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Fourier transform infrared spectroscopic identification of gel phase domains in reconstituted phospholipid vesicles containing Ca^{2+}-ATPase

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Ca^{2+}-ATPase from rabbit sarcoplasmic reticulum has been isolated, purified, and reconstituted into lipid environments containing as primary components 1,2-dielaidoylphosphatidylcholine (DEPC) and acyl-chain perdeuterated 1,2-dimyristoylphosphatidylcholine (DMPC-d_{54}). Differential scanning calorimetry (DSC) has been used to elucidate the phase behavior of this lipid pair while Fourier transform infrared spectroscopy (FT-IR) has been used to monitor the state of each lipid component in the presence of protein. The lipid mixture shows gel state miscibility over at least most of the composition range, a result in good accord with Van Dijck et al. (Biochim. Biophys. Acta 470, 58–69 (1977)), for the binary mixture with proteated DMPC. Acyl chain perdeuteration thus does not greatly alter the miscibility properties of the lipid pair. Reconstitution of Ca^{2+}-ATPase with this lipid pair proceeds with moderate efficiency. Up to 80% of the endogenous lipid can be replaced depending on the lipid composition. Unusual composition-dependent protein-induced effects on lipid melting properties are noticed. At low levels of DMPC-d_{54}, both the DEPC and DMPC-d_{54} components have their melting processes broadened and shifted to lower temperatures, compared with binary lipid mixtures of the same composition. This suggests that protein perturbs both lipids in similar fashion. At high levels of DMPC-d_{54}, the DEPC component exhibits a highly cooperative melting process at temperatures close to that for pure DEPC. This strongly indicates that domains of DEPC are present (at least at low temperatures) in the bilayer, and that Ca^{2+}-ATPase is excluded from these domains. The protein thus exhibits preferential interaction with the DMPC-d_{54} component. This work demonstrates the utility of FT-IR for identification of the molecular origin of particular domains in reasonably complex lipid mixtures. The relevance of this work to native membrane systems where lipid domains have been observed by several groups is discussed.

Introduction

The dependence of membrane-bound enzyme activity upon the chemical structure or physical state of the phospholipid environment around the protein is well documented [1,2]. In view of the possible functional consequences of this dependence, many physical studies of the effect of membrane proteins on the melting characteristics and dynamics of both the protein and lipid components in reconstituted systems have been undertaken (for a review, see Ref. 2). Most experiments have involved the reinsertion of previously purified membrane proteins into vesicles composed of a single lipid component – not infrequently a disaturated phosphatidylcholine. Yet such sim-

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plified model lipid systems cannot mimic the possibilities for complex phase behavior which may arise due to lipid heterogeneity in native membrane lipid preparations. An example of the existence of discrete lipid domains (thermodynamically impossible for a single lipid to achieve over a finite temperature range) in native membrane systems was provided by the recent studies of Schlegel et al. [3]. They demonstrated that insertion of the lipids of the human erythrocyte membrane into the plasma membrane of baby hamster kidney fibroblasts can produce discrete domains which persist for long periods of time after fusion. Fluorescence spectroscopy has been the technique of choice thus far [4–6] for monitoring the existence of domains in native membranes. The method is very sensitive for domain identification, but cannot shed insight into the molecular components of the domain. The current study proposes FT-IR spectroscopy for identification of gel phase lipid domains in appropriate model systems, with possible extensions to native environments.

The simplest systems which exhibit some of the properties of multicomponent environments such as phase separation while remaining amenable to detailed physical measurements are binary lipid mixtures. The requisite thermodynamics has been summarized by Lee [7,8]. In previous studies, [9–11], we have demonstrated that Fourier transform infrared spectroscopy (FT-IR) provides a powerful approach for monitoring the configuration of each lipid as well as the secondary structure of the protein in ternary complexes formed from two phospholipids plus protein. The experiment requires the use of an acyl-chain perdeuterated phospholipid in binary combination with a proteated (normal) phospholipid as components of the lipid mixture. The configuration-sensitive C-^{2}H stretching vibrations arising from the deuterated component appear in an infrared spectral region (2000–2300 cm^{-1}) distinct from the configuration-sensitive C-H stretching vibrations from the proteated component that absorb near 2800–3000 cm^{-1}. Thus both lipids can be simultaneously and separately monitored for protein-induced alterations in structure.

In the current experiment, the protein chosen for reconstitution is Ca^{2+}-ATPase (ATP phosphohydrolase, EC 3.6.1.3) from rabbit sarcoplasmic reticulum. This protein has several advantages for the current work. Several groups, including our own, have published [10,12–15] protocols for delipidation of the native enzyme followed by reconstitution into exogenous lipid environments with retention of function. The ability of changed lipid composition to modulate Ca^{2+}-ATPase activity has also been clearly demonstrated. Hidalgo et al. [16] demonstrated that blocking the amino group of PE in sarcoplasmic reticulum vesicles resulted in inhibition of Ca^{2+} transport, but not of Ca^{2+}-dependent ATP hydrolysis. Navarro et al. [17] reconstituted the protein with a variety of lipids and lipid mixtures and concluded that the coupling ratio of Ca^{2+} pumped/ATP hydrolyzed is stabilized by the presence of cone-shaped lipid molecules such as dioleoyl PC and monogalactosyldiacylglycerol. One goal of this work is to develop methods to monitor the tendency of Ca^{2+}-ATPase to partition into particular regions of chemical structure or physical order in a complex lipid environment. In light of the ability of lipids to alter the rate of Ca^{2+}-ATPase activity, such partitioning, if it occurs, might serve as a means of control of membrane-bound enzyme activity. In previous studies, we have reconstituted Ca^{2+}-ATPase into lipid mixtures where the lipids were either well-mixed or immisicible in the gel state over a large fraction of their composition range. In the current work, the protein has been reconstituted with complex mixtures of lipids that included dielaidoylphosphatidylcholine (DEPC) and acyl-chain perdeuterated dimyristoylphosphatidylcholine (DMPC-d_{54}) as two main components. These lipids were emphasized as each component possesses a unique infrared spectral signature enabling it to be identified in a complex lipid environment and thus be potentially useful for another goal of this work, namely the development of a means to identify particular lipid domains. In addition, it was necessary as a preliminary experiment in the current study to determine the phase properties of the binary phospholipid system in the absence of protein. This arose as the miscibility properties (for two proteated components) as determined by differential scanning calorimetry (DSC) [18] were in conflict with those found [19] from partitioning of a TEMPO spin label.

