Surfactant Protein SP-B Induces Ordering at the Surface of Model Membrane Bilayers†

John E. Baatz, Barry Elledge, and Jeffrey A. Whitsett*
University of Cincinnati College of Medicine, Cincinnati, Ohio 45267-0541
Received October 13, 1989; Revised Manuscript Received April 6, 1990

ABSTRACT: The effects of bovine pulmonary surfactant-associated protein B (SP-B) on molecular packing of model membrane lipids (7:1 DPPC/DPPG) were studied by fluorescence anisotropy. The bilayer surface was markedly ordered by SP-B below the gel to fluid phase transition temperature (T_c) while it was only slightly ordered above this temperature as indicated by surface-sensitive probes 6-NBD-PC and 6-NBD-PG. The effects of SP-B on fluorescence anisotropy were concentration dependent, reaching maximal activity at 1–2% protein to phospholipid by weight. Anisotropy measurements of interior-selective fluorescent probes (*cis*-parinaric acid and DPH) imply that addition of SP-B into the phospholipid shifted the T_c of the model membrane but did not alter lipid order at the membrane interior. Since fluorescence anisotropy studies with *trans*-parinaric acid, an interior-sensitive probe with high affinity for gel-phase lipids, did not detect any changes in lipid packing or T_c, it is likely that SP-B resides primarily in fluid-phase domains. Fluorescence lifetime measurements indicated that two conformers of the NBD-PC probe exist in this DPPC/DPPG model membrane system. Fluorescence intensity measurements generated with NBD-PC and NBD-PG, in conjunction with information from lifetime measurements, support the concept that SP-B increases the distribution of the short-lifetime conformer in the gel phase. In addition, the anisotropy and intensity profiles of NBD-PG in the model membrane indicate that bovine SP-B interacts selectively with phosphatidylglycerol.

Pulmonary surfactant, a complex material that lines alveolar surfaces, is critical for maintenance of lung volume during the respiratory cycle (Goerke, 1974; Goerke & Clements, 1986; Notter & Finkelstein, 1984; Notter, 1984). This material consists predominantly of phospholipids (primarily phosphatidylcholine and phosphatidylglycerol) but also contains small amounts of associated proteins (Hallman et al., 1982; Tanaka et al., 1983). Although phospholipids alone are capable of reducing surface tension during dynamic compression, they are incapable of spreading rapidly at lower surface tensions to prevent alveolar collapse (Goerke & Clements, 1986; Notter & Finkelstein, 1984; Notter, 1984). In contrast, preparations utilizing organic solvent extracts of natural surfactant impart surface-active properties comparable to those of natural surfactant.

Two hydrophobic surfactant-associated proteins, SP-B[1] and SP-C, have been purified from pulmonary surfactant by organic extraction (Whitsett et al., 1986a; Yu & Possmayer, 1986; Hawgood et al., 1987). SP-C, with an apparent M_r = 4000 under reducing conditions, is abundant in valine and leucine, thus conferring an unusually high hydrophobicity (Whitsett et al., 1986a,b; Glasser et al., 1988; Johansson et al., 1988). Proteolipid SP-B, with M_r = 8000 (monomeric form), is also rich in the hydrophobic amino acids valine, leucine, and isoleucine; however, it contains more polar and more positively charged residues than SP-C (Glasser et al., 1987; Olafson et al., 1987; Curstedt et al., 1988). Reconstitution of either SP-B or SP-C with surfactant phospholipid mixtures results in material possessing full surfactant-like biophysical properties (Takahashi & Fujiwara, 1986; Tanaka et al., 1986; Notter et al., 1987). This characteristic may make these hydrophobic proteins useful components of surfactant mixtures in surfactant replacement therapy for surfactant-deficient diseases such as respiratory distress syndrome (RDS) in infants and adult respiratory distress syndrome (ARDS).

The mechanisms by which SP-B and SP-C enhance the surface activity of the phospholipids have yet to be elucidated. The present work utilizes fluorescence anisotropy for the determination of the molecular basis by which bovine SP-B alters lipid organization in a model system consisting of 7:1 (mol/mol) DPPC/DPPG, similar to the composition of the phospholipids in natural pulmonary surfactant. Fluorescent membrane probes specific for the membrane bilayer surface and interior were used to detect changes in lipid order at the respective bilayer regions as a function of temperature and SP-B concentration to determine the mechanism(s) by which SP-B enhances surfactant activity of the phospholipids in the lung.

Materials and Methods

Fluorescent Membrane Probes. Dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) were purchased from Sigma Chemical Co. (St. Louis, MO). *trans*-Parinaric acid (*trans*-PnA), *cis*-parinaric acid (*cis*-PnA), 1-hexadecanoyl-2-[*N*-(7-nitro-2-benzoxa-1,3-diazol-4-yl)aminohexanoyl]phosphatidylcholine (6-NBD-PC), and 1-acyl-2-[*N*-(7-nitro-2-benzoxa-1,3-diazol-4-yl)aminohexanyol]phosphatidylglycerol (6-NBD-PG) were acquired from Avanti Polar Lipids, Inc. (Pelham, AL). Di-

† Supported by Programs of Excellence in Molecular Biology [HL41496 and HL38859 (J.A.W.)] and Research Training in Perinatology [HD07200 (J.E.B.)].
* Address correspondence to this author at the University of Cincinnati, College of Medicine, Pediatrics/Neonatology Division, 231 Bethesda Ave., Cincinnati, OH 45267-0541.

[1] Abbreviations: SP-B, pulmonary surfactant-associated protein B; 6-NBD-PC, 1-hexadecanoyl-2-[*N*-(7-nitro-2-benzoxa-1,3-diazol-4-yl)-aminohexanoyl]phosphatidylcholine; 6-NBD-PG, 1-acyl-2-[*N*-(7-nitro-2-benzoxa-1,3-diazol-4-yl)aminohexanoyl]phosphatidylglycerol; DPH, 1,6-diphenylhexa-1,3,5-triene; *cis*-PnA, *cis*-parinaric acid; *trans*-PnA, *trans*-parinaric acid.

0006-2960/90/0429-6714$02.50/0 © 1990 American Chemical Society

phenylhexatriene (DPH) was purchased from Molecular Probes, Inc. (Eugene, OR).

