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FOURIER TRANSFORM INFRARED SPECTROSCOPIC STUDIES OF LIPID-PROTEIN INTERACTION IN NATIVE AND RECONSTITUTED SARCOPLASMIC RETICULUM *

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Fourier transform infrared spectroscopy has been used to monitor lipid-protein interaction and protein secondary structure in native and reconstituted sarcoplasmic reticulum vesicles. Studies of the temperature dependence of the CH_2 symmetric stretching frequency reveal no cooperative phase transitions in purified sarcoplasmic reticulum or in vesicles reconstituted with dioleoylphosphatidylcholine, although a continuous introduction of disorder into the lipid acyl chains is observed as the temperature is raised. In addition, temperature-dependent changes are observed in the Amide I and Amide II vibrations arising from protein peptide bonds. A comparison of lipid order in native sarcoplasmic reticulum and its lipid extract showed that the introduction of protein is accompanied by a slight increase in lipid order. Reconstitution of Ca^{2+}-ATPase from sarcoplasmic reticulum with dipalmitoylphosphatidylcholine (lipid/protein ratio 30:1), reveals a perturbed lipid melting event broadened and reduced in midpoint temperature from multilamellar lipid vesicles. The onset of melting (27–28°C) correlates well with the onset of ATPase activity and confirms a suggestion (Hesketh, T.R., Smith, G.A., Houslay, M.D., McGill, K.A., Birdsall, N.J.M., Metcalfe, J.C. and Warren, G.B. (1976) Biochemistry 15, 4145–4151) that a liquid crystalline environment is a requirement for optimal protein function. Finally, Ca^{2+}-ATPase has been reconstituted into binary lipid mixtures of DOPC and acyl-chain perdeuterated DPPC. The effect of protein on the structure and melting behavior of each lipid component was monitored. The protein appears to preferentially interact with the DOPC component.

Introduction

Sarcoplasmic reticulum is a specialized membrane network which acts as a repository for Ca^{2+} in resting muscle. Excitation leads to rapid release of this ion into the sarcoplasm, and myofibril contraction then occurs.

Sarcoplasmic reticulum has been widely used in studies of lipid-protein interaction and its relationship to the activity of membrane-bound proteins (for a general review of lipid-protein interactions, see Protein-Lipid Interactions in Membranes – Biophysical Discussions [1]; see also Ref. 2). The main protein of the native membrane is (Mg^{2+} + Ca^{2+})-ATPase (ATP phoshohydrolase, EC 3.6.1.3) which is known to be responsible for Ca^{2+} transport [3]. Techniques have been developed for replacing more than 95% of the endogenous lipid with external lipid, while retaining enzymatic activity [4–6]. The lipid/protein ratios in the recon-

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Abbreviations: Ca^{2+}-ATPase, Mg^{2+}-, Ca^{2+}-activated ATP phosphohydrolase, EC 3.6.1.3; DPPC, 1,2-dipalmitoylphosphatidylcholine; DOPC, 1,2-dioleoylphosphatidylcholine; DPPC-d_{62}, acyl-chain perdeuterated DPPC.

stituted systems may be varied, making it possible to study the effect of protein on lipid phase behavior as well as the effect of lipid composition and physical state on enzyme activity.

Many physical methods have been applied to the study of both native and reconstituted vesicle systems containing Ca^{2+}-ATPase, with conflicting results. Investigations as to the nature of the lipid environment in reconstituted systems, were interpreted in terms of two distinct lipid populations, one with characteristics only slightly perturbed from pure lipid, the other with characteristics of an immobilized component [7,8]. However, recent studies [9–11] involving ^{31}P-NMR and ^{2}H-NMR spectroscopy produced contradictory results and have concluded that a single homogeneous lipid environment exists, at least on the time scale of the NMR experiments.

Vibrational spectroscopy offers several advantages for the study of lipid-protein interaction. In native systems and in systems reconstituted with a single lipid component, both the lipid configuration and the protein secondary structure may be monitored without the use of a possibly perturbing probe molecule. When proteins are reconstituted into binary lipid mixtures, one of the lipids selected may have its acyl chains perdeuterated. The C-^{2}H modes of the perdeuterated species are sensitive to the physical state of the lipids [12]. As their vibrational frequencies occur in a spectral region free from interference from other lipid components, the conformation of each lipid in the system may be individually determined [12].

