Self-Aggregation of Surfactant Protein A†

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ABSTRACT: Environmental factors of physiological relevance such as pH, calcium, ionic strength, and temperature can affect the state of self-aggregation of surfactant protein A (SP-A). We have studied the secondary structure of different SP-A aggregates and analyzed their fluorescence characteristics. (a) We found that self-aggregation of SP-A can be Ca^{2+}-dependent. The concentration of Ca^{2+} needed for half-maximal self-association ($K_a^{Ca^{2+}}$) depended on the presence of salts. Thus, at low ionic strength, $K_a^{Ca^{2+}}$ was 2.3 mM, whereas at physiological ionic strength, $K_a^{Ca^{2+}}$ was 2.35 μM. Circular dichroism and fluorescence measurements of Ca^{2+}-dependent SP-A aggregates indicated that those protein aggregates formed in the absence of NaCl are structurally different from those formed in its presence. (b) We found that self-aggregation of SP-A can be pH-dependent. Self-aggregation of SP-A induced by H^+ was highly influenced by the presence of salts, which reduced the extent of self-association of the protein. The presence of both salts and Ca^{2+} attenuated even more the effects of acidic media on SP-A self-aggregation. (c) We found that self-aggregation of SP-A can be temperature-dependent. At 20 °C, SP-A underwent self-aggregation at physiological but not at low ionic strength, in the presence of EDTA. All of these aggregates were dissociated by either adding EDTA (a), increasing the pH to neutral pH (b), or increasing the temperature to 37 °C (c). Dissociation of Ca^{2+}-induced protein aggregates at low ionic strength was accompanied by an irreversible loss of both SP-A secondary structure and SP-A-dependent lipid aggregation properties. On the other hand, temperature-dependent experiments indicated that a structurally intact collagen-like domain was required for either Ca^{2+}- or Ca^{2+}/Na^+-induced SP-A self-aggregation but not for H^+-induced protein aggregation.

Of the four known surfactant proteins, SP-A[1] is the most abundant. It is a multifunctional protein capable of binding several ligands, including phospholipids, carbohydrates, and Ca^{2+}, and it belongs to the Ca^{2+}-dependent lectin family (for reviews, see refs *1* and *2*). SP-A is a large oligomeric protein of approximately 650 kDa, composed of 18 nearly identical subunits. Each SP-A subunit contains an amino-terminal collagen-like domain and a carboxyl-terminal carbohydrate recognition domain (CRD) that are linked by a more hydrophobic domain (neck). This monomeric form is about 228 amino acid long with an approximate molecular weight of 28 000. Several post-translational modifications, including glycosylation, sulfation, and hydroxylation of prolines, result in a monomeric protein with a heterogeneous molecular mass ranging from 30 to 40 kDa. The functional form of SP-A is assembled through interactions in the collagen-like domain into a complex octadecameric structure, which resembles a floral bouquet, where the amino-terminal and part of collagen-like regions form a common stalk.

Nearly all alveolar SP-A is complexed with phospholipids. The CRD and the neck region are important protein domains involved in SP-A−lipid interactions (*3*, *4*). SP-A preferentially interacts with dipalmitoylphosphatidylcholine (DPPC) (*5*−*7*), the main surfactant lipid (*1*). The interaction of DPPC with SP-A leads to a conformational change on the protein molecule (*6*) and a marked protection of SP-A from trypsin degradation (*8*). The interaction of SP-A with phospholipid vesicles induces vesicle aggregation in the presence of Ca^{2+} or H^+ (*6*, *8*−*10*). SP-A also induces aggregation of liposomes containing SP-B or SP-C or both hydrophobic proteins (*9*). The association of SP-A with lipids appears to be essential for the conversion of lipid aggregates from multilamellar forms present in the exocytic granule to dispersed ordered arrays known as tubular myelin (*11*). This physical transformation also requires the hydrophobic surfactant protein SP-B and Ca^{2+} (*12*). SP-A enhances adsorption of lipids along the air−liquid interface in a concerted action with SP-B (*13*). In addition, SP-A as well as SP-D, another water-soluble surfactant protein, participates in lung-specific host innate defense (*14*, *15*).

The solubility of lipid-free SP-A in stock solutions changes significantly with the buffer conditions. SP-A is insoluble in physiological saline at room temperature, but solubilizes readily in low-ionic strength buffers (*16*). Others and we

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[1] Abbreviations: CD, circular dichroism; CRD, carbohydrate recognition domain; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol; K_{sv}, Stern−Volmer dynamic quenching constant; $K_a^{Ca^{2+}}$, calcium activation constant; SDS−PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; SP-A−D, surfactant proteins A−D, respectively.

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previously found that Ca^{2+} induced a rapid self-aggregation of pig, dog, or human SP-A at neutral pH and in low-ionic strength buffers (*8*, *16*). The threshold concentration of Ca^{2+} required to induce self-aggregation of SP-A from different species was 0.5 mM (*8*, *16*). However, the presence of trace amounts of Ca^{2+} was enough to induce SP-A self-aggregation in physiological-ionic strength buffers at 37 °C (*17*). In addition, we recently found that SP-A could undergo pH-dependent self-aggregation in the absence of Ca^{2+} and salts in the pH range of 4.5−6.0 (*9*). The state of self-aggregation of SP-A may have important effects on its functions. Soluble SP-A did not stimulate macrophage production of reactive oxygen but did stimulate when SP-A was adhered to a surface, resulting in a multivalent presentation (*18*, *19*).

To better characterize the process of self-aggregation of SP-A, we have investigated the effect of ionic strength, Ca^{2+}, pH, and temperature on this phenomenon. The structural characteristics of different protein aggregates were studied by circular dichroism and fluorescence spectroscopy.

EXPERIMENTAL PROCEDURES

Isolation of SP-A. Pulmonary surfactant was prepared from pig or human bronchoalveolar lavage as described previously (*20*). SP-A was purified from isolated surfactant using sequential butanol and octyl glucoside extractions (*13*). The purity of SP-A was checked by one-dimensional SDS−PAGE in 16% acrylamide under reducing conditions (50 mM dithiothreitol). Quantification of SP-A was carried out by amino acid analysis in a Beckman System 6300 High Performance analyzer. The protein hydrolysis was performed with 0.2 mL of 6 M HCl, containing 0.1% (w/v) phenol, in evacuated and sealed tubes at 108 °C for 24 h. Norleucine was added to each sample as the internal standard. Experiments presented in this paper were performed with at least four different preparations of porcine SP-A. Some experiments were repeated with human SP-A from multiple-organ donor lungs.

