins (Levine & Williams, 1982). Sr^{2+}, an ion with an irregular coordination shell like Ca^{2+}, is at least as potent as Ca^{2+} in inducing both SP-A aggregation and surfactant lipoprotein aggregation and partially competes with Ca^{2+} for divalent cation binding sites in the binding assay. In contrast, Mg^{2+} with a smaller ionic radius and a more regular coordination shell than Ca^{2+} does not induce surfactant lipoprotein aggregation and competes less effectively than Sr^{2+} for cation binding sites.

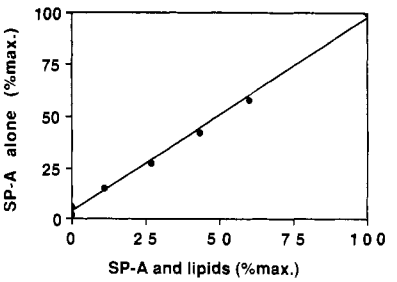

FIGURE 10: Correlation between the Ca^{2+} requirement for SP-A aggregation and surfactant lipoprotein aggregation. The data for reconstituted surfactant lipoprotein aggregation have been taken from our previous study (Efrati et al., 1987). At each Ca^{2+} concentration (range 0.01–5 mM), the extent of protein or lipoprotein aggregation was expressed as a percent of the maximal aggregation induced by Ca^{2+}.

The Ca^{2+} concentration required for half-maximal protein or lipoprotein aggregation was 1 mM. This value is well below the free Ca^{2+} concentration in the alveolar space of the adult animal (Nielson & Lewis, 1988) but is above the value reported for fetal lung liquid (Nielson & Lewis, 1988). Although surfactant and SP-A are secreted into fetal lung liquid, relatively less of the secreted lamellar body content appears to be transformed into tubular myelin in the fetus compared to the adult (Williams, 1977). This observation is consistent with the hypothesis that the free Ca^{2+} concentration in the alveolar space regulates surfactant structure and possibly functions by determining the extent of SP-A self-association.

The collagen-like region in SP-A is important in the assembly of SP-A subunits into the oligomeric form (Haagsman et al., 1989). This domain may also be involved in the Ca^{2+}-induced association of SP-A oligomers and therefore the aggregation of phospholipid membranes. The denaturation temperature for half-maximal activity of SP-A in the lipoprotein aggregation assay is 48 °C (Figure 3). This value is very close to the melting temperature of the collagen-like triple helix in SP-A (Voss et al., 1988; King et al., 1989; Haagsman et al., 1989), suggesting a structurally intact collagen-like domain may be required for spefific protein self-association. It is also possible that changes in the structure of other regions in SP-A contribute to the observed temperature dependence of lipoprotein aggregation.

Localization of a Calcium Binding Site to the Noncollagenous Domain of SP-A. The specific locations of the two proposed Ca^{2+} binding sites in the SP-A molecule are not known, but our results indicate at least one of the sites is located in the COOH-terminal fragment of SP-A. This fragment contains the ligand binding domains for carbohydrates (Drickamer et al., 1986) and putatively for type II cells (Wright et al., 1989). Because the tendency of intact SP-A to aggregate in Ca^{2+} may potentially complicate the interpretation of studies of the conformational change induced by binding Ca^{2+}, we have concentrated our attention on the COOH-terminal fragment of SP-A. The results of the binding studies suggest this fragment, CRF, has a lower total capacity for Ca^{2+} than the intact oligomeric protein within the range of free Ca^{2+} concentrations tested (0.01–7 mM). The estimated affinity of CRF for Ca^{2+} is slightly less than that of the high-affinity site on the intact protein. The homologous carbohydrate recognition domain of the asialoglycoprotein receptor also has a lower apparent affinity for Ca^{2+} than the

intact receptor (Loeb & Drickamer, 1988). It is possible that the structure of CRF is altered or becomes less stable when the collagen-like stem of SP-A is removed.

Our previous circular dichroism measurements of CRF in EDTA and Ca^{2+} did not suggest a major change in the secondary structure of CRF in response to binding Ca^{2+} (Haagsman et al., 1989). The results of the intrinsic fluorescence measurements and studies with proteases reported here, however, suggest a change in conformation of the COOH-terminal end of SP-A when Ca^{2+} is bound. Similar findings have recently been reported for the homologous carbohydrate recognition domain of the asialoglycoprotein receptor (Loeb & Drickamer, 1988). The Ca^{2+} concentration required for half-maximal resistance of CRF to digestion by trypsin was approximately 60 μM. In contrast, the Ca^{2+} concentration required for half-maximal protein or lipoprotein aggregation was 1 mM.

On the basis of our interpretation of the results of this investigation, we propose a model in which a single high-affinity binding site for Ca^{2+} is located in the COOH-terminal, ligand binding end of SP-A. Occupancy of this site induces a significant conformational change in this region of the protein without inducing protein self-association. Additional lower affinity sites, presumably occupied in the range of free Ca^{2+} concentrations found in the alveolar space, either are located in the collagen-like domain or are dependent on the presence of this domain. Occupancy of both the high- and the low-affinity site appears to be required for protein self-association, a step we propose to be of importance in the transformation of secreted lamellar body contents into tubular myelin. Confirmation of this model will require more direct information on the nature and location of the Ca^{2+} binding sites.

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