Experimental

Isolation, purification, and reconstitution of Ca^{2+}-ATPase

Ca^{2+}-ATPase was isolated and purified from the sarcoplasmic reticulum of rabbit skeletal muscle according to the procedure of MacLennan [20]. Exchange of endogenous lipids was accomplished by a deoxycholate-mediated exchange protocol first described by Warren et al. [12] and later modified by Hidalgo et al. [14]. The weight ratios (protein/deoxycholate/phospholipid) used in all samples were 1 : 0.5 : 2.0. Composition control was attempted by varying the lipid/lipid mole ratios of the starting DEPC/DMPC-d_{54} mixtures. The following lipid/lipid mole ratios were used in the samples indicated: A, 100 : 0; B, 90 : 10; C, 73 : 27; D, 50 : 50; E, 33 : 67; F, 20 : 80. Samples were purified by layering them on a discontinuous sucrose density gradient (15%/50%) and centrifuging overnight at 150 000 × *g*.

Vesicles were assayed for chain length distribution by gas chromatography of their methylated acyl chains. The lipids were extracted according to the procedure of Bligh and Dyer [21], esterified with a BF_3-methanol solution and extracted with diethyl ether. The methyl esters were analyzed on a Hewlett-Packard 5890A gas chromatograph equipped with a methyl-silicone gum capillary column by using a temperature program of 100 –183°C.

ATPase activities were measured with a coupled enzyme assay system [12]. Lipid concentrations were determined as lipid phosphorus [22]. Protein concentrations were determined by the method of Lowry et al. [23].

Binary lipid complexes

The binary lipid complexes for the reconstitutions and for the controls were prepared by dissolving the appropriate mole ratios of the lipids in $CHCl_3$, dried under a stream of N_2 gas, evacuated in a dessicator (< 1 torr) for 3–10 h to remove residual traces of solvent, and rehydrated at temperatures greater than the transition temperature of either component.

FT-IR

Samples for FT-IR were examined in a Harrick cell (12 μm pathlength) equipped with CaF_2 windows. Spectra were recorded on a Mattson Instruments Inc., Sirius 100 spectrometer equipped with a Mercury-Cadmium-Telluride detector. Routinely, 100 interferograms were collected, coadded, 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 A80 circulating bath and monitored via a Bailey BAT-12 digital thermometer, with a thermocouple sensor placed close to the cell windows. Frequencies were measured with a center of gravity routine [24]. The spectrum of water (matched for temperature and pathlength) was subtracted from all spectra. Residual sloping baselines were removed by a linear baseline leveling routine.

DSC

Calorimetry experiments were performed in a Micro-cal MC1 unit. Sample volumes (0.7 cc) containing approx. 5 mg of lipid material were injected into the sample cell, with the same amount of water used in the reference cell. Samples were heated at 24 Cdeg/h following an equilibration period of 1–2 h. Enthalpies were determined by cutting and weighing.

Materials

Lipids were purchased from Avanti Polar Lipids (Birmingham, AL) BF_3 methanol was purchased from Supelco Inc. Solvents were of the highest purity commercially available. Doubly distilled water was used in all buffers.

Results

(1) Biochemical results

The compositions of the various complexes (acyl chain length distribution and lipid/protein ratios) used in the FT-IR experiments are summarized in Table I, along with ATPase activities. Several points are noteworthy. First, the reconstitution of either DEPC alone or in binary combination with DMPC-d_{54} did not proceed with efficiencies seen in prior studies [10,11]. For example, the current results (showing 55–81% incorporation of the desired components) are to be contrasted with studies of Ca^{2+}-ATPase-DPPC complexes [10] where

TABLE I

BIOCHEMICAL CHARACTERIZATION OF THE DEPC/DMPC-d_{54} COMPLEXES

Complex	Lipid/protein ratio	Activity [a] (I.U./mg)	Fatty acid composition [b]						
			14:0-h_{54}	14:0-d_{54}	16:0	18:0	18:1 *trans*	18:1 *cis*	18:2
A	97:1	0.91	–	–	12.7	16.5	59.4	11.4	–
B	8:1	4.12	0.8	3.2	7.1	–	70.6	8.2	10.1
C	37:1	17.55	–	31.6	6.9	–	49.5	2.1	9.8
D	66:1	1.17	–	9.5	6.8	6.4	66.5	10.8	–
E	133:1	1.17	0.8	34.2	13.3	–	31.1	8.7	11.9
F	10:1	1.45	–	25.9	12.3	12.4	29.3	20.2	–

[a] Activity measured at 37°C.

[b] Number of carbon atoms: number of C=C bonds.

95% of the lipids could be replaced with a single exchange protocol. Due to the substantial expense of DMPC-d_{54}, it was not feasible to subject the multicomponent systems to multiple exchanges with deoxycholate. Second, the origin of the substantial variation in the final lipid/protein ratio is difficult to understand, especially as previous studies [10,11] using the same protocols led to reasonably predictable values.