SP-A Self-Aggregation Assays. Self-aggregation assays were performed at 37 °C (unless otherwise stated) by measuring the change in protein absorbance at 360 nm in a Beckman DU-640 spectrophotometer, induced by the presence of calcium ions, H^+, or low temperature. Ca^{2+}-dependent self-aggregation experiments were carried out as described previously (*8*). The calcium requirement for self-aggregation was studied by titration experiments in which an increasing amount of a concentrated solution of $CaCl_2$ was added to the protein solution (40 μg/mL, final concentration) in 50 μM EDTA, 5 mM Tris-HCl buffer (pH 7.4) either with or without 150 mM NaCl. The absorbance at 360 nm was registered once it was stabilized after each change of Ca^{2+} concentration. The free Ca^{2+} concentration in each point of the titration experiments was estimated by a computer program (CHELATOR) (*21*), which also permits correction for ionic strength, temperature, pH, and other competing ions. pH-dependent self-aggregation experiments were carried out as previously described (*9*) The variation of SP-A self-aggregation as the pH was reduced was studied at 37 °C in the presence or absence of 5 mM Ca^{2+} and either with or without salts (150 mM NaCl or 150 mM KCl). pH titration was started at neutral pH in 5 mM Tris, 5 mM MES, 5 mM acetate buffer (pH 7.2) in the presence or the absence of $CaCl_2$ and/or salts. From the starting pH, SP-A was titrated to pH 4.2 by addition of 1−2 μL aliquots of 0.05 or 0.1 M HCl. During titration, the medium pH was monitored with a micro-pH electrode. The absorbance at 360 nm at each pH was registered once it was stabilized after each change of pH. To find out if SP-A was able to undergo self-aggregation at neutral pH by lowering the temperature, the sample and reference cuvette were filled with 0.1 mM EDTA, 5 mM Tris-HCl buffer (pH 7.4) either with or without 150 mM NaCl. After equilibration for 10 min at 20 °C, SP-A (30 μg/mL, final concentration) was added to the sample cuvette and the absorbance was monitored at 1 min intervals over 10 min. Next, the temperature was raised to 37 °C, and the absorbance was recorded again. In another set of experiments, self-aggregation of SP-A was studied as a function of temperature. The temperature was lowered from 40 to 15 °C or raised in reverse order. The absorbance at 360 nm was registered once it was stabilized after each temperature change.

Phospholipid Vesicle Aggregation Assay. DPPC vesicles were prepared in a buffer containing 150 mM NaCl and 5 mM Tris-HCl (pH 7.4) by sonication as described previously (*8*, *9*). The DPPC vesicle diameter at 37 °C, determined by quasielastic light scattering, was around 130 nm.

The vesicle aggregation assay was carried out as a continuation of Ca^{2+}-dependent self-aggregation experiments after dissociation of protein aggregates by addition of EDTA. Then, 1,2-dipalmitoylphosphatidylcholine (DPPC) vesicles (200 μg/mL) (lipid:SP-A weight ratio of 10:1) were added to both the sample and the reference cuvette, and the turbidity change at 360 nm was monitored at 1 min intervals over 10 min. Afterward, Ca^{2+} (1 mM, final concentration) was added to both cuvettes, and the change in absorbance was monitored again. EDTA was used again for reversing vesicle aggregation.

CD Measurements. CD spectra were obtained on a Jasco J-715 spectropolarimeter fitted with a 150 W xenon lamp (*9*). Quartz cells with a 1 mm path length were used, and the spectra were recorded in the far-UV region (190−260 nm) with a scanning speed of 50 nm/min and at the indicated temperature. Four scans were accumulated and averaged for each spectrum. The acquired spectra were corrected by subtracting the appropriate blanks, subjected to noise-reduction analysis, and presented as molar ellipticities (degrees cm^2 $dmol^{-1}$) assuming 110 Da as the average molecular mass per amino acid residue. At least three independent preparations of SP-A were measured. The final SP-A concentration was 80 μg/mL. Measurements at acidic or neutral pH were carried out in 5 mM acetate buffer (pH 4.5) or in 5 mM Tris-HCl buffer (pH 7.2), respectively, with or without either NaCl or Ca^{2+}.

Fluorescence Measurements. All fluorescence experiments were carried out on a Perkin-Elmer MPF-44E spectrofluorimeter operated in the ratio mode as described in ref *6*. Cells with a 0.2 cm optical path length were used. The slit widths were 7 and 5 nm for the excitation and emission beams, respectively. Fluorescence spectra of SP-A were measured at 20 or 37 °C in 0.3 mL of 5 mM Tris-HCl buffer (pH 7.4) in the absence or presence of either 5 mM Ca^{2+} or 5 μM Ca^{2+}/150 mM NaCl. The final protein concentration of SP-A was in the range of 6.7−10 μg/mL. The blanks and protein samples were excited at 275 nm for measuring the total

protein fluorescence spectrum or at 295 nm to preferentially excite tryptophan residues. Emission spectra were recorded from 300 to 400 nm.

The change in fluorescence emission intensity of SP-A upon addition of millimolar concentrations of Ca^{2+} was determined as follows. First, the fluorescence spectrum of SP-A (2 μg) at an excitation wavelength of 275 nm was recorded in 0.3 mL of 5 mM Tris-HCl buffer (pH 7.4) and 5 μM Ca^{2+}, either with or without 150 mM NaCl. Subsequently, the titration experiment was started by adding increasing amounts of a concentrated solution of $CaCl_2$ to the protein solution in the cuvette. The fluorescence intensity readings were corrected for the dilution caused by aliquot addition. The change in fluorescence emission intensity of SP-A on addition of NaCl was analyzed as described above in 5 mM Tris-HCl (pH 7.4) and 5 μM Ca^{2+} either with or without 0.1 mM EDTA.

Quenching experiments with acrylamide were performed at an excitation wavelength of 295 nm to preferentially excite tryptophan residues and to reduce the absorbance by acrylamide. The quenching experiments were carried out as follows. SP-A (7 μg/mL) was added to the cell containing 5 mM Tris-HCl buffer (pH 7.4) with or without either 2 mM Ca^{2+} or 5 μM Ca^{2+}/150 mM NaCl. After equilibration for 10 min at 20 °C, the spectrum of SP-A was recorded. Afterward, aliquots from a stock solution of acrylamide in water were added to the protein solution, and fluorescence spectra were recorded. The values of fluorescence intensity at 330 nm were corrected for dilution and the scatter contribution derived from acrylamide titration of a blank. In addition, an inner filter correction for the absorbance by acrylamide was applied. This correction factor was determined according to the equation $F_c = F_m \times 10^{(A_{ex}+A_{em})/2}$ (*22*), in which F_c is the corrected fluorescence intensity, F_m is the measured fluorescence intensity after correction for scattering, and A_{ex} and A_{em} are the absorbances measured at the excitation wavelength (295 nm) and at the emission wavelength (330 nm), respectively. The absorbance of the samples was measured after each addition of acrylamide by using a Beckman DU-640 spectrophotometer.

Quenching studies were analyzed according to the classical Stern−Volmer equation for collisional quenching (*23*), $F_o/F = 1 + K_{sv}[Q]$, where F_o and F are the corrected emission intensities in the absence and presence of the quencher Q, respectiveky, and K_{sv} is the Stern−Volmer dynamic quenching constant. K_{sv} values were calculated by regression of the initial linear portion of the Stern−Volmer plot.

RESULTS

Effect of Ionic Strength on Ca^{2+}-Dependent Self-Aggregation of SP-A. Figure 1A shows that addition of SP-A (50 μg/mL) to 5 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl and micromolar concentrations of Ca^{2+} (5 μM) resulted in a marked increase in light absorbance due to self-aggregation of the protein. Light absorbance did not change after a further addition of Ca^{2+} (5 mM, final concentration). The addition of EDTA (10 mM) reversed completely the process. Furthermore, the presence of EDTA (1 mM) in the buffer prevented the self-aggregation of SP-A occurring at physiological ionic strength and micromolar concentrations of Ca^{2+} (data not shown). Figure 1A also shows that in the absence of NaCl, SP-A did not aggregate unless Ca^{2+} was added at millimolar concentrations. The extent of Ca^{2+}-dependent self-aggregation reached at low ionic strength is higher than that reached at physiological ionic strength. Panels B and C of Figure 1 show the calcium dependence of the self-aggregation process of pig SP-A in the absence (B) and presence (C) of NaCl. The Ca^{2+} activation constant ($K_a^{Ca^{2+}}$) for self-aggregation of pig SP-A depended on the presence of salts. At low ionic strength, the concentration of Ca^{2+} required for half-maximal self-association was 2.3 ± 0.2 mM as previously reported (*8*). In contrast, at physiological ionic strength, $K_a^{Ca^{2+}}$ was 2.4 ± 0.5 μM. Thus, the presence of salts drastically decreased the concentration of Ca^{2+} required to set out this process. To find out whether the effect of ionic strength on Ca^{2+}-dependent self-aggregation of pig SP-A occurred also in other SP-A species, we repeated the experiments with human SP-A isolated from multiple-organ donor lungs. The Ca^{2+} concentration for half-

