Biochimica et Biophysica Acta 896 (1987) 1–10
Elsevier

BBA 73181

Fourier transform infrared and differential scanning calorimetric studies of a surface-active material from rabbit lung

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(Received 1 July 1986)

Key words: Pulmonary surfactant; Fourier transform; Infrared spectroscopy; Phase transition; Surface-active material; Differential scanning calorimetry; (Rabbit lung)

A surface-active material with a chemical composition consistent with lung surfactant and with the ability to lower surface tension on a Wilhelmy balance to about 6 mN/m, has been isolated from rabbit pulmonary lavage. The thermotropic properties have been characterized with the techniques of Fourier transform infrared spectroscopy (FT-IR) and Differential Scanning Calorimetry (DSC). FT-IR melting curves were constructed from the temperature-dependence of the lipid CH_2 symmetric stretching vibrational frequencies near 2850 cm^{-1}. A broad gel-liquid crystal phase transition with an onset temperature of about 22°C, and a completion temperature of about 38°C was observed, with slight sample-to-sample variations in temperatures. A similar completion temperature was noted in DSC endotherms. Ca^{2+} (5–10 mM) increased the onset temperature of the lipid-melting event, and induced an ordering of surfactant and of its lipid extract at all temperatures studied. The effect on the lipids was suggestive of a Ca^{2+}-induced phase separation caused by ion binding to phosphatidylglycerol and other acidic components. Evidence for a direct interaction between Ca^{2+} and the phosphate groups was suggested through small Ca^{2+}-induced shifts in the 1090 cm^{-1} symmetric PO_2 stretching frequency. Removal of most of the protein component from a 10:1 (lipid/protein, w/w) sample caused an ordering of the resultant lipid fractions. In contrast, removal of most of the protein component from a 20:1 sample resulted in no change in lipid order or thermotropic behavior. These observations are discussed in light of the roles played both by Ca^{2+} and protein in the spreading of surfactant. The power of FT-IR to acquire useful structural information from complex biological tissues is demonstrated.

Introduction

Pulmonary surfactant functions by reducing the surface tension at the air-liquid interface in the alveoli of mammalian lung. The physiological requirements for an effective surfactant have been discussed [1,2]. These include the ability of a compressed monolayer to produce a surface tension of less than 9 dyn/cm, to sustain a low surface tension for sufficient time to avoid al-

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Abbreviations: FT-IR, Fourier-transform infrared spectroscopy; DSC, differential scanning calorimetry; DPPC, 1,2-dipalmitoylphosphatidylcholine; POPG, 1-palmitoyl,2-oleoylphosphatidylglycerol; DMPC, 1,2-dimyristoylphophatidylcholine; PC, phosphatidylcholine; PG, phosphatidylglycerol.

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veolar collapse and to spread rapidly from the subphase. Chemical analysis of surfactant reveals the presence of lipids, primarily phospholipids, and protein. The predominant lipid of mammalian lung surfactant is dipalmitoylphosphatidylcholine (DPPC). In bilayer systems, DPPC undergoes its gel-liquid crystal transition at 41°C. Therefore, under high compression at 37°C, the rigidity of the molecule enables it to sustain high surface pressures. However, the very rigidity of the acyl chains (necessary for the first two requirements above), constitutes a detriment to the third requirement, namely, the ability of surfactant to spread rapidly. It has been suggested [3] that a delicate balance of fluid lipids (such as unsaturated phospholipids) and rigid lipids is to be expected, and indeed, appears to occur [4]. In addition, it has recently been shown [5] that specific Ca^{2+}-protein–lipid interactions are important for determining surfactant structure and function. Notter, et al. [6], showed that whole lung surfactant will form a surface film when Ca^{2+} (but not Mg^{2+}) is present in the subphase, while Hawgood, et al. [5] suggest that the threshold concentration for Ca^{2+}-induced aggregation of unilamellar liposomes was reduced from 13 to 0.5 mM by a protein fraction isolated from surfactant. It has been previously suggested [7,8] that both Ca^{2+} and specific proteins modify the state of dispersion of surfactant lipids. As a Ca^{2+} concentration of 1.6 mM has been recently measured for adult rabbit lungs [9], the protein-induced reduction of the Ca^{2+} threshold could have functional consequences. Physical studies of the interaction between the components in this complex system are therefore indicated.