Residual levels of deoxycholate (measured with radioactive isotopes) are typically less than one to two moles/mole protein. Electron micrographs (negative staining) showed the presence of multilamellar vesicles with little evidence of fragmentation.

(2) DEPC/Ca^{2+}-ATPase

FT-IR spectra of the C-H stretching region (2800–3000 cm^{-1}) at various temperatures are shown (as overlays) in Fig. 1A for pure DEPC and in figure 1b for a DEPC/Ca^{2+}-ATPase complex at a lipid/protein mole ratio of 97:1. Analysis of the lipid composition of complex A (Table I) indicates that 60% of the endogenous lipid has

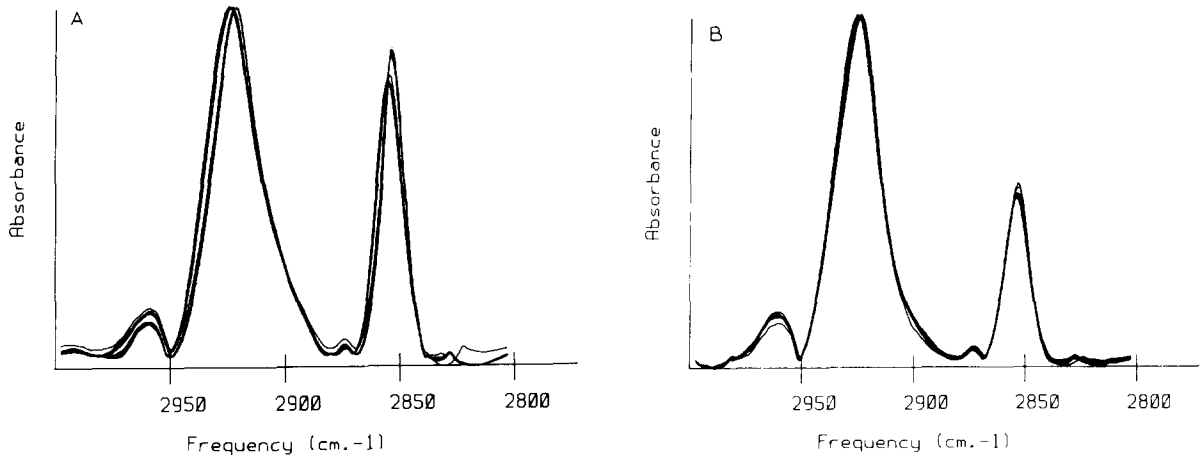

Fig. 1. (A) FT-IR spectral data for the C-H spectral region (2800–3000 cm^{-1}) for pure DEPC. Spectra are plotted at intervals of about 1–2 Cdeg from 4 to 16°C. Peak height at 2850 cm^{-1} decreases with increasing temperature. The spectral features are assigned in the text. (B) FT-IR spectral data for the C-H spectral region (2800–3000 cm^{-1}) for a lipid-protein complex (lipid/Ca^{2+}-ATPase = 97:1) where about 60% of the lipid is DEPC. The fatty acid distribution is given as that for sample A in Table I. Details as in (A). The discontinuity in the data occurs at lower temperatures than in (A).

been exchanged for DEPC. Residual fractions of palmitate, oleate, and stearate are noted. The origin of the observed FT-IR spectral features is well established [25,26]. The asymmetric and symmetric methyl C-H stretching vibrations appear near 2956 and 2872 cm^{-1}, respectively, while antisymmetric and symmetric methylene CH_2 stretching vibrations are observed near 2920 and 2850 cm^{-1}, respectively. Also evident is a broad Fermi resonance band which appears as an asymmetry at 2900 cm^{-1} [27]. The protein component of the complexes contributes no distinct peaks to the contour in Fig. 1B, although part of the intensity underlying the band at 2920 cm^{-1} is derived from Ca^{2+}-ATPase (Compare the 2920 cm^{-1} band in Figs. 1A and 1B).

As is evident from the spectra overlaid in Fig. 1, small alterations in the position and intensities of the spectral features are noted as the temperature is increased. As the spectra are reproduced for approximately constant temperature intervals, the discontinuity in the data for pure DEPC shown in Fig. 1A implies a rapid change in spectral properties with temperature which is reduced or abolished by protein incorporation (Fig. 1B). The spectral parameter most useful for following structural alterations in the lipid component is the methylene symmetric CH_2 stretching frequency near 2850 cm^{-1}. This band suffers little or no underlying contribution from protein and is useful for monitoring proteated lipid components.

Temperature-induced variations in the frequency of this band for pure DEPC and for this complex with Ca^{2+}-ATPase are shown in Fig. 2. The sharp discontinuity at 12°C for pure DEPC coincides with the calorimetrically observed (see below) gel-liquid crystal phase transition. The change in this parameter at the transition is about 2 cm^{-1}. The precision with which frequency shifts may be observed is much better than the resolution of the experiment (4 cm^{-1}) or the width of the bands (10–15 cm^{-1}). Frequencies may in fact be measured with a precision approaching the magnitude of the drift in the He-Ne laser (0.01 cm^{-1}), whose frequency stability controls the accuracy of the interferometer. The measurement was the center-of-gravity algorithm developed by Cameron et al. [24]. The origin of the increase in the symmetric stretching frequency upon the introduction of *gauche* rotamers into the hydrocarbon chains has been traced [28] to alterations in the interaction constant between C-H stretching coordinates on adjacent CH_2 groups when the lipid physical state is altered.