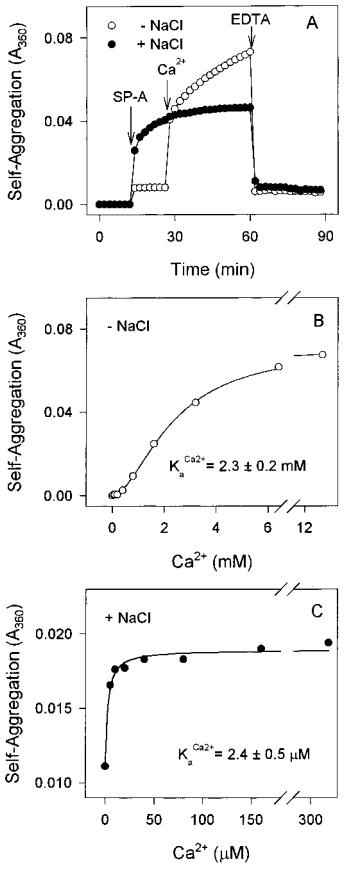

FIGURE 1: Ca^{2+}-dependent self-aggregation of porcine SP-A in the presence and absence of NaCl. (A) Kinetics of Ca^{2+}-dependent self-aggregation of SP-A. SP-A (50 μg/mL) was added to the sample cuvette filled with 5 μM Ca^{2+}, 5 mM Tris-HCl buffer (pH 7.4) either with (●) or without (○) 150 mM NaCl. The turbidity change at 360 nm was monitored at 37 °C at 1 min intervals. After stabilization, 5 mM Ca^{2+} (final concentration) was added to both the sample and reference cuvette and the turbidity changes were monitored again. Addition of EDTA (10 mM, final concentration) completely dissociated SP-A aggregates induced by either Ca^{2+} or Ca^{2+} and NaCl. (B) Calcium dependence of SP-A self-aggregation in the absence of NaCl. (C) Calcium dependence of SP-A self-aggregation in the presence of 150 mM NaCl. In panels B and C, the final concentration of SP-A was 40 μg/mL. Experiments were carried out at 37 °C. The results of a representative experiment of four experiments from four different preparations of porcine SP-A are shown.

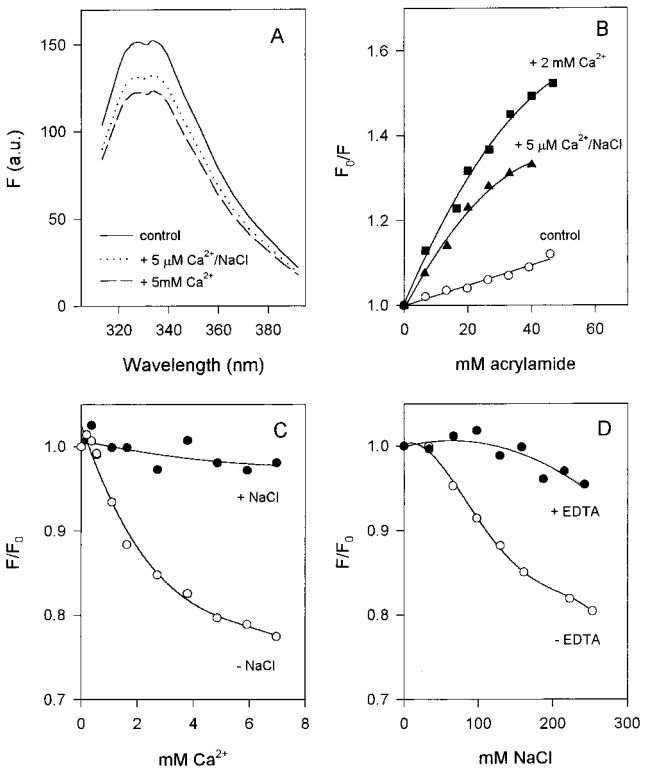

FIGURE 2: Fluorescence measurements of nonaggregated and aggregated forms of SP-A induced by either Ca^{2+} or Ca^{2+} and NaCl. (A) Fluorescence emission spectra of SP-A on excitation at 275 nm in the absence or the presence of either 5 mM Ca^{2+} or 5 μM Ca^{2+} and 150 mM NaCl. Fluorescence (*F*) is expressed in arbitrary units. (B) Stern−Volmer plots of aqueous quenching by acrylamide of SP-A. F_o and *F* are the corrected emission intensities at 330 nm in the absence and presence of acrylamide, respectively. Experiments were carried out in 5 mM Tris-HCl buffer (pH 7.4) (control, ○) with or without either 2 mM Ca^{2+} (■) or 5 μM Ca^{2+} and 150 mM NaCl (▲). (C) Changes in fluorescence emission intensity of SP-A on addition of $CaCl_2$. *F* and F_o are the corrected fluorescence emission intensities at 330 nm in the presence and absence of $CaCl_2$, respectively. SP-A (6.7 μg/mL) was initially in 5 μM Ca^{2+}, 5 mM Tris-HCl buffer (pH 7.4) with (●) or without (○) 150 mM NaCl. (D) Changes in fluorescence emission intensity of SP-A on addition of NaCl. *F* and F_o are the corrected fluorescence emission intensities at 330 nm in the presence and absence of NaCl, respectively. SP-A (6.7 μg/mL) was initially in 5 μM Ca^{2+}, 5 mM Tris-HCl buffer (pH 7.4) with (●) or without (○) 0.1 mM EDTA. The results of a representative experiment of four are shown.

maximal self-aggregation of human SP-A was 3 ± 0.1 mM at low ionic strength and 12 ± 1.8 μM at physiological ionic strength.

Figure 2A shows fluorescence emission spectra of porcine SP-A in its nonaggregated form and in its aggregated form induced by either 5 mM Ca^{2+} at low ionic strength or 5 μM Ca^{2+} and 150 mM NaCl. The fluorescence emission spectrum of SP-A on excitation at 275 is characterized by two maxima at about 326 and 337 nm as previously reported for porcine and human SP-A (*6*). The two maxima are blue-shifted in comparison to that of free tryptophan model systems, revealing a partially buried character for these fluorophores. Self-aggregation of the protein induced by either 5 μM Ca^{2+} and 150 mM NaCl or 5 mM Ca^{2+} and 0 mM NaCl led to a decrease in fluorescence emission intensity without any shift in the wavelength of the emission maxima. The observed decrease in fluorescence intensity can be explained in terms of changes in the tryptophan environment, which probably implies a higher level of quenching of the tryptophan fluorescence by polarizable groups in the proximity of these residues. The decrease in fluorescence emission intensity caused by 5 mM Ca^{2+} and 0 mM NaCl was greater than that caused by 5 μM Ca^{2+} and 150 mM NaCl, indicating a more drastic change in the tryptophan environment when the protein is self-aggregated at millimolar concentrations of Ca^{2+} in the absence of salts.