The physical techniques used to date for the study of lung surfactant have centered around surface balance determination of monolayer film properties (for a review see Notter [10]) and the use of (most frequently) turbidimetric [5,11] or DSC determination [12] of surfactant phase properties. Yet these methods do not provide information about the molecular configuration or conformation of surfactant components. A recent exception to this [13] involved electron paramagnetic spectroscopic studies of lipid fluidity in surfactant. While this method has provided much useful data on lipid structure in general [14], the requirement of the technique for a probe molecule such as 5-doxylmethylstearate in the aforementioned study, leads to the possibility of probe-induced alterations in lipid order [15], and partitioning of the probe into particular regions of the lipidic system. Thus, the application of additional physical methods to the problem of interacting components in lung surfactant is in order.

The techniques of FT-IR spectroscopy offer several advantages for the study of surfactant, as follows:

(1) Both lipid configuration and protein secondary structure are monitored in a single experiment.

(2) No probe molecules are required.

(3) Small amounts of material (1–500 μg) may be studied in a variety of physical states, including monolayers both on IR substrates such as germanium (via the techniques of attenuated total reflectance) and on an aqueous surface [16,17].

The current study reports the first FT-IR investigations of rabbit lung surfactant. The study focuses on the thermotropic properties of surfactant and their modification by Ca^{2+} and surfactant protein. Complementary DSC investigations are also presented.

Materials and Methods

Isolation and purification of surfactant

New Zealand white rabbits were anesthetized with sodium pentobarbital (30 mg/kg of body weight, intravenously) and exsanguinated by severing the abdominal aorta. The chest was opened and the lungs lavaged through a tracheal cannula with a volume of cold saline equal to the functional residual capacity, i.e., 30 ml/kg of body weight. The saline was gently infused and withdrawn five times. This procedure was repeated five times, the total volume of saline in the lungs never exceeding the functional residual capacity.

Surfactant was isolated by the method of Suzuki, et al. [18]. Briefly, lavage fluid was centrifuged at $100 \times g$ for 10 min to remove cells and debris. The supernatant was centrifuged at 16000 $\times g$ for 1 h at 0°C. The precipitate was resuspended in 0.145 M NaCl containing 0.01 M Tris-HCl and 1 mM EDTA (buffered NaCl), layered over a discontinuous gradient of 0.25 M and 0.68

M sucrose and centrifuged at 78 000 × *g* for 1 h at 0°C. The material at the interface was collected by aspiration and washed three times with buffered NaCl. The pellet was resuspended in buffered NaCl and stored at −20°C.

Chemical characterization

Total phosphorus was determined by the method of Chen et al. [19]. Protein was determined by the method of Lowry [20]. Lipid and protein components were separated using Bligh-Dyer or Folch [21] extraction protocols. The lipid classes were determined by analytical thin-layer chromatography using a $CHCl_3$/MeOH/NH_4OH (65 : 25 : 5) solvent system. The phospholipid spots were scraped, and total phosphorus for each spot was determined as per Chen et al. [19]. Acyl chain lengths of the lipid component were assayed by gas chromatography on a Hewlett Packard 5890A gas liquid chromatograph following formation of the methyl esters. Protein molecular weights were evaluated by 10% SDS-polyacrylamide gel electrophoresis.

Dynamic surface tension

A 1 ml aliquot of surfactant with a lipid/protein ratio of 10 : 1 (w/w) and containing 1.8 mg total lipid/ml was gently spread on the surface of buffered Ringer's solution (pH 7.4) in a vertical film surface balance [22] at 37°C, 100% humidity, and the subphase was gently stirred. The minimum surface area was 30.2 cm^2 and maximal surface area was 161.4 cm^2. The rate of cycling between the minimum and maximum surface areas was 5.25 min/cycle. Surface tension was measured via a Cahn electrobalance (Cahn Instrument Co., Paramont, CA, U.S.A.). Cycling was continued until the maximum and minimum surface areas were stable.

FT-IR spectroscopy

Samples were prepared for FT-IR examination as follows:

(1) Surfactant without calcium-surfactant in NaCl buffer was centrifuged at 100 000 × *g* for 1 h at 0°C.

(2) Surfactant with calcium-surfactant was centrifuged at 10 000 × *g* for 1 h at 0°C. The pellet was resuspended in 0.145 M NaCl containing 0.01 M Tris-HCl and 5 mM or 10 mM $CaCl_2$ (calcium buffer) and recentrifuged at 100 000 × *g* for 1 h at 0°C.

(3) Lipid extract without calcium-lipid component was extracted as above and the organic solvent evaporated. The lipids were resuspended in buffered NaCl and centrifuged as for surfactant

(4) Lipid extract with calcium-prepared in the same manner as the sample without calcium except that the lipids were resuspended in calcium buffer and then centrifuged.

