Tryptophan fluorescence study on the interaction of pulmonary surfactant protein A with phospholipid vesicles

Cristina CASALS,* Eugenio MIGUEL and Jesus PEREZ-GIL
Department of Biochemistry and Molecular Biology, Faculty of Chemistry, Complutense University of Madrid, 28040 Madrid, Spain

The fluorescence characteristics of surfactant protein A (SP-A) from porcine and human bronchoalveolar lavage were determined in the presence and absence of lipids. After excitation at either 275 or 295 nm, the fluorescence emission spectrum of both proteins was characterized by two maxima at about 326 and 337 nm, indicating heterogeneity in the emission of the two tryptophan residues of SP-A, and also revealing a partially buried character for these fluorophores. Interaction of both human and porcine SP-A with various phospholipid vesicles resulted in an increase in the fluorescence emission of tryptophan without any shift in the emission wavelength maxima. This change in intrinsic fluorescence was found to be more pronounced in the presence of dipalmitoyl phosphatidylcholine (DPPC) than with dipalmitoyl phosphatidylglycerol (DPPG), DPPC/DPPG (7:3, w/w) and 1-palmitoyl-sn-glycerol-3-phosphocholine (LPC). Intrinsic fluorescence of SP-A was almost completely unaffected in the presence of egg phosphatidylcholine (egg-PC). In addition, we demonstrated a shielding of the tryptophan fluorescence from quenching by acrylamide on interaction of porcine SP-A with DPPC, DPPG or LPC. This shielding was most pronounced in the presence of DPPC. In the case of human SP-A, shielding was only observed on interaction with DPPC. From the intrinsic fluorescence measurements as well as from the quenching experiments, we concluded that the interaction of some phospholipid vesicles with SP-A produces a conformational change on the protein molecule and that the interaction of SP-A with DPPC is stronger than with other phospholipids. This interaction appeared to be independent of Ca²⁺ ions. Physiological ionic strength was found to be required for the interaction of SP-A with negatively charged vesicles of either DPPG or DPPC/DPPG (7:3, w/w). Intrinsic fluorescence of SP-A was sensitive to the physical state of the DPPC vesicles. The increase in intrinsic fluorescence of SP-A in the presence of DPPC vesicles was much stronger when the vesicles were in the gel state than when they were in the liquid-crystalline state. The effect produced by SP-A on the lipid vesicles was also dependent on temperature. The aggregation of DPPC, DPPC/DPPG (7:3, w/w) or dimyristoyl phosphatidylglycerol (DMPG) was many times higher below the phase-transition temperature of the corresponding phospholipids. These results strongly indicate that the interaction of SP-A with phospholipid vesicles requires the lipids to be in the gel phase.

INTRODUCTION

Pulmonary surfactant is a heterogeneous lipid–protein complex that overlies the alveolar epithelium and consists of about 90% lipids and 10% surfactant-associated proteins. This material is synthesized and assembled into lamellar bodies by alveolar type II cells. After secretion, the content of lamellar bodies expands into large ordered tubular aggregates known as tubular myelin. This structure is widely thought to be the immediate precursor of a lipid film at the alveolar air/liquid interface which modifies the surface tension in a manner that depends on alveolar surface area. Thus the reduction in surface tension at the alveolar surface protects the alveoli against collapse at end expiration (Goerke, 1974; van Golde et al., 1988; Haagsman and van Golde, 1991).

Although the composition of the alveolar surface film has not been assessed biochemically, biophysical studies (Schürch et al., 1989) support the concept that in vivo it is greatly enriched in dipalmitoyl phosphatidylcholine (DPPC).

Surfactant protein A (SP-A) is the most abundant protein of pulmonary surfactant. The primary structure of SP-A (White et al., 1985; Floros et al., 1985) indicated the presence of two different structural domains in each SP-A subunit: an N-terminal domain containing a collagen-like sequence and a C-terminal domain with a sequence similar to several Ca²⁺-dependent lectins. These domains assemble to a complex hexameric structure resembling a flower bouquet, which is composed of 18 polypeptide chains (Voss et al., 1988; King et al., 1989). In one of the initial steps of the assembly of SP-A, three subunits of SP-A probably form a triple-helical stem which is stabilized by interchain disulphide bonds. In the final stage of the assembly, the hexamers appear to be formed by lateral aggregation of the N-terminal half of the triple-helical stems (Haas et al., 1991).

One of the most important properties of SP-A is its ability to bind phospholipids. SP-A co-isolates with surfactant lipids and appears to be highly associated with them. Deglycosylation of SP-A does not affect the lipid-binding properties of SP-A (Haagsman et al., 1991; Kuroki and Akino, 1991), whereas removal of the collagenous domain of SP-A results in a marked loss of its ability to bind phospholipids (Ross et al., 1991; Kuroki and Akino, 1991). A specific lipid-binding site has been proposed to be located at the linking region of SP-A, a segment of non-collagenous sequence between the collagen-like region and the carbohydrate-recognition domain (Ross et al., 1986). SP-A can also bind glycosphingolipids and the binding site has been proposed to be at the carbohydrate-recognition domain (Childs et al., 1992).