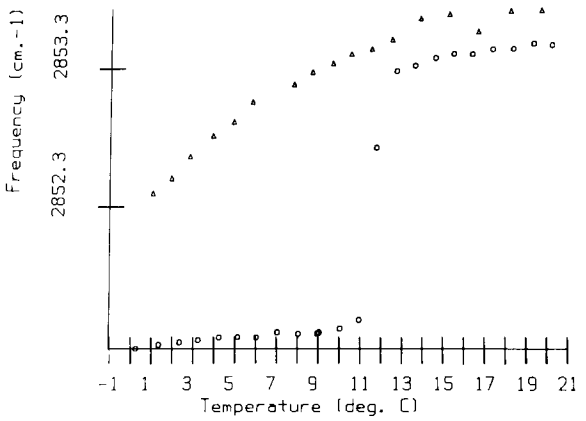

Fig. 2. Temperature-induced variation for the 2850 cm^{-1} CH_2 symmetric stretching mode for (○) pure DEPC and for (△) the 97 : 1 complex (A in Table I). Note the sharp melt at 12°C for the pure lipid and its elimination and concomitant protein-induced lipid disordering in the complex.

The introduction of Ca^{2+}-ATPase into the vesicles at a lipid/protein mole ratio of 97 : 1 produces substantial changes in the FT-IR melting profile of the lipid component (Fig. 2) compared with pure DEPC. The cooperative phase transition is completely abolished and a gradual increase in the CH_2 frequency is observed as the temperature is raised. In addition, the increase in CH_2 frequency in the complex at temperatures corresponding to the gel phase of the lipid suggests that insertion of protein induces substantial disorder into that domain. The results are similar to those observed with Ca^{2+}-ATPase complexes with 1-palmitoyl-2-oleoylphosphatidylethanolamine and 1-stearoyl-2-oleoylphosphatidylcholine [29].

(3) DEPC/DMPC-d_{54} binary systems

The phase behavior of binary lipid mixtures is efficiently determined by differential scanning calorimetry. Experimental DSC curves for pure DEPC, pure DMPC-d_{54} and seven binary lipid mixtures are shown in Fig. 3. A rather slow scan rate (24 Cdeg/h) was utilized in order to minimize instrument-induced lineshape distortion. The main gel-liquid crystal phase transition in DEPC

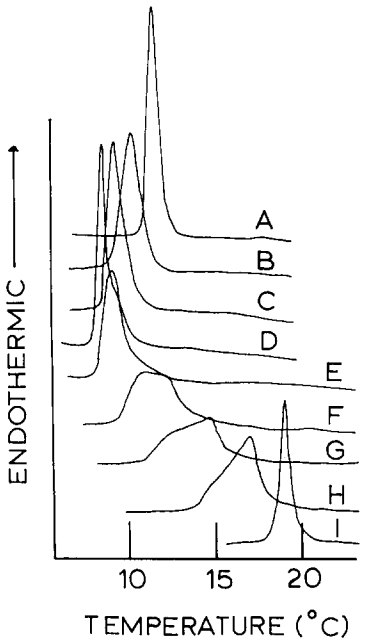

Fig. 3. Differential scanning calorimetry traces for DEPC, DMPC-d_{54} and binary mixtures of the two lipids. The letters on each trace refer to DEPC/DMPC-d_{54} mole ratios as follows: A, 100:0; B, 90:10; C, 80:20: D, 67:33; E, 50:50; F, 33:67; G, 20:80; H, 7:93 I, 0:100. Scan rates were 24 Cdeg/h. The vertical scales differ from trace to trace.

has onset, midpoint, and completion temperatures of 10.7, 11.5 and 13.6°C, respectively. These data are in good accord with those of van Dijck et al. [18] who also used DSC and Wu and McConnell [19] who utilized partitioning of a TEMPO spin label. The transition width at half height is 0.8°C. The calorimetric enthalpy, is 5.5 kcal/mol, 20% lower than seen by Van Dijck et al. [18]. For pure DMPC-d_{54}, the onset, midpoint and completion temperatures are 18.1, 19.1 and 21.0°C, respectively. Acyl chain perdeuteration reduces the midpoint temperature by about 5 Cdeg compared with the proteated species. A similar result has been observed for dipalmitoylphosphatidylcholine [30,31]. Unilamellar vesicles of DEPC and 1:1 mixtures of DEPC/DMPC-d_{54} showed transition characteristics similar to their multilamellar counterparts. A slight broadening of the DEPC melt was noted, but T_m was unchanged. Thus, although the lipid/protein complexes whose melting characteristics are discussed in the previous section are unilamellar, the use of multilamellar vesicle dispersions as controls is justified.

As the mole fraction of DMPC-d_{54} (the higher melting component) in the mixture is increased from 0% to 25%, the onset of melting is initially decreased in temperature. This is followed by a region of composition over which the onset temperature increases (50–100% DMPC-d_{54}). In the 25–50% range, changes are very slight. The phase diagram for the system is determined from the collection of onset and completion temperature data. These have been plotted in two ways. In Fig. 4A, the uncorrected experimental data are shown, while in Fig. 4B, the data have been corrected for the finite width of the melting of the pure components as per Mabrey and Sturtevant [32]. Their protocol minimizes the effects of impurities and instrumental response on the measured melting profiles, although little fundamental justification exists for its application. The shape of the phase diagram is in reasonable accord with that of Van Dijck et al. [18] for the binary (both system proteated) system. One interpretation of the data is that the lipids are miscible over the entire concentration range, although substantial deviation from ideal behavior is apparent. A second interpretation of these data is possible. Since changes in the onset temperatures from about 25 to 50 mol% DMPC-d_{54} are small, a horizontal line (indicating gel phase immiscibility) might be drawn over this narrow composition range. The minimum observed in the current data between 25 and 30% coincides well with the earlier calorimetric study and indicates that isotopic substitution does not substantially alter the miscibility properties of