An additional criterion for revealing structural changes in the protein after self-aggregation is the analysis of the accessibility of SP-A fluorophores to acrylamide, an efficient neutral collisional quencher of indole derivates, capable of permeating the protein matrix (*24*). Figure 2B shows Stern−Volmer plots of the effect of acrylamide on fluorescence emission intensity of porcine SP-A, measured in the presence and absence of either 5 μM Ca^{2+} and 150 mM NaCl or 2 mM Ca^{2+} and 0 mM NaCl. Self-aggregation of SP-A markedly increased the accessibility of SP-A fluorophores to acrylamide. SP-A in its nonaggregated form exhibited an acrylamide quenching constant (K_{sv} = 2.3 M^{-1}) that was lower than in its self-aggregated form induced by either 5 μM Ca^{2+} and 150 mM NaCl (K_{sv} = 10.5 M^{-1}) or 2 mM Ca^{2+} and 0 mM NaCl (K_{sv} = 16.2 M^{-1}). From these results, it is clear that the quenching of tryptophan residues by acrylamide was more pronounced when the protein underwent Ca^{2+}-dependent self-aggregation in the absence than in the presence of salts.

Figure 2C shows the fluorescence emission intensity of SP-A at 330 nm as a function of Ca^{2+} concentration. At low ionic strength, the fluorescence emission intensity of SP-A started to decrease at concentrations of Ca^{2+} higher than 0.5 mM. The change in the fluorescence emission intensity of SP-A as a function of Ca^{2+} might reflect the conformational change of SP-A at different levels of self-aggregation. Figure 2C also shows that at physiological ionic strength and micromolar concentrations of Ca^{2+} the fluorescence of SP-A did not change as the Ca^{2+} concentration increased in the millimolar range. These results support the concept that once SP-A was self-aggregated by micromolar concentrations of Ca^{2+} in the presence of salts (Figure 1A,C), the addition of further amounts of Ca^{2+} does not induce changes in both the self-aggregation state and fluorescence emission intensity of the protein.

Figure 2D shows the fluorescence emission intensity of SP-A at 330 nm as a function of NaCl concentration in the presence of micromolar concentrations of calcium. SP-A fluorescence progressively decreased as the NaCl concentration increased, reflecting a conformational change of SP-A at different levels of self-aggregation induced by micromolar concentrations of both calcium and NaCl. When the Ca^{2+} present in the buffer solution (5 μM) was chelated by addition of EDTA, the fluorescence emission intensity of SP-A was affected little by increasing ionic strength.

Figure 3 shows the change in the circular dichroism spectrum of SP-A as a function of Ca^{2+} concentration in the absence of salts (Figure 3A) and as a function of NaCl concentration in the presence of 20 μM Ca^{2+} (Figure 3B). Addition of Ca^{2+} at concentrations higher than 0.5 mM to the protein solution led to a marked decrease of the negative ellipticity and a shift of the minimum from 207 to 210 nm (Figure 3A). CD spectra of SP-A at Ca^{2+} concentrations

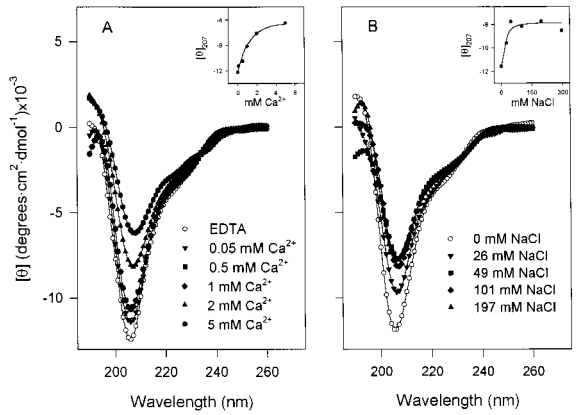

FIGURE 3: Changes in the circular dichroism spectrum of porcine SP-A as a function of Ca^{2+} (A) or NaCl concentration (B). Experiments with Ca^{2+} or NaCl titration were performed at 37 °C. (A) SP-A (80 μg/mL) was in 5 mM Tris-HCl buffer (pH 7.4) in the absence of NaCl. (B) SP-A (80 μg/mL) was in 20 μM Ca^{2+}, 5 mM Tris-HCl buffer (pH 7.4). The insets show ellipticity at 207 nm of SP-A as a function of Ca^{2+} (A) or NaCl (B) concentration. The results of a representative experiment of three are shown.

higher than 0.5 mM must reflect the secondary structure of SP-A at different levels of self-aggregation. At concentrations of Ca^{2+} lower than 0.5 mM, there was a small but significant decrease of the contribution of the 207 nm minimum to the spectrum without any shift of this minimum. At these Ca^{2+} concentrations, the protein was not self-aggregated. The molar ellipticity at 207 nm (θ_{207}) as a function of the Ca^{2+} concentration is shown in the inset of Figure 3A. The Ca^{2+}-dependent decrease of θ_{207} reached a maximum at around 5 mM. On the other hand, Figure 3B shows a decrease of the negative ellipticity as the concentration of NaCl increased in the presence of 20 μM Ca^{2+}. However, that decrease in the CD signal of SP-A was much less pronounced than that induced by millimolar concentrations of Ca^{2+} in the absence of salts (Figure 3A). The molar ellipticity at 207 nm (θ_{207}) as a function of the Na^+ concentration is shown in the inset of Figure 3B.

Figure 4A shows that those changes in the secondary structure of porcine SP-A produced by 5 mM Ca^{2+} and 0 mM NaCl were not reversed when aggregates of protein were dissociated by addition of EDTA. Removing Ca^{2+} produced additional important changes in the CD spectrum of SP-A. Figure 4C shows that the addition of DPPC vesicles to this nonaggregated form of porcine SP-A after chelating Ca^{2+} with EDTA (Figure 4B) resulted in a marked increase in light absorbance due to lipid aggregation. That vesicle aggregation induced by this form of SP-A at 37 °C was completely independent of Ca^{2+} and irreversible. Thus, removing Ca^{2+} also affected the SP-A property to aggregate lipid vesicles in a Ca^{2+}-dependent manner.

In contrast, Figure 5 shows that addition of EDTA to porcine SP-A aggregates formed in the presence of 50 μM Ca^{2+} and 150 mM NaCl caused dissociation of protein aggregates (Figure 5B) with little change in the CD spectrum of SP-A (Figure 5A). This nonaggregated form of SP-A obtained by dissociation of SP-A aggregates with EDTA was able to mediate the process of Ca^{2+}-dependent vesicle aggregation, which could be reversed by addition of EDTA (Figure 5C). These results indicated that aggregation of SP-A induced by Ca^{2+} and NaCl was reversible and that after dissociation of protein aggregates, SP-A retained its ability to induce phospholipid vesicle aggregation in the presence of Ca^{2+} and NaCl.

Effect of the Ionic Strength on pH-Dependent Self-Aggregation of SP-A. Figure 6A shows the effect of lowering the pH on self-aggregation of the protein in the presence or absence of 150 mM NaCl. At pH <6.0, the level of protein aggregation was lower in the presence than in the absence of salts. CD spectra of SP-A at neutral and acidic pH, in the presence of EDTA, and either with or without NaCl are shown in Figure 6B. At neutral pH and at room temperature, the presence of NaCl in the medium slightly reduced the negative ellipticity. The change of pH from 7.4 to 4.5 led to a drastic change in the CD spectra of SP-A characterized by a marked decrease of the negative ellipticity and a shift of