The samples were examined in a Harrick cell (pathlength 25 μM) equipped with CaF_2 windows. Spectra were recorded on a Mattson Instruments Sirius 100 spectrometer equipped with a mercury-cadmium-telluride detector. Routinely, 200 interferograms were collected, co-added, apodized with a triangular function and Fourier transformed to give a resolution of 4 cm^{-1} with data encoded every 2 cm^{-1}. Temperature was controlled with a Haake circulating bath and monitored with a Bailey BAT-12 digital thermometer, with a thermocouple sensor placed close to the IR windows in the cell. Frequencies were measured by fitting a parabola to the top three data points followed by finding the maximum in the parabola. The spectrum of water (matched for temperature and path length) was subtracted from all data sets. Residual sloping baselines were removed with a linear baseline leveling routine supplied with the instrument software.

Differential scanning calorimetry

Calorimetry experiments were performed in a Micro-cal MC1 unit. Sample volumes were 0.70 ml. Samples were heated at about 24°C/h following 1–2 h of equilibration in the instrument.

Results

Biochemical characterization of surfactant

The phospholipid composition (only components present in > 1% quantities are listed) of surfactant as determined by quantitative TLC is summarized in Table I. The results are in general accord with those of Hook et al. [13], whose value for phosphatidylglycerol (9.4%) was somewhat lower. The fatty acid composition of the phospholipids is also given in Table I. Palmitic acid

TABLE I
PHOSPHOLIPID COMPOSITION OF SURFACTANT

Phospholipid Class	% of P recovered
PC	78.5
PG	15.1
PE	3.7
LysoPC	2.7

FATTY ACID COMPOSITION OF TOTAL LIPID EXTRACT

Chain length: C = C bonds	(%)
14:0	3.0
16:0	64.9
16:1	4.6
18:0	3.8
18:1	15.5
18:2	6.2
Unidentified	2.0

was the major fatty acid, accounting for 64.9% of the total fatty acid present. Substantial amounts of oleic and linoleic acid were also noted. These results are in excellent accord with those of Magoon et al. [23], who observed that palmitic and oleic acids constituted the main fatty acid constituents in both the PC and PG fractions of rabbit lamellar bodies.

Five major protein fractions were observed with SDS-polyacrylamide gel electrophoresis. The molecular weights (kDa) were 15, 24, 32, 45 and 66. Wright, et al. [24] observed and characterized two fractions of protein with apparent molecular mass 30–36 kDa and 10–15 kDa. In addition, they observed prominent bands (possibly serum derived) with approximate molecular masses of 25, 50, 65–67 and 75 kD, in excellent accord with the current preparation.

Some sample-to-sample variation in the lipid/protein ratio has been noted. Values ranged from 10:1 to 5:1 with occasional samples falling outside these levels. Our variation was similar to that noted by Keough et al. [12], who noted variation in the lipid/protein ratio from 10.8:1 to 6.6:1 depending on the number of centrifugation steps used in the isolation protocol.

Dynamic surface tension studies

Slow cycling of surface area resulted in a stable maximum surface tension of 50 mN/m and minimum surface tension of 6 mN/m. This fits the criterion for surface tension lowering ability of surfactant as discussed in the Introduction.

TABLE II

ASSIGNMENTS OF FT-IR FREQUENCIES IN THE 1000–1800 cm^{-1} REGION OF SURFACTANT

Symbols: s, m, w, sh, v stand for strong, medium, weak, shoulder and very, respectively.

Frequency in cm^{-1}	Assignment
1018 sh	
1065 s	ester C-O sym. stretch
1088	phosphate sym. stretch
1114 sh	
1148 sh	
1175 w	ester C-O asym. stretch
1200	CH_2 wagging progression superimposed on the phosphate
1225	
1242	
1263	
1287	antisymmetric stretch at 1225 cm^{-1}
1342 w	CH_2 wagging
1379 w	CH_2 symmetric bend
1403 vs	choline CH_3 sym. bend
1420 w	alpha CH_2 scissoring
1437 sh	
1458 sh	CH_3 asym. bend
1468 s	CH_2 scissoring
1487 sh	choline asym. bend
1543	protein amide II
1550	
1557	
1638 sh	protein amide I + H_2O
1651 s	
1738 s	C = O stretch

FT-IR and DSC studies

Typical FT-IR spectra from the 1000–1800 and 2700–3100 cm^{-1} spectral regions are shown in Fig. 1. The observed intense spectral features arise from the phospholipid components of surfactant with two exceptions – the contour near 1650 cm^{-1} is due to overlapped solvent bending and protein Amide I (peptide C = O stretch) modes, and the features between 1540 and 1560 cm^{-1} arise from protein Amide II (mixed N-H in-plane bend and C-N stretch) modes. Spectral assignments are given in Table II.