A drawback of conventional infrared and Raman spectroscopy is the time-consuming nature of the measurements on dispersive spectrometers. In order to achieve sufficiently high signal/noise ratios so that small changes in spectral parameters may be confidently reported, samples must be examined for rather lengthy periods of time. Raman studies of native and reconstituted sarcoplasmic reticulum [13,14] revealed both the utility of vibrational spectroscopy for the study of this system and the limitations on signal/noise ratios inherent in the method.

The recent popularity of Fourier transform infrared spectroscopy has taken place because the technique offers the advantages of rapid data collection and high sensitivity. This results in high signal/noise levels and the concomitant possibility of accurate monitoring of small changes in frequency, intensity and bandwidth [15]. Our laboratories have applied this technique to studies of pure lipid systems [16,17], and to glycophorin/lipid interaction in reconstituted systems [12,18], while other investigators have examined bacteriorhodopsin and rhodopsin [19,20] in a variety of environments. A conventional IR study of Ca^{2+}-ATPase has also been reported [21].

The current investigation reports the first Fourier transform infrared spectroscopy measurements of purified native sarcoplasmic reticulum, as well as systems in which the native lipid has been exchanged for a variety of selected lipid environments.

Materials and Methods

Methods

Isolation and purification of sarcoplasmic reticulum. Crude sarcoplasmic reticulum was purified from the back and leg muscles of albino rabbits [22]. Dithiothreitol (5 mM) and phenylmethylsulphonyl fluoride (5 μM) were added to all buffer systems.

Sarcoplasmic reticulum was purified further as described (step A2 in Ref. 22), with a deoxycholate level of 0.05 mg/mg protein. For final purification, sarcoplasmic reticulum was layered on a discontinuous (30%/50%) sucrose gradient and spun overnight at $150\,000 \times g$. Two main bands were collected which had different lipid/protein ratios. In general, data shown are for the bottom fraction. Fourier transform infrared spectroscopy studies showed the lipid order characteristics of each fraction to be similar. After recovery, all preparations were diluted in 20 mM Tris-maleate, pelleted by centrifugation and frozen at solid CO_2 temperatures until infrared spectral examination. ATPase activity measurements were carried out using the coupled enzyme assay system of Warren et al. [4]. Lipid concentrations were determined as lipid phosphorus using the assay of Chen et al. [23], following extraction of the lipids with organic solvents. Protein concentration was determined using the method of Lowry et al. [24].

Native lipids were extracted using organic

solvents, dried in vacuo for several hours to remove the last traces of solvent, and stored at solid CO_2 temperatures prior to spectral analysis.

Lipid exchange procedures. The endogenous lipids of sarcoplasmic reticulum were exchanged using a deoxycholate-mediated exchange procedure first described by Warren et al. [5], as modified by Hidalgo et al. [6]. The following weight ratios (protein/deoxycholate/phospholipid) were used for the indicated samples: DPPC, (2 : 1 : 4); DOPC, (1 : 1 : 1); equimolar DPPC-d_{62}/DOPC, (1.5 : 1 : 1.2). Samples containing DPPC or DPPC-d_{62} were incubated at 37 °C for 1.0 h prior to layering on the discontinuous sucrose gradient described above, while the DOPC-Ca^{2+}-ATPase complex was incubated for 2.0 h at 0 °C. Studies with radioactive deoxycholate showed less than 1 mol detergent/mol protein in the final complexes.

Vesicles were assayed for lipid chain length distribution by gas chromatography of their methylated acyl chains. The lipids were transesterified with 5% (w/w) HCl in MeOH and extracted with diethyl ether. The methyl esters were then analyzed on a Hewlett-Packard 5750 gas chromatograph equipped with a column of 5% DEGS on Chromosorb W, 80–100 mesh, by using a temperature program of 80–260 °C.