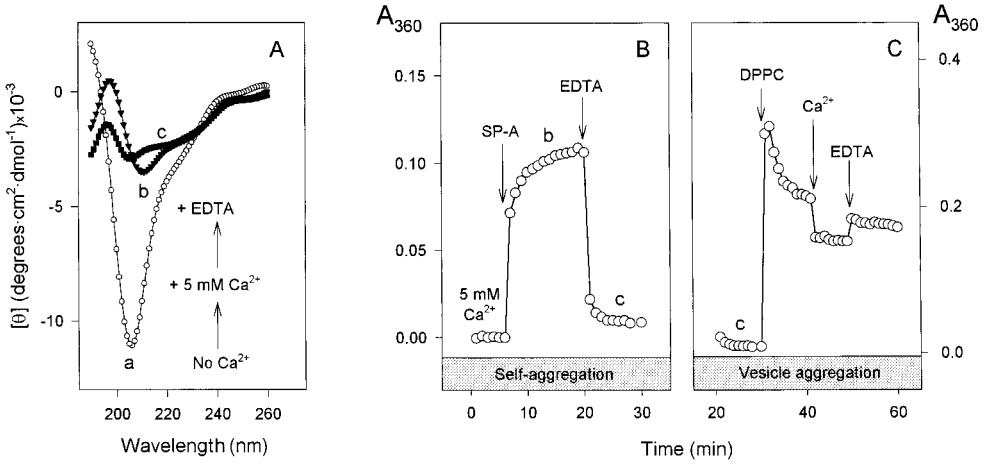

FIGURE 4: Changes in the secondary structure of SP-A and in its lipid vesicle aggregation activity after dissociation of Ca^{2+}-induced protein aggregates by addition of EDTA. (A) Circular dichroism spectra of porcine SP-A (80 μg/mL) at 37 °C under different conditions: (a) 5 mM Tris-HCl buffer (pH 7.4) containing 0.1 mM EDTA (○), (b) after addition of $CaCl_2$ (5 mM free Ca^{2+}) (▼), and (c) after addition of EDTA (10 mM, final concentration) (■). (B) Self-aggregation (absorbance change at 360 nm) of porcine SP-A at 37 °C. The protein (40 μg/mL, final concentration) was added to the sample cuvette containing 5 mM Ca^{2+}, 5 mM Tris-HCl buffer (pH 7.4) (b). After stabilization, self-aggregation was reverted with EDTA (10 mM) (c). (C) The SP-A-induced vesicle aggregation assay was performed as a continuation of the SP-A self-aggregation experiment described for panel B. Thus, SP-A was in a nonaggregated form after dissociation of protein aggregates by addition of EDTA (c). Vesicle aggregation was performed as described in Experimental Procedures. The results of a representative experiment of four are shown.

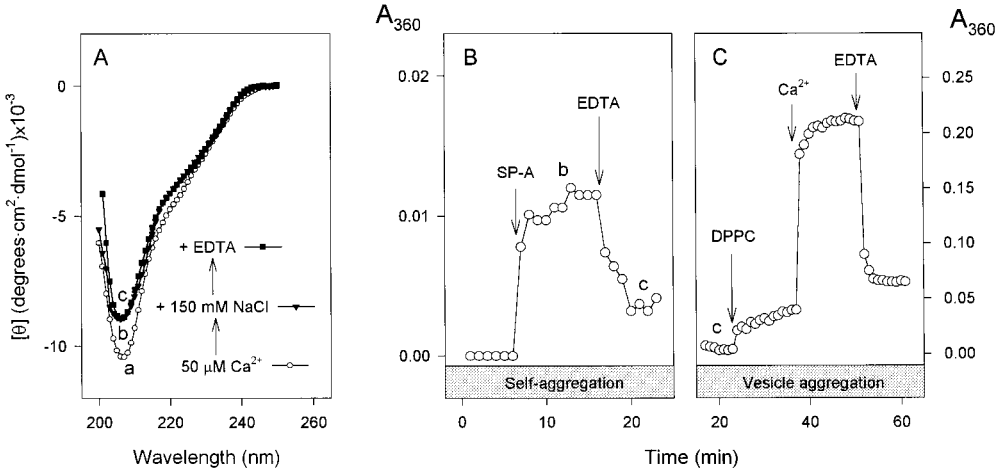

FIGURE 5: Effect of dissociation of Ca^{2+}/NaCl-induced SP-A aggregates by addition of EDTA on the secondary structure of the protein and on its lipid vesicle aggregation activity. (A) Circular dichroism spectra of porcine SP-A (80 μg/mL) at 37 °C under different conditions: (a) 50 μM Ca^{2+}, 5 mM Tris-HCl buffer (pH 7.4) (○), (b) after addition of NaCl (150 mM, final concentration) (▼), and (c) after addition of EDTA (■). (B) Self-aggregation (absorbance change at 360 nm) of porcine SP-A at 37 °C. The protein (40 μg/mL, final concentration) was added to the sample cuvette containing 50 μM Ca^{2+}, 150 mM NaCl, 5 mM Tris-HCl buffer (pH 7.4) (b). Once the absorbance was stabilized, self-aggregation was reverted with EDTA (0.5 mM) (c). (C) The SP-A-induced vesicle aggregation assay was performed as a continuation of the SP-A self-aggregation experiment described for panel B. Vesicle aggregation was performed as described in Experimental Procedures. The results of a representative experiment of four are shown.

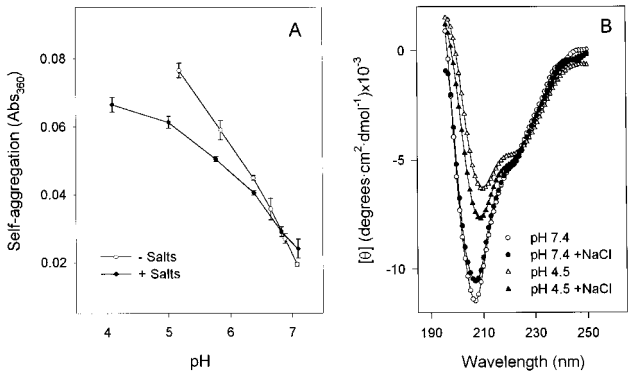

FIGURE 6: Effect of NaCl on SP-A self-aggregation (A) and SP-A secondary structure (B) at acidic and neutral pH in the absence of Ca^{2+}. (A) pH titration started at pH 7.4 in 5 mM Tris-HCl, 5 mM MES, 5 mM acetate, and 0.1 mM EDTA buffer either with (●) or without (○) 150 mM NaCl. From the starting pH, SP-A (30 μg/mL) was titrated by addition of 1−2 μL aliquots of a 0.05 or 0.1 M HCl solution. During titration, the medium pH was monitored with a micro-pH electrode. The absorbance at 360 nm was registered at 37 °C once it was stabilized after each change of pH. Values are expressed as means ± the standard deviation of four experiments. (B) Far-UV circular dichroism spectra of SP-A (80 μg/mL) in 5 mM acetate, 5 mM Tris-HCl buffer at neutral (circles) or acidic (triangles) pH, either with (black symbols) or without (white symbols) 150 mM NaCl. The results of a representative experiment of four are shown.

the minimum from 207 to 209 nm as previously reported (*9*). In the presence of NaCl, the change of pH from 7.4 to 4.5 led to a less pronounced decrease of the negative ellipticity. This is consistent with the lower extent of pH-dependent self-aggregation of SP-A in the presence of salts.

Figure 7A shows that at low ionic strength there was a marked aggregation of the protein in the pH range of 6−6.5 provided Ca^{2+} was present in millimolar concentrations, in agreement with previous results (*9*). The extent of protein aggregation was greater as the pH was reduced. Thus, there was a synergy between H^+ and Ca^{2+} on self-aggregation of SP-A in the pH range of 5−6.5. However, at physiological ionic strength, there was an antagonism between H^+ and Ca^{2+} below pH 6.5 (Figure 7B). The extent of pH-dependent self-aggregation of SP-A was lower in the presence than in the absence of Ca^{2+}, provided physiological concentrations of either KCl or NaCl were present in the medium.