Lipid thermotropic behavior is conveniently monitored [26] through temperature-induced alterations in the frequency of the CH_2 symmetric

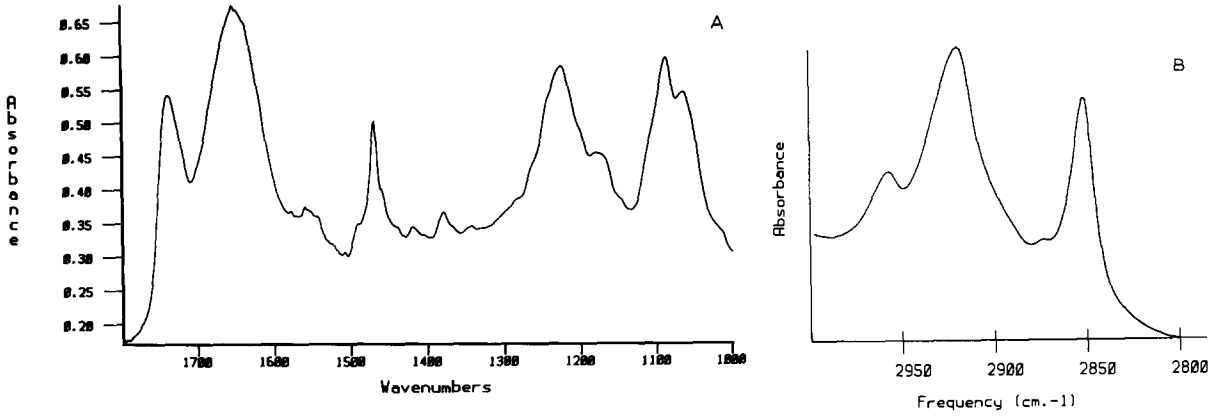

Fig. 1. Typical FT-IR spectra for surfactant. (A) The 1000–1800 cm^{-1} spectral region of a sample of surfactant with 5 mM Ca^{2+}. The experiment was performed at 5.7°C. The majority of the spectral features arise from the lipids; exceptions to this are the weak protein features between 1500–1580 cm^{-1}, and some of the intensity underlying the 1650 cm^{-1} band. (B) The 2800–3000 cm^{-1} spectral region of a sample of surfactant with 5 mM Ca^{2+}. The experiment was performed at 5.7°C. The spectral features arise primarily from the lipidic components. The band near 2850 cm^{-1} had little underlying contribution from protein and, therefore, was the band of choice for monitoring the thermotrophic behavior of phospholipids.

stretching bands of the lipid acyl chains near 2850 cm^{-1}. This spectral feature is one of the strongest in the spectrum and suffers no interference from overlapping protein bands. Although frequency increases are small (1–3 cm^{-1}) during gel-liquid crystal phase transitions, they may be monitored with a precision of 0.01–0.05 cm^{-1} through use of the center of gravity algorithm for band positions developed by Cameron et al. [27]. The origin of the frequency increase on melting has been traced by Snyder et al. [28] to changes in the interaction

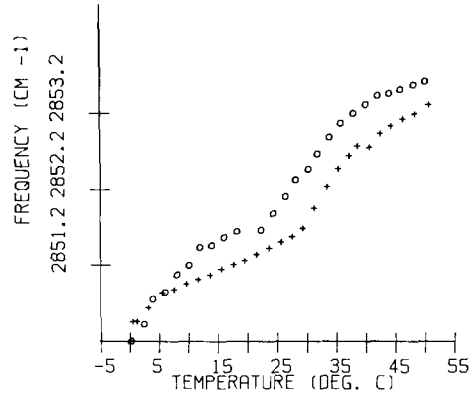

Fig. 2. Thermotropic behavior for surfactant (O) and surfactant+10 mM Ca^{2+} (+) with lipid/protein ratios (w/w) of 7:1. The parameter monitored is the frequency of the CH_2 symmetric stretching modes of the lipid acyl chains near 2850 cm^{-1}.

constants between C-H stretching coordinates on adjacent methylene groups when the lipid physical state is altered through, for example, the formation of *gauche* rotamers in the acyl chains. Although the procedures of Snyder et al. [28] permit no rigorous correlation between the frequency and the number of *gauche* rotamers in the chain, procedures have been developed [29] for conversion of FT-IR melting curves into quantitative indicators of fractions of lipid melted.