SDS-polyacrylamide gel electrophoresis was carried out according to the method of Weber and Osborn [25]. 8% cylindrical gels were used, stained with Coomassie blue, and scanned on a densitometer. The absorbance at 600 nm was monitored along the length of the gel. A typical trace from a densitometer scan is shown in Fig. 1.

Fourier transform infrared spectroscopy. Samples for Fourier transform infrared spectroscopy were examined in a Harrick cell (50 μm pathlength) equipped with CaF_2 windows. Spectra were recorded on a Digilab FTS-15 instrument equipped with a HgCdTe detector. 250 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 control was as described [12]. Frequencies were determined using a center of gravity routine and are accurate to ± 0.01 cm^{-1} [15]. Occasionally, subtraction of gently sloping H_2O or ^2H_2O bands was accomplished in order to improve the background prior to the frequency measurement. Fourier deconvolution was accomplished as described [26]. Samples for which the C-H stretching region was to be examined were prepared in ^2H_2O-containing buffer, while samples for which the C-^{2}H stretching region was to be observed were made up in H_2O-containing buffer.

Materials

Lipids were purchased from Avanti Polar Lipids (Birmingham, AL) and evaluated for purity by thin-layer chromatography. Solvents were the highest quality commercially available and were used without further purification. Water was doubly distilled.

Results

Biochemical characterization of complexes

The fatty acid composition of the lipid acyl chains for several preparations studied in the current work are given in Table I.

The fatty acid composition for native purified sarcoplasmic reticulum is in good agreement with data obtained by Hidalgo et al. [16]. The data for those preparations (DPPC/Ca^{2+}-ATPase and DOPC/DPPC-d_{62}/Ca^{2+}-ATPase) involving deoxycholate-mediated lipid exchange followed by sucrose density gradient purification of the lipid-protein complexes shows that high levels of incorporation of the desired phospholipid species have been achieved.

TABLE I

FATTY ACID COMPOSITIONS FOR SOME PREPARATIONS USED IN THE CURRENT WORK

SR, sarcoplasmic reticulum.

Fatty acid	Purified SR	DPPC/ Ca^{2+}-ATPase	DOPC/ DPPC-d_{62}/ Ca^{2+}-ATPase
Lipid/protein mole ratio	30 : 1	30 : 1	55 : 1
12 : 0	0.9	1.3	1.7
14 : 0	0.9	0.5	8.0
16 : 0	30.4	95.8	43.2 [a]
18 : 0	8.2	0.9	2.9
18 : 1	22.6	0.8	41.0
18 : 2	34.7	0.7	3.2
18 : 3	2.4	–	–

[a] This was measured as perdeuterated palmitate.

Typical ATPase activities for our preparations of native, purified sarcoplasmic reticulum ranged between 5–10 IU as determined by the method of Warren et al. [4]. Activity profiles for native sarcoplasmic reticulum show a temperature-dependence similar to those published [13]. It is noted that samples stored at −20 °C in sucrose occasionally showed substantially higher activities after having been frozen for up to 2 months. Densitometer traces of our polyacrylamide gels (Fig. 1) demonstrate two main peaks, as shown. The intense band (80–85% of total protein) occurs at a molecular weight of approx. 115000 and corresponds to Ca^{2+}-ATPase. The smaller peak occurs at a molecular weight of 55000. Overall, our data are in excellent agreement with those of Fernandez et al. [27]. Upon reconstitution with exogenous lipids as described in the text followed by discontinuous sucrose density gradient fractionation, the relative intensity of the Ca^{2+}-ATPase peak is increased and corresponds to greater than 90% of the total protein.

Fourier transform infrared spectroscopy

Native sarcoplasmic reticulum and its lipid extract. Fourier transform infrared spectra of the C-H stretching region at various temperatures for native sarcoplasmic reticulum and its lipid extract are shown in Figs. 2A and B, respectively. The main spectral features in Fig. 2A arise from the lipid acyl chains. The asymmetric and symmetric methyl C-H stretching bands appear near 2956 and 2872 cm^{-1}, while the antisymmetric and symmetric CH_2 stretching bands are observed near 2920 and 2850 cm^{-1}. Also seen are C-H stretching bands near 3010 cm^{-1} arising from -CH = CH- moieties and a broad Fermi resonance band centered at about 2900 cm^{-1} [28]. Underlying the 2920 cm^{-1} feature are protein C-H vibrations. Similar features are noted in the spectra of the lipid extract, with the exception of the underlying protein bands.