SP-B Purification. Bovine SP-B was isolated and purified from chloroform/methanol extract of bovine lung according to the method described by Kogishi et al. (1988) with the following modifications. The organic lipid/protein extract was dried by evaporation and suspended in 10 mM Tris buffer (pH = 8.0) with 1% Triton X-100. This suspension was applied directly to the anion-exchange column without any centrifugation step.

Purification of the SP-B (M_r = 8000) was ascertained by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) utilizing tricine buffers (Schagger & von Jagow, 1987). Under nonreducing conditions, SP-B existed primarily as an oligomer with an M_r = 18000–28000, whereas in the reduced form, the SP-B existed as a monomer of M_r = 8000. For additional verification of SP-B purity, amino-terminal sequence analysis of the protein was performed by using an Applied Biosystems Model 470A vapor-phase protein sequencer as described elsewhere (Ross et al., 1986). SP-B concentration was determined by analysis of amino acid composition (Ross et al., 1986).

Membrane Reconstitution. Reconstitution of model membranes was performed in the following manner. First, appropriate volumes of DPPC and DPPG stock solutions in chloroform and 98:1 chloroform/methanol, respectively, were mixed to achieve a 7:1 molar ratio of DPPC to DPPG (0.60 mg total lipid). Bovine SP-B stock solution (in ethanol/0.01 M HCl) was added to the lipid preparations in volumes necessary to yield 0, 0.5, 1.0, and 2.0% (w/w) SP-B in lipid. The mixture was then dried to completeness under a stream of nitrogen. Two milliliters of buffer consisting of 120 mM NaCl and 10 mM MOPS [3-(*N*-morpholinopropane)sulfonic acid], pH 7.0, was then added to the dried lipid/protein film and incubated at 53 °C for 10 min. The sample was then vortexed for 30 s to form multilamellar vesicles. The fluorescent probes were incorporated into the vesicles in amounts necessary to yield a lipid/probe mole ratio of 500:1.

Fluorescence Measurements. Anisotropy and intensity measurements were effected on a Perkin-Elmer 650-10S fluorescence spectrophotometer with a polarization accessory unit. Temperatures of the samples were regulated by a Neslab Endocal RTE 110 circulating bath and determined by a thermistor submersed in the samples. Excitation and emission wavelengths used for each fluorescent probe are

probe	λ_{ex} (nm)	λ_{em} (nm)
6-NBD-PC	468	533
6-NBD-PG	465	530
trans-PnA	330	420
cis-PnA	332	425
DPH	362	427

6-NBD-PC and 6-NBD-PG were used to detect alterations in membrane lipid organization near the bilayer surface, whereas *trans*-PnA, *cis*-PnA, and DPH were used for the detection of changes in lipid organization in the bilayer interior. Fluorescence anisotropy r was calculated by using the equation

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} C$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities parallel and perpendicular to the plane of polarization of the excitation beam, respectively, and C is a correction factor for instrumental anisotropy. Total fluorescence intensity was calculated as

$$I_{tot} = I_{\parallel} + 2I_{\perp}$$

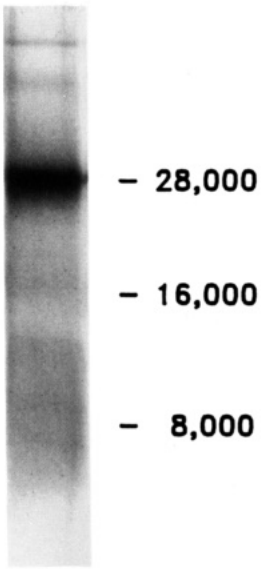

FIGURE 1: Silver-stained SDS–PAGE gel (14% acrylamide) of purified nonreduced bovine SP-B. Under nonreducing conditions SP-B exists primarily as oligomers of M_r = 28K and 16K.

Fluorescence Lifetimes. Fluorescence lifetime measurements were performed at the Center for Fluorescence Dynamics, University of Illinois at Urbana–Champaign, on a modified instrument utilizing SLM 480 T-format optics mated to a variable-frequency modulator. Excitation light at 325 nm, provided by a nitrogen laser, was modulated at a minimum of 8 frequencies between 1 and 160 MHz to permit resolution of multiexponential lifetime decays (Gratton & Limkeman, 1984). Fluorescence phase shift and demodulation data were analyzed by several multiexponential decay models using a least-squares fit software program, ISS187 (ISS Instruments, Urbana).

Results

Protein Analysis. Bovine SP-B migrated primarily in larger oligomers of M_r 18000 and 26000 (unreduced) (Figure 1). A single N-terminal amino acid sequence was identified by a gas-phase amino acid sequenation: Phe-Pro-Ile-Pro-Ile-Pro-Tyr-[?]-[?]-Leu-Leu-Arg-Thr-Leu-Ile-Lys-Arg-Ile-Gln-Ala-Val-Ile-Pro-Lys-Gly. With the exception of Arg-17 and the undetermined amino acid residues in positions 8 and 9, this sequence corresponds to the N-terminal residues Phe-1 to Gly-25 of bovine SP-B as reported by Olafson et al. (1987). The amino acid residue at position 17 was identified as lysine by Olafson et al. (1987). Amino acid composition was consistent with the entire amino acid sequence of the bovine peptide.

Fluorescence Anisotropy. The fluorescence anisotropy of *trans*-PnA in the 7:1 DPPC/DPPG model membrane was unaltered below T_c upon the introduction of SP-B at all concentrations studied (Figure 2). The phase transition of the membrane from the solid to fluid phase was very sharp, as indicated by the slope of the change in anisotropy at the phase transition, with a T_c of 40 °C. When *trans*-PnA was used as the reporter, neither the cooperativity of the melt nor the transition temperature was affected by the incorporation of SP-B into the membrane.