The temperature dependence of the 2850 cm^{-1} band for a surfactant preparation (lipid/protein ratio 7:1) is shown in Fig. 2. Although some scatter in the data points is evident at low temperatures, a broad phase transition with an onset temperature of 22–23°C and a completion temperature of about 36–39°C is clearly discernable. As the monitored IR frequency arises from the lipid component of the preparation, the observed transition evidently arises from a gel-liquid crystal transition of the surfactant phospholipids, which are probably in multibilayer form in the current preparation. Thus, at physiological temperatures, the lipids are almost entirely in the liquid crystalline state. The magnitude of the change in the spectral parameter used is similar to, although slightly smaller than, that usually observed in single component phospholipid systems (e.g. Ref. 30). Slight variations in the onset and completion

temperatures were noted from sample to sample. Further evidence for the temperature-induced disordering of the acyl chains comes from examination of the 1200–1300 cm^{-1} spectral region (Table II). At low temperatures, the observed series of bands at 1200, 1225, 1242, 1262 and 1284 cm^{-1} arises from a progression in the CH_2 wagging mode. The series arises essentially from coupling between similar vibrations along a sequence of unit cells and only occurs if the acyl chain configuration is all-*trans*. Thus at 5°C (data of Table II), a substantial all-*trans* character exists in the chains. As the temperature is raised (data not shown) the shoulders of the series progressively diminish in intensity so that at 48°C, only a single broad peak at 1225 cm^{-1} due to the antisymmetric PO_2 double bond stretching mode remains. The elimination of the band progression arises due to disruption of the coupling between adjacent unit cells upon *gauche* rotamer formation.

The effect of Ca^{2+} on the 7:1 surfactant sample is also shown in Fig. 2. Addition of 10 mM Ca^{2+} produces a 5–7 Cdeg increase in the onset temperature with no large alteration of the completion temperature. The midpoint of the transition is also shifted to higher temperatures. In addition, the CH_2 frequency is lowered from the Ca^{2+}-free surfactant at all temperatures, indicating a Ca^{2+}-induced ordering of the acyl chains.

The results from DSC experiments are shown in Fig. 3. Surfactant (lipid/protein ratio 6:1) shows a broad endotherm with an onset temperature of about 20°C, a completion temperature of

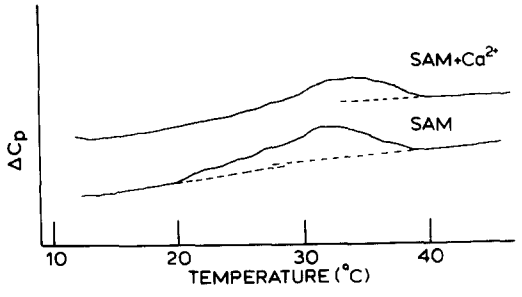

Fig. 3. Differential scanning calorimetry endotherms for surfactant (SAM) and surfactant +10 mM Ca^{2+}. The lipid/protein (w/w) ratio was 6:1 The dashed lines are estimated baselines. Note that for the calcium-containing sample the onset temperature was difficult to estimate due to the continuous curvature of the trace.

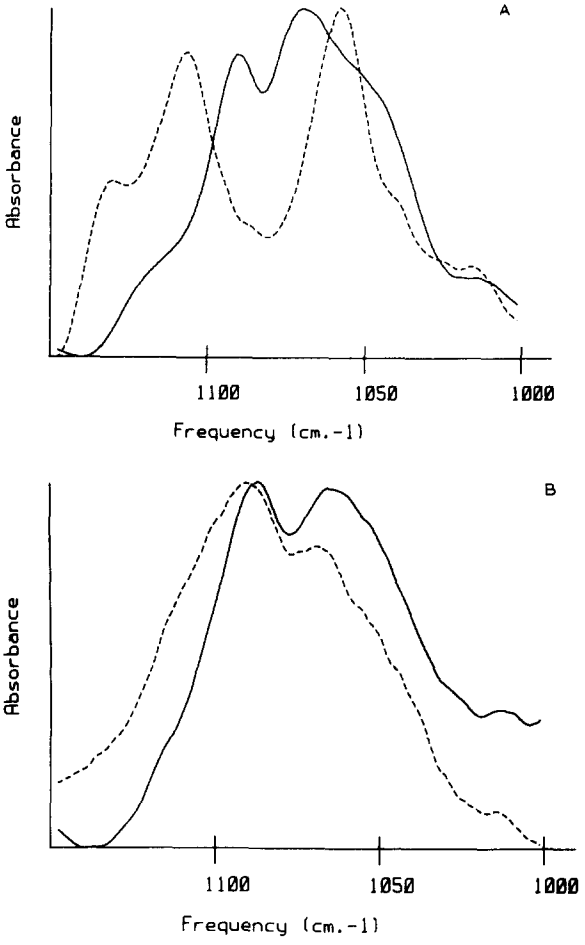

Fig. 4. FT-IR evidence for the interaction of calcium with the phosphate group. (A) The spectral region of 1000–1200 cm^{-1} for POPG (———) and for POPG+ 10 mM Ca^{2+} (- - - - -). (B) The spectral region of 1000–1200 cm^{-1} for surfactant (———) and for surfactant + 10 mM Ca^{2+} (- - - - -).

