Reversible Calcium-dependent Interaction of Liposomes with Pulmonary Surfactant Protein A

ANALYSIS BY RESONANT MIRROR TECHNIQUE AND NEAR-INFRARED LIGHT SCATTERING*

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Surfactant protein A (SP-A) is crucial for lung function, including tubular myelin formation and lipid uptake by type II pneumocytes. Known properties of SP-A in vitro are its Ca\(^{2+}\)-dependent interaction with phospholipids and its role in the aggregation of liposomes. To dissect and to analyze these processes, we have immobilized SP-A and measured binding of liposomes by the resonant mirror technique. Liposome aggregation was followed separately by kinetic light scattering in suspensions. It was found that SP-A-mediated binding and aggregation of liposomes have a common \(K_{M}\) of 20 \(\mu\)M for free Ca\(^{2+}\), independent of the species (sheep, rat, or cow) and of the phospholipid composition, and that both reactions exhibit the same high cooperativity (Hill coefficients of 6–9) for Ca\(^{2+}\) ions. However, binding of liposomes to SP-A is >10-fold faster than aggregation. Both processes are completely reversed by low Ca\(^{2+}\) concentrations, but liposomes dissociate from SP-A in <0.3 s, whereas disaggregation of the liposomes takes ~30 s. At equilibrium, the level of aggregation depends on the concentration of free SP-A. We interpret these results to be a rapid and reversible sequence of three reactions: (i) a cooperative Ca\(^{2+}\)-dependent conformational change in SP-A, (ii) binding of Ca\(^{2+}\)-bound SP-A to liposomes, and (iii) aggregation of the Ca\(^{2+}\)/SP-A-bound liposomes.

Pulmonary surfactant stabilizes the lung by regulating the surface tension of the alveoli. A key element is thereby the formation of a surface monolayer of phospholipids, mainly dipalmitoylphosphatidylcholine, which lowers surface tension. The monolayer is generated from an “aqueous” compartment, the extracellular alveolar hypophase, which contains surfactant lipids. The key element is thought to play an important role in the phospholipid organization of the alveolar fluid and in surfactant lipid exchange in type II pneumocytes.

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§ The abbreviations used are: SP-A, surfactant protein A; NIR, near-infrared.

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content was determined using the Bio-Rad protein assay and bovine serum albumin as protein standard. The purity of the SP-A preparation was assessed by SDS-polyacrylamide gel electrophoresis according to Laemmli (14).

Liposome Preparation—All lipids used were obtained from Sigma. Phospholipid mixtures in CH<sub>3</sub>OH/CHCl<sub>3</sub> (1:2) consisting of dipalmitoylphosphatidylcholine/egg phosphatidylcholine/phosphatidylglycerol/cholesterol (55:25:10:10) were dried under vacuum using a Speed Vac system (Eppendorf GmbH, Germany) and stored at 4°C.

Lipid films were hydrated in 5 mM Tris, pH 7.4, containing 100 mM NaCl and 50 μM EGTA to a phospholipid concentration of 10 mg/ml. Samples were sonicated 8 × 15 s at 50°C with a Bandelin Sonopuls BM 70 disintegrator. Multilamellar liposomes were obtained by 10 repeated freeze-thaw cycles. They were extruded through a polycarbonate membrane of defined pore size (200 nm) using a Lipo-Fast extrusion set. Liposomes produced by extrusion are unilamellar as described (20–22).

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Spectrophotometric Assays—Liposome aggregation was monitored at 400 nm by measurement of turbidity changes using a diode array spectrophotometer (Hewlett-Packard, Waldbronn, Germany). Liposomes, prepared as described above, were dissolved in a glass cuvette containing 5 mM Tris, 100 mM NaCl, and 50 μM EGTA, pH 7.4, to a final concentration of 10 μg/ml. The buffer was exchanged in Microcon separation kits (Amicon, Inc.). After immobilization of SP-A, the remaining binding sites were blocked with 1% Tween 20, and 5 mM Tris, pH 7.4. Signals were obtained using 100 μl of liposome solution in 100 mM NaCl, 50 μM EGTA, and 5 mM Tris, pH 7.4, with the addition of a small sample volume of Ca<sup>2+</sup> from different stock solutions (100:10:1 mM). Cuvettes were washed with 10 mM EDTA, 0.1% Tween 20, and 5 mM Tris, pH 7.4, and with 100 mM NaCl and 5 mM Tris, pH 7.4, with or without EGTA, depending on the experiment.