Analysis of the phase transition using the fluorescence anisotropy of *cis*-parinaric acid and DPH probes in 7:1 DPPC/DPPG bilayers indicated that addition of SP-B did not affect the anisotropy of these probes in the bilayer interior (Figures 3 and 4, respectively). However, SP-B increased the

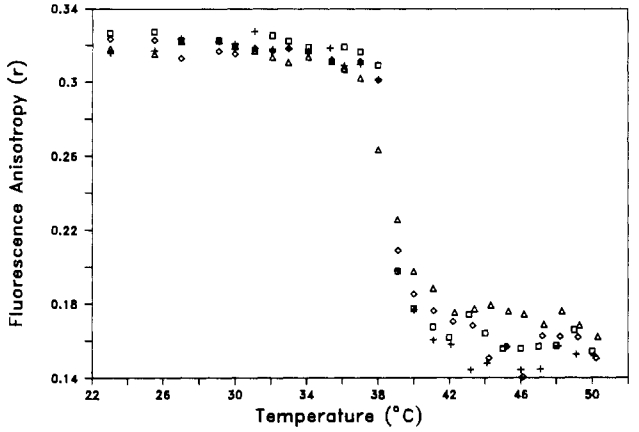

FIGURE 2: Fluorescence anisotropy of *trans*-parinaric acid in 7:1 DPPC/DPPG vesicles as a function of temperature and bovine SP-B concentration. The fluorescence anisotropy of *trans*-PnA as a function of temperature is shown for multilamellar vesicles containing 0% (□), 0.5% (+), 1.0% (◇), and 2.0% (△) bovine SP-B (weight SP-B per weight total lipid). Excitation was at 330 nm and emission recorded at 420 nm.

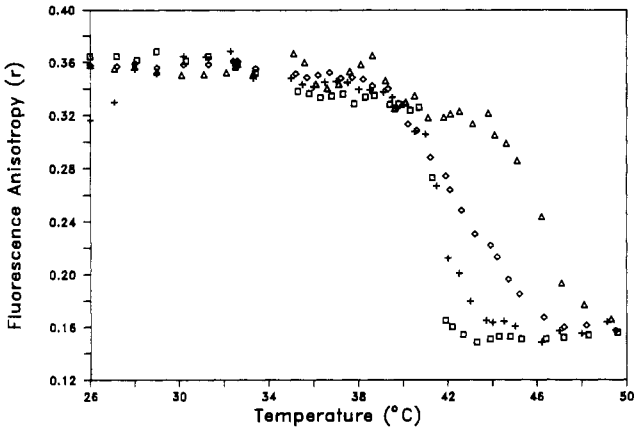

FIGURE 3: Fluorescence anisotropy of *cis*-parinaric acid in 7:1 DPPC/DPPG multilamellar vesicles as a function of temperature and bovine SP-B concentration. The temperature dependence of *cis*-PnA fluorescence anisotropy is shown for model membrane vesicles containing SP-B in concentrations (weight percent with respect to weight total lipid) of 0% (□), 0.5% (+), 1.0% (◇), and 2.0% (△). Excitation was at 332 nm, and emission was recorded at 425 nm.

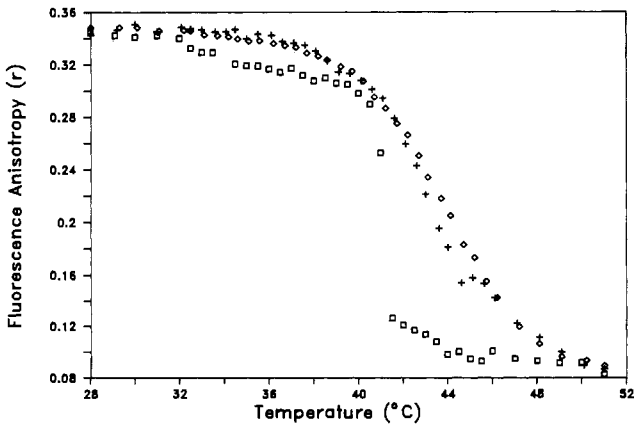

FIGURE 4: Fluorescence anisotropy of DPH in 7:1 DPPC/DPPG multilamellar vesicles as a function of temperature and bovine SP-B concentration. The fluorescence anisotropy of DPH in DPPC/DPPG liposomes is shown for preparations containing 0% (□), 1.0% (+), and 2.0% (◇) bovine SP-B (weight percent with respect to weight of total lipid). Excitation was performed at 362 nm, and emission was recorded at 427 nm.

T_c dramatically from 41.2 °C (0% SP-B) to 45.1 °C (2.0% SP-B) and broadened the phase transition. The anisotropy

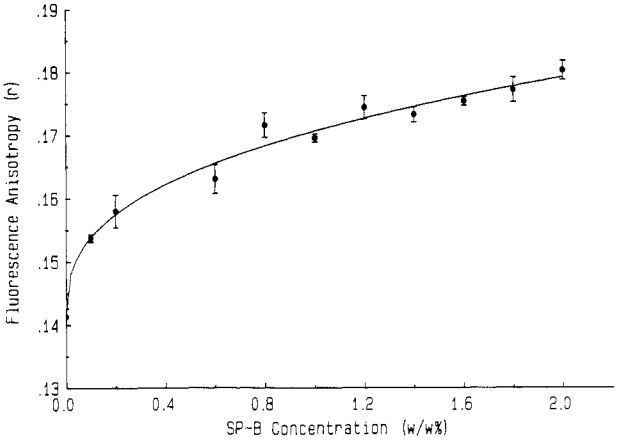

FIGURE 5: Fluorescence anisotropy of NBD-PC in 7:1 DPPC/DPPG multilamellar vesicles as a function of bovine SP-B concentration (weight percent with respect to total lipid weight); T = 32 °C. Excitation was at 468 nm, and emission was recorded at 533 nm.