38.5°C and a maximum at about 31°C. The onset and completion temperatures are in reasonable accord with those determined from FT-IR data. We note that our DSC results differ slightly from those recently reported by Keough et al. [12], who observed higher completion temperatures although the temperature of the maximum was about the same. Part of the discrepancy may arise from difficulties in establishing accurate baselines for samples which have broad, weak endotherms. Ca^{2+}-induced effects, similar to those seen in the FT-IR melting profiles, were observed in the DSC experiments (Fig. 3). A comparison of the endotherms generated in the absence and presence of

Ca^{2+} (10 mM) shows a 1–2 Cdeg increase in the position of maximum excess enthalpy as well as a 1 Cdeg increase in the completion temperature induced by the ion. The occurrence of changes in the onset temperatures were difficult to determine (Fig. 3) due to continually changing baselines in the low temperature (single phase?) regions of surfactant in the presence of Ca^{2+}.

Direct FT-IR evidence for the interaction of Ca^{2+} with lipid head-groups was sought from an experiment whose results are depicted in Fig. 4. The vibrational mode known to respond most strongly to Ca^{2+}/phosphate interaction [31] is the symmetric PO_2^- double bond stretching mode near 1090 cm^{-1}. The control system (Fig. 4a) shows the effect of addition of 10 mM Ca^{2+} to POPG. Substantial alterations in the pattern of frequencies and intensities in the 1000–1150 cm^{-1} region are noted. Much smaller changes are observed when Ca^{2+} is added to surfactant (Fig. 4b). A frequency increase of about 4 cm^{-1} is seen in the 1090 cm^{-1} band and a frequency increase of 2 cm^{-1} is seen in the 1067 cm^{-1} band along with an intensity increase in the shoulder near 1115 cm^{-1}. Direct interaction of the ion with (at least) some of the phospholipid head-groups is therefore suggested.

The effect of calcium on the lipidic components alone is shown in Fig. 5. Lipids were extracted either with a Folch wash or with Bligh-Dyer extraction protocols. Rehydration of the dried lipids was accomplished either in an EDTA-containing buffer or in a buffer containing Ca^{2+}. FT-IR melting curves (Fig. 5) reveal a slight Ca^{2+}-induced ordering as measured from the CH_2 symmetric stretching frequency and upward shifts of the onset and completion temperatures of the lipid phase transition. An attempt was made to

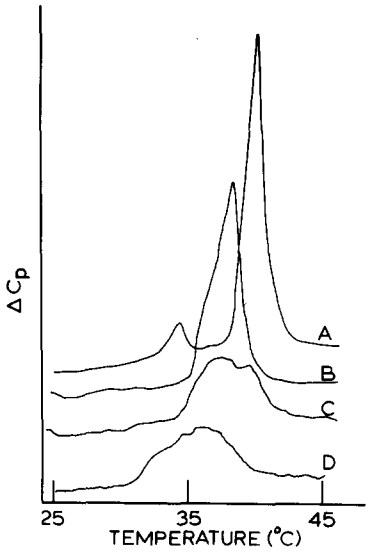

Fig. 6. Differential scanning calorimetry traces for:
(A) a 9:1 DPPC/POPG mixture in the presence of 10 mM Ca^{2+}
(B) a 9:1 DPPC/POPG mixture in the absence of Ca^{2+}
(C) a 4:1 DPPC/POPG mixture in the presence of 10 mM Ca^{2+}
(D) a 4:1 DPPC/POPG mixture in the absence of Ca^{2+}

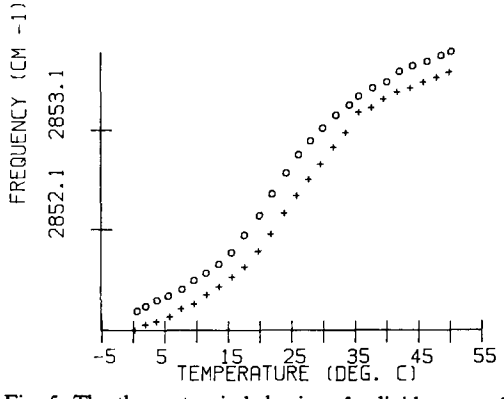

Fig. 5. The thermotropic behavior of a lipid extract from the surface-active material in the absence (O) and presence (+) of 10 mM Ca^{2+}. The parameter monitored is the same as that in Fig. 1.