When the temperature is increased, changes occur in the frequencies and halfwidths of the various features, which have been used to monitor

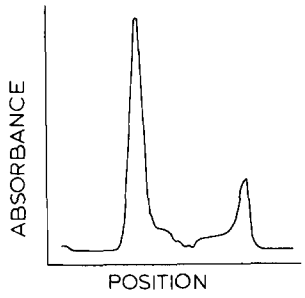

Fig. 1. Densitometer trace from the gel electrophoresis pattern obtained after fractionation of sarcoplasmic reticulum in discontinuous sucrose density gradients. Gel electrophoresis was carried out according to the method of Weber and Osborn [25] using 8% polyacrylamide. Staining was carried out with Coomassie blue. The intense peak on the left (80–85% of total protein) arises from Ca^{2+}-ATPase, and corresponds to a molecular weight in the 110–120 kDa range.

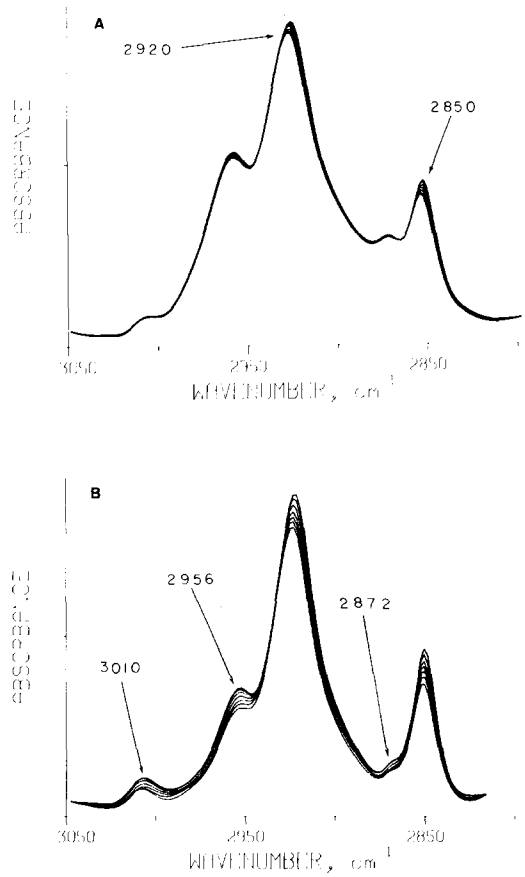

Fig. 2. A series of infrared spectra of the C-H stretching region as a function of temperature for (A) native sarcoplasmic reticulum (purified vesicles, lipid/protein ratio 35:1 in ^2H_2O) and (B) lipid extract of native sarcoplasmic reticulum (in ^2H_2O). Spectra decrease in peak height with increasing temperature and are plotted in approx. 5 deg. C intervals over the range 6–50 °C.

alterations in the physical state of the lipid acyl chains [16,17]. The CH_2 symmetric stretching mode near 2850 cm^{-1} is free from interference from underlying protein features and so is useful for studies of the lipid components of the system. This procedure has been followed for glycophorin-lipid recombinants [18]. Temperature-induced variations in the frequency of this band in sarcoplasmic reticulum and its lipid extract are plotted in Fig. 3.

Upon heating native sarcoplasmic reticulum, a continuous increase in the frequency of the band at 2852 cm^{-1} is noted. There is no evidence for a cooperative lipid melting process. Rather, the increase of frequency with temperature reveals the gradual introduction of disorder into the lipid hydrocarbon chains as the temperature is raised. The physical origin of the increase in the frequency can be traced to changes in the interaction constants between C-H stretching coordinates on adjacent methylene groups when the lipid physical state is altered [29].

Comparison of the temperature dependence for native sarcoplasmic reticulum and its lipid extract (Fig. 3) shows that at all temperatures the frequency of the symmetric CH_2 stretching band for the lipid extract is slightly higher than that for native preparation.