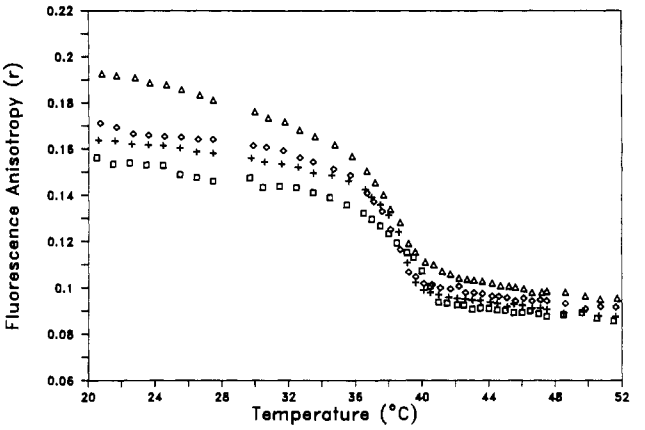

FIGURE 6: Fluorescence anisotropy of NBD-PC in 7:1 DPPC/DPPG multilamellar vesicles as a function of temperature and bovine SP-B concentration. The fluorescence anisotropy of NBD-PC is shown for DPPC/DPPG vesicles containing bovine SP-B at concentrations of 0% (□), 0.5% (+), 1.0% (◇), and 2.0% (△) (weight percent with respect to weight of total lipid). Excitation was at 468 nm, and emission was recorded at 533 nm.

of DPH in the liposomes is slightly enhanced by SP-B in the gel state. As in the studies with *cis*-PnA, DPH detected a substantial increase in T_c (from 41.1 to 43.9 °C), and a decreased cooperativity as SP-B content was increased from 0 to 2.0% (w/w). These data suggest that SP-B causes minimal ordering of the membrane interior.

The fluorescence anisotropy of 6-NBD-PC in 7:1 DPPC/DPPG liposomes as a function of SP-B concentration at 32 °C is shown in Figure 5. An SP-B concentration dependent increase in anisotropy of 6-NBD-PC in the model membrane was observed over a range of 0 to 2.0% (w/w) SP-B, with the increase readily observable at SP-B levels as low as 0.1% (w/w). Upon incorporation of increasing amounts of SP-B into the liposomes, the anisotropy of the probe increased dramatically at temperatures below the phase transition temperature, T_c (40.2 °C), and increases slightly above the T_c (Figure 6). These results are consistent with an ordering effect of SP-B at the membrane surface. Changes in T_c or cooperativity of the melt were not observed after incorporation of increasing amounts of SP-B.

Fluorescence anisotropy of NBD-PG, as a function of temperature and concentration of SP-B, differs from that of 6-NBD-PC (Figure 7). Incorporation of 0.5% (w/w) SP-B into the model membrane increased the anisotropy of 6-NBD-PG at temperatures below T_c. The anisotropy of NBD-PG in

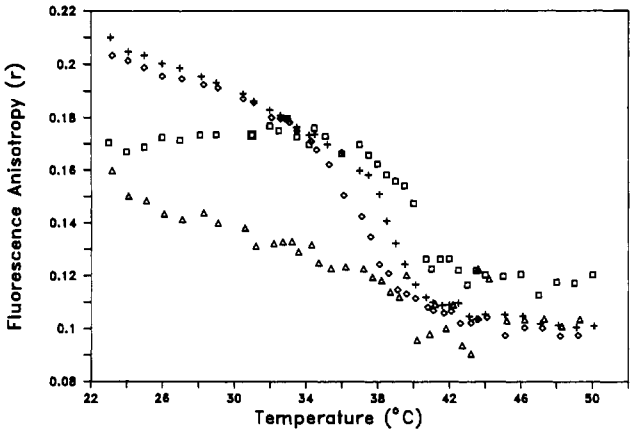

FIGURE 7: Fluorescence anisotropy of NBD-PG in 7:1 DPPC/DPPG multilamellar vesicles as a function of temperature and bovine SP-B concentration. The fluorescence anisotropy of NBD-PG is shown for DPPC/DPPG vesicles containing 0% (□), 0.5% (+), 1.0% (◇), and 2.0% (△) bovine SP-B (weight percent with respect to weight of total lipid). Excitation was performed at 465 nm, and emission was recorded at 530 nm.

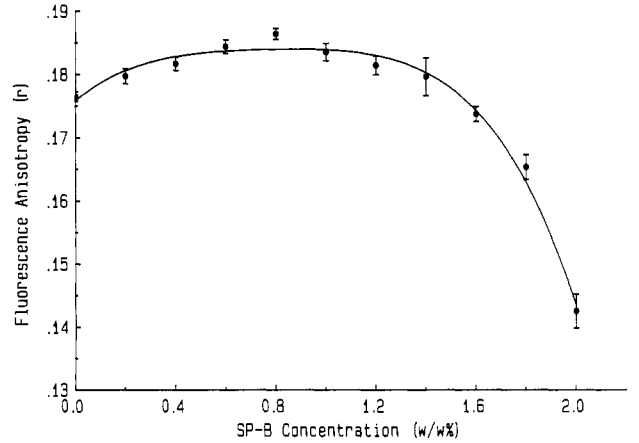

FIGURE 8: Fluorescence anisotropy of NBD-PG in 7:1 DPPC/DPPG multilamellar vesicles as a function of bovine SP-B concentration (weight percent with respect to total weight); $T = 32$ °C. Excitation was at 465 nm, and emission was recorded at 530 nm.

liposomes containing 1.0% (w/w) SP-B was lower than that containing 0.5% SP-B, but higher than that of the vesicles alone. The slope of the change in anisotropy at the phase transition ("melt") was also reduced by the addition of 1% SP-B. Incorporation of 2% SP-B into the membrane caused a large reduction in fluorescence anisotropy compared to the samples containing 0, 0.5, and 1.0% SP-B. The presence of 2% SP-B completely abolished an identifiable phase transition. The SP-B concentration-dependent trend in fluorescence anisotropy of 6-NBD-PG is more clearly illustrated in Figure 8, which displays the anisotropy of NBD-PG as a function of SP-B concentration at 32 °C.