One of the effects of SP-A on phospholipid vesicles is that it modifies the thermotropic behaviour of either DPPC (King et al.,

Abbreviations used: DPPC, dipalmitoyl phosphatidylcholine; SP-A, surfactant protein A; DPPG, dipalmitoyl phosphatidylglycerol; DMPG, dimyristoyl phosphatidylglycerol; egg-PC, egg phosphatidylcholine; egg-PG, egg phosphatidyglycerol; LPC, 1-palmitoyl-sn-glycero-3-phosphocholine.

* To whom correspondence should be addressed.
1986) or DPPC/dipalmitoyl phosphatidylglycerol (DPPG) (7:3, w/w) vesicles (Reilly et al., 1989; Oosterlaken-Dijkstra, 1991). A second effect of SP-A on phospholipids is that it induces phospholipid vesicle aggregation in the presence of Ca"++. (Hawgood et al., 1985; Efrati et al., 1987; Haagsman et al., 1990).

Two important roles related to the ability of SP-A to bind Ca"++ and phospholipids have been postulated: (a) participation in the transformation of lamellar body content into tubular myelin (Suzuki et al., 1989; Williams et al., 1991), and (b) promotion of the formation of a stable surface film of phospholipids in a concerted action with the hydrophobic surfactant protein SP-B (Hawgood et al., 1987). SP-A may also be important in the regulation of the secretion and clearance of surfactant lipids [see Wright and Dobbs (1991) for review] and in alveolar defence (Tenner et al., 1989; van Iwaarden et al., 1990).

The first aim of the present work was to study the effect of phospholipids on SP-A by c.d. and by steady-state fluorescence measurements. The amino acid sequence of SP-A appears to be highly conserved in all species studied so far, and contains only two tryptophans, which are located in the C-terminal region [White et al. (1985) and Floros et al. (1985) (human); Benson et al. (1985) (dog); Sano et al. (1987) (rat); Boggaram et al. (1988) (rabbit)]. We describe here the intrinsic fluorescence characteristics of porcine and human SP-A (fluorescence emission intensity, emission maximum wavelength and susceptibility to quenching by acrylamide) in the absence and presence of lipids. The second objective of this work was to analyse the effect on the lipid/SP-A interaction of (a) the nature of the polar head group and acyl chains of the phospholipids, (b) the physical state of the phospholipid vesicles and (c) the ionic strength of the medium, by means of intrinsic fluorescence techniques as well as by SP-A-induced vesicle aggregation measurements.

**EXPERIMENTAL**

**Purification of SP-A**

SP-A was isolated from porcine lung lavage. Pig lungs were obtained from the slaughterhouse and lavaged three times with a solution of 0.15 M NaCl. Pulmonary surfactant was prepared from bronchoalveolar lavage and separated from blood components by NaBr density-gradient centrifugation as previously described (Casals et al., 1989). Porcine SP-A was purified from isolated surfactant using sequential butanol and octyl glucoside extractions (Haagsman et al., 1987). Human SP-A, isolated from bronchoalveolar lavage of patients with alveolar proteinosis, was kindly supplied by Dr. H. P. Haagsman (University of Utrecht, Utrecht, The Netherlands). SP-A concentrations were estimated by quantitative amino acid analysis as described below. The protein was stored in small portions in 5 mM Tris/HC1 buffer, pH 7.4, at -20 °C. Electrophoretic analysis was performed under reducing conditions (50 mM dithiothreitol) by one-dimensional SDS/PAGE as described by Laemmli (1970). Gels were stained with Coomassie Brilliant Blue R250.

**Amino acid analysis**

The amino acid analyses of porcine and human SP-A were carried out on a Beckman System 6300 high-performance amino acid analyser. 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 both 24 and 120 h. The cysteine content was determined as cysteic acid in tubes in which performic acid oxidation was carried out as described by Hirs (1967). The tryptophan content was determined as described by Bean and Holiday (1952).

**Preparation of lipid vesicles**

Synthetic phospholipids, DPPC, DPPG and dimyristoyl phosphatidylglycerol (DMPG) were purchased from Avanti Polar Lipids (Birmingham); egg phosphatidylincholine (egg-PC), egg phosphatidylglycerol (egg-PG) and 1-palmitoyl-sn-glycerol-3-phosphocholine (LPC) were from Sigma (St. Louis, MO, U.S.A.), and their homogeneity was routinely tested by t.l.c. The organic solvents (methanol and chloroform) used to dissolve lipids were h.p.l.c. grade (Scharlau, Barcelona, Spain).

Unilamellar vesicles were used throughout all spectroscopy experiments. The different lipid vesicles were prepared at a phospholipid concentration of 3 mg/ml by hydrating dry lipid films in a buffer containing 150 mM NaCl and 5 mM Tris/HCl, pH 7.4, and allowing them to swell for 1 h at a temperature above the phase-transition temperature of the corresponding phospholipid. Next the lipid dispersion (1 ml) was sonicated at 240 W with 10 bursts of 30 s (15 s between bursts) in an MSE tip sonifier, with a microtip (2 mm diameter). The temperatures of sonication were: 4 °C for egg-PC and LPC; 35 °C for DMPG; and 45 °C for DPPC, DPPG and DPPC/DPPG (7:3, w/w) and DPPC/egg-PG (7:3, w/w) mixtures. All vesicles were prepared freshly each day, just before the start of the experiment, and were kept at 37 °C during the course of titration experiments (unless otherwise stated). The phospholipid concentration was determined by measuring phosphorus by the method of Rouser et al. (1966).

For vesicle-size analysis in solution, quasielastic light scattering was used as described by Koppel (1972). Light-scattering measurements were performed in an Autosizer IIC Photon Correlation Spectrometer (Malvern Instruments) using a helium–neon laser as a source of incident light (λ = 632.8 nm) operating at 5 mW. Measurements were performed at 25 °C and 45 °C. Vesicle diameter for DPPC, DPPG and for the binary mixtures DPPC/DPPG (7:3, w/w) and DPPC/PG (7:3, w/w) was around 160–200 nm with a polydispersity index of 0.2. Vesicles were stable during the time of experiment.

**C.d.**

Spectra were obtained on a Jobin Yvon Mark III dichrograph fitted with a 250 W xenon lamp. Cells of 0.1 cm optical path were used, and the spectra were recorded in the far-u.v. region (250–200 nm) at 0.2 nm/s scanning speed. Five scans were accumulated and averaged for each spectrum. Experiments were repeated at least twice with different protein preparations. The results were expressed in molar ellipticities (degrees·cm²·dmol⁻¹) assuming 110 Da as the average molecular mass per residue of both porcine and human SP-A. The effect of phospholipid vesicles on the c.d. spectrum of SP-A was checked, recording the spectra after the addition of increasing amounts of a concentrated suspension of DPPC/DPPG (7:3, w/w) vesicles or LPC micelles up to a lipid/protein weight ratio of 10:1. Similar amounts of lipid were added to a buffer sample in order to subtract the appropriate baseline for each spectrum.

**Fluorescence measurements**

All fluorescence experiments were carried out on a Perkin–Elmer MPF-44E spectrofluorimeter operated in the ratio mode. Cells of 0.2 cm optical path were used. The slit widths were 7 nm and 5 nm for the excitation and emission beams respectively.
Fluorescence spectra of SP-A were measured at 37 °C (unless otherwise stated) in 0.3 ml of 5 mM Tris/HCl buffer, pH 7.4, in the presence or absence of 150 mM NaCl. The final protein concentration of SP-A was in the range 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. Evaluation of the tyrosine and tryptophan contributions to the spectra excited at 275 nm was achieved as previously described (Eisinger, 1969). Briefly, the spectrum obtained after excitation at 295 nm was normalized at 380 nm with the spectrum obtained after excitation at 275 nm. As the protein fluorescence emission above 380 nm only arises from tryptophan residues, the differences between the two emission spectra gave the contribution of tryptophan residues to the total protein fluorescence spectrum.

Titration experiments

The phospholipid/SP-A interaction was studied by monitoring the changes in the total protein fluorescence spectrum on addition of different types of phospholipid vesicles or micelles of LPC. First, the fluorescence spectrum of SP-A (2 μg) was recorded in 0.3 ml of 5 mM Tris/HCl buffer, pH 7.4, in the presence or absence of 150 mM NaCl, after 10 min of equilibration of the protein at 37 °C (unless otherwise stated). Subsequently, the titration experiment was started by adding increasing amounts of a concentrated vesicle suspension (typically 3 mg/ml, maintained at 37 °C) to the protein solution in the cuvette. The fluorescence intensity readings were corrected for (i) the dilution caused by vesicle addition, (ii) the vesicle blank (scattering) and (iii) the inner filter effect. The latter correction factor was determined according to the equation

\[ F_c = F_m 10^{(-A_{ex}+A_{em})/2} \]

(Lakowicz, 1983) 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 (275 nm) and the emission wavelength 330 nm respectively. The absorbance of the samples was measured during the titration experiments after each addition of vesicles by using a Beckman DU-8 spectrophotometer with cell holder thermostatically controlled at the same temperature as that of the fluorescence experiment (typically 37 °C). In all lipid-titration experiments, the \( A_{295} \) was always less than 0.1.

Quenching experiments

Quenching by acrylamide was 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: phospholipid vesicles were added to the protein solution [lipid/SP-A weight ratio of 4:1 (200:1 molar ratio, considering SP-A monomer)], and 10 min after equilibration at 37 °C the spectrum of SP-A was recorded. Afterwards samples 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 vesicle blank. In addition, an inner filter correction for the absorbance by acrylamide was performed.

Quenching studies were analysed by the classical Stern–Volmer equation for collisional quenching (Lehrer, 1971)

\[ F_0/F = 1 + K_{sv}[Q] \]

where \( F_0 \) and \( F \) are the corrected emission intensities in the absence and presence of the quencher [Q], and \( K_{sv} \) is the Stern–Volmer dynamic quenching constant. \( K_{sv} \) values were calculated by the regression of the initial linear portion of the Stern–Volmer plot. We did not calculate the static quenching constant by acrylamide (Eftink and Ghiron, 1976, 1981).

Phospholipid vesicle aggregation assay

SP-A-induced phospholipid vesicle aggregation assays were performed at 37 °C on a Beckman DU-8 spectrophotometer, measuring the change in \( A_{400} \) as follows: phospholipid vesicles (30 μg) and Ca\(^{2+}\) were added to both the sample (S) and the reference (R) cuvette in a total volume of 0.3 ml of 5 mM Tris/HCl buffer, pH 7.4, in the presence or absence of 150 mM NaCl. After 10 min equilibration at 37 °C, SP-A (3 μg) was added to the sample cuvette and the change in \( A_{400} \) was monitored for 1 min intervals. The process was reversed by adding EDTA. The scatter contribution derived from Ca\(^{2+}\)-dependent self-aggregation of SP-A was also analysed in experiments without lipids. In addition, the extent of Ca\(^{2+}\)-dependent aggregation of lipid vesicles in the absence of SP-A was evaluated.

All data reported in the Figures of this work were obtained from three or four different preparations of porcine SP-A, and from two different preparations of human SP-A. For each preparation, experiments were repeated twice.

RESULTS

Characterization of porcine and human SP-A

SDS/PAGE analysis of SP-A prepared from pig lung showed a major band at 40 kDa as well as a high-molecular-mass band at 66 kDa similar to that found for human SP-A.

The amino acid compositions obtained from porcine and human SP-A were similar and correlated with the composition calculated from the genomic sequence of White et al. (1985). The amino acid composition of porcine SP-A was also similar to that reported by Suzuki et al. (1989). The tryptophan content of porcine and human SP-A was calculated as described by Beaven and Holiday (1952). Porcine SP-A contains only two tryptophans like SP-A from other species [human (White et al., 1985); dog (Benson et al., 1985); rat (Sano et al., 1987); and rabbit (Boggaram et al.; 1988)]. The two tryptophans in porcine SP-A are presumably located in the globular C-terminal region as in SP-A from other species studied so far.

Fluorescence emission spectra of porcine and human SP-A on excitation at 275 nm are shown in Figure 1. In order to analyse the contribution of both tryptophan and tyrosine fluorescence when excited at 275 nm, the fluorescence emission spectrum on excitation at 295 nm (where the absorbance of tyrosine is almost zero) was also recorded and normalized with the spectrum for excitation at 275 nm by considering that the fluorescence emission above 380 nm only arises from tryptophan residues (Eisinger, 1969). The normalized spectrum given in Figure 1 as W indicates the contribution of tryptophan residues to the protein fluorescence. The difference between the emission spectrum obtained after excitation at 275 nm and the normalized spectrum gave the contribution of tyrosine residues to the protein fluorescence and resulted in a band centred at about 305 nm (band Y in Figure 1). The protein fluorescence is dominated by the contribution of tryptophan residues. The spectrum of SP-A is characterized by two maxima at about 326 and 337 nm, indicating heterogeneity in the emission of the two tryptophan residues for SP-A. The maxima as 326 and 337 nm are blue-shifted in comparison with that of free tryptophan model systems, revealing a partially
buried character for these fluorophores, which is typical of folded proteins. When SP-A is denatured in 6 M guanidinium chloride, the fluorescence emission intensity decreases and the position of fluorescence emission maxima of tryptophan residues shifts to approx. 350 nm, which indicates that, under these conditions, the tryptophan residues become exposed to the solvent.

Far-u.v. c.d. spectra of porcine and human SP-A are shown in Figure 2. Both spectra are characterized by a shoulder at 220 nm and a strong negative extremum at 205 nm. They are comparable in shape and magnitude with published spectra for canine, human and recombinant SP-A (Voss et al., 1988; King et al., 1988; Haagsman et al., 1989).

Interaction of SP-A with lipid vesicles

First the interaction of SP-A with various lipids was investigated by measuring the protein fluorescence intensity and wavelength of emission maxima in the absence and presence of different concentrations of phospholipids. The lipid systems tested were DPPC, DPPG, DPPC/DPPG (7:3, w/w), egg-PC and LPC.

The interaction of SP-A with various phospholipid vesicles results in an increase in fluorescence emission intensity of the tryptophan residues without any shift in the wavelength of the emission maxima. Figure 3 shows that the fluorescence emission intensity of both porcine (a) and human (b) SP-A markedly increases with increasing amounts of DPPC present, whereas it is virtually unaffected by egg-PC. A less pronounced increase in the fluorescence emission intensity of SP-A occurs on addition of increasing amounts of either DPPG or DPPC/DPPG (7:3, w/w) instead of DPPC vesicles, indicating that the presence of negatively charged phospholipids decreases lipid–SP-A interaction. On the other hand, the interaction of SP-A with LPC micelles is also less pronounced than with DPPC vesicles, indicating that the sn-2-positioned acyl chain could be important in this interaction. The absence of the fatty acid at the sn-2 position determines the molecular shape of these amphipathic molecules and the form in which they self-associate in water.

In order to exclude the occurrence of artifacts caused by scattering by the membranes, we titrated solutions of N-acetyl-L-tryptophanamide with increasing amounts of DPPC, DPPG, egg-PC and LPC. At all LPC concentrations, spectra recorded were superimposable on those of N-acetyl-L-tryptophanamide alone. For DPPC, DPPG and egg-PC there was only a slight increase in the quantum yield for tryptophan emission at lipid concentrations higher than 33 µg/ml (i.e. at a lipid/protein weight ratio higher than 5:1 in our assay conditions).

The two tryptophan residues of SP-A seem to be partially buried in the protein matrix. The quantum yield of buried tryptophans is controlled mainly by static quenching processes, which depend on the proximity and orientation of quencher groups such as disulphide, thiol and amide. Thus the observed increase in the fluorescence emission intensity of SP-A produced by interaction of SP-A with phospholipid vesicles could arise from a decreased static quenching caused by the resulting conformational change. This conformational change seems to occur without modification of the polarity in the environment of the tryptophan residues, as the position of fluorescence emission maxima of tryptophan residues in SP-A did not change on interaction with phospholipids, and it is known that the fluor-
nescence of the indole group shows blue or red shifts when the polarity of the microenvironment decreases or increases respectively (Lakowicz, 1983).

An additional criterion for revealing phospholipid–SP-A interaction is the analysis of the accessibility of SP-A fluorophores to acrylamide, an efficient neutral collisional quencher of indole derivatives capable of permeating the protein matrix (Eftink and Ghiron, 1976). Figure 4 shows Stern–Volmer plots of the effect of acrylamide on fluorescence emission intensity in both porcine and human SP-A, measured in the presence and absence of phospholipids at a lipid/protein weight ratio of 4:1 (200:1 molar ratio). The interaction of lipid vesicles with both porcine and human SP-A does not cause a complete shielding of tryptophan residues from quenching by acrylamide, and the extent of shielding depends on the type of lipid interacting. The interaction of DPPC vesicles with porcine SP-A markedly decreases the accessibility of SP-A fluorophores to acrylamide, whereas in the presence of either negatively charged DPPG vesicles or LPC micelles this shielding from quenching by acrylamide is less pronounced. Human SP-A shows a lower acrylamide quenching constant (Ksv) in the absence of phospholipids than porcine SP-A (Ksv values for human and porcine SP-A at physiological ionic strength are 6.5 M⁻¹ and 9.3 M⁻¹ respectively). In the presence of DPPC, human SP-A shows less shielding from quenching by acrylamide than porcine SP-A, and no shielding is observed in the presence of DPPG vesicles.

Figure 4 also illustrates a downward-curving Stern–Volmer plot in the absence of phospholipid vesicles, indicating that tryptophan residues in both porcine and human SP-A have widely different accessibilities to quencher at physiological ionic strength (Eftink and Ghiron 1976). However, on interaction with DPPC vesicles, a linear Stern–Volmer plot is found, indicating that SP-A fluorophores differ slightly in accessibility to quencher, probably as a consequence of the conformational change on the protein molecule induced by lipids.

The effect of phospholipids on the structure of SP-A was also investigated by c.d. The c.d. spectra of porcine SP-A in the absence and presence of increasing amounts of DPPC/DPPG (7:3, w/w) or LPC micelles were superimposable in the interval between 210 and 250 nm, whereas below 210 nm a decrease in ellipticity in the presence of both types of lipids was observed (results not shown). Because the presence of phospholipids decreases the signal-to-noise ratio in the region below 210 nm, the changes in ellipticity induced by lipids were not significant enough to infer that phospholipids produce changes in the secondary structure of SP-A.

All fluorescence experiments were carried out without the addition of Ca²⁺ because, in the presence of millimolar concentrations of Ca²⁺, both SP-A-induced vesicle aggregation and self-association of SP-A take place (King et al., 1983; Hawgood et al., 1985, Efstrati et al., 1987). Analysis of Ca²⁺-dependent aggregation of different types of phospholipids induced by human SP-A is shown in Figure 5(a). Maximum aggregation is observed for DPPC/egg-PG (7:3, w/w) vesicles. The extent of aggregation of DPPC is similar to that of DPPG, whereas aggregation of egg-PC vesicles is very low and that of LPC micelles does not occur as the increase in absorbance found with LPC corresponds to the scatter contribution of Ca²⁺-dependent self-association of SP-A. Similar results were found for porcine SP-A. In the absence of SP-A (Figure 5b), at the same Ca²⁺ concentration (5 mM), maximum vesicle aggregation is found for DPPG. DPPC/egg-PG (7:3, w/w) vesicles also aggregate in a Ca²⁺-dependent manner, but no aggregation is registered for DPPC, egg-PC or LPC. It is clear that the extent of Ca²⁺-dependent aggregation of phospholipid vesicles, before the addition of SP-A, depends on the amount of negatively charged phospholipids in the vesicle. Thus the change in absorbance found after the addition of SP-A clearly reflects the selective interaction of SP-A with vesicles containing DPPC.

**Effect of the Ionic Environment on Phospholipid–SP-A Interaction**

From the intrinsic fluorescence measurements as well as the quenching experiments, it is clear that the interaction of SP-A with DPPC vesicles is more pronounced than with DPPG vesicles. To find out whether the interaction of SP-A with DPPG could be enhanced by decreasing the ionic strength of the medium, we next performed titration experiments at very low ionic strength (Figure 6). Interestingly, negatively charged vesicles of either DPPG or DPPC/DPPG (7:3, w/w) hardly interact with porcine or human SP-A at low ionic strength.
Phospholipid vesicles were prepared at a phospholipid concentration of 3 mg/ml in 5 mM Tris/HCl buffer, pH 7.4, containing 150 mM NaCl. For titration experiments at low ionic strength, 1 µl portions of concentrated vesicle suspension were added to the protein solution which was in 5 mM Tris/HCl buffer, pH 7.4, and experiments were performed at 37 °C as described in the Experimental section. ○, DPPC; ■, DPPG; □, DPPC/DPPG (7:3, w/w); ○, egg-PC; ▲, LPC.

Effect of the physical state of the vesicles

The preferential interaction of SP-A with DPPC compared with egg-PC could be explained by either a specific interaction of SP-A with disaturated molecular species of phospholipids or a preference for the gel state of the lipid. At the temperature of the fluorescence experiments (37 °C), DPPC exists in a gel state (Tm = 41.5 °C), whereas egg yolk PC exists in a fluid state above -10 °C (Ladbrooke and Chapman, 1969). To investigate whether the protein fluorescence was sensitive to the physical state of DPPC vesicles, we performed titration experiments at 20 °C and 45 °C. No loss of triple-helical structure of SP-A is expected to occur at 45 °C as the midpoint transition ‘melting’ temperature was reported to be about 52 °C for canine and human SP-A (Haagsman et al., 1989). Figure 8 shows that the increase in the relative fluorescence intensity of both porcine and human SP-A with increasing amounts of DPPC is much more pronounced at 20 °C than at 45 °C, indicating that the interaction of SP-A with DPPC vesicles is stronger in the gel phase than in the liquid-crystalline phase. Similar results were obtained when SP-A was heated for 10 min at 45 °C and cooled to 20 °C before the addition of DPPC vesicles and the changes in the fluorescence spectrum of SP-A monitored at 20 °C.
residues of SP-A (Figure 1). The blue-shifted tryptophan emission maxima when compared with that of free tryptophan revealed a shielding of the tryptophan residues from water by the protein matrix.

The effect of different phospholipids on the intrinsic fluorescence characteristics (fluorescence emission intensity, emission maximum wavelength and susceptibility to quenching by acrylamide) of porcine and human SP-A has been studied. The fluorescence emission intensity of SP-A increased on interaction with DPPC, DPPG, DPPC/DPPG (7:3, w/w) vesicles and LPC micelles. These changes in intrinsic fluorescence were found to be more pronounced in the presence of DPPC vesicles (Figure 3). In addition, quenching experiments demonstrated a shielding of tryptophan fluorescence from quenching by acrylamide on interaction of SP-A with phospholipid vesicles. This shielding was also more pronounced in the presence of DPPC vesicles (Figure 4).

Interestingly, the interaction of SP-A with phospholipids was not accompanied by a blue shift in maximum emission wavelengths. The only two tryptophan residues found in SP-A from human, dog, rat and rabbit, are located in the C-terminal 38 amino acids (at positions 191 and 213 in all species studied until now) (Benson et al., 1985; White et al., 1985; Sano et al., 1987; Boggaram et al., 1988). On the other hand, the lipid-binding site of SP-A is supposed to be located at the linking region of SP-A according to Ross et al. (1986), or more likely at the end of the N-terminal segment of SP-A (Voorhout et al., 1991). Therefore tryptophan residues are not involved directly in the lipid–SP-A interaction. However, the interaction of SP-A with phospholipids, especially with DPPC vesicles, seems to affect protein conformation, as both intrinsic fluorescence of SP-A and tryptophan fluorescence quenching by acrylamide markedly changed on addition of DPPC vesicles. The observed increase in the intrinsic fluorescence of SP-A on interaction with DPPC vesicles could indicate a larger shielding of SP-A fluorophores from the polarizable groups of either the protein itself (disulphide, thiol and amine) or the solvent, which are responsible for tryptophan quenching. The observed decrease in the extent of quenching by acrylamide in the presence of DPPC might confirm this hypothesis. On the other hand, the absence of any significant blue shift in the position of the fluorescence emission maxima of tryptophan residues could be interpreted in terms of no polarity changes in the environment of the tryptophan residues on interaction of SP-A with DPPC vesicles, which is in agreement with the fact that SP-A tryptophan residues are not located at the lipid-binding site of the protein. Similar effects, i.e. increased tryptophan quantum yield with no significant shift in the position of its emission maximum, have been reported for the tryptophan fluorescence of both the antitumour protein α-sarcine interacting with DMPC vesicles (Gasset et al., 1991) and the myelin proteolipid protein when complexed with lyssolecithin (Cockle et al., 1978). In both cases, hydrophobic protein–lipid interactions were concluded.

Different methods have been used to study lipid–SP-A interactions. King et al. (1983) analysed the interaction of SP-A with different mixtures of lipids by sedimentation methods that separated associated and free constituents. They concluded that maximum association of the apolipoprotein SP-A occurred with DPPC/DPPG (17:3, w/w) vesicles, which was especially clear in the presence of 3 mM Ca\(^{2+}\). We found that Ca\(^{2+}\)-dependent vesicle aggregation induced by SP-A was higher with this binary mixture than with pure DPPC (Figure 5a), in agreement with King’s results. On the other hand, Kuroki and Akino (1991) reported the direct binding of \(^{125}\)I-SP-A to different classes of phospholipids adsorbed to silica gel, indicating that SP-A spec-

---

**Figure 9** Effect of temperature on SP-A-induced vesicle aggregation

Sample and reference cuvettes (SR) were filled with 30 µg of either DPPC (○), or DPPC/DPPG (7:3, w/w) (△), or DMPC (□) in a total volume of 0.3 ml of 5 mM Tris/HCl buffer, pH 7.4, containing 1 mM Ca\(^{2+}\) and 150 mM NaCl. After 10 min equilibration at the indicated temperatures, aggregation induced by SP-A was started by the addition of 3 µg of porcine SP-A into the sample cuvette, and the absorbance was monitored for a further 10 min at the corresponding temperature. The aggregation rate was expressed as a percentage of the rate observed at 20 °C.

The effect of temperature on vesicle aggregation induced by SP-A has also been studied (Figure 9). Vesicle aggregation is higher below the phase-transition temperature of DPPC (41.5 °C), DPPC/DPPG (7:3, w/w) (41.5 °C) or DMPC (23 °C), than above it. In other experiments, SP-A was heated at 50 °C for 10 min in the absence of lipids. After cooling to 37 °C, SP-A was added to DPPC vesicles and vesicle aggregation at 37 °C assessed. The rate and extent of SP-A-induced vesicle aggregation is similar before and after mild heat treatment of SP-A at 50 °C.

**DISCUSSION**

SP-A from different species (human, dog, rat, rabbit) as well as recombinant SP-A have been previously characterized with respect to amino acid composition, amino acid sequence, electrophoretic migration under denaturing and non-denaturing conditions, isoelectric point, secondary structure and susceptibility to bacterial collagenase (White et al., 1985; Benson et al., 1985; Sano et al., 1987; Boggaram et al., 1988; Voss et al., 1988; King et al., 1989; Haagsman et al., 1989). Few studies have been carried out with porcine SP-A. Thus, as a preliminary to studying phospholipid–SP-A interactions, we have determined some structural characteristics of both porcine and human SP-A, such as amino acid composition, electrophoretic migration and secondary structure. We found that the two proteins were comparable and the data were similar to those reported by others (White et al., 1985; Suzuki et al., 1989; King et al., 1989; Haagsman et al., 1989).

In addition, we have determined the fluorescence characteristics of porcine and human SP-A. The fluorescence emission spectrum of both proteins on excitation at either 275 or 295 nm was characterized by two maxima at about 326 and 337 nm, indicating heterogeneity in the emission of the two tryptophan
ifically bound DPPC and that DPPC-binding activity of SP-A was dependent on Ca\(^{2+}\) ions. In those experiments, the Ca\(^{2+}\) concentration in the binding medium was 2 mM. Under these conditions, self-aggregation of SP-A takes place. Therefore the detection of the binding of SP-A to DPPC might be enhanced in the presence of Ca\(^{2+}\) by self-aggregation of SP-A on the lipid-binding site, although a possible Ca\(^{2+}\)-dependent lipid–protein interaction cannot be excluded from these experiments. Our results partially differ from those of Kuroki and Akino. We did not find a specific interaction of SP-A with DPPC, but a preferential interaction with this phospholipid. In addition, we found that SP-A interacted with DPPC vesicles in a Ca\(^{2+}\)-independent manner. The discrepancies between the current results and those of Kuroki and Akino could be due to the different experimental systems used.

Another point of interest is the effect of the ionic strength on phospholipid–SP-A interaction. Physiological ionic strength appears to be required for the association of SP-A with phospholipids, as at low ionic strength the interaction of SP-A with DPPC vesicles decreased and that with negatively charged vesicles of either DPPG or DPPC/DPPG (7:3, w/w) completely disappeared (Figure 6). In addition, Oosting et al. (1991) reported that SP-A did not bind to DPPC/egg-PG (7:3, w/w) vesicles in water. The absence of interaction of SP-A with negatively charged phospholipid vesicles at low ionic strength might be due to electrostatic repulsion between the negative charge of phospholipids and the surface charge on the protein contributed by carboxyl groups [pI 4.8–5.2 according to Benson et al. (1985)]. It is of interest to note that the fluorescence characteristics of SP-A (i.e., tryptophan fluorescence intensity and susceptibility to quenching by acrylamide) in the absence of NaCl were different from when the buffer contained 100 or 150 mM NaCl (C. Casals, E. Miguel and J. Perez-Gil, unpublished work). SP-A seems to exhibit a different conformation and/or aggregation state at physiological ionic strength, which allows acidic phospholipid–protein interactions. Furthermore, we also observed that the ionic strength of the medium had an effect on the phospholipid aggregation activity of SP-A which decreased at low ionic strength (Figure 7).

The physical state of phospholipid vesicles seems to influence lipid–SP-A interaction. We have demonstrated here that the effect of DPPC vesicles on the fluorescence emission intensity of SP-A was much stronger when vesicles were in the gel state than when they were in the liquid-crystalline state (Figure 8). In addition, SP-A-induced aggregation of phospholipid vesicles was also dependent on the physical state of the vesicles (Figure 9). The extent of aggregation of DPPC, DPPC/DPPG (7:3, w/w) or DMPO was many times higher below than above the phase-transition temperature of the corresponding phospholipid. These strongly indicate that the interaction of SP-A with phospholipid vesicles depends on the physical state of the vesicles and could partially explain the poor interaction of SP-A with egg-PG vesicles, which at the temperature of the fluorescence and aggregation experiments (typically 37 °C) were in a fluid state. These results further confirm previous studies of King et al. (1986) who demonstrated by sedimentation methods that SP-A had a marked preference for binding to disaturated phospholipids in the gel state. SP-A is not unique in its preference for interacting with lipid vesicles in the gel phase. Phospholipase A\(_2\) binds to DPPC bilayer (or DPPC monolayer) in a Ca\(^{2+}\)-independent process that requires the lipid to be in the gel phase. After binding, activation of the enzyme–substrate complex requires Ca\(^{2+}\) and the existence of structural irregularities in the lipid bilayer (Lichtenberg et al., 1986; Grainger et al., 1990).

From these studies it is clear that the interaction of SP-A with phospholipids causes a conformational change in the protein molecule, and that the nature of the polar head group of phospholipids as well as their physical state in aqueous systems influence phospholipid–SP-A interaction. The interaction of SP-A with DPPC was more pronounced than with other phospholipids such as DPPG (with the same acyl chains and \(T_m\) but different polar head group) or egg-PC (with the same polar head group but different acyl chains and \(T_m\)). SP-A seems to show preference for interacting with the specific head group/backbone conformation of highly orderly DPPC vesicles at 37 °C. However, whether SP-A can penetrate the bilayer and interact specifically with saturated acyl chains of phospholipids is currently unresolved. Studies of Reilly et al. (1989) on SP-A/phospholipid mixtures [SP-A/(DPPC/DPPG, 7:3, w/w)] molar ratio 1:25] indicated that SP-A induced a shift of Fourier transform–i.r. melting curve with 5–6 °C increase from the protein-free system, without any change in the enthalpy of the transition. The marked SP-A-mediated shift of the peak maximum of the transition, at high protein to lipid molar ratio, without change in the enthalpy of the transition could suggest that only electrostatic forces are involved in the interaction of SP-A with phospholipids. On the other hand, Kuroki and Akino (1991) found that SP-A bound strongly to DPPC immobilized on silica but poorly to egg-PC. Because acyl chains of phospholipids immobilized on silica are not buried in the interior of a bilayer, they concluded that SP-A bound to phospholipids in an acyl-chain-specific way. The physical state of phospholipids in a silica matrix is not understood. However, it is conceivable that lipids form aggregates, such as monolayers or sheets of monolayers. Owing to its high hydrophobicity and its uniform size, DPPC is better suited to forming high-order aggregates on a silica matrix than unsaturated PC. The packing of phospholipids in lipid aggregates imposes certain constraints on the lipid conformation (including head group and backbone). If SP-A does recognize the specific head group/backbone conformation of high-order DPPC aggregates, it might not be able to recognize egg-PC provided that unsaturated PC does not form such aggregates. This is an alternative interpretation of Kuroki and Akino’s results which could also explain why SP-A did not bind to palmitic acid immobilized on silica.

The preferential interaction of SP-A with DPPC could be important in the metabolic cycle of pulmonary surfactant. First, SP-A may directly phospholipids, preferentially DPPC, to lamellar bodies and maybe other secretion vesicles that are highly enriched in this saturated phospholipid in comparison with other subcellular membranes of type II cells (Schlame et al., 1988). We have reported here that, at physiological ionic strength, SP-A interacts strongly with DPPC in a Ca\(^{2+}\)-independent process. Therefore at any intracellular concentration of Ca\(^{2+}\), SP-A would be associated with DPPC and both would probably follow the same intracellular path until being secreted or the same intracellular path after reuptake by type II cells. Second, the selective interaction of SP-A with DPPC could be important in the generation of a DPPC-enriched film at the alveolar air/fluid interface during the adsorption process. The posterior ‘squeeze-out’ of other remaining lipids and maybe proteins during film compression might give rise to maximum enrichment of DPPC in the monolayer which is essential for withstanding high surface pressures, and thus for preventing alveolar collapse.
REFERENCES


Received 29 April 1993/14 July 1993; accepted 22 July 1993

Rouser, G., Slakotis, A. N. and Fliesser, S. (1968) Lipids 12, 505–510