Effect of Temperature on Self-Aggregation of SP-A. Figure 8A shows that SP-A underwent self-aggregation at 20 °C at neutral pH in the presence of EDTA. This aggregation process required the presence of NaCl and was reversed by increasing the temperature to 37 °C. We found that the extent of self-aggregation of SP-A progressively increased as the temperature was decreasing from 40 to 20 °C, provided NaCl was present in the medium (data not shown). The extent of this type of aggregation is much lower than that induced by Ca^{2+} or H^+. Figure 8B shows the effect of decreasing temperature on CD spectra of SP-A. In the presence but not in the absence of NaCl, there was a small but progressive decrease of the negative ellipticity without any shift of the minimum at 207 nm.

Figure 9A shows that heat treatment of porcine SP-A resulted in a pronounced decrease in the magnitude of the negative band at 207 nm without any shift of this band as previously reported for canine and human SP-A (*25*, *26*). The molar ellipticity at 207 nm (θ_{207}) as a function of temperature is shown in the inset of Figure 9A. On the other hand, we have calculated the molar fraction of native and denatured collagen components of CD spectra of porcine SP-A as a function of temperature (inset of Figure 9A) using an algorithm developed by Perczel et al. (*27*), called convex constraint analysis (CCA). Deconvolution of SP-A CD spectra obtained at different temperatures by CCA reveals that the change in ellipticity on going from 37 to 60 °C can be fitted to a structural transition between two components, which closely follows the denaturation profile of collagen. This suggests that there is no additional contribution of changes affecting other structural components in that range of temperatures. The inset of Figure 9A indicates that the midpoint transition "melting" temperature of porcine SP-A

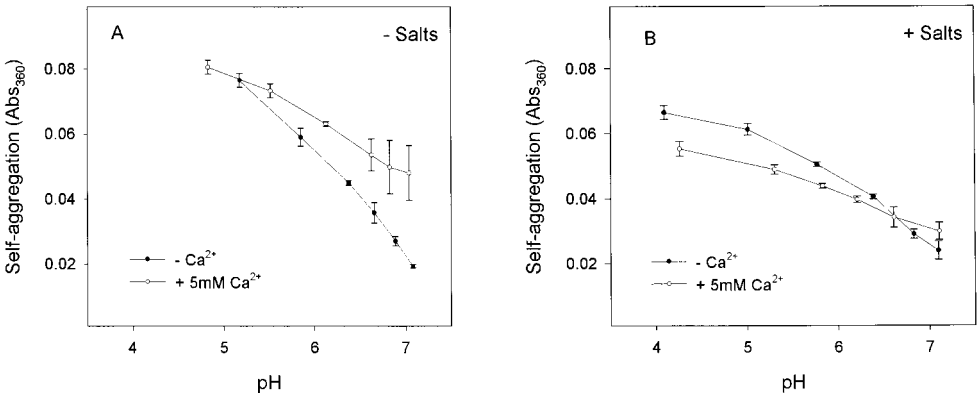

FIGURE 7: Effect of ionic strength and $CaCl_2$ on SP-A self-aggregation at different pHs. pH titration was carried out at 37 °C as described in the legend of Figure 6 either in the absence (A) or in the presence (B) of 150 mM NaCl. The same results were obtained with KCl: (●) no calcium and (○) 5 mM $CaCl_2$. Values are expressed as means ± the standard deviation of four experiments.

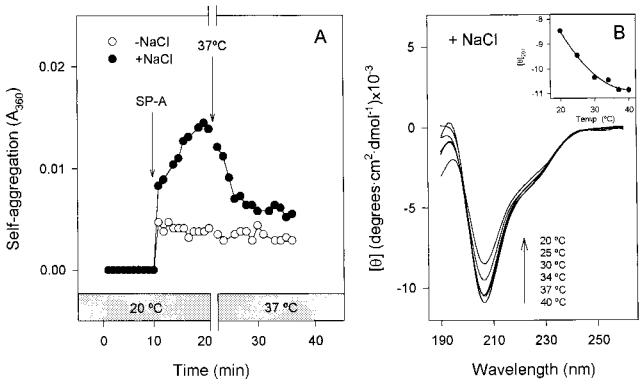

FIGURE 8: Effect of low temperature on SP-A self-aggregation (A) and SP-A secondary structure (B) in the presence of NaCl and EDTA. (A) Self-aggregation experiments were carried out as described in Experimental Procedures. (B) Far-UV circular dichroism spectra of SP-A (80 μg/mL) were recorded from 40 to 20 °C in 150 mM NaCl, 0.1 mM EDTA, 5 mM Tris-HCl buffer (pH 7.4). The inset shows the molar ellipticity at 207 nm as a function of temperature.

was 51 °C. Comparable values were found for canine and human SP-A (*26*).

Panels B and C of Figure 9 show kinetics of Ca^{2+}/Na^{+}- and Ca^{2+}-dependent protein self-aggregation at 37 °C, respectively, using porcine SP-A previously heated for 10 min at different temperatures and then cooled to 37 °C before assessment of self-aggregation activity. Both Ca^{2+}- and Ca^{2+}/Na^{+}-dependent SP-A self-aggregation were completely abrogated when the protein was preheated at 56 °C, indicating that a structurally intact collagen-like domain is required for both processes. The denaturation temperature at which SP-A showed half-maximal Ca^{2+}-dependent self-aggregation was 50 °C in the presence of NaCl and 54 °C in its absence (Figure 9D). These data suggest that Ca^{2+}/Na^{+}-induced SP-A self-aggregation is more susceptible to partial unfolding of the collagen-like triple-helical domain of SP-A than Ca^{2+}-induced self-aggregation. On the other hand, H^{+}-induced SP-A self-aggregation was not affected by partial or total unfolding of the collagen-like domain of SP-A (Figure 9D).

DISCUSSION

Ca^{2+}- and H^{+}-Dependent Self-Aggregation of SP-A. It is well established that SP-A self-aggregates to form higher-order structures in the presence of Ca^{2+} (*8*, *16*) or at mildly acidic pH (*9*). The biological significance of this process is unknown. Ca^{2+}-dependent self-aggregation of SP-A has typically been studied in buffers without physiological saline (150 mM NaCl) (*8*, *16*). Under these conditions, this process

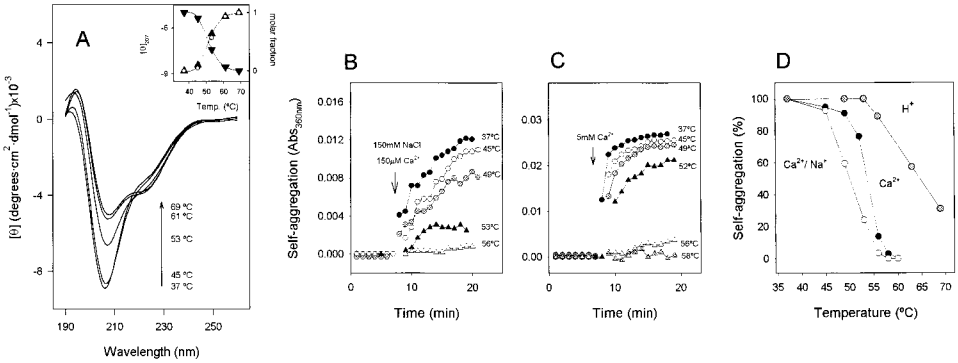

FIGURE 9: Effect of heat treatment of SP-A on the secondary structure of the protein and on its self-aggregation activity induced by either Ca^{2+} and Na^{+}, Ca^{2+}, or H^{+}. (A) Far-UV circular dichroism spectra of SP-A (80 μg/mL) were recorded from 37 to 69 °C in 5 mM Tris-HCl buffer (pH 7.4). The inset shows molar ellipticity at 207 nm as a function of temperature (○). The molar fractions of native (▼) and denatured (▲) collagen components of CD spectra of SP-A at different temperatures are also given. (B) Comparison of the kinetics of Ca^{2+}/Na^{+}-induced self-aggregation of SP-A at 37 °C using different protein preparations previously heated for 10 min at different temperatures in 5 mM Tris-HCl buffer (pH 7.4). SP-A was cooled to 37 °C before assessment of self-aggregation at 37 °C was carried out. (C) Comparison of the kinetics of Ca^{2+}-induced self-aggregation of heated SP-A as indicated for panel B. (D) Self-aggregation activity induced by either Ca^{2+} and Na^{+}, Ca^{2+}, or H^{+} at 37 °C performed with different SP-A preparations heated for 10 min at different temperatures as indicated for panel B. The rate of self-aggregation was determined with each SP-A preparation and expressed as a percentage of the rate observed at 37 °C. The results of a representative experiment of three are shown.