Fluorescence Intensity Measurement. The relationships between fluorescence intensity and temperature of 6-NBD-PC and 6-NBD-PG in the model membranes containing 0, 0.5, 1.0, and 2.0% (w/w) bovine SP-B are shown in Figures 9 and 10. The patterns of these profiles for the lipid preparations are atypical for membrane probes in that the intensity increases with increasing temperature below T_c, rather than decreasing with increasing temperature as with most membrane probes. Incorporation of 0.5, 1.0, or 2.0% (w/w) SP-B into the model membrane caused a large (and approximately equivalent) enhancement in fluorescence intensity of 6-NBD-PC at temperatures lower than the gel–liquid crystalline transition temperature, indicating that the probe is less quenched in the

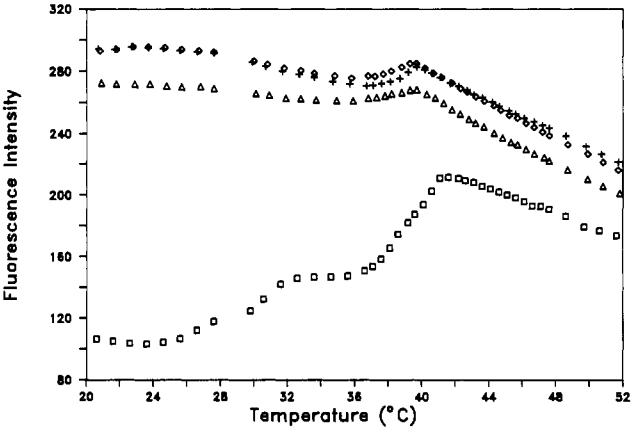

FIGURE 9: Fluorescence intensity of NBD-PC in 7:1 DPPC/DPPG multilamellar vesicles as a function of temperature and bovine SP-B concentration. The fluorescence intensity of NBD-PC as a function of temperature is shown for DPPC/DPPG vesicles containing 0% (□), 0.5% (+), 1.0% (◇), and 2.0% (△) bovine SP-B (weight percent with respect to weight of total lipid). Excitation was at 468 nm and emission at 533 nm. Intensity was calculated as $I_{\parallel} + 2I_{\perp}$.

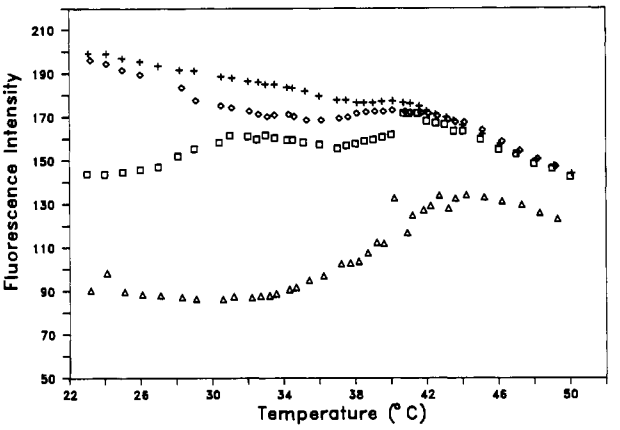

FIGURE 10: Fluorescence intensity of NBD-PG in 7:1 DPPC/DPPG multilamellar vesicles as a function of temperature and bovine SP-B concentration. The fluorescence intensity of NBD-PG as a function of temperature is shown for DPPC/DPPG liposomes containing 0% (□), 0.5% (+), 1.0% (◇), and 2.0% (△) bovine SP-B (weight percent protein with respect to weight of total lipid). Excitation was at 465 nm and emission at 530 nm. Intensity was calculated as $I_{\parallel} + 2I_{\perp}$.

presence of SP-B. In contrast, 0.5% SP-B has a larger effect on fluorescence intensity of 6-NBD-PG below T_c as noted from the increase in intensity between 20 and 38 °C. Introduction of 1.0% (w/w) SP-B yields slightly increased fluorescence of 6-NBD-PG over the lipid mixture, although to a lesser extent compared to the preparation containing 0.5% (w/w) SP-B. SP-B at 2% (w/w) caused a dramatic decrease in intensity of the 6-NBD-PG probe with respect to that of the lipid preparation below T_c.

Fluorescence Lifetimes. Fluorescence lifetime data for 6-NBD-PC in 7:1 DPPC/DPPG liposomes were compatible with either a two- or three-component decay model but could not be adequately modeled by one specific lifetime (Table I). The Gaussian distribution of the two-component model produced a better fit than the discrete lifetime values, as indicated by the decrease in χ^2. In the three-component model, Gaussian distribution did not improve the quality of the fit as compared to discrete lifetimes. Analysis of the experimental data based on three-component models yielded better fits than either discrete or distributional two-component models. Guassian distributions of both the two- and three-component models assigned a very narrow distributional width to the long-lifetime component and a broader, but modest, width to the short-

Table I: Fit Parameters for Two- and Three-Lifetime Models of NBD-PC Fluorescence Decay in 7:1 DPPC/DPPG Multilamellar Vesicles[a]

	f_1	τ_1	Δ_1 (α_1)	f_2	τ_2	Δ_2 (α_2)	f_3	τ_3	Δ_3 (α_3)	χ^2
Gaussian	0.74	6.63	0.06	0.26	1.60	1.07				3.35
Gaussian	0.46	8.30	0.08	0.41	3.76	1.14	0.13	1.05	1.24	0.43
uniform	0.74	6.60	0.05	0.26	1.60	1.74				3.28
discrete	0.77	6.44 ± 0.11	(0.41)	0.23	1.35 ± 0.05	(0.59)				4.91
discrete	0.50	8.09 ± 0.32	(0.20)	0.40	3.44 ± 0.28	(0.38)	0.11	0.84 ± 0.07	(0.42)	0.42

[a] Symbols are as follows: $f_{1,2,3}$ = fraction of respective lifetime component of Gaussian, uniform, or discrete term; $\tau_{1,2,3}$ = lifetime centers (ns); $\Delta_{1,2,3}$ = half-widths (ns) for Gaussian and uniform fits; ($\alpha_{1,2,3}$) = mole fractions of the fluorophore decaying at the respective lifetime for the discrete fits. Data were acquired at 11 °C.

lifetime component(s). Therefore, the distributions are not overlapping and indicate well-resolved components.