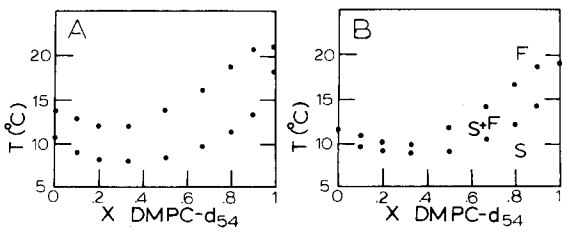

Fig. 4. Phase diagrams for the DEPC/DMPC-d_{54} system. (A) Uncorrected data based on the set of onset and completion temperatures from Fig. 3. (B) Data from (A) corrected by the procedures of Mabrey and Sturtevant [32], which allows for the finite width of melting of the pure components. F, fluid phase; S, solid phase.

this lipid pair. The existence regime of 'rippled' phases defined by pretransitions of the pure components is deleted from the current phase diagram. The current data differ from those of Wu and McConnell [19] obtained from partitioning of a TEMPO spin label. They indicated regions of gel state immiscibility over the 0–75 mol% DMPC range. However, no data are shown over the range 0–25 mol% DMPC, where a substantial decrease in onset temperature with increasing fractions of DMPC-d_{54} is clearly noted in the current work (Fig. 3), consistent with the observations of Van Dijck et al. [18] in this composition range.

FT-IR studies of selected binary lipid mixtures support the calorimetric results. Typical data are shown in Fig. 5 for the pure lipid components and for each of the components in a 73:27 (DEPC/DMPC-d_{54}) mixture. Melting of the DMPC-d_{54} component in Fig. 5B is monitored by temperature-induced alterations in the antisymmetric C^2H_2 stretching vibrations of the deuterated acyl chains near 2200 cm^{-1}. For a sample composition of 73:27 (DEPC/DMPC-d_{54}), which is near the observed minimum in the phase diagram, the melting of each of the lipid components occurs over a very narrow range (8 to 10.5–11 Cdeg) which corresponds very well to that deduced from the phase diagram in Fig. 4. At compositions away from the minimum (data not shown), the observed transition ranges are broadened in a fashion consistent with the phase diagram.

(4) Ternary complexes with Ca^{2+}-ATPase

The advantage of FT-IR for investigation of multicomponent lipid-containing complexes lies in the ability of the method to examine the configurations of individual lipid species, much as was done for the binary lipid mixtures above. In addition, under conditions of low enthalpy changes and broadened phase transitions where DSC data cannot be acquired, FT-IR spectra can be easily obtained. Six lipid-protein complexes of varying complexities were prepared and examined in the current work. The biochemical characterizations of each are summarized in Table I. Whereas in previous work from this laboratory [11] two dominant lipid components were noted, in the current work a greater number of components were frequently observed in the reconstituted complexes.

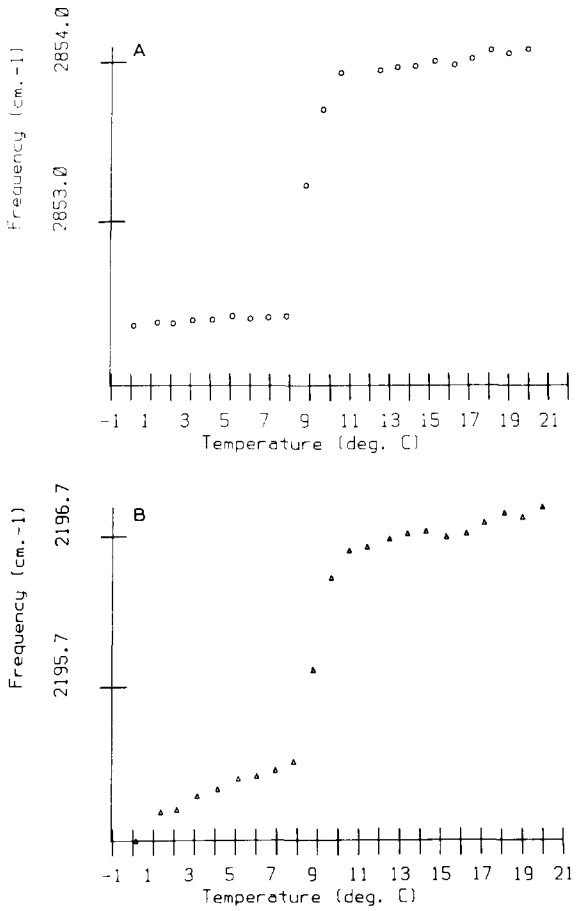

Fig. 5. Temperature dependence of lipid melting in a 73:27 (DEPC/DMPC-d_{54}) binary lipid mixtures for (A) the DEPC component as monitored by the symmetric CH_2 stretching frequency. (B) The DMPC-d_{54} component, as monitored by the antisymmetric C^2H_2 stretching mode. The sharp discontinuity in each case matches the temperature deduced from the phase diagram.

In all cases, the main fatty acyl ester components were elaidate and myristate-d_{27}, indicating the main lipid species present to be DEPC and DMPC-d_{54}. Analysis of the FT-IR data from these systems is based on the following framework:

(i) Characterization of the deuterated component (DMPC-d_{54}) is unambiguously determined from the temperature dependence of the C-^{2}H stretching vibrations.