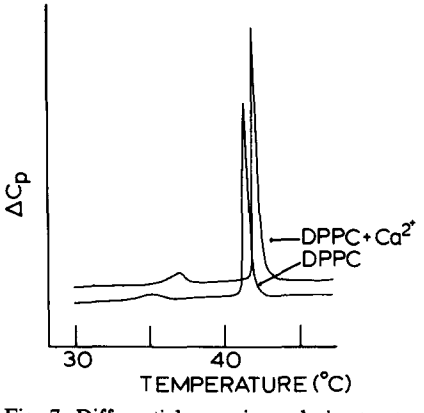

Fig. 7. Differential scanning calorimetry traces for DPPC in the presence and absence of 10 mM Ca^{2+}. The slight Ca^{2+}-induced increases in melting temperatures of both the pre-transition and main transition are evident.

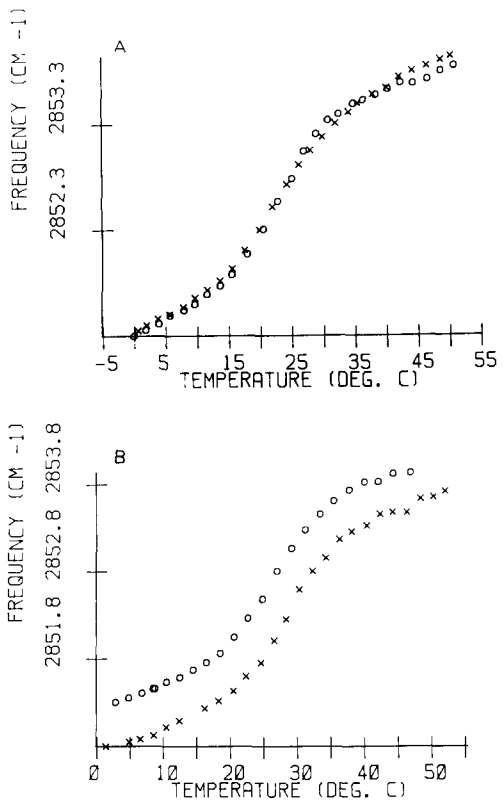

Fig. 8. The effect of protein on the thermotropic behavior of surfactant. (A) A sample with a lipid/protein ratio of 20:1. The curves for the surfactant (O) and for the lipid extract (x) are virtually superimposed. (B) A sample with a lipid/protein ratio of 10:1. The curve for the lipid extract (x) shows increased order, onset temperature and midpoint temperature from that of surfactant (O). The parameter monitored is the same as that in Fig. 1.

model this effect using DSC with phospholipid samples containing DPPC and POPG at molar ratios of 9 : 1 and 4 : 1, respectively. The results are shown in Figs. 6 and 7. In the absence of Ca^{2+}, the DSC traces for the 9 : 1 and 4 : 1 samples show (Fig. 6b and d) a broadening and shift to lower temperatures compared with pure DPPC (Fig. 7). Addition of Ca^{2+} to pure DPPC results in minor changes in the endotherms. As shown in Fig. 7, the main transition and pre-transition are shifted up in temperature by 0.6 Cdeg and about 1.6 Cdeg, respectively. In contrast, addition of 10 mM Ca^{2+} to the 9 : 1 sample results in major changes and leads to a trace (Fig. 6a) in which the pretransition is restored and the main endotherm is markedly reduced in width and increased in temperature. An upward shift is onset and completion temperatures are also noted for the 4 : 1 sample upon the addition of Ca^{2+} (Fig. 6c).

The final experiments involve the effect of protein on lipid melting. Extraction of the lipid component was followed by rehydration and FT-IR examination of samples found to have two different lipid/protein ratios, 20 : 1 and 10 : 1 (w/w), respectively. The FT-IR thermotropic data for the 20 : 1 sample and for its lipid extract are shown in Fig. 8a. The melting curve for the lipid fraction is virtually superimposed on that for intact surfactant. However, for the sample containing higher levels of protein (10 : 1 w/w), the removal of protein reveals (Fig. 8b) a general ordering of the lipid component. This is revealed by an increase in the CH_2 symmetric stretching frequency at all temperatures as well as a 3–5 Cdeg increase in the onset and mid-point temperatures of the phase transition. The completion temperature is less altered.

Discussion

The qualitative nature of the interactions between Ca^{2+}, surfactant lipids and the 35 kDa protein fraction of surfactant has been addressed by King and coworkers [8,11,32]. The efficiency of formation of complexes reconstituted from purified apoprotein and phospholipid mixtures was enhanced both by PG and Ca^{2+} [11]. Reconstitution studies of the protein with DMPC using DSC suggest that 9 mol of DMPC are immobilized upon complexation [32]. Whether similar effects occur in native surfactant is unclear. This and several additional issues would appear to require further reconstitution experiments and molecular spectroscopic techniques for their resolution. These include the effects of Ca^{2+} on the secondary structure of the various protein fractions and lipid-induced alterations therein, and the structure of tubular myelin.

The ability of FT-IR spectroscopy to determine the molecular nature of the interaction between surfactant components and hence resolve some of the issues alluded to above is evident from the current preliminary studies. The sites of Ca^{2+} action and its effect on surfactant lipid thermo-

tropic properties are revealed in Figs. 2, 3 and 4. The interaction between the ion and the lipid head-group is revealed by alterations in the PO_2 symmetric stretching region. While the spectral perturbations (Fig. 4) are weaker than those produced by Ca^{2+} on phosphatidylserine [31] or on POPG, (Fig. 4) they nevertheless are indicative of the site of action. A possible explanation for the relatively small magnitude of the ion-induced shifts is that mainly the PG is directly affected by Ca^{2+}. The IR spectrum, however is dominated by the (less-affected) PC component, hence, is not strongly perturbed. The effect of Ca^{2+} on acidic lipid phase behavior is to rigidify the lipids and either to increase the midpoint temperature of the thermotropic transition [33] or, as noted in our recent FT-IR studies of Ca^{2+}-phosphatidylserine interaction [31], to abolish the cooperative melting event completely. Our current FT-IR and DSC observations of an increased ordering and melting temperatures suggest a calcium-induced sequestering (phase separation) of the acidic components. If this is indeed the case, the remainder of the surfactant would be enriched in DPPC compared with the Ca^{2+}-free preparation and would melt at higher temperatures. Data for the model systems formed from DPPC/POPG are consistent with this interpretation. The restoration of a pre-transition upon Ca^{2+} addition to the 9 : 1 DPPC/POPG mixture provides (Fig. 6) particularly good evidence for phase separation as the pre-transition is abolished by small amounts of a second component in an otherwise pure phase. In addition, this description would help explain the speculation [34] that during compression of a surface film of surfactant, non-DPPC components are selectively eliminated from the monolayer. Our data suggest that Ca^{2+} plays a key role in this process.

The two proteins expected to interact hydrophobically with phospholipids are the 32 and 14 kDa species [24], while the other species observed by SDS-polyacrylamide gel electrophoresis in the current work have been tentatively suggested (in studies of other species) as arising from serum [24]. The effect of protein on the thermotropic behavior of the lipid fraction is minimal at a lipid/protein ratio of 20 : 1 (Fig. 7). At higher protein levels, the observed protein-induced disordering of the lipid fraction is very similar to that observed by FT-IR in studies of Ca-ATPase reconstituted with a variety of lipid classes [30]. We note that the current FT-IR spectral parameter, which measures *gauche* rotamer formation in the acyl chains, reflects in part the bending of the hydrocarbon chains to accommodate the protein surface, in addition to the rates of acyl chain motion. Thus, the effect of protein on lipid order/mobility as revealed by FT-IR differs substantially compared with other techniques that monitor events that occur on slower time scales.

What can be concluded from the current work about the roles of Ca^{2+} and protein in surfactant function? The observation that PG liposomes rapidly aggregate and fuse in the presence of 10 mM Ca^{2+} [35], coupled with the indication from the current experiments of direct interaction of Ca^{2+} with PG head-group phosphates, is suggestive of a mechanism for calcium-induced aggregation of surfactant liposomes [5]. In addition, the observation of protein-induced disordering of surfactant lipids at relatively high protein levels suggests a means for control of lipid order. Not all of the molecular details of the interactions are accessible from the current experiments. It is possible that one or more of the several proteins present interacts strongly with a small subclass of lipid molecules or, alternatively, that more non-specific events occur. These possibilities can only be distinguished with reconstitution experiments. Further, it is reasonable to assume that ternary interactions between the lipids, Ca^{2+} and protein take place. In any event, there appear to be a number of possibilities by which the order of the lipids (hence their ease of spreading and rigidity) may be controlled. Further details may become available when interactions between purified proteins and lipid components are analyzed. FT-IR spectroscopy promises to be useful in studies of Ca^{2+} or lipid-induced alterations in protein secondary structure.

Acknowledgements

This work was supported by a grant (GM 29864) to R.M. from the National Institutes of Health. We thank Mr. A. Hassankhani for acquiring some of the DSC data. Additional support was provided from the Busch Memorial Fund of Rutgers University.

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