RESULTS

Rate and Reversibility of SP-A/Liposome Interaction

To investigate the interaction of lipids with SP-A, liposome binding to immobilized SP-A and their release were studied by the resonant mirror technique. The aggregation of liposomes by SP-A was assayed using the kinetic NIR light scattering method (16). The two methods enabled us to distinguish between the binding of liposomes to SP-A (Fig. 1A) and the SP-A-mediated aggregation of the liposomes (Fig. 1B).

Binding of Liposomes to Immobilized SP-A—Fig. 1A shows changes in refractive index within the sensitive layer on the surface of the resonant mirror apparatus. These sensorgrams, measured in units of arc seconds (arc s), reflect changes during calcium-dependent binding or release of liposomes to SP-A bound to the surface chip. It is shown that the addition of Ca<sup>2+</sup> leads to a binding signal, indicating transfer of liposomes to immobilized SP-A.
The addition of EGTA during the rising phase of the binding signal (Fig. 1A) produces rapid negative deflections, indicating partial dissociation of the liposomes. The first two additions maintain the full amplitude of the resonant mirror signal of SP-A-bound liposomes, presumably due to free Ca$^{2+}$ still available. The subsequent back-titration of Ca$^{2+}$ dissociates the liposomes from SP-A very rapidly, as seen in the dramatic drop of the resonant mirror signal. Full complexation of Ca$^{2+}$ with equimolar amounts of EGTA recovers the baseline signal. The binding reaction is fully reversible.

Fig. 1A (inset) displays the downward deflection of the resonant mirror signal in real time recording. The measured dissociation of 0.3 s is an upper limit of the actual reaction time since the time resolution of the instrument is in this order of magnitude.

Sensorgrams obtained upon coupling of SP-A on the sensor chip were reproducible for liposome binding in nine immobilization experiments. Minor alterations of base-line levels did occur, probably caused by nonspecific liposome interaction on the surface of the sensor chip. After SP-A complex formation under saturating conditions of liposomes, the addition of SP-A and/or more Ca$^{2+}$ did not change the resonant mirror signal. The affinity sensor monitors changes in the refractive index limited to a surface thickness of ~200 nm. The observations indicate that liposome aggregation does not interfere with the binding signal from surface-localized SP-A. Neither empty cuvettes without bound SP-A nor heat-denatured SP-A produced any liposome binding signal.

Although one can never exclude an influence on the binding kinetics, immobilization of proteins has been applied to a number of investigation purposes. However, precise and rapid Ca$^{2+}$-dependent liposome binding and its complete reversibility strongly suggest that the SP-A function is maintained after immobilization on the sensor surface. The measured rates are likely to be lower limits for the actual reaction rates in free solution due to limited accessibility of the protein on the sensor surface.

**SP-A-mediated Aggregation of Liposomes**—Aggregation manifests itself in a change in light scattering (see “Experimental Procedures”). The data in Fig. 1B are from a typical experiment, with the SP-A-liposome interaction started by addition of Ca$^{2+}$. The amplitude of the scattering signal ($\Delta I_d$) is the recorded intensity change (in volts) at the detector output. The signal arises predominantly from the gain of particle volume by Ca$^{2+}$-induced, SP-A-mediated liposome aggregation. Controls performed without SP-A or Ca$^{2+}$ did not show any light scattering changes.

The addition of calcium chelators (EDTA or EGTA) during the rise of the signal sets the scattering intensity back to the starting value. As was observed for SP-A binding, partial titration of free Ca$^{2+}$ reduces the aggregation only partially, and a subsequent second addition of excess Ca$^{2+}$ elicits again a full aggregation signal (data not shown). These results indicate that, in a certain range of Ca$^{2+}$ concentrations, the equilibrium level of SP-A-mediated liposome aggregation is sensitive to free Ca$^{2+}$. The aggregation is completely reversible, with a halftime of complete reversal of aggregation (which we may term “disaggregation”) on the order of 18 s (see Fig. 1B, inset).