requires millimolar concentrations of Ca^{2+} (*8*, *16*). The Ca^{2+} concentration required for half-maximal self-association is 2.3 ± 0.2 mM for porcine SP-A and 3 ± 0.1 mM for human SP-A. These values are above the free Ca^{2+} concentration in the alveolar space of adult animals (approximately 1.5 mM) (*28*). The Na^+ and Cl^- concentrations of the lung liquid are similar to those in plasma (*29*). Therefore, we have studied here the Ca^{2+}-dependent self-aggregation of SP-A in the presence of physiological saline (150 mM NaCl). The Ca^{2+} concentration required to initiate self-aggregation of SP-A is highly reduced at physiological ionic strength. Under these conditions, the concentration of Ca^{2+} required for half-maximal self-aggregation is 2.4 ± 0.5 μM for porcine SP-A and 12 ± 1.8 μM for human SP-A. In the presence of 150 mM NaCl, addition of higher concentrations of Ca^{2+} (in the millimolar range) neither increases the extent of protein aggregation nor changes the fluorescent characteristics of SP-A. Addition of EDTA completely reverses the process of Ca^{2+}/Na^+-dependent self-aggregation. The presence of EDTA in the medium prevents that process at 37 °C. Therefore, NaCl does not promote self-association of the protein in the absence of Ca^{2+}at 37 °C.

We previously reported that the phospholipid vesicle aggregation activity of SP-A also required micromolar concentrations of Ca^{2+} for canine and porcine SP-A (*8*). This finding was confirmed for other SP-A species (*30*). Vesicle aggregation studies were carried out at physiological ionic strength. We suggest that Ca^{2+}/Na^+-dependent lipid vesicle aggregation is likely mediated by Ca^{2+}/Na^+-induced self-aggregation of the protein on different vesicles. Haasgman et al. (*16*) suggested that the process of lipid aggregation induced by SP-A could be correlated with that of self-association of the protein occurring at supramillimolar concentrations of Ca^{2+} and low ionic strength. However, these two processes have very different requirements for Ca^{2+} (*8*) unless physiological concentrations of NaCl are present in the medium.

Circular dichroism and fluorescence measurements of Ca^{2+}-dependent SP-A aggregates indicate that those protein aggregates formed in the absence of NaCl are structurally different from those formed in its presence. Changes in the secondary structure of SP-A, in the fluorescence emission intensity of the protein, and in the accessibility of SP-A tryptophan residues to acrylamide are much more pronounced when the protein undergoes Ca^{2+}-induced self-aggregation in the absence than in the presence of NaCl. In addition, the extent of Ca^{2+}-dependent self-aggregation of SP-A is much higher in the absence than in the presence of NaCl. Transmission electron microscopy studies (*31*) indicate that SP-A octadecamers become compacted (closed bouquet) and the stem becomes more detectable in the presence of millimolar concentrations of Ca^{2+} and in the absence of NaCl. Interestingly, when calcium and NaCl are present in the medium, the SP-A octadecamer retains more of an opened-bouquet structure and the stem is less detectable. Thus, the effect of Ca^{2+} on SP-A structure appears to be modified by the presence of NaCl. The compacted SP-A conformation induced by millimolar concentrations of Ca^{2+} in the absence of NaCl could contribute to the formation of larger protein aggregates in comparison to a more opened-bouquet structure.

Addition of EDTA to Ca^{2+}-dependent SP-A aggregates causes dissociation of those aggregates formed either in the presence or in the absence of NaCl. However, when the protein is in the absence of NaCl, addition of EDTA leads to a marked loss of ellipticity. Furthermore, SP-A loses its ability to aggregate lipid vesicles in a Ca^{2+}-dependent manner. In contrast, when EDTA is added to Ca^{2+}/Na^+-induced protein aggregates, the resulting nonaggregated SP-A hardly changes its secondary structure and retains its vesicle aggregation properties. All together, these data indicate the strong effect of the ionic strength on the Ca^{2+}-dependent self-aggregation process of SP-A.

On the other hand, SP-A undergoes a marked self-aggregation as the pH is reduced below 6.5. The protein encounters a mildly acidic pH along its exocytic/endocytic pathway (*9*, *32*). Acidic compartments along this pathway have millimolar concentrations of Ca^{2+} and physiological ionic strength. SP-A becomes immunologically unreactive under these conditions (*33*), and its lipid binding properties changed (*9*, *34*). We show here that the presence of salts in the medium is a regulatory element defining the level of pH-dependent self-aggregation. In the presence of salts (either 150 mM NaCl or 150 mM KCl), the extent of self-aggregation as a function of pH is reduced. The effect of salts is also visible in the change in the secondary structure of the protein. When both NaCl (or KCl) and $CaCl_2$ are present in the medium, the level of self-aggregation decreases even more as the pH is reduced in the range of 4.2−6.5, suggesting different effects of Ca^{2+} and H^+ ions on the protein in the presence of salts. H^+ could induce self-aggregation of the protein by protonation of the carboxyl groups, which would reduce the negative surface charge on the protein. The action of Ca^{2+} on the protein in the presence of salts might be more specific and not simply related to neutralization of the negatively charged carboxyl groups on the protein.

Temperature Effect on Self-Aggregation of SP-A. At physiological temperature, SP-A self-aggregation can be induced either by Ca^{2+} or by H^+. By contrast, at low temperatures, SP-A undergoes self-aggregation in the presence of EDTA at neutral pH, provided physiological saline is present in the medium. Increasing the temperature to 37 or 40 °C reverses this process. In the absence of NaCl, the negative surface charge on the protein would likely prevent protein aggregation at low temperatures unless charge stabilization occurred in the presence of NaCl. The negatively charged CRD regions of SP-A would approach each other more easily if charge stabilization occurred. The same process would happen as the pH is reduced. The headgroups of SP-A would move closer to each other. The effect of low temperature on SP-A self-aggregation can be explained by a decrease in solubility. Purified lipid-free SP-A is soluble in a presumably native octadecameric form in very low ionic strength buffers and at neutral pH. Other conditions affecting ionic strength, pH, temperature, or even the presence of trace amounts of calcium in the protein solution could lead to some type of self-aggregation of the protein in the aqueous phase. The state of SP-A self-aggregation could account for some differences in the results from different laboratories.

On the other hand, both Ca^{2+}- and Ca^{2+}/Na^+-dependent self-aggregation activity of SP-A is completely inhibited by unfolding of the collagen-like domain of porcine SP-A at

56 °C. Haagsman et al. (*16*) reported that the extent of lipid aggregation induced by canine SP-A, in the presence of Ca^{2+} and salts, decreased as the temperature was increased to 60 °C, indicating that an intact collagen domain may be required for specific SP-A-induced lipid aggregation. The similar temperature dependence of both self-aggregation and lipid aggregation processes also supports the concept that Ca^{2+}-dependent self-aggregation and SP-A-induced lipid aggregation are related phenomena.