In addition to data concerning the lipid acyl chains, the current experiments contain information pertaining to the lipid interfacial region and to protein secondary structure. The relevant spectral region, 1500–1800 cm^{-1}, is shown at various temperatures in Fig. 4. In order to improve visualization of the highly overlapped spectral features, the data have been subjected to Fourier deconvolution [26].

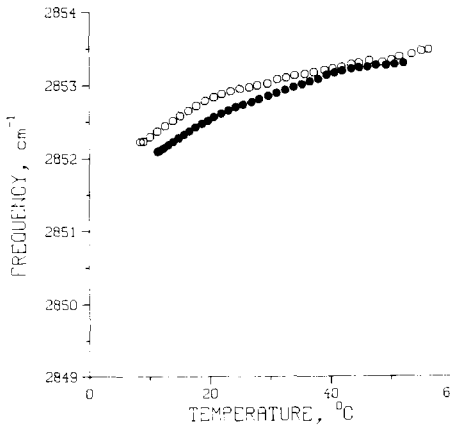

Fig. 3. Plots of the temperature dependence of the CH_2 symmetric stretching frequency near 2852 cm^{-1} for the acyl chains of native sarcoplasmic reticulum (●) and its lipid extract (○).

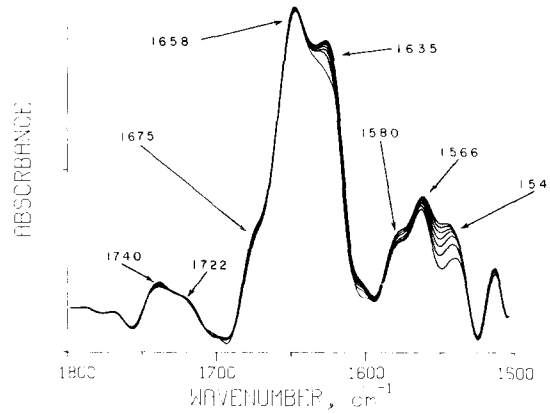

Fig. 4. The spectral region 1500–1800 cm^{-1} is plotted for native sarcoplasmic reticulum as a function of temperature. The data have been subject to Fourier deconvolution [26] in order to effectively improve the resolution. A 25 cm^{-1} Lorentzian band has been deconvolved from the data and the resolution increased by a factor of 1.8. As the temperature increases, the shoulder near 1635–1640 cm^{-1} becomes less well defined. Spectra are plotted in approx. 5 deg. C intervals from 6 to 50 °C.

The spectral region 1700–1750 cm^{-1} contains the C = O stretching vibrations of the lipid ester carbonyls. Deconvolution reveals two spectral features, near 1740 and 1722 cm^{-1}, the intensity of the former decreasing as the temperature is raised. The higher frequency band is assigned to the C = O stretching mode of the *sn*-1 chain with a *trans* conformation in the C-C bond adjacent to the ester grouping, while the lower frequency vibration arises from the *sn*-2 chain and suggests the presence of a *gauche* bend in that position [30,31].

Also seen in Fig. 4 are an intense band and two shoulders in the Amide I (peptide bond C = O stretching) region between 1600 and 1680 cm^{-1}. the main feature at 1658 cm^{-1} probably reflects contributions from α-helical and random coil secondary structures, while the shoulders at 1675 and 1635–1640 cm^{-1} suggest the occurrence of a small proportion of β-sheet forms [32]. The Amide II (mixture of C-N stretch and N-H in-plane bend) spectral region shows features at 1580, 1566 and

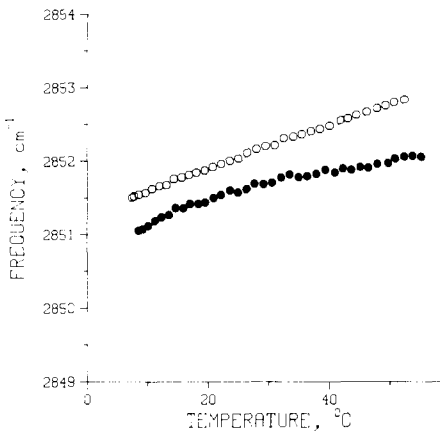

Fig. 5. Plots of the temperature dependence of the CH_2 symmetric stretching frequency near 2851 cm^{-1} for the lipid acyl chains of pure DOPC (○) and for a 35:1 DOPC-Ca^{2+}-ATPase complex (●).