Discussion

Changes in lipid organization within a model membrane similar to pulmonary surfactant were determined by static fluorescence anisotropy. Deviations in fluorescent probe rotational motions (fluorescence anisotropic motions), which are coupled to membrane lipid acyl chain orientational fluctuations, were utilized to ascertain variations in membrane order. Three probes, *trans*-PnA, *cis*-PnA, and DPH, were used specifically to assess perturbations in lipid order at the bilayer interior via fluorescence anisotropy. The fluorescent moeities of *cis*- and *trans*-parinaric acid are situated at the fatty acyl chain terminus and are sensitive to changes in lipid organization at the interior plane of a membrane bilayer (Sklar et al., 1975; Welti & Silbert, 1982), while DPH is distributed more randomly about the hydrophobic regions of a bilayer and is sensitive to changes in lipid order at various depths in the hydrophobic bilayer interior (Lentz, 1989). *trans*-Parinaric acid favors regions of gel-phase lipids, while *cis*-parinaric acid and DPH are distributed equally throughout the gel- and fluid-phase lipids (Sklar et al., 1975; Welti & Silbert, 1982; Lentz, 1989). Insertion of SP-B into the model membrane did not alter the fluorescence anisotropy of any of the three membrane interior probes at gel-phase or fluid-phase temperatures, indicating that SP-B had little or no effect on the organization at the bilayer interior. Since *trans*-PnA resides predominantly in gel-phase lipid domains and SP-B had no effect on *trans*-PnA fluorescence anisotropy within the model membrane, it is possible that SP-B either creates or occupies regions of fluid-phase lipid. Although the fluorescence anisotropy of *cis*-PnA or DPH as a function of temperature was unaltered upon the addition of SP-B, the phase transition temperature increases proportionally to SP-B concentration. In contrast, outer membrane-spanning proteins, such as rhodopsin and the M13 coat protein, tend to decrease the fluorescence anisotropy of the parinaric acid probes (Sklar et al., 1979; Kimelman et al., 1979) and demonstrate the disordering effects of these proteins on the membrane interior. The inability of SP-B to affect the properties of the interior-specific probes supports the concept that SP-B is not a membrane-spanning protein. The ability of SP-B to cause a shift in the observed T_c as measured by DPH and *cis*-PnA is intriguing and will be addressed in forthcoming work.

Alterations in the anisotropy NBD-PC and NBD-PG upon incorporation of SP-B into the model membrane can be attributed to changes in lipid order at the bilayer surface. Fluorescence lifetime measurements of NBD-PC in 7:1 DPPC/DPPG liposomes revealed at least two lifetimes for the fluorophore which correspond to different conformers. The dominant component has a relatively short lifetime (approximately 1.5 ns), suggeting that the NBD group of this conformer is more accessible to water (a quencher) and therefore is located near the polar/hydrocarbon interface of the bilayer. The longer lifetime component (τ_c = 6.6 ns) is less exposed to water and penetrates the hydrophobic portion of the bilayer. Relative changes in the distribution of each of the conformers are apparent in the plots of fluorescence intensity as a function of temperature. Addition of 0.5% (w/w) SP-B increased the distribution of the NBD-PC short lifetime component at gel-phase temperatures. Higher concentrations of SP-B [1.0 and 2.0% (w/w)] did not significantly alter the distribution of the NBD-PC fluorescence lifetime components observed for 0.5% (w/w) SP-B, but incorporation of 1.0 and 2.0% (w/w) SP-B resulted in an apparent reversal in the distribution of the NBD-PC lifetime components. Chattopadhyay and London (1987, 1988) concluded from membrane depth measurements that the NBD ring of NBD-PC, owing to its polarity, folds back toward the surface of the bilayer in dioleoylphosphatidylcholine liposomes at liquid-crystalline-phase temperatures. The short-lifetime species of NBD-PC that we have identified in the DPPC/DPPG model membrane at gel-phase temperatures presumably exists in this conformation as presumably do the majority of the NBD-PG molecules. Any changes in lipid organization near the bilayer surface consequently influence the fluorescence properties of both NBD-PC and NBD-PG.

Bovine SP-B markedly ordered the surface of the 7:1 DPPC/DPPG membrane bilayer as indicated by the increase in anisotropy of NBD-PC and NBD-PG upon the incorporation of the protein into the membrane. Ordering at the polar/hydrophobic interface is maximal at temperatures below that of the phase transition but is also apparent above the phase transition. The ability of SP-B to cause such a marked increase in surface order, while minimally altering fatty acyl chain structure, is consistent with an amphipathic structure previously predicted for SP-B (Glasser et al., 1987). In addition, the increased organization of the phospholipid polar head groups, as detected by the fluorescence anisotropy of NBD-PC, was found to be dependent upon SP-B concentration over a range of 0–2% SP-B (w/w protein to lipid). This SP-B concentration range is inclusive of an amount corresponding to the SP-B content estimated for natural surfactant (Tanaka et al., 1986).

Phosphatidylglycerol (PG) is present in pulmonary surfactant as 10–20% (w/w) of the total phospholipid, second in abundance only to phosphatidylcholine (75%) (Tanaka et al., 1986; Post & van Golde, 1988). This relatively high amount of PG, as compared to the minute quantities found in other mammalian tissues (Post & van Golde, 1988), suggests that phosphatidylglycerol has a very important role in the surfactant system; its precise function, however, is as yet unknown. Fluorescence anisotropy and fluorescence intensity measurements using 6-NBD-PG suggest a specific interaction between the phosphatidyl head group of the probe and SP-B. Although NBD-PG anisotropy data indicate an initial decrease in surface order upon the addition of 0.5% SP-B, a decrease in surface order and a concomitant increase in broadening of the phase transition occur upon the incorporation of 1.0 and 2.0% SP-B.

Since the fluorescence intensity of NBD-PG decreases significantly with incorporation of 1.0 or 2.0% SP-B into the model membrane, the NBD ring was quenched either by becoming more exposed to water at the membrane surface or by interacting with the SP-B. A concept that would support the fluorescence anisotropy and fluorescence intensity data for NBD-PG is that there is a distinct interaction between the negative charge of the phosphoglycerol head group and one or more of the 10 positive charges on the bovine SP-B. Consequently, at lower protein concentrations NBD-PG molecules are more dispersed throughout the model membrane. Increasing amounts of SP-B would provide more positive charges at sites for interaction with the PG head group, an interaction that would result in quenching of the NBD fluorophore.