Fully assembled SP-A is a hexamer of trimers. Strong noncovalent intermolecular forces in the N-terminal segment and the first part of the collagen-like region of SP-A, and not interchain disulfide bonds, are the major determinants of SP-A assembly into supratrimeric oligomers (*35*). Deoligomerization is expected to lead to loss or alteration of biological functions of SP-A (*36*). Because interactions between collagen triple helices are important in SP-A oligomerization, the formation of smaller forms of SP-A after mild heat treatment could lead to inhibition of self-aggregation and lipid aggregation activities of SP-A. However, the possibility that a collagen-like domain is involved in the process of protein−protein aggregation induced by Ca^{2+} cannot be ruled out.

By contrast, self-aggregation induced by H^+ is not affected by unfolding of the collagen-like domain, indicating that neither this domain nor oligomerization of SP-A is absolutely required for this type of self-aggregation. This finding strongly suggests that H^+ and Ca^{2+} ions induce SP-A self-aggregation by different mechanisms. This idea is additionally supported by the finding that the presence of Ca^{2+} and NaCl attenuates the effect of H^+ ions on protein self-aggregation.

Physiological Implications. SP-A aggregation mediated by Ca^{2+} and NaCl might have physiological significance since alveolar hypophase contains Na^+ (150 mM) and Ca^{2+} (1.5 mM) ions. The interaction of SP-A with pathogens or with its receptors on macrophages or on type II cells or the ability of SP-A to aggregate lipids has been studied under these ionic conditions. Thus, SP-A could interact with different ligands in a Ca^{2+}/Na^+-dependent self-aggregated form, which would retain an opened-bouquet structure (*31*). We suggest that SP-A bound to lipids, carbohydrates, or cell surfaces in the alveolar subphase is in a self-aggregated form induced by physiological concentrations of Ca^{2+} and NaCl, resulting in a multivalent presentation of SP-A to its ligands.

REFERENCES

1. Johansson, J., and Curstedt, T. (1997) *Eur. J. Biochem. 244*, 675−693.
2. McCormack, F. X. (1998) *Biochim. Biophys. Acta 1408*, 109−131.
3. Kuroki, Y., McCormack, F. X., Ogosawara, Y., Mason, R. J., and Voelker, D. R. (1994) *J. Biol. Chem. 269*, 29793−29800.
4. McCormarck, F. X., Kuroki, Y., Stewart, J. J., Mason, R. J., and Voelker, D. R. (1994) *J. Biol. Chem. 269*, 29801−29807.
5. Kuroki, Y., and Akino, T. (1991) *J. Biol. Chem. 266*, 3068−3073.
6. Casals, C., Miguel, E., and Pérez-Gil, J. (1993) *Biochem. J. 296*, 585−593.
7. Ruano, M. L. F., Nag, K., Worthman, L., Casals, C., Pérez-Gil, J., and Keough, K. M. W. (1998) *Biophys. J. 74*, 1101−1109.
8. Ruano, M. L. F., Miguel, E., Pérez-Gil, J., and Casals, C. (1996) *Biochem. J. 313*, 683−689.
9. Ruano, M. L. F., Pérez-Gil, J., and Casals, C. (1998) *J. Biol. Chem. 273*, 15183−15191.
10. Efrati, H., Hawgood, S., Williams, M. C., Hong, K., and Benson, B. J. (1987) *Biochemistry 26*, 7986−7993.
11. Korfhagen, T. R., Bruno, M. D., Ross, G. F., Huelsman, K. M., Ikegami, M., Jobe, A. H., Wert, S. E., Stripp, B. R., Morris, R. E., Glasser, S. W., Bachurski, C. J., Iwamoto, H. S., and Whitsett, J. A. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 9594−9599.
12. Suzuki, Y., Fujita, Y., and Kogishi, K. (1989) *Am. Rev. Respir. Dis. 140*, 75−81.
13. Hawgood, S., Benson, B. J., Schilling, J., Damm, D., Clements, J., and White, R. T. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 66−70.
14. Tino, M. J., and Wright, J. R. (1998) *Biochim. Biophys. Acta 1408*, 241−263.
15. Haagsman, H. P. (1998) *Biochim. Biophys. Acta 1408*, 264−277.
16. Haagsman, H. P., Sargeant, T., Hauschka, P. V., Benson, B. J., and Hawgood, S. (1990) *Biochemistry 29*, 8894−8900.
17. Casals, C., Miguel, E., Pérez-Gil, J., and Fernández-Ruano, M. L. (1994) *Prog. Respir. Res. 27*, 39−43.
18. Gaynor, C. D., McCormack, F. X., Voelker, D. R., Mcgowan, S. E., and Schlesinger, L. S. (1995) *J. Immunol. 155*, 5343−5351.
19. Weissbach, S., Neuendank, A., Pettersson, M., Schaberg, T., and Pison, U. (1994) *Am. J. Physiol. 267*, L660−L666.
20. Casals, C., Herrera, L., Miguel, E., García-Barreno, P., and Municio, A. M. (1989) *Biochim. Biophys. Acta 1003*, 201−203.
21. Schoenmakers, T. J. M., Visser, G. J., Flik, G., and Theuvenet, A. P. R. (1992) *BioTechniques 12*, 870−879.
22. Lakowicz, J. R. (1983) *Principles of Fluorescence Spectroscopy*, Plenum Press, New York and London.
23. Lehrer, S. S. (1971) *Biochemistry 10*, 3254−3263.
24. Eftink, M. R., and Ghiron, C. A. (1981) *Anal. Biochem. 114*, 199−227.
25. Voss, T., Eistetter, H., and Schafer, K. P. (1988) *J. Mol. Biol. 201*, 219−227.
26. Haagsman, H. P., White, R. T., Schilling, J., Lau, K., Benson, B. J., Golden, J., Hawgood, S., and Clements, J. A. (1989) *Am. J. Physiol. 257*, L421−L429.
27. Perzcel, A., Hollosi, M., Tusnady, G., and Fasman, G. D. (1991) *Protein Eng. 4*, 669−679.
28. Nielson, D. W., and Lewis, M. B. (1988) *Pediatr. Res. 24*, 322−325.
29. Nielson, D. W., Goerke, J., and Clements, J. A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 7119−7123.
30. Meyboom, A., Maretzki, D., Stevens, P. A., and Hofmann, K. P. (1997) *J. Biol. Chem. 272*, 14600−14605.
31. Palaniyar, N., Ridsdale, R. A., Holterman, C. E., Inchley, K., Possmayer, F., and Harauz, G. (1998) *J. Struct. Biol. 122*, 297−310.
32. Osanai, K., Mason, R. J., and Voelker, D. R. (1998) *Am. J. Respir. Cell Mol. Biol. 19*, 929−935.
33. Voorhout, W. F., Veenendaal, T., Haagsman, H. P., Verkleij, A. J., van Golde, L. M. G., and Geuze, H. J. (1991) *J. Histochem. Cytochem. 39*, 1331−1336.
34. Ruano, M. L. F., Nag, K., Casals, C., Pérez-Gil, J., and Keough, K. M. W. (1999) *Biophys. J. 77*, 1469−1476.
35. McCormack, F. X., Damodarasamy, M., and Elhalwagi, B. M. (1999) *J. Biol. Chem. 274*, 3173−3181.
36. Hickling, T. P., Malhotra, R., and Sim, R. B. (1998) *Mol. Med. 4*, 266−275.

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