(ii) Melting processes monitored in the C-H stretching region reflect all proteated lipid species present. Each species contribution to the observed

temperature profiles is in proportion to its occurrence (weighted slightly by the number of CH_2 groups in the acyl chains). Since DEPC is the dominant proteated lipid, changes in the C-H stretching vibration reflect alterations in its configuration. Further evidence for specific melting of the elaidic components (see below) comes from the temperature of the observed events, as well as from changes in a spectral region (950–980 cm^{-1}), which contains =C-H out-of-plane deformations from alkene chains containing *trans* C=C bonds.

FT-IR melting data for complex C (Table I) are depicted in Fig. 6. This complex contains primarily DMPC-d_{54} and DEPC, with all other components totalling less than 20% of the lipids. From the data of Fig. 6, it is evident that the melting of the two dominant lipid components is affected by protein in approximately the same fashion. The sharp discontinuities that characterize the binary mixtures (Fig. 5) are gone, and are replaced by broad non-cooperative melting events that are completed by approx. 8 Cdeg. The onset temperatures seem to be below zero and are not sampled in this experiment due to difficulties anticipated with vesicle stability upon formation of ice crystals. The main effect of Ca^{2+}-ATPase is to disorder each of the lipid components as well as to broaden the melting processes. Similar effects were noted for samples B and D.

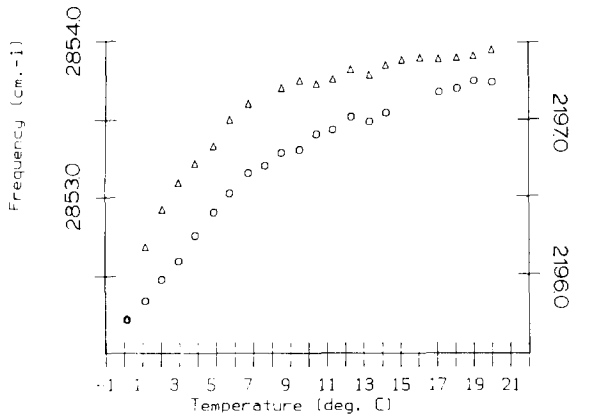

Fig. 6. FT-IR melting data for ternary complex C in Table I. Melting of the DEPC component is given by the left-hand ordinate scale and the symbol ○. Melting of the DMPC-d_{54} component is given by the right-hand ordinate scale and the symbol △. Note that each component has its melt shifted to lower temperature than in the appropriate binary lipid mixture (Fig. 4). In addition, each melt begins (apparently) below 0°C.

At lower levels of DEPC (samples E and F in Table I), unexpected effects are noted in the melting profiles. These are demonstrated in Figs. 7 and 8. The deuterated component in each instance (Figs. 7B, 8B) demonstrates a broad melting event not unlike those seen in samples B-D. The C-H component in Fig. 7A, however, exhibits a sharp transition at 12°C in addition to a gradual increase in the CH_2 frequency between 0 and 11.5°C. Above the sharp transition, the frequency increase with temperature is negligible. Similar results are observed with the C-H component in sample F (Fig. 8A) except that the temperature of

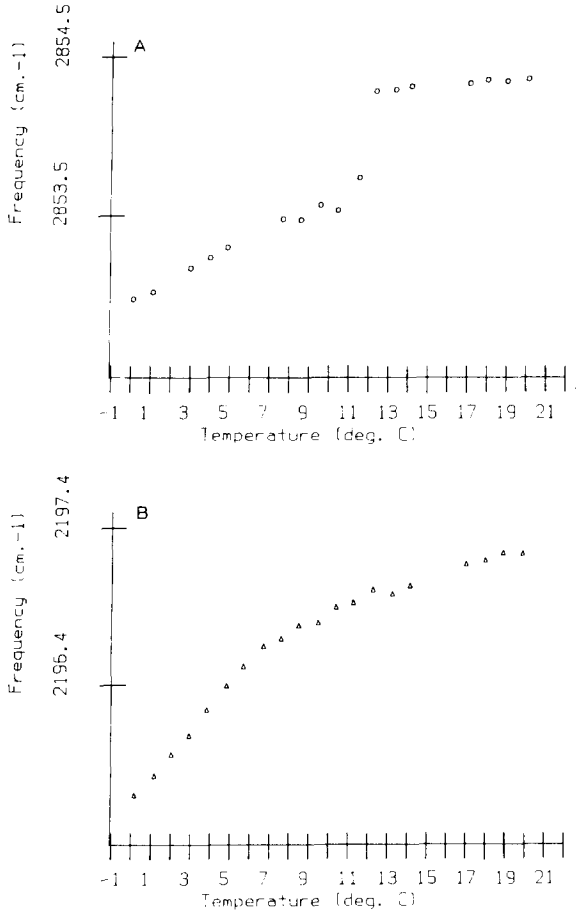

Fig. 7. FT-IR melting data for ternary complex E in Table I. (A) Melting of the DEPC component. (B) Melting of the DMPC-d_{54} component. Note now that the deuterated component has its melted shifted to low temperatures, while the DEPC component retains a sharp melt near 12°C. These data suggest that domains of DEPC exist below T_m.