1545 cm^{-1}. As the temperature is increased, the band at 1545 cm^{-1} (consistent with an α-helical conformation) is reduced in intensity. Whether this is due to conformational alterations or increased accessibility of the peptide bond residues to H-^{2}H exchange (which would shift the frequency to about 1450 cm^{-1}) cannot be determined from the current experiments.

DOPC / Ca^{2+}-ATPase. The temperature-induced variation in the CH_2 symmetric stretching frequency for vesicles reconstituted with DOPC

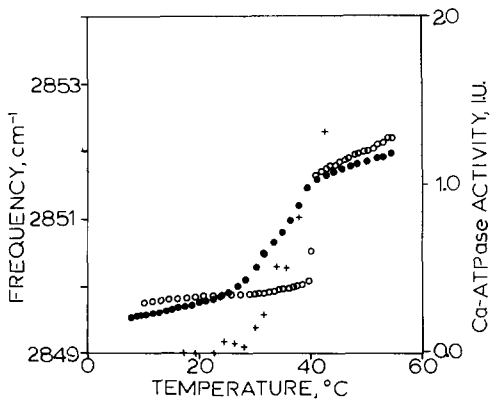

Fig. 6. Plots of the temperature dependence of the lipid CH_2 symmetric stretching frequency for a DPPC-Ca^{2+}-ATPase complex (●), as well as for pure DPPC multibilayers (○). The lipid/protein ratio in the complexes is 30:1. Included for comparison are data for the temperature dependence of ATPase activity (+).

(lipid/protein, 35:1) is plotted in Fig. 5. As for the native sarcoplasmic reticulum, there is no sign of a cooperative melting event. When the temperature is raised, a gradual (noncooperative) increase in the frequency of the 2850 cm^{-1} band is noted. Included for comparison in Fig. 5 are data for the thermotropic behavior of pure DOPC multibilayers. At all temperatures, the CH_2 stretching frequency for the free lipid is higher than that for the lipid-protein complex. This suggests that, as in the native sarcoplasmic reticulum, the presence of protein induces a slight ordering in the lipid acyl chains.

DPPC / Ca^{2+}-ATPase. The deoxycholate-mediated lipid exchange procedure of Hidalgo et al. [6] followed by discontinuous sucrose density gradient centrifugation leads to a purified reconstituted system greatly enriched in DPPC as shown in Table I. The level of enrichment is about the same as reported by Hidalgo et al. [6]. In addition, gel electrophoresis data (not shown) indicate that the Ca^{2+}-ATPase constitutes at least 90% of the protein in the purified preparation. Finally, electron micrographs of the reconstituted systems reveal the occurrence of unilamellar vesicles.

Detailed plots of the temperature-induced variation in the CH_2 symmetric stretching frequency for DPPC/Ca^{2+}-ATPase vesicles as well as for pure DPPC multibilayers are plotted in Fig. 6. Pure DPPC bilayers exhibit the well-known gel to liquid-crystal phase transition at 41°C. In the reconstituted system (lipid/protein, 30:1), the transition is broadened and shifted to lower temperatures, the onset of cooperative melting appearing at about 27–28°C, the completion at 41°C. There is no evidence for cooperative lipid melting occurring above 41°C. Also included in Fig. 6 are data for the temperature-dependence of ATPase activity in the reconstituted system. Little activity is noted below 29°C, at which point a rapid increase occurs as the temperature is raised. The data are in good accord with those of Hesketh et al. [33].