One problem that exists in the utilization of the NBD-PC and NBD-PG probes is the high sensitivity of these probes to self-quenching effects due to increased contacts with one another (Arvinte et al., 1986). As self-quenching occurs, the fluorescence lifetime of the NBD probe decreases with a concomitant increase in anisotropy (Hoekstra, 1982). This phenomenon could explain the increases in anisotropy of NBD-PC as a function of SP-B concentration if SP-B promotes lateral phase separation, thereby concentrating NBD-PC probes in phase-separated regions (increased collisions and/or formation of clusters of NBD-PC probes would be enhanced). In this case, an increase in SP-B concentration would most likely promote an increase in lateral phase-separated regions in the membrane, thus increasing probe self-quenching and decreasing the NBD fluorescence lifetime. As a result, a decrease in fluorescence intensity and an increase in anisotropy would occur. The results obtained for the NBD-PC probe indicate that SP-B does increase anisotropy from 0.1 to 2.0% (w/w) protein; however, a concomitant decrease in the probe fluorescence intensity above 0.5% (w/w) SP-B does not occur. Therefore, the results reported here support the concept that SP-B orders the membrane surface rather than inducing lateral phase separations. While no evidence for SP-B-induced phase separations has been noted with NBD-PC and NBD-PG, further investigation into this possibility is currently underway.

SP-B has unique protein–lipid interactions as compared to those of two other surfactant-associated proteins, SP-A and SP-C. SP-A, a glycoprotein of M_r = 35 000, promotes aggregation of phospholipid vesicles in the presence of Ca^{2+} and is a necessary component of tubular myelin (Ross et al., 1986; King, 1984). SP-A is not as effective as SP-B or SP-C in enhancing biophysical activity of surfactant phospholipids (Ross et al., 1986). SP-C, a very hydrophobic protein of M_r = 4000, causes increased fluorescence of the NBD-PC probe, similar to that observed with SP-B. However, SP-C does not demonstrate a selective interaction with NBD-PG, even though the amino terminus of SP-C contains three cationic charges (B. Elledge and J. A. Whitsett, unpublished data).

The precise mechanism by which SP-B enhances the biophysical activity of surfactant phospholipids remains to be clarified. The present findings, however, indicate that SP-B reorganizes membrane lipids to yield a more ordered bilayer surface and appears to have a distinct interaction with the PG head group. These characteristics would yield a lipid/protein mixture with surfactant activity higher than that of the lipid mixture in the absence of SP-B.

Acknowledgments

We thank Dr. Friedhelm Schroeder of the Department of Pharmacy, University of Cincinnati, for many helpful discussions. We acknowledge the Laboratory for Fluorescence Dynamics, University of Illinois at Urbana–Champaign, where lifetime experiments were performed.

References

Arvinte, T., Cudd, A., & Hildenbrand, K. (1986) *Biochim. Biophys. Acta 860*, 215–228.

Chattopadhyay, A., & London, E. (1987) *Biochemistry 26*, 39–45.

Chattopadhyay, A., & London, E. (1988) *Biochim. Biophys. Acta 938*, 24–34.

Curstedt, T., Johansson, J., Barros-Soderling, J., Robertson, B., Nilsson, G., Westberg, M., & Jornvall, H. (1988) *Eur. J. Biochem. 172*, 521–525.

Glasser, S. W., Korfhagen, T. R., Weaver, T. E., Pilot-Matias, T., Fox, J. L., & Whitsett, J. A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4007–4011.

Glasser, S. W., Korfhagen, T. R., Weaver, T. E., Clark, J. C., Pilot-Matias, T., Meuth, J., Fox, J. L., & Whitsett, J. A. (1988) *J. Biol. Chem. 263*, 9–12.

Goerke, J. (1974) *Biochim. Biophys. Acta 344*, 241–261.

Goerke, J., & Clements, J. (1986) in *Handbook of Physiology* (Macklem, P. T., & Mead, J., Eds.) Vol. III, pp 247–261, American Physiological Society, Bethesda, MD.

Gratton, E., & Limkeman, M. (1984) *Biophys. J. 44*, 315–324.

Hallman, M., Spragg, R., Harell, J. H., Moser, K. M., & Gluck, L. (1982) *J. Clin. Invest. 70*, 673–683.

Hawgood, S., Benson, B. J., Schilling, J., Damm, D., Clements, J. A., & White, R. T. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 66–70.

Hoekstra, D. (1982) *Biochemistry 21*, 1055–1061.

Johansson, J., Jornvall, H., Eklund, A., Christensen, N., Robertson, B., & Curstedt, T. (1988) *FEBS Lett. 232*, 61–64.

Kimelman, D., Tecoma, E. S., Wolber, P. K., Hudson, B. S., Wickner, W. T., & Simoni, R. D. (1979) *Biochemistry 18* (26), 5874–5880.

King, R. J. (1984) *Exp. Lung Res. 6*, 237–253.

Kogishi, K., Kurozumi, M., Fujita, Y., Murayama, T., Kuze, F., & Suzuki, Y. (1988) *Am. Rev. Respir. Dis. 137*, 1426–1431.

Lentz, B. R. (1989) *Chem. Phys. Lipids 50*, 171–190.

Notter, R. H. (1984) in *Pulmonary Surfactant* (Robertson, B., Van Golde, L. M. G., & Batenburg, J. J., Eds.) pp 17–65, Elsevier Science Publishers, Amsterdam.

Notter, R. H., & Finkelstein, J. N. (1984) *J. Appl. Physiol.: Respir., Environ. Exercise Physiol. 57*, 1613–1624.

Notter, R. H., Shapiro, D. L., & Whitsett, J. A. (1987) *Chem. Phys. Lipids 44*, 1–7.

Olafson, R. W., Rink, U., Kielland, S., Yu, S.-H., Chung, J., Harding, P. G. R., & Possmayer, F. (1987) *Biochem. Biophys. Res. Commun. 148* (3), 1406–1411.

Post, M., & van Golde, L. M. G. (1988) *Biochim. Biophys. Acta 947*, 249–286.

Ross, G. F., Notter, R. H., Meuth, J., & Whitsett, J. A. (1986) *J. Biol. Chem. 261*, 14283–14291.

Schagger, H., & von Jagow, G. (1987) *Anal. Biochem. 166*, 368–379.

Sklar, L. A., Hudson, B. S., & Simoni, R. D. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1649–1653.

Sklar, L. A., Miljanich, G. P., Bursten, S. L., & Dratz, E. A. (1979) *J. Biol. Chem. 254*, 9583–9591.

Takahashi, A., & Fujiwara, T. (1986) *Biochem. Biophys. Res. Commun. 135*, 527–532.

Tanaka, Y., Takei, T., & Masuda, K. (1983) *Chem. Pharm. Bull. 31*, 4110–4115.

Tanaka, Y., Takei, T., Aiba, T., Masuda, K., Kiuchi, A., & Fujiwara, T. (1986) *J. Lipid Res. 27*, 475–485.
Welti, R., & Silbert, D. F. (1982) *Biochemistry 21*, 5685–5689.
Whitsett, J. A., Ohning, B. L., Ross, G., Meuth, J., Weaver, T., Holm, B. A., Shapiro, D. L., & Notter, R. H. (1986a) *Pediatr. Res. 20*, 460–467.
Whitsett, J. A., Hull, W. H., Ohning, B. L., Ross, G. F., & Weaver, T. E. (1986b) *Pediatr. Res. 20*, 744–749.
Yu, S.-H., & Possmayer, F. (1986) *Biochem. J. 236*, 85–89.

Evaluation of Membrane Phase Behavior as a Tool To Detect Extrinsic Protein-Induced Domain Formation: Binding of Prothrombin to Phosphatidylserine/Phosphatidylcholine Vesicles†

Susan W. Tendian and Barry R. Lentz*
Department of Biochemistry and Nutrition, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7260

Received August 29, 1989; Revised Manuscript Received March 26, 1990

ABSTRACT: The temperature–composition phase diagram of mixed dimyristoylphosphatidylserine (DMPS) and dimyristoylphosphatidylcholine (DMPC) small unilammelar vesicles was determined in the presence and absence of bound bovine prothrombin by monitoring the phospholipid order–disorder phase separation using diphenylhexatriene (DPH) fluorescence anisotropy. The shape of the membrane temperature–composition diagram was essentially unaltered by the binding of prothrombin in the presence of Ca^{2+} although the two-phase (gel/fluid) region was slightly narrowed and shifted by 1–10 °C to higher temperatures. This result does not support the popular idea that extensive domains rich in negatively charged phospholipid are induced in response to prothrombin binding. Instead of implying domain formation, our results demonstrate that the observed increase in melting temperature associated with binding of prothrombin to acidic phospholipid membranes can be accounted for by the observed altered membrane order both in the fluid and in the solid lamellar phases. The membrane order in the liquid-crystalline phase increased with increased acidic lipid content, and much more so for DMPS than for dipentadecanoylphosphatidylglycerol ($DC_{15}PG$). These results demonstrate that simple shifts in membrane phase behavior cannot be properly interpreted to prove the existence of charged lipid domains. In addition, we report the unexpected observation that prothrombin increased the anisotropy of DPH in DMPS/DMPC vesicles in the liquid-crystalline phase in the absence of Ca^{2+} as well as in its presence. This effect was seen to a lesser extent and only at a much higher charged-lipid content for $DC_{15}PG$/DMPC vesicles. Prothrombin fragment 1 with or without Ca^{2+} did not alter the packing in $DC_{15}PG$/DMPC or DMPS/DMPC membranes in this manner. These observations are discussed in terms of the possibility that phosphatidylserine interacts in a specific and Ca^{2+}-independent manner with at least one site on prothrombin.

Crucial proteolytic steps in the blood coagulation cascade are catalyzed by multiprotein complexes that assemble on platelet-derived membranes (Nelsestuen, 1978; Mann, 1987). The protein components of these proteolytic complexes are thought to bind to platelet membranes via electrostatic interactions with negatively charged phospholipids. One such protein complex, the prothrombinase complex, catalyzes the proteolytic conversion of prothrombin to thrombin. The enzyme, factor X_a, associates with its cofactor, factor V_a, on the surface of a platelet or phospholipid vesicle to accelerate conversion.

Factor X_a, and prothrombin are vitamin K dependent proteins containing doubly negatively charged γ-carboxyglutamic acid (GLA)[1] residues. Binding of these proteins to membranes containing negatively charged phospholipids requires Ca^{2+} and has been proposed to occur via "calcium bridging" of GLA residues to negatively charged phospholipids in the membrane (Lim et al., 1977; Dombrose et al., 1979; Wei et al., 1982), although there is no direct evidence in support of this bridging hypothesis. Irrespective of whether "bridging" is involved, the binding site for these proteins is currently envisioned by some as a local pool or "domain" of negatively charged phospholipid that condenses under the surface-bound protein (Lim et al., 1977; Dombrose et al., 1979; Mayer & Nelsestuen, 1981). This model for the formation of domains of negatively charged phospholipids has also been suggested for the binding of other extrinsic membrane proteins (Birrell & Griffith, 1976; Hartmann et al., 1977; Wiener et al., 1985).

We have questioned the concept of negative lipid domain formation in response to binding of extrinsic membrane proteins, in particular the binding of GLA-containing proteins

†Supported by the UNC SCOR in Thrombosis and Hemostasis (USPHS HL 26309).
*To whom correspondence should be addressed.

[1] Abbreviations: GLA, γ-carboxyglutamic acid; DMPS, 1,2-dimyristoyl-3-*sn*-phosphatidylserine; $DC_{15}PG$, 1,2-dipentadecanoyl-3-*sn*-phosphatidylglycerol; POPA, 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidic acid; DMPC, 1,2-dimyristoyl-3-*sn*-phosphatidylcholine; PS, phosphatidylserine; PC, phosphatidylcholine; PA, phosphatidic acid; PG, phosphatidylglycerol; SUV, small unilamellar vesicle(s); TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; Na_2EDTA, disodium ethylenediaminetetraacetate; Tris, tris(hydroxymethyl)aminomethane; DEAE-cellulose, (diethylaminoethyl)cellulose; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPHpPC, 1-palmitoyl-2-[[2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl]ethyl]carbonyl]-3-*sn*-phosphatidylcholine; K_d, equilibrium dissociation constant; T_m, melting temperature.

0006-2960/90/0429-6720$02.50/0 © 1990 American Chemical Society