the sharp transition is lowered to 8.5–9°C. Since the main proteated lipid in these preparations is DEPC, it seems reasonable to assume that the sharp discontinuity arises from that component. Two lines of evidence support this contention. First, the temperature of the discontinuity is close to that for the pure DEPC (Fig. 1). Second, there is a =C-H out-of-plane bending vibration near 965 cm^{-1} which arises only from *trans*-substituted C=C bonds [33]. Although the spectral region is complicated by the presence of antisymmetric stretching modes of the choline headgroup near 970 cm^{-1} [34], discontinuities in this spectral region would be suggestive of participation by the DEPC component. This spectral region for pure DEPC multibilayers is illustrated in Fig. 9. At temperatures below T_m, two features are observed in the spectrum, a main band near 965 cm^{-1} and a shoulder near 973 cm^{-1}. At T_m, these bands merge into a single contour at about 971 cm^{-1}. Similar patterns (Fig. 10) occur in this spectral region in ternary complex E. A shoulder seen at 965 cm^{-1} at low temperatures merges with higher frequency components at temperatures close to but slightly lower than the discontinuity observed in the C-H stretching region (Fig. 7). In addition, a weak shoulder near 975 cm^{-1} merges into the main contour at approx. 12°C. The weakness of these bands makes it impossible to quantitatively plot these spectral alterations. However, the observation of two discontinuous changes is consistent with a particular domain melting, and comparison with Fig. 9 implicates the DEPC. The increased intensity of the feature at 971 cm^{-1} in the ternary system compared with the binary system (Fig. 9) arises because of the greater proportion of choline head group compared with trans C=C bonds in the former. The perdeuterated lipid component in the former still contributes its proteated antisymmetric N-$(CH_3)_3$ modes to the spectrum. It is not entirely clear why the temperature at which the 965 cm^{-1} band merges differs slightly from the discontinuity in the C-H stretching region (Fig. 7). Different vibrational modes

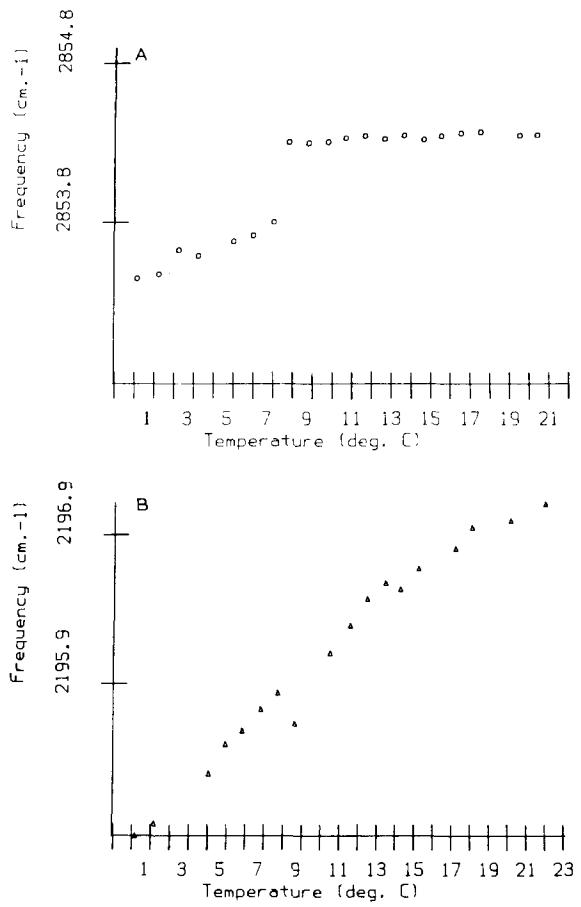

Fig. 8. FT-IR melting data for ternary complex F. (A) Melting of the DEPC component. (B) Melting of the DMPC-d_{54} component. Again (see Fig. 7) the DEPC retains a sharp melting event.

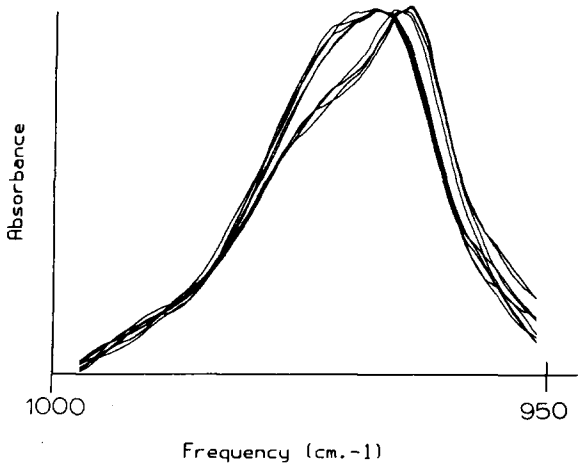

Fig. 9. The spectral region 950–1000 cm^{-1} for the pure DEPC. This region contains C=C-H out-of-plane deformations as well as choline modes. The merging of the main peak and shoulder occurs at T_m.

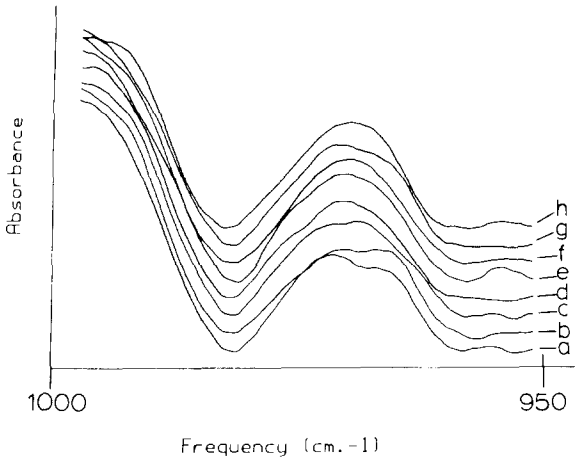

Fig. 10. The spectral region 950–1000 cm^{-1} for ternary complex E. The relative intensity of the C=C-H modes is reduced from Fig. 9 because of the reduced amount of elaidic acid. Temperatures of the various spectra are as follows: a, 0.2°C; b, 4.1°C; c, 7.8°C; d, 10.6°C; e, 11.6°C; f, 12.6°C; g, 18.3°C; h, 20.4°C.

may respond with different sensitivities to different aspects of lipid structure. The overall contour is virtually unchanged above 12°C, consistent with the C-H stretching data.