DOPC / DPPC-d_{62} / Ca^{2+}-ATPase. As noted in Introduction, a primary goal of the current study is the development of methods for determining whether membrane proteins select particular regions of chemical structure or physical order in a complex lipid environment. The use of deuterated

lipid permits this determination [12]. Ca^{2+}-ATPase from sarcoplasmic reticulum has been reconstituted with binary lipid mixtures of DOPC and DPPC-d_{62} (total lipid/protein, 55 : 1). The data must be compared with the appropriate control system, i.e., a 1 : 1 binary mixture of DOPC/DPPC-d_{62}. Melting of the proteated DOPC chains is shown in Fig. 7 for the ternary (two lipids + protein) mixture, binary lipid mixture and pure DOPC. Melting of the deuterated chains of DPPC-d_{62} is shown for three analogous systems (pure DPPC-d_{62} replacing pure DOPC as a control) in Fig. 8. The parameter plotted in Fig. 8 is the frequency of the symmetric C-$^2H_2$ stretching modes of the deuterated acyl chains which occurs near 2090 cm^{-1} [12]. The melting of pure DPPC-d_{62} occurs sharply at 37 °C, in good agreement with calorimetric data [34]. In the binary mixture, DOPC/DPPC-d_{62}, a rapid variation in the frequency vs. temperature profile is still noted from 18–29 °C. Introduction of protein into the binary mixture extends the range of this rapidly varying component, so that cooperative processes begin at about 22 °C and end at about 38 °C. The DOPC component alone (fig. 7) shows no sign of a cooperative phase transition above 7 °C. This is expected as the molecule has its gel to liquid-crystal phase transition at −22 °C [34]. Hence, it is always in the disordered state in the current experiments. In the 1 : 1 mixture with DPPC-d_{62}, a change in the slope of the DOPC melting profile is noted at around 26 °C, but no sign of a cooperative melting event is seen. Similar trends are noted for the DOPC component in the ternary system, with the change in slope appearing at around 39 °C (see Fig. 7).

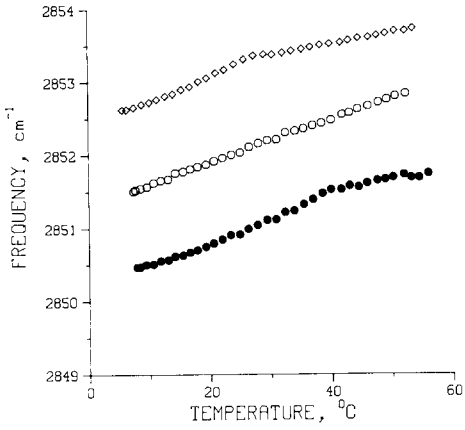

Fig. 7. Plots of the temperature dependence of the CH_2 symmetric stretching frequency for the DOPC component in DOPC/DPPC-d_{62}/Ca^{2+}-ATPase ternary system (●), DOPC/DPPC-d_{62} 1:1 binary lipid mixture (◇) and pure DOPC multibilayers (○). Relative concentrations for the components in the ternary system are given in the text.

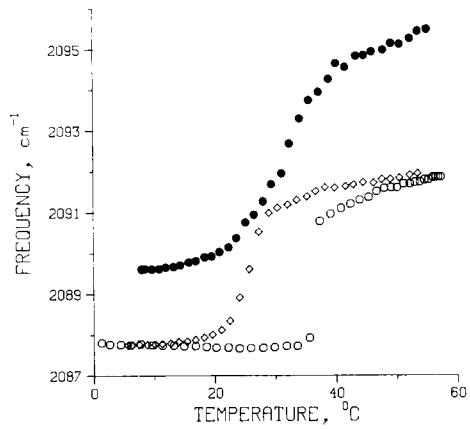

Fig. 8. Plots of the temperature dependence of the C^2H_2 symmetric stretching frequency for the DPPC-d_{62} component in DOPC/DPPC-d_{62}/Ca^{2+}-ATPase ternary system (●), DOPC/DPPC-d_{62} 1:1 binary lipid mixture (◇) and pure DPPC-d_{62} multibilayers (○). Relative concentrations of components are given in the text for the ternary system.

Discussion

The current investigation reveals the utility of Fourier transform infrared spectroscopy for addressing three aspects of lipid-protein interaction. First, what is the effect of protein on lipid order in native sarcoplasmic reticulum? