Comparison of DPPC and DPPG Environments in Pulmonary Surfactant Models

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ABSTRACT Deuterium nuclear magnetic resonance was used to monitor lipid acyl-chain orientational order in suspensions of dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) containing Ca^{2+} and the lung surfactant proteins SP-A and SP-B separately and together. To distinguish between protein-lipid interactions involving the PC and PG lipid headgroups and to examine whether such interactions might influence spatial distribution of lipids within the bilayer, acyl chains on either the DPPC or the DPPG component of the mixture were deuterated. The lipid components of the resulting mixtures were thus either DPPC-d_{62}/DPPG (7:3) or DPPC/DPPG-d_{62} (7:3), respectively. SP-A had little effect on DPPC-d_{62} chain order but did narrow the temperature range over which DPPG-d_{62} ordered at the liquid-crystal-to-gel transition. No segregation of lipid components was seen for temperatures above or below the transition. Near the transition, though, there was evidence that SP-A promoted preferential depletion of DPPG from liquid crystalline domains in the temperature range over which gel and liquid crystal domains coexist. SP-B lowered average chain order of both lipids both above and below the main transition. The perturbations of chain order by SP-A and SP-B together were smaller than by SP-B alone. This reduction in perturbation of the lipids by the additional presence of SP-A likely indicated a strong interaction between SP-A and SP-B. The competitive lipid-lipid, lipid-protein, and protein-protein interactions suggested by these observations presumably facilitate the reorganization of surfactant material inherent in the transformation from lamellar bodies to a functional surfactant layer.

INTRODUCTION

Pulmonary surfactant is a complex mixture of lipids and proteins synthesized and secreted by type II pneumocytes in the lining of the alveoli. The role of this surfactant is to decrease the work associated with breathing by reducing the surface tension at the air-water interface in the lung (1). The main constituents of pulmonary surfactant are lipids and proteins, which account for ~90% and 10% of the surfactant mass, respectively (2–4). Of the lipids, ~85–90% are phospholipids of which ~80% are phosphatidylcholine (PC) lipids and ~10% are phosphatidylglycerol (PG) lipids (4,5). The fraction of PC accounted for by dipalmitoylphosphatidylcholine (DPPC), a disaturated PC, ranges from ~36% in rabbit to 54% in humans (5). Formation of a DPPC-rich monolayer at the interface is thought to reduce surface tension and thus facilitate respiration. An alternative model for surfactant surface dynamics (6) implies that selective enrichment of the monolayer is not necessary to achieve ultra-low surface tensions. Nevertheless, unless there is selective depletion of DPPC in the monolayer by some mechanism that has not been observed in model surfactant, the monolayer is still expected to be enriched in saturated PC, especially DPPC. Regardless of the extent to which DPPC is responsible for surface activity, surfactant physiological effectiveness depends on the presence of other components, including unsaturated lipids, anionic lipids such as PG, surfactant proteins, and Ca^{2+}, that increase the rate of adsorption to the air-water interface (7).

The most abundant of all surfactant proteins by mass is surfactant protein A (SP-A), a large, water-soluble glycoprotein with a collagen-like N-terminus and variable glycosylation of the C terminus (8–13). Trimers comprising 35 kDa monomers associate into octadecamers with a molecular mass of ~700 kDa (14,15). SP-A has an isoelectric point of 4–5 and is thus negatively charged at physiological pH (16). SP-A has a higher affinity for DPPC than for 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG) (13) but DPPG enhances the ability of SP-A to interact with mixtures of lipids in the presence of Ca^{2+} (17). SP-A has been reported to associate with phosphatidylcholine bilayers via its C-terminal region (18,19) and there is evidence suggesting that SP-A interacts at the corners of bilayer tubules in a unique structure, tubular myelin, that can form in surfactant material (19,20). SP-A also enhances the ability of SP-B to promote uptake of DPPC into monolayers (21). SP-A is also reported to have a prominent role in the lung innate host defense system (22,23). Like SP-A, surfactant protein D (SP-D) is a member of the Ca^{2+}-dependent carbohydrate-binding collectin family. Most evidence to date implicates SP-D in host immune defense and not normally in surfactant biophysical function (22–25).

Surfactant protein B (SP-B) has a monomeric molecular mass of 8.7 kDa but, in its native state, forms cysteine-linked dimers with an apparent molecular mass of 17.4 kDa. It contains regions of amphipathic, α-helical structure and has a charge of +12 at physiological pH (26,27). SP-B has been shown to promote lipid mixing and fusion of vesicles

Submitted December 6, 2006, and accepted for publication March 14, 2007.
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Editor: Lukas T. Tamm.

0006-3495/07/07/164/12 $2.00
doi: 10.1529/biophysj.106.102681

containing anionic phospholipids (28). SP-B has also been reported to enhance the squeeze-out of acidic phospholipids from the air-water interface during compression and the subsequent respreading of these lipids during expansion (29). The way in which SP-B inserts into phospholipid bilayers is sensitive to the mode of reconstitution (30).

Surfactant protein C (SP-C) is a small, hydrophobic protein containing 35 residues with a molecular mass of 4.2 kDa. The N-terminal region contains two thioester-linked palmitoyl groups (26,31), which increase the hydrophobic nature of the protein, and in the C-terminal region, two-thirds of the protein is α-helical.

Native surfactant material undergoes structural reorganization during the transformation from lamellar bodies to the surface-active monolayer. Tubular myelin, suggested as a possible precursor for the active film, is characterized in electron micrographs by the appearance of a lattice-like pattern (32–35). The in vitro requirements for production of tubular myelin structure include SP-A, SP-B, DPPC, PG, and Ca^{2+} (28,36,37). Our own studies of model surfactant material containing DPPC, egg-PG, SP-A, SP-B, and Ca^{2+} (M. R. Morrow, S. Harris, A. Dico, J. Stewart, S. Taneva, and K. M. W. Keough, unpublished) have found lattice structures with a characteristic spacing of ~50 nm, a length scale consistent with previous reports of tubular myelin structure (37).

The apparent requirement for SP-A, a hydrophilic protein, plus anionic lipid to produce tubular myelin structures implies that interactions at the bilayer surface are important for the formation of this structure and thus for the reorganization of lung surfactant material from lamellar bodies to a functional layer at the air-water interface. In the current study, we have used ^{2}H-NMR to observe two saturated-chain lipids, 1,2-perdeuterodipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC-d_{62}) and 1,2-perdeuterodipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG-d_{62}), in model surfactant material containing both SP-A and SP-B in the presence of Ca^{2+}. In each sample, the acyl chains of one or the other lipid species were perdeuterated. Because DPPC and DPPG differ only in headgroup charge and structure, comparison of their behaviors within a given model system allows us to isolate the headgroup-specific effects from those due to lipid chain unsaturation. It thus provides a way to distinguish between headgroup-specific protein-lipid interactions and to examine whether such interactions can influence the spatial distribution of lipid components within the bilayer.

Except for the PG component being in the form of a saturated-chain lipid rather than a mixture of unsaturated lipids, the proportions of lipid and protein used in this work are based on model systems, containing DPPC, egg-PG, SP-A, and SP-B, in which tubular myelin has been observed (36,37). Similarly-proportioned mixtures containing DPPC in place of egg-PG do not show the characteristic tubular myelin lattice structure but multilamellar structures with layer separations of ~25 nm can be seen (J. Stewart, M. R. Morrow, and K. M. W. Keough, unpublished). This spacing likely reflects accommodation of SP-A between adjacent bilayer surfaces (37). While the absence of unsaturated-chain lipids apparently precludes formation of tubular myelin, interactions within the saturated-lipid model system are still relevant to knowing how interactions at the bilayer surface can contribute to organization of surfactant material.

Because the system studied here lacks unsaturated chains, it is not intended to mimic all aspects of natural lung surfactant. However, comparison of specific interactions involving PC and PG groups in such mixtures would be effectively impossible without using a synthetically labeled PG component and comparing otherwise identical, labeled PC and PG components in identical mixtures. In particular, differences between the interactions of PC and PG headgroups with surfactant proteins would be much less apparent in model systems containing unsaturated PG such as those that produce tubular myelin. Egg-PG, for example, is a mixture of saturated and unsaturated lipids with a broad transition lower than that of DPPC. Even without protein present, the main liquid-crystal-to-gel-phase transition in a mixture of DPPC and egg-PG (7:3) is $>5°$ wide (38) and differences in chain length and degree of unsaturation likely influence the spatial distribution of lipid components, particularly near the transition, independently of protein-lipid interactions. In the model system containing DPPC and DPPG plus SP-A and SP-B, the liquid-crystal-to-gel transition temperatures of the two saturated lipid components, in the presence of Ca^{2+}, are only slightly different. As a result, small perturbations resulting from interaction with the protein components of the mixture are more apparent.

^{2}H-NMR has been used previously to study interactions of individual lung surfactant proteins with single lipids or mixtures of lipids. One such study showed that in a bilayer of DPPC, the presence of SP-B, at a concentration of 17% (w/w), reduced chain order in both the gel and liquid crystalline phases (39). Lower concentrations ($\leq$11% (w/w)) of either synthetic SP-B monomers in DPPC (40) or natural SP-B in DPPC/DPPG mixed bilayers (41) had previously been found to have less effect on chain order but were found to significantly affect bilayer motions observable by quadrupole echo decay. ^{2}H-NMR was also used to study the effect of SP-A on mixtures of DPPC and egg-PG in the presence of Ca^{2+} (38). SP-A was found to reduce DPPC chain order in the gel phase but not in the liquid crystalline phase. Quadrupole echo-decay time studies showed that SP-A also altered bilayer motions in the liquid crystalline phase.

Samples for which ^{2}H-NMR spectra have been obtained here are suspensions of DPPC-d_{62}/DPPG (7:3) and DPPC/DPPG-d_{62} (7:3) in the presence of Ca^{2+} without protein and with SP-A and SP-B together. Because previous ^{2}H-NMR studies of bilayer systems with a single lung surfactant protein were carried out on differing compositions or combinations of components, samples of the lipid mixture interacting with either SP-A or SP-B separately have also been examined for comparison.

MATERIALS AND METHODS

Lipids

Chain-perdeuterated DPPC-d_{62} and DPPG-d_{62} were purchased from Avanti Polar Lipids (Alabaster, AL). Unlabeled DPPC and DPPG were obtained from Sigma Chemical (St. Louis, MO). The lipids were used without further purification and the concentration of lipid in each sample was determined by phosphorus analysis (42,43).

Protein preparation

SP-B

Surfactant protein B was obtained from extracts of porcine lung lavage (44–46). The protein was isolated and purified using gel exclusion chromatography on Sephadex LH-60 media (Pharmacia, Uppsala, Sweden) in chloroform/methanol 1:1 (v/v) containing 2% by volume of 0.1 M HCL. SDS-polyacrylamide gel electrophoresis (45) under nonreducing conditions yielded a band at ~18 kDa.

SP-A

Surfactant protein A was purified by injecting a suspension of porcine surfactant into 1-butanol and stirring for 30 min (46,47). The mixture was then centrifuged at 10,000 × *g* for 20 min at 4°C. The supernatant was removed and the precipitate dried under N_2 and then washed twice with 20 mL of buffer (20 mM *n*-octyl-β-D-glucopyranoside, 100 mM NaCl, 10 mM HEPES, pH 7.4). After centrifugation twice at 100,000 × *g* for 30 min at 4°C, the resultant pellet was resuspended in a small volume of 5 mM HEPES buffer, pH 7.4 and dialyzed against the same buffer for 48 h. Finally, the sample was centrifuged again at 100,000 × *g* for 30 min. The supernatant containing SP-A was stored in small aliquots at −20°C. Protein concentrations were estimated via the fluorescamine method using bovine serum albumin as the standard (48) and were verified by amino acid analysis (49).

Sample preparation

All samples were prepared in the same way except as noted below. The lipids, DPPC and DPPG at a molar ratio of 7:3 with one or the other chain-perdeuterated, were first dissolved and mixed in chloroform/methanol 1:1 (v/v). For each sample, the initial amount of lipid was ~20 mg. Lipid concentration in the solution was determined by phosphorus analysis (42,43). Except for samples containing lipids only or lipids plus SP-A alone, the amount of SP-B needed to obtain the required lipid/SP-B ratio was also added to this solution. The solution containing lipids, and SP-B if present, was then dried under N_2 and further dried overnight under vacuum. The lipids, plus SP-B if present, were then hydrated in 10 mL of buffer (10 mM TRIS/145 mM NaCl/1 mM EDTA/pH 7.4) at 46°C with periodic vortexing for 1 h. This suspension was then centrifuged at 50,000 × *g* and 4°C for 30 min. The resulting pellet was resuspended in 1 ml of the supernatant and lipid concentration was again determined using phosphorus analysis. Except for samples containing lipids only or lipids plus SP-B alone, the amount of SP-A needed to obtain the required SP-A/lipid ratio was then added to this suspension and allowed to mix with stirring by rotation of the sample container for 30 min at 37°C. Calcium in solution was then added to bring the overall Ca^{2+} concentration to 5 mM in a volume of 4 ml for samples without SP-A or 8–10 ml for samples containing SP-A. After further mixing by stirring overnight, the sample was compacted by light centrifugation in a bench-top centrifuge (<5000 × *g*) and transferred to an NMR sample tube in a volume of ~400 μl. This approach is intended to expose bilayers to SP-A before any calcium-induced aggregation of SP-A (50).

^{2}H-NMR

Wide-line ^{2}H-NMR spectra and echo-decay measurements were obtained using a locally constructed spectrometer and a 9.4 *T* superconducting solenoid (Magnex Scientific, Concord, CA). Spectra were acquired using a quadrupole echo pulse sequence (51) with π/2 pulse lengths of 3.7–4 μs. The two π/2 pulses in the quadrupole echo sequence were separated by 35 μs and the free-induction decays were oversampled by a factor of two to give effective dwell times of 4 μs and 2 μs for the liquid-crystalline and gel phase samples, respectively (52). The number of transients averaged was typically 8000 for samples containing DPPC-d_{62} and between 16,000 and 100,000 for samples containing DPPG-d_{62}. More transients were averaged in the latter case to compensate for the smaller concentration of deuterated lipid in samples containing DPPG-d_{62}. Transients that were averaged for a given spectrum were collected with a repetition time of 0.9 s. To facilitate calculation of first spectral moments, spectra were symmetrized by zeroing the imaginary channel before Fourier transformation.

The sample tube and probe coil were enclosed within a copper oven and temperatures were maintained to within ±0.1° of the set temperature using a digital temperature controller. Experiments were carried out for series of temperatures beginning at 55°C and decreasing in steps of 4°C or 2°C except near the transition where the steps were reduced to 1°C. Samples were allowed to reach thermal equilibrium before the start of each experiment by waiting at least 30 min after each cooling step before beginning data collection.

RESULTS

In this work, interactions involving surfactant protein have been inferred from comparisons between protein-lipid mixtures and protein-free lipid mixtures having the same lipid component deuterated. Nevertheless, it is useful to begin with a comparison of the protein-free DPPC-d_{62}/DPPG (7:3) and DPPC/DPPG-d_{62} (7:3) systems in the presence of Ca^{2+}. This DPPC/PG ratio is often used in model studies and was originally selected to conform to the DPPC/unsaturated-PG ratio in Artificial Lung Expanding Compound (ALEC) (53).

Fig. 1 shows selected ^{2}H NMR spectra for bilayers of DPPC-d_{62}/DPPG (7:3) and DPPC/DPPG-d_{62} (7:3) in the presence of 5 mM Ca^{2+} and the absence of surfactant protein. At higher temperatures, the spectra reflect the fast, axially symmetric reorientation characteristic of the liquid crystalline bilayer phase. In the liquid crystal phase, ^{2}H NMR spectra obtained from bilayers containing chain-perdeuterated lipid are superpositions of Pake doublets. The splitting between prominent edges of each doublet is proportional to the orientational order parameter, $S_{CD} = \frac{\langle 3\cos^2\theta - 1\rangle}{2}$, where θ is the angle between the carbon-deuterium bond and the symmetry axis for reorientation and the average is over motions that modulate the orientation-dependent quadrupole interaction with correlation times shorter than the characteristic timescale (~10^{-5} s) of the experiment (54). The orientational order parameter is smaller for CD bonds near the bilayer center, where motions are less constrained. In the gel phase, the broader spectra reflect chain motions that are slower and no longer axially symmetric on the timescale of the experiment. For both of the samples represented by Fig. 1, the spectra between 45°C and 41°C are superpositions of spectral components characteristic of liquid crystal and gel phases. From Fig. 1, *A* and *B*, the temperature range over which intensity shifts from liquid crystal to gel components on cooling through the two-phase region is roughly similar whether the observed component is DPPC or DPPG.

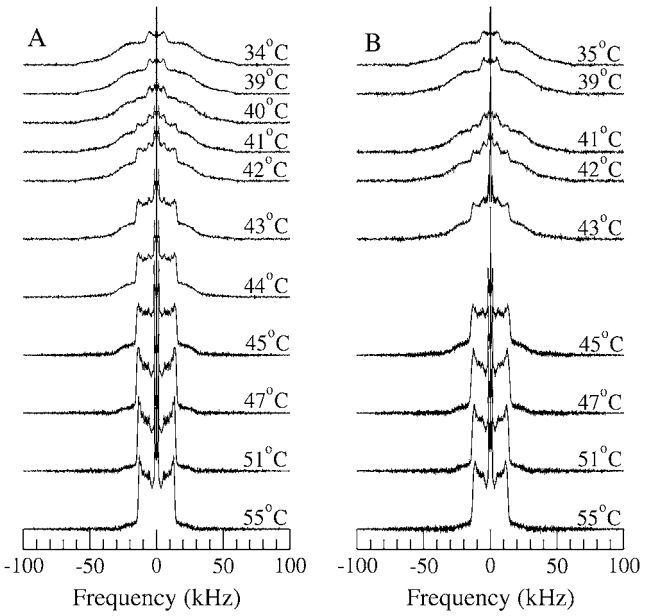

FIGURE 1 Deuterium NMR spectra at selected temperatures for (*A*) DPPC-d_{62}/DPPG (7:3) and (*B*) DPPC/DPPG-d_{62} (7:3) both hydrated in buffer containing 5 mM Ca^{2+} at pH 7.4.

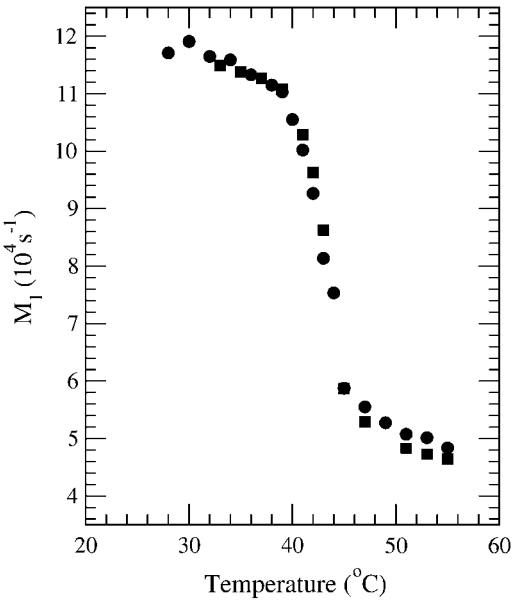

FIGURE 2 Temperature dependence of ^{2}H-NMR first spectral moments for ●, DPPC-d_{62}/DPPG (7:3) and ■, DPPC/DPPG-d_{62} (7:3) both hydrated in buffer containing 5 mM Ca^{2+} at pH 7.4.

The first spectral moment is defined as

$$M_1 = \frac{\int_0^\infty \nu f(\nu) d\nu}{\int_0^\infty f(\nu) d\nu}, \quad (1)$$

where $f(\nu)$ is the spectral intensity as a function of frequency and the integral is over half of the symmetric ^{2}H NMR spectrum. For spectra comparable to those obtained in this work, reproducibility of the first spectral moment is estimated to be ±3%. For chain-perdeuterated lipid bilayers, the first spectral moment is proportional to the intensity-weighted mean quadrupole splitting and thus to the average orientational order parameter. Fig. 2 shows the temperature dependence of M_1 obtained from spectra for bilayers of DPPC-d_{62}/DPPG (7:3) and DPPC/DPPG-d_{62} (7:3) in the presence of 5 mM Ca^{2+}. The rise in M_1 for DPPC/DPPG-d_{62} (7:3), on cooling through the transition, is slightly sharper than for DPPC-d_{62}/DPPG (7:3). Aside from this small difference, the two samples display very similar dependences of deuterated-component chain order on temperature through the transition.

From Figs. 1 and 2, chain order and the transition in either protein-free mixture displays little dependence on which of the lipid components is deuterated. This similarity facilitates the comparisons described below, but effects of differential deuteration and Ca^{2+} on the underlying temperature-composition phase diagrams should be noted.

In the absence of Ca^{2+}, DPPC, and DPPG bilayers undergo their main liquid-crystal-to-gel transitions at effectively identical temperatures. Complete chain perdeuteration of either lipid alone lowers its transition by 3–4°. In a mixture of one chain-perdeuterated lipid component and one normal lipid component, suppression of the transition midpoint temperature is approximately scaled in proportion to the overall extent of deuteration. It was previously observed, for example, that in the absence of Ca^{2+} the transition for DPPC-d_{62}/DPPG (7:3), with 70% lipid deuteration, is ~1° lower than that for DPPC/DPPG-d_{62} (7:3), with 30% lipid deuteration (41). Spectra displayed in that earlier work were consistent with coexistence of liquid crystal and gel domains over a narrow temperature range (≤1°). This reflects the slight difference in component transition temperatures resulting from deuteration of one component.

The effect of Ca^{2+} on the phase behavior of DPPC/DPPG mixtures was previously studied using mixtures with both lipids deuterated simultaneously (55). In the absence of Ca^{2+}, the DPPC-d_{62}/DPPG-d_{62} (7:3) transition was sharp and unshifted relative to that for DPPC-d_{62} alone (55). Excess Ca^{2+} had little effect on the transition temperature for bilayers of DPPC-d_{62} alone. However, because Ca^{2+} raises the DPPG-d_{62} transition temperature relative to that of DPPC-d_{62}, the DPPC-d_{62}/DPPG-d_{62} (7:3) mixture in excess Ca^{2+} displayed liquid crystal/gel coexistence over a range of ~3° and the midpoint of the transition was ~4° above that of DPPC-d_{62} alone (55).

DPPC and DPPG display effectively ideal mixing. Because deuteration affects both lipids similarly, the midpoints of the two phase coexistence regions in the temperature-composition phase diagrams for DPPC-d_{62}/DPPG and DPPC/DPPG-d_{62} are effectively the same. Also, because this region is narrow, any coexistence observable near the transition midpoint involves liquid crystal and gel domains whose compositions do not depart significantly from the overall lipid

composition of the sample. The result, as seen in Figs. 1 and 2, is that partitioning of the deuterated component between liquid crystal and gel domains through the narrow two-phase coexistence range of the transition is not strongly dependent on whether it is the higher-melting or lower-melting component that is deuterated.

The compositions of the protein-containing samples studied were DPPC/DPPG (7:3) +16% SP-A, DPPC/DPPG (7:3) + 13% SP-B, or DPPC/DPPG (7:3) + 16% SP-A + 13% SP-B all by weight and all hydrated in buffer containing Ca^{2+}. These proportions were selected to be comparable to those of DPPC/egg-PG/SP-A/SP-B systems that have been observed to form tubular myelin and tubular-myelin-like structures ((36,37), M. R. Morrow, S. Harris, A. Dico, J. Stewart, S. Taneva, and K. M. W. Keough, unpublished).

Fig. 3 shows selected spectra for (Fig. 3 *A*) DPPC-d_{62}/DPPG (7:3) + 16% SP-A and (Fig. 3 *B*) DPPC/DPPG-d_{62} (7:3) + 16% SP-A, both in buffer containing 5 mM Ca^{2+}. In Fig. 4, *A* and *B*, the first spectral moments (M_1) for these samples are compared to first moments for the corresponding lipid mixtures in the absence of protein. Fig. 4 *C* compares first moments for the two samples containing SP-A with one or the other lipid component deuterated. For DPPC-d_{62}/DPPG (7:3) + 16% SP-A, Fig. 3 *A* shows that there is a small amount of gel phase coexisting with the liquid crystalline phase at 45°C. The lowest temperature for which a liquid crystalline spectral component persists in this sample is 39°C. For DPPC/DPPG-d_{62} (7:3) + 16% SP-A, Fig. 3 *B* shows a larger fraction of coexisting gel phase at 45°C. For this sample, the spectral component characteristic of liquid crystalline DPPG-d_{62} disappears between 43°C and 42°C. The apparent range of two-phase coexistence is thus ~3° narrower for DPPC/DPPG-d_{62} (7:3) + 16% SP-A than for DPPC-d_{62}/DPPG (7:3) + 16% SP-A.

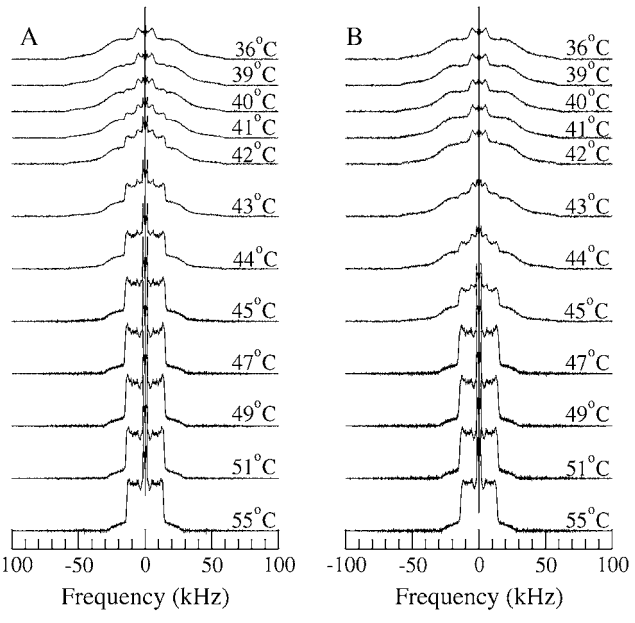

FIGURE 3 Deuterium NMR spectra at selected temperatures for (*A*) DPPC-d_{62}/DPPG (7:3) + 16% SP-A and (*B*) DPPC/DPPG-d_{62} (7:3) + 16% SP-A. Both samples are hydrated in buffer containing 5 mM Ca^{2+} at pH 7.4.

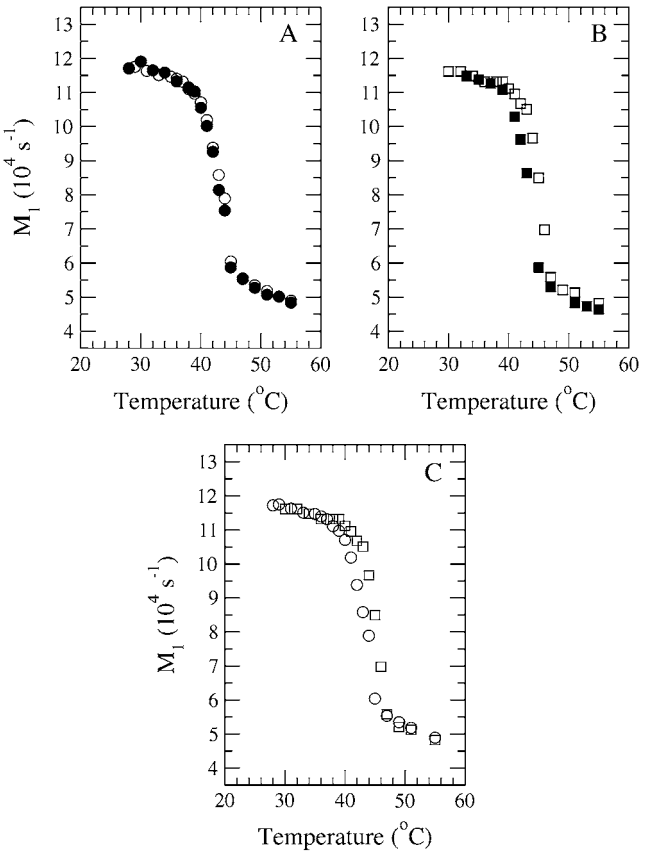

FIGURE 4 (*A*) Temperature dependence of ^{2}H-NMR first spectral moments for ●, DPPC-d_{62}/DPPG (7:3) and (○) DPPC-d_{62}/DPPG (7:3) + 16% SP-A. (*B*) Temperature dependence of ^{2}H-NMR first spectral moments for ■, DPPC/DPPG-d_{62} (7:3) and □, DPPC/DPPG-d_{62} (7:3) + 16% SP-A. (*C*) Temperature dependence of ^{2}H-NMR first spectral moments for ○, DPPC-d_{62}/DPPG (7:3) + 16% SP-A and □, DPPC/DPPG-d_{62} (7:3) + 16% SP-A. All samples are in buffer containing 5mM Ca^{2+} at pH 7.4.

The spectra in Fig. 3, *A* and *B*, for temperatures above and below the transition region show only single liquid crystal or gel spectral components, respectively, regardless of which lipid component is deuterated. This shows that, for temperatures away from the main transition, SP-A did not induce significant separation of the two lipid components. Comparison of corresponding spectra in Figs. 1 *A* and 3 *A*, and of first moments in Fig. 4 *A*, also shows that SP-A did not alter the temperature range over which the transition occurred or the mean DPPC chain order in the DPPC-d_{62}/DPPG (7:3) mixture. This differs from earlier observations on lipid mixtures in which the anionic lipid component, egg-PG, was predominantly unsaturated (38). In that case, SP-A reduced observed chain order in the gel phase of DPPC-d_{62}/egg-PG (7:3) while having little effect on the midpoint temperature of the DPPC-d_{62}/egg-PG transition.

Comparison of spectra in Fig. 1 *B* and Fig. 3 *B* and of first moments in Fig. 4 *B* shows that SP-A sharpened and slightly raised the transition as seen by observing the DPPG-d_{62} component in the DPPC/DPPG-d_{62} (7:3) mixture. Fig. 4 *C* compares first spectral moments for the two lipid components in the presence of SP-A and Ca^{2+}. On cooling, the DPPG-d_{62} spectra reflected the emergence of an ordered phase spectral component at ~1° higher than in the DPPC-d_{62} series of spectra. Also, the liquid crystal phase component disappeared more rapidly on cooling in the DPPG-d_{62} series of spectra than in the DPPC-d_{62} series. DPPG has a higher transition temperature than DPPC in the presence of calcium and these differences may indicate a slight SP-A-induced transfer of DPPG from liquid crystalline phase domains to gel domains in the temperature range close to the mixture transition.

In effect, SP-A seems to have slightly enhanced compositional differences between coexisting gel and liquid crystal domains in the narrow temperature range over which the two phases coexist. This suggests that the partitioning of components between domains is sensitive to a weak but potentially significant difference between the interaction of SP-A with DPPC and with DPPG in these mixtures. Above the transition, spectra were characteristic of a single phase indicating that any such difference was too weak to cause segregation of lipid components within the liquid crystalline phase itself.

As discussed below, the difference between the effect of SP-A on DPPC-d_{62} seen here and in the previous study involving DPPC/egg-PG mixtures (38) demonstrates that in mixtures with PG, the perturbation of DPPC by SP-A is sensitive to properties, like chain unsaturation, of the PG. This is complemented by direct observation of the DPPG-d_{62} component here and together they extend our understanding of how SP-A interacts with PC/PG mixtures.

Fig. 5 shows spectra for (Fig. 5 *A*) DPPC-d_{62}/DPPG (7:3) + 13% SP-B and (Fig. 5 *B*) DPPC/DPPG-d_{62} (7:3) + 13% SP-B in buffer containing 5 mM Ca^{2+}. In Fig. 6, *A* and *B*, the first moments (M_1) of these spectra are compared to first moments for the corresponding lipid mixtures in the absence of protein. Fig. 6 *C* compares first moments for the two samples containing SP-B with one or the other lipid component deuterated. The spectrum for DPPC-d_{62}/DPPG (7:3) + 13% SP-B at 45°C in Fig. 5 *A* is primarily liquid crystalline but the reduced intensity of the axially symmetric spectral component, relative to intensity beyond ±15 kHz, indicates the presence of small amounts of gel phase at this temperature. As the temperature is lowered further, the liquid crystalline fraction decreases and disappears just below 39°C. For DPPC/DPPG-d_{62} (7:3) + 13% SP-B, the spectra in Fig. 5 *B* also indicate the onset of two-phase coexistence at ~45°C. However, DPPG-d_{62} in this sample completely transforms into the gel phase over a significantly narrower temperature range and by 43°C the spectral component characteristic of DPPG-d_{62} in a liquid crystalline environment is barely detectable.

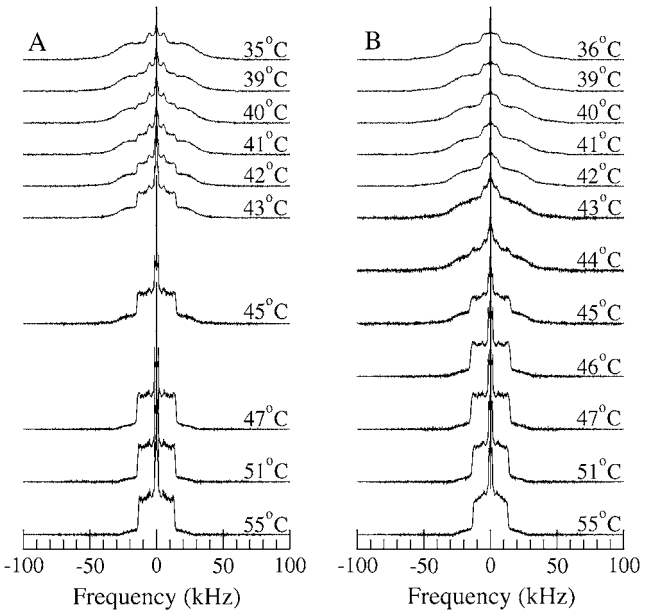

FIGURE 5 Deuterium NMR spectra at selected temperatures for (*A*) DPPC-d_{62}/DPPG (7:3) + 13% SP-B and (*B*) DPPC/DPPG-d_{62} (7:3) + 13% SP-B. Both samples are hydrated in buffer containing 5 mM Ca^{2+} at pH 7.4.

Observation of either the DPPC or the DPPG components in Figs. 5 and 6 indicates similar, but not identical, perturbations of the DPPC/DPPG (7:3) mixture by SP-B. Comparison of spectra in Fig. 5 *A* with those in Fig. 1 *A* and of first moments in Fig. 6 *A* indicate that SP-B did not substantially change the temperature range over which gel-liquid crystal coexistence was observed in the DPPC-d_{62}/DPPG (7:3) mixture but that it did reduce DPPC-d_{62} chain order in the gel phase and, to a lesser extent, in the liquid crystalline phase. A corresponding examination of Figs. 5 *B*, 1 *B*, and 6 *B* shows that SP-B similarly reduced DPPG-d_{62} chain order in the liquid crystal and gel phases but that it also narrowed the temperature range over which the DPPG-d_{62} spectra from the mixture showed gel-liquid crystal coexistence and thus effectively raised the midpoint of the transition slightly as represented by the behavior of the DPPG component in the mixture. In the gel phase, SP-B caused slightly more disordering of DPPC-d_{62} chains than of DPPG-d_{62} chains. This exaggerates the apparent difference in transition midpoints, as indicated by the two components in Fig. 6 *C*.

Earlier studies of lipid bilayers containing SP-B in the absence of Ca^{2+} suggested that perturbation of lipid chain order by SP-B is sensitive to protein concentration (39,41). DPPC/DPPG bilayers containing 11% (w/w) SP-B, a slightly lower concentration than used here, showed significant perturbation of bilayer motions that influence quadrupole echo decay times but little perturbation of either DPPC-d_{62} or DPPG-d_{62} chain order in the liquid crystalline phase (41). On the other hand, SP-B at a concentration of 17% (w/w), higher than that used here, significantly reduced chain order in both

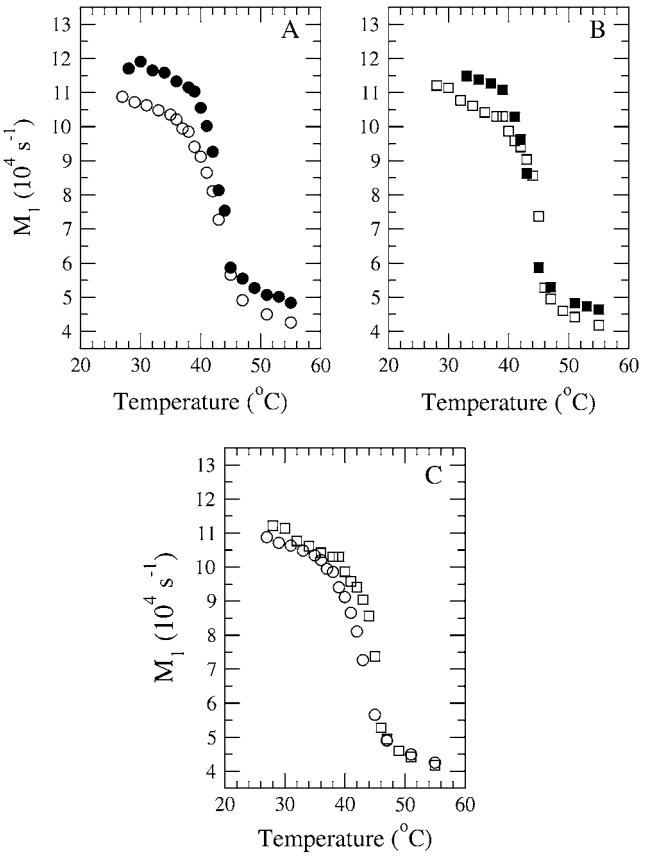

FIGURE 6 (*A*) Temperature dependence of ^{2}H-NMR first spectral moments for ●, DPPC-d_{62}/DPPG (7:3) and ○, DPPC-d_{62}/DPPG (7:3) + 13% SP-B. (*B*) Temperature dependence of ^{2}H-NMR first spectral moments for ■, DPPC/DPPG-d_{62} (7:3) and □, DPPC/DPPG-d_{62} (7:3) + 13% SP-B. (*C*) Temperature dependence of ^{2}H-NMR first spectral moments for ○, DPPC-d_{62}/DPPG (7:3) + 13% SP-B and □, DPPC/DPPG-d_{62} (7:3) + 13% SP-B. All samples are in buffer containing 5 mM Ca^{2+} at pH 7.4.

the liquid crystal and gel phases of DPPC-d_{62} bilayers (39). In the current study, with Ca^{2+} present, an SP-B concentration of 13% (w/w) reduced lipid chain order both above and below the main transition. The perturbation was slightly larger for the DPPC-d_{62} component than for the DPPG-d_{62} in the mixture, but the current study demonstrates that both components of the mixture are affected by SP-B and that the presence of a PG component does not substantially alter the strong perturbation of DPPC previously observed in the presence of a higher SP-B concentration (39).

The SP-B monomer contains regions of amphipathic helix and the tendency for SP-B to reduce chain order in both phases could reflect an SP-B-induced increase in the average separation of lipid headgroups resulting in weaker lateral interaction between chains. The apparent sensitivity of the perturbation to changes in SP-B concentration between ~11% and 17% SP-B (w/w) may indicate that lower concentrations of SP-B can be accommodated in the bilayer in a less perturbing way than higher concentrations but the microscopic origin of this apparent threshold behavior is not understood.

Figs. 7 and 8 display results for mixtures containing both SP-A and SP-B. Fig. 7 *A* shows spectra for DPPC-d_{62}/DPPG (7:3) + 16% SP-A + 13% SP-B in the presence of Ca^{2+}. The DPPC-d_{62} spectra for this mixture between 45°C and 41°C are superpositions of liquid crystalline and gel spectral components. The range of two-phase coexistence as indicated by DPPC-d_{62} in this mixture containing both proteins was thus similar to that seen in Fig. 1 *A* for the lipid mixture without protein. Fig. 8 *A* compares first spectral moments obtained from the spectra of Fig. 7 *A* to those obtained from DPPC-d_{62}/DPPG (7:3) in the absence of protein. DPPC-d_{62} chain order in the gel phase of the mixture containing both proteins was reduced relative to that in the protein-free lipid mixture, but DPPC-d_{62} chain order in the liquid crystalline phase and the width of the transition as reflected by DPPC-d_{62} ordering were similar in the protein-free sample and in the sample containing SP-A plus SP-B.

Comparison of Fig. 8 *A* with Fig. 6 *A* shows that, relative to the protein-free case, the presence of SP-B alone caused larger perturbations to three aspects of DPPC-d_{62} behavior than did the presence of SP-A and SP-B together. These were: the width of the temperature range over which the DPPC-d_{62} chains ordered; the DPPC-d_{62} chain order above the transition; and the DPPC-d_{62} chain order below the transition.

Fig. 7 *B* shows spectra obtained from DPPC/DPPG-d_{62} (7:3) + 16% SP-A + 13% SP-B in the presence of Ca^{2+}. The DPPG-d_{62} spectrum at 45°C for this sample is a superposition of a gel phase spectrum with a small liquid crystalline spectral component. By 44°C, the DPPG-d_{62} component

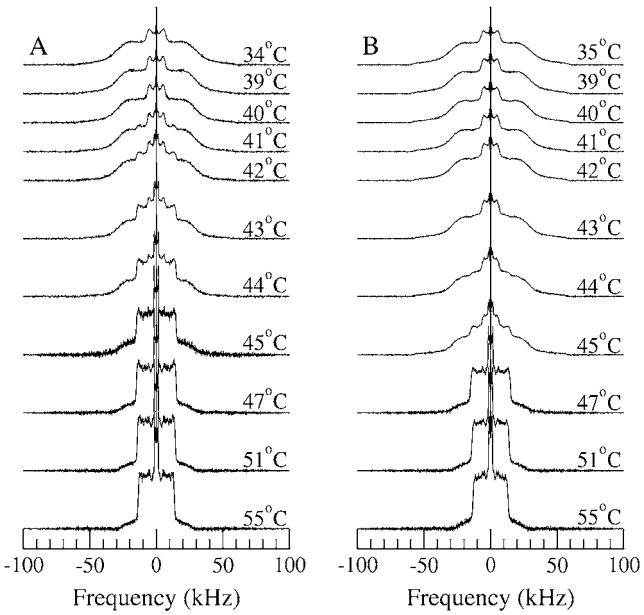

FIGURE 7 Deuterium NMR spectra at selected temperatures for (*A*) DPPC-d_{62}/DPPG (7:3) + 16% SP-A + 13% SP-B and (*B*) DPPC/DPPG-d_{62} (7:3) + 16% SP-A + 13% SP-B. Both samples are hydrated in buffer containing 5 mM Ca^{2+} at pH 7.4.

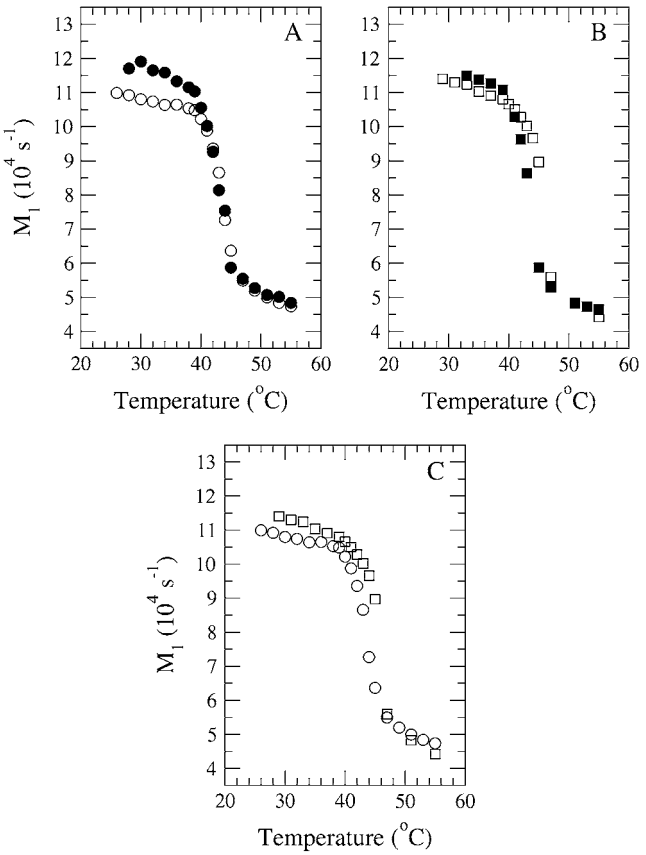

FIGURE 8 (*A*) Temperature dependence of ^{2}H-NMR first spectral moments for ●, DPPC-d_{62}/DPPG (7:3) and ○, DPPC-d_{62}/DPPG (7:3) + 16% SP-A + 13% SP-B. (*B*) Temperature dependence of ^{2}H-NMR first spectral moments for ■, DPPC/DPPG-d_{62} (7:3) and □, DPPC/DPPG-d_{62} (7:3) + 16% SP-A + 13% SP-B. (*C*) Temperature dependence of ^{2}H-NMR first spectral moments for ○, DPPC-d_{62}/DPPG (7:3) + 16% SP-A + 13% SP-B and □, DPPC/DPPG-d_{62} (7:3) + 16% SP-A + 13% SP-B. All samples are in buffer containing 5 mM Ca^{2+} at pH 7.4.

of the sample is almost entirely in a gel phase environment. The range of temperatures over which the DPPG-d_{62} spectra were characteristic of two-phase coexistence was thus narrower in the presence of SP-A and SP-B together than in the absence of protein. Fig. 8 *B* compares first spectral moments obtained from DPPC/DPPG-d_{62} (7:3) + 16% SP-A + 13% SP-B to those from DPPC/DPPG-d_{62} (7:3) in the absence of protein. DPPG-d_{62} chain order in the gel phase of the mixture containing both proteins was lower than that of DPPG-d_{62} in the gel phase with no protein present. As was the case for SP-A alone, the two proteins together reduced the temperature range over which DPPG-d_{62} was observed to coexist in the liquid crystal and gel phases. This resulted in a slight increase in effective midpoint of the transition as represented by the behavior of the DPPG-d_{62} component in the mixture.

Fig. 8 *C* compares first spectral moments for DPPC-d_{62} and DPPG-d_{62} in the corresponding mixtures containing both lipids and both proteins in the presence of Ca^{2+}. As was seen in Fig. 4 *C* for SP-A alone and in Fig. 6 *C* for SP-B alone, the apparent transition midpoint as reflected by DPPG-d_{62} spectra in the mixture with both proteins was slightly higher than that shown by the DPPC-d_{62} spectra in the same mixture. It should be noted, though, that in Figs. 4 *C* and 8 *C*, which show comparisons for samples containing SP-A or SP-A plus SP-B, respectively, this difference reflects an increase in the midpoint of the transition as seen in the DPPG-d_{62} spectra relative to the protein-free case. In Fig. 6 *C*, which compares samples containing SP-B alone, the difference also reflects a decrease in the midpoint of the transition as seen from the DPPC-d_{62} spectra. It should also be noted that while SP-B alone perturbed both lipids more than the combination of SP-A plus SP-B, comparison of Figs. 6 *C* and 8 *C* suggests that the differences between DPPC-d_{62} and DPPG-d_{62} chain orders over the full temperature range observed were roughly the same in the two cases. This implies that the way in which SP-A altered the SP-B-lipid interaction was similar for both lipid components.

Because the spectra of the two lipid components in the absence of protein indicated very similar behaviors, as seen in Fig. 2, the differences between DPPC-d_{62} and DPPG-d_{62} seen in Figs. 4 *C*, 6 *C*, and 8 *C* suggest that interactions with SP-A, SP-B, and both proteins together modified the distribution of DPPC and DPPG between liquid crystal and gel domains in the narrow temperature range about the transition temperature over which such domains coexist.

DISCUSSION

These observations point to a rich set of interactions between the two lipid and two protein components in these systems. In the absence of protein, the average chain orders for DPPC-d_{62} and DPPG-d_{62} in the lipid mixtures were close throughout the range of temperatures studied. Both lipids displayed similar ranges of two-phase coexistence and the midpoints of the transition displayed by observing each lipid were the same.

It is interesting to consider separately the way in which the behavior of each lipid component was perturbed by SP-A and SP-B alone and together. In doing so, it is important to bear in mind that all observations were made with calcium present in the system and that the interactions observed, particularly those involving SP-A, are likely influenced by the presence of calcium.

SP-A had little effect on DPPC-d_{62} chain order either above or below the transition. SP-B lowered the average chain order of DPPC-d_{62} both above and below the transition. When both SP-A and SP-B were present, the average DPPC-d_{62} chain order was reduced in the gel phase but its behavior was otherwise nearly unchanged from that in the sample containing no protein.

SP-A reduced the temperature range over which DPPG-d_{62} ordered at the transition. SP-B lowered the average DPPG-d_{62} chain order both above and below the transition but the perturbation, particularly in the gel phase, was slightly

smaller than for DPPC-d_{62}. Compared to the corresponding protein-free samples, the sample containing both proteins displayed a sharper increase in DPPG-d_{62} chain order at the transition and lower DPPG-d_{62} chain order in the gel phase. The latter perturbation was smaller than for DPPC-d_{62} in the corresponding sample. Average DPPG-d_{62} chain order in the liquid crystalline phase, like that of DPPC-d_{62}, was nearly the same in the presence of both proteins together as in their absence.

These observations suggest some interesting aspects to the interactions between protein and lipid in lung surfactant material. In the liquid crystalline phase, the perturbation of chain order by SP-B was effectively removed when SP-A was also present even though the molar ratio of SP-B to lipid was the same in both cases. In effect, SP-A interfered with the SP-B-lipid interaction. It is interesting to note that earlier observations of monolayers containing DPPC, egg-PG, and SP-B spread on a subphase containing SP-A showed evidence for SP-A-induced redistribution of SP-B and segregation of a phase enriched in the two proteins (56). The current observations demonstrate that interactions between SP-A and SP-B can significantly alter the extent to which SP-B perturbs both lipid components in the mixture. The possibility that the interaction between SP-A and SP-B might compete with that between SP-B and the lipid components in the liquid crystalline phase would be particularly interesting in light of evidence for SP-A self-aggregation on monolayer or bilayer surfaces (57–60).

Binding studies of SP-A with a variety of lipid mixtures, including DPPC/DPPG at a molar ratio of 85:15 with calcium present, have been interpreted as indicating that interaction of SP-A with lipid assemblies is facilitated by disruptions in lipid packing (57). It has also been observed that SP-A in a calcium-free aqueous phase accumulates at boundaries between liquid-expanded and liquid-condensed domains of DPPC/DPPG monolayers (58). Epifluorescence microscopic observations have shown that the organization of porcine lung surfactant lipid extract (PLSE) spread as a monolayer on an aqueous phase containing SP-A is sensitive to calcium in the aqueous phase. With SP-A present in the aqueous phase, calcium promoted aggregation of condensed phase domains in the PLSE monolayer and affected the distribution of fluorescently-labeled SP-A interacting with the monolayer (59). In addition to some association of SP-A with more fluid domains in the PLSE monolayer, calcium was also found to promote aggregation of SP-A at domain boundaries (60). While SP-A has not been found to alter lipid chain packing significantly, these reports suggest that it can influence the spatial distribution of components and domain boundaries within lipid-protein assemblies. The tendency for SP-A to aggregate on regions where lipid packing is perturbed might provide a mechanism whereby SP-A can differentially influence the distribution of other components, like SP-B and lipids, with which it interacts. This in turn, may indirectly influence the interactions between these components. In particular, SP-A-induced aggregation of SP-B might account for the reduced average perturbation of chain order by SP-B seen in the mixtures containing both proteins.

Another aspect of the observations reported here is that the temperature range over which DPPG-d_{62} in the lipid mixture ordered at the transition was narrower in the presence of either SP-A or SP-A plus SP-B than it was in the absence of protein. DPPC-d_{62} in corresponding mixtures did not respond similarly. This may reflect a tendency for SP-A to promote enrichment of ordered phase domains in PG lipids near the transition.

SP-A is known to interact strongly with DPPC (13,19) and it is possible that any tendency to partially exclude DPPG from liquid crystalline domains for temperatures close to the transition might reflect a preferential interaction of SP-A with fluid phase DPPC. Studies involving fluorescently-labeled SP-A found that, at pH 7.4 in the absence of calcium, SP-A associated somewhat with the liquid-expanded phase of a DPPC monolayer and accumulated at boundaries between liquid-expanded and liquid-condensed phases but did not accumulate within liquid-condensed phases (58). The same study found that for mixed DPPC/DPPG monolayers, labeled SP-A appeared only at the boundaries of the liquid-expanded and liquid condensed phases. The interactions responsible for the observed partitioning of SP-A at monolayer surfaces might be related to those that promoted the apparent preferential depletion of DPPG from liquid crystalline domains near the transition as seen in the current experiments. It must be noted, though, that the presence of calcium modifies the interaction of SP-A with DPPG (17) so that the observations reported here may reflect a difference in the way that SP-A interacts with DPPC and DPPG in the presence of calcium rather than a strong preferential interaction with one lipid or the other.

While SP-A may influence the spatial distribution of lipid components in the bilayer near the transition, it did not have a significant effect on average chain order of either lipid in the liquid crystalline phase of the mixture. This is similar to observations from an earlier study that showed SP-A to have little effect on chain order in the liquid crystalline phase of DPPC-d_{62}/egg-PG mixtures (38). The earlier study did, however, show a substantial effect on bilayer motions detectable through quadrupole echo decay and it was suggested that because of its size, the interaction of SP-A with the bilayer surface might be distributed over large numbers of lipids.

The earlier study also found that SP-A, in the presence of Ca^{2+}, reduced chain order in the gel phase of DPPC-d_{62}/egg-PG (7:3) (38), an observation that is qualitatively different from the effect of SP-A on gel phase chain order in the DPPC/DPPG mixtures observed in the current work. This difference might be reconciled by taking into account the difference between the anionic lipids in the two studies. Egg-PG lipids typically contain one unsaturated acyl chain and have a much lower main transition temperature than DPPC. In the current study, the transition temperature of the anionic lipid component

DPPG, in the presence of Ca^{2+}, was slightly higher than that of DPPC. If SP-A does tend to promote depletion of the PG component in liquid crystalline domains near the transition, the effect on DPPC-d_{62}/egg-PG bilayers would be a relative enrichment, of gel phase domains, in unsaturated lipids having a lower melting point and intrinsically less-ordered chains. On the other hand, enriching gel phase domains with DPPG near the transition would have little effect on gel phase order, since ordered DPPG and DPPC chains are effectively equivalent, but would reduce the fraction of DPPG present in fluid domains at a given temperature near the transition resulting in an apparently sharper transition for the sample based on the DPPC/DPPG-d_{62} (7:3) mixture.

While interactions involving SP-A, in the presence of calcium, appear to have been sufficient to influence the distribution of lipid components between coexisting liquid crystal and gel phase domains near the transition, they do not appear to have resulted in significant segregation of the lipid components in the gel phase. The lack of sharp features in the gel phases of chain-perdeuterated lipids makes it difficult to rule out the coexistence of ordered phases having different lipid compositions. Nevertheless, substantial mixing of the lipid components in the ordered phases is strongly suggested by the observation that all of the spectra for temperatures below the transition, regardless of the sample's protein content, showed only a single spectral component with a splitting ($\sim$5–7 kHz) characteristic of the deuterated chain methyl group.

For this work, as has been noted above, samples containing SP-A were prepared by allowing SP-A to interact with the lipid components before the addition of calcium. It is thus important to consider the extent to which calcium-induced self-aggregation of SP-A might be reflected by the observations reported here. In the presence of calcium, SP-A alone was found to have little effect on the DPPC-d_{62} component of the bilayer mixture. The behavior of DPPG-d_{62} was affected only over the narrow temperature range around the main transition. While we cannot rule out the possibility that calcium-induced SP-A self-aggregation might reduce lipid perturbation, these observations do not suggest that SP-A aggregation amplifies any such perturbation. SP-A does, however, reduce average perturbation of the bilayer by SP-B and it is possible that the effects of SP-A self-aggregation on the bilayer might be mediated by SP-B under the conditions of the experiments reported here. While SP-A-induced aggregation of SP-B might account for the reduced average effect of SP-B on the lipids observed here, it might also result in more perturbed regions of lipid that are not easily detected by these experiments. Highly perturbed regions could, however, affect the energetics of surfactant material reorganization and thus be relevant to the understanding of surfactant function.

The picture that emerges from these comparisons is as follows. In the liquid crystalline phase, the DPPC and DPPG components of the mixture were well-mixed. The two lipid components displayed similar average chain orders in the liquid crystalline phase of all three samples containing protein(s). The interaction of SP-A with SP-B seems to be such that it interfered with perturbation of the liquid crystalline lipid components that resulted from the presence of SP-B alone. This may reflect an SP-A-induced spatial redistribution of SP-B. At the liquid-crystal-to-gel transition, differences in the way that SP-A interacted with the two lipid components likely resulted in a preferential depletion of PG from liquid crystal domains. In the gel phase, SP-A modified but did not appear to completely remove the effect of SP-B on chain order of the two components. While the microscopic understanding of these interactions is not complete, it is likely that they have important implications for the way in which surfactant material is reorganized in the transformation from lamellar bodies to a functional surfactant layer.

This research was supported by grants from the Natural Science and Engineering Research Council of Canada to M.R.M. and from the Canadian Institutes of Health Research to K.M.W.K.

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