**Influence of Lipid Concentration**

Fig. 2A shows sensorgrams that were obtained when the amount of liposomes in the cuvette was varied. Again, binding to immobilized SP-A was measured. The amount of liposomes is expressed in units of phospholipid concentration. The sensorgrams generally consist of two phases, namely a rapid association phase and a subsequent slower phase. Two exponential functions are sufficient to fit the two phases of the binding signals (Table I). They can be understood as the initial occupation of free binding sites on surface-bound SP-A and a slower...

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**Table I**

| Liposome conc | $A_1$ | $k_1$ | $A_2$ | $k_2$ | Liposome | $A_1$/Liposome |
|--------------|-------|-------|-------|-------|-----------|---------------|
| $\mu$M       | (arc s) s$^{-1}$ | (arc s) s$^{-1}$ | nM    |        |            |               |
| 100          | 160   | 337   | 0.0449| 431    | 0.0029   | 0.32          | 2.7           |
| 200          | 320   | 407   | 0.0751| 310    | 0.0054   | 0.64          | 1.3           |
| 400          | 640   | 515   | 0.1050| 269    | 0.0081   | 1.28          | 0.7           |
| 1280         | 1280  | 587   | 0.1719| 234    | 0.0082   | 2.56          | 0.33          |
| 2560         | 2560  | 688   | 0.1925| 180    | 0.0095   | 5.12          | 0.17          |

**Table II**

| Liposome conc | $A$   | $k$   | Liposome conc | $A$/Liposome |
|--------------|-------|-------|--------------|--------------|
| $\mu$M       | V     | s$^{-1}$ | nM            |              |
| 80           | 2.72  | 0.0005 | 0.16          | 28           |
| 160          | 3.21  | 0.0069 | 0.32          | 14           |
| 320          | 4.36  | 0.0011 | 0.64          | 7            |
| 640          | 4.20  | 0.0015 | 1.28          | 3.5          |
| 1280         | 4.30  | 0.0021 | 2.56          | 1.8          |
equilibration. The initial fast phase takes only a few seconds.

Each record is from a new sample, started with the same amount of Ca$^{2+}$. The resonant mirror signal increases with the concentration of the liposomes, which can be approximately taken from the concentration of lipid, the space occupied by one lipid molecule in a bilayer (0.5 nm$^2$), and the diameter of liposomes on the order of 200 nm. Sensorgrams were analyzed by the equation given in the legend to Table I. The initial reaction rate ($k_{on}$) depends only slightly on liposome concentration (4-fold change for 16-fold change in lipid). Generally and at any concentration of the lipid, binding to SP-A (Fig. 2A) is much faster than SP-A-mediated liposome aggregation (Fig. 2B).

The estimated volumetric equivalent concentration of SP-A in the resonant mirror cuvette, as calculated from the amount of SP-A coupled on the surface of the sensor chip and the cuvette volume, is on the order of 1 nM. The light scattering experiments employed 4.5 nM SP-A. Thus, although the SP-A concentration in the resonant mirror experiments was lower (one-fifth), the $k_{on}$ of the binding signals was at least an order of magnitude higher than that of aggregation. This is consistent with fast binding between SP-A and liposomes, followed by slower aggregation.

**Influence of SP-A Concentration**

Higher SP-A levels in the liposome NIR scattering experiments give rise to higher aggregation signals. Fig. 3 shows light scattering signals at constant Ca$^{2+}$ and lipid concentrations. It is shown that the rate and extent of aggregation depend on the concentration of SP-A, indicating that the SP-A bound after a short time (see Figs. 1A and 2A) governs the aggregation process. The amplitude and $k_{on}$ value of liposome aggregation rise in proportion to the amount of SP-A present. $k_{on}$ values become higher because the more SP-A is present, the faster SP-A/liposome complexes are formed. Different amplitudes reflect that size and/or number of liposome aggregates depends on the amount of SP-A in solution. Analogous observations were made with SP-A from different species (rat, sheep, and cow).

**Calcium Dependence of SP-A/Liposome Interaction**

In Fig. 4 (A and B), the dependence on Ca$^{2+}$ of liposome binding to SP-A and aggregation is plotted. The original concentration in the saline buffer (typically 20 µM) was measured spectrophotometrically, using arsenazo III (19). Ca$^{2+}$ was first adjusted to a low initial concentration by EGTA (50 µM). Each point represents the signal amplitude 200 or 400 s after the addition of the respective amount of Ca$^{2+}$.

Liposome binding and aggregation start to be visible in the same range of Ca$^{2+}$ concentrations added. This is consistent with the conclusion reached above that aggregation is dependent on and follows SP-A/lipid association induced by micromolar Ca$^{2+}$. It does not exclude an additional influence of Ca$^{2+}$ at higher concentrations; the aggregation process by itself may well depend on high Ca$^{2+}$ concentrations as described previously (10, 20, 21).
Cooperativity of Liposome Binding to SP-A

The data in Fig. 4 demonstrate that SP-A does not interact with liposomes at low free Ca\(^{2+}\) concentrations. Within a narrow range of concentration, the addition of Ca\(^{2+}\) starts to induce the structural transitions reflected in the SP-A-dependent interactions, indicating a cooperative mode of SP-A action in both binding and aggregation. We conclude that SP-A exists in (at least) two states, namely a calcium-depleted form that does not interact with liposomes and one or several interacting forms that bind calcium cooperatively.

To obtain more quantitative information, the coupled SP-A on the aminosilane surface of the resonant mirror offers the possibility of a treatment with high concentrations of EGTA to reduce the Ca\(^{2+}\) bound to SP-A to a great extent. The actual concentration of free Ca\(^{2+}\) can then be estimated after Ca\(^{2+}\) determination in buffer and known addition of EGTA and Ca\(^{2+}\). Fig. 5 is a plot of the resulting levels of lipid binding depending on free Ca\(^{2+}\). The Hill coefficient, based on a computer fit to the data, was on the order of 6–9 (see Table IV), and \(K_0\) was \(\sim 20 \mu M\) free Ca\(^{2+}\) for all species investigated (sheep, rat, and cow; data not shown).

**DISCUSSION**

In this study, we have analyzed the functions of SP-A. The biophysical techniques applied enabled us to differentiate between its Ca\(^{2+}\)-dependent interaction with phospholipids and its role in the aggregation of liposomes. Both these processes are closely coupled in situ. Our analysis shows how they are concatenated as two partial steps of one Ca\(^{2+}\)-dependent overall process.

*Liposome Binding to SP-A*—We used immobilized SP-A to monitor the binding and release of liposomes to and from the protein by changes in the refractive index at an affinity sensor surface (binding signals). Binding of liposomes to SP-A occurs upon addition of Ca\(^{2+}\) concentrations in the micromolar range \((K_{0.5} = 22 \pm 3 \mu M\) free Ca\(^{2+}\)). The association rate constants \((k_{\text{on}})\) rise in proportion to the lipid concentration, which is consistent with a mechanism of collisional coupling between liposomes and immobilized SP-A (Fig. 2A and Table I). Successful coupling requires a Ca\(^{2+}\)-dependent form of SP-A, presumably a specific conformation. We have also seen that removal of Ca\(^{2+}\), e.g. by addition of EGTA, switches SP-A back to an inactive form, leading to very rapid dissociation of liposomes. This Ca\(^{2+}\)-dependent switch of the SP-A/liposome interaction operates in a wide range of phospholipid compositions and for SP-A from different species. The observed switch of the SP-A conformation fits well with a class of binding sites with \(K_D\) on the order of 10 \(\mu M\) (22). Conformational changes in SP-A that result from Ca\(^{2+}\) binding in the absence of lipids were detected by fluorescence spectroscopy by Sohma et al. (23). They found increased fluorescence intensities of SP-A with half-maximal changes of \(-60 \mu M\) Ca\(^{2+}\) added.

In terms of the subunit structure of SP-A, it is now an interesting question of how Ca\(^{2+}\) triggers the change in structure of SP-A that leads to the observed effect. Suggested regions for binding of lipid include the neck plus carbohydrate recognition domains of the SP-A subunits (6–8), which form a bouquet-like structure in the octadecamer (5).

*Liposome Aggregation*—Kinetic light scattering has revealed that liposome aggregation is similar in its Ca\(^{2+}\)-dependence, but always lags behind the liposome binding reaction. Remarkably, the rise and equilibrium level of aggregation depend on the concentration of SP-A in the sample, which shows that the protein controls the aggregation process. Our results confirm that reversal of aggregation (disaggregation) of SP-A-liposome complexes can be achieved by the mere addition of EGTA or EDTA in excess of calcium concentrations, as reported by others (11, 22, 24). We have further seen that the decay of the aggregates is much delayed compared with the very rapid dissociation of the SP-A-liposome complex (18 s versus 0.3 s). This is consistent with a two-step mechanism in which the release of the SP-A/liposome interaction triggers disaggregation. It is interesting to estimate the average number of SP-A molecules/liposome for the aggregation data (Table III). Assuming that each SP-A molecule with a diameter of 20 nm (5) occupies an area of 300 nm\(^2\), it turns out that approximately two to four SP-A molecules/vesicle (125 \(\times 10^3\) nm\(^2\) allow a substantial aggregation (\(-50%\) of maximum), although only 1% of the vesicle surface (corresponding to 400 SP-A molecules) is occupied by the protein. These numbers make it likely that SP-A forms bridges between liposomes by a vesicle/SP-A/vesicle coupling mechanism. Together with the difference in kinetics between SP-A/liposome binding and aggregation, this argues for an asymmetric bivalent model of SP-A function, in agreement with a recent analysis of different functional regions in the SP-A structure (6).

*Calcium Cooperativity*—The results of this study are consistent with a model of the SP-A/liposome interaction that involves two states of the protein. The inactive protein does not interact, whereas the active protein, in concert with cooperative binding of Ca\(^{2+}\), interacts with liposomes. Conceivably, bound Ca\(^{2+}\) switches SP-A into a conformation, termed SP-\(\hat{A}\), that exposes...
hydrophobic phospholipid-binding sites not available in the calcium-free protein. Such a Ca\textsuperscript{2+}-dependent protein switch would induce both liposome binding to SP-A and SP-A-mediated liposome aggregation in the same affinity range (K\textsubscript{0.5} \approx 20 \mu M) in a time-ordered sequence. It is characteristic for this reaction sequence that liposome association/dissociation with SP-A is much faster than the respective aggregation/dissociation of the liposomes.

We propose the following hypothesis for the SP-A/liposome interaction with SP-A as the active conformation.

\[
\frac{n}{5} = 6 \\
\text{K}_{0.5} = 20 \mu M
\]

From the binding data, a high cooperativity for Ca\textsuperscript{2+} is calculated with a Hill coefficient of \(-6\)–9 (Table IV). The maximum value of \(n\) is equal to the number of effective sites. Although the number \(x\) of calcium atoms bound to SP-A is unknown, the high value for the Hill coefficient implies a strong cooperativity between multiple subunits. The complex multimeric structure of SP-A is compatible with this idea.

Coupled equilibrium, such as described above, can be decoupled at one or more steps, provided enough driving energy is available for the remaining reactions. It is tempting to relate this model to results with SP-A, which was capable of binding lipids without aggregation activity. Such a loss of function was observed with SP-A altered by mutations in the carbohydrate recognition domain (6) and was also reported for SP-A damaged by free radicals (25, 26).

**Physiological Implications**—There are numerous physiological functions of SP-A, which depend on calcium and involve interaction with lipids. They include the following: (i) SP-A-dependent enhancement of phospholipid uptake (12), (ii) inhibition of surfactant secretion (27, 28), (iii) binding of SP-A to type II pneumocytes (29), (iv) aggregation of liposomes (10, 11, 22, 24, 30), and (v) enhanced adsorption and surface sorting of surfactant lipids (31). Their relation to the Ca\textsuperscript{2+}-dependent switch of the SP-A/liposome interaction remains to be investigated. The protein should remain in its active lipid-binding conformation as long as calcium-binding sites are saturated in the alveolar hypophase, which contains 1.5 mM free Ca\textsuperscript{2+} (32). The operation of the switch to the inactive state and the concurrent release of lipids may depend on intracellular free Ca\textsuperscript{2+} concentrations in the micromolar range.

**TABLE IV**

| Resonant mirror (added Ca\textsuperscript{2+}) | NIR light scattering, added Ca\textsuperscript{2+} | Resonant mirror (free Ca\textsuperscript{2+}) |
|---------------------------------------------|-----------------------------------------------|-------------------------------------------|
| A                                           | 865 ± 13 (arc s)                              | 4.5 ± 0.14 (relative units) 408 ± 5.16 (arc s) |
| \(n\)                                       | 8.8 ± 1.2                                    | 5.5 ± 0.62                                |
| \(K_{0.5} (\mu M)\)                         | 40 ± 0.6                                     | 62.7 ± 1.15                               |
| \(K_{0.5} (\mu M)\)                         | 22 ± 3.0                                     |                                           |

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