Thus, there appear to be lipid compositions and lipid/protein ratios where sharp transitions are still observable from the DEPC lipid component. It is concluded that gel phase domains of DEPC exist in the bilayer, at temperatures below these transitions. As the effect of protein on DEPC alone (Fig. 2) is to eliminate the transition at 12°C, the fact that the transition is still observed in samples E and F, suggests that DEPC is excluded from the Ca^{2+}-ATPase environment.

Discussion

A variety of studies have suggested that the lipids of the plasma membranes of living cells can be segregated into domains of differing organization. The techniques used for these studies have usually been based upon fluorescence spectroscopy (e.g. lifetime decay [4], polarization [5], fluorescence recovery after photobleaching [6], and the use of merocyanine 540 [3]). The advantage of this approach is the high sensitivity of the fluorescence technique, coupled with the ability to insert the fluorophore into the system of interest. The drawbacks of the method are that (i) the reporter molecules may perturb the system of interest and (ii) the nature of the phases about which the molecules report cannot be unambiguously determined. Thus the molecular origin of the putative domains (e.g. Are they based on chemical structures of particular lipids or the physical state of the system?) cannot easily be determined.

FT-IR spectroscopy lacks the sensitivity of fluorescence spectroscopy for the observation of domains of lipids in native plasma membranes. It may in the near future, however, be possible to overcome this problem by incorporation of deuterated lipids into intact cells and observation of the deuterated components [35]. The current work demonstrates the power of FT-IR for observation of lipid domains in fairly complex model systems, consisting of 2–4 lipid components plus a functioning membrane protein. The data show that the existence of domains depends very strongly upon the lipid composition in the vesicle. The existence of independent domains rich in DEPC is strongly suggested from the data in Figs. 7 and 8. The sharp melting event at temperatures close to the melting of pure DEPC (Figs. 7 and 8) coupled with the observation of spectral changes in regions containing a substantial contribution from vibrations unique to the DEPC species (=C-H (*trans*) deformation as plotted in Figs. 9 and 10) provides ample evidence for this. In the same samples, the DMPC-d_{54} component (Figs. 7 and 8) showed only broad, non-cooperative melting phenomena indicating this component not to be laterally segregated. We further suggest that the DEPC component is excluded from the vicinity of Ca^{2+}-ATPase in samples E and F. Evidence for this is provided from the control experiment in Fig. 2. Reconstitution of protein into vesicles highly enriched in DEPC (which presumably forces the protein to interact with that lipid) leads to the elimination of the sharp transition and its replacement by a broadened melt shifted to lower temperatures. Thus, when the DEPC interacts with protein, no sharp melt is observed. Conversely, the presence of a sharp melt implies the exclusion of the DEPC component from the protein surface.

The observation of an excluded DEPC component (samples E and F) occurs in samples containing relatively low levels of this lipid (31.1 and

29.3% of the fatty acid, respectively), with widely varying levels of protein (lipid protein ratios 133 : 1 and 10 : 1, respectively). Samples with relatively high levels of DEPC (49.5, 66.5 and 70.7%) show this component to be well-mixed. To rationalize the lipid dependence, we suggest that sufficient DMPC-d_{54} and proteated lipids (excluding DEPC) must be present to accommodate the protein surface, and it is these lipids that interact with the Ca^{2+}-ATPase with greater affinity than DEPC. In samples B–D, there is insufficient lipid other than DEPC to form a layer around the protein, thus DEPC is then utilized, and its cooperative melt abolished.

To rationalize the wide variation in lipid/protein ratio for which gel state phase separation is induced (samples E, F), we suggest that at high protein levels (sample F) substantial protein-protein contact occurs and that fewer lipids/protein are required to accommodate the entire exposed protein surface than for monomeric Ca^{2+}-ATPase (sample E). Thus, there is still sufficient (non-DEPC) lipid available for this purpose. This speculation must be verified with techniques sensitive to protein aggregation.

The final issue to be discussed is the relevance of these results to native membrane systems. It is noted that elaidic acid is not a natural constituent of phospholipids in biological membranes. It was selected in the current experiment (i) because of the conflicting conclusion concerning its interesting phase properties when mixed with DMPC and (ii) because of the availability of infrared spectra features unique to it. The selection was successful in that the ability of FT-IR to follow the melting characteristics of DEPC alone and hence the occurrence of domains of particular components was unambiguously demonstrated. The melting of both the deuterated component and the component with the trans C=C bond could be followed unambiguously. This suggests that it will be feasible to follow such components (especially deuterated ones) which have been inserted into plasma membranes. It will then be possible to begin to map those features of lipid structure which are responsible for domain formation.

The functional significance of phase segregation in native systems has yet to be determined. It has been suggested [36,37] that membrane proteins might display a greater affinity for disordered rather than ordered domains of the bilayer. It has already been shown that affinities of particular membrane proteins exist for particular lipid classes [1]. In reconstituted systems containing Ca^{2+}-ATPase, a particular requirement has been demonstrated [16] for phosphatidylethanolamine in order that the Ca^{2+} pumping function be maintained. It is not necessary that the physical basis for a particular affinity be linked to a functional requirement. Such a correlation would however, provide a means for control of membrane-bound protein function. In any case, the development of techniques such as FT-IR which can monitor discrete domains and give clues about those lipids responsible for their existence is of importance. The persistence of domains [3] suggests that a study of their relevance to function is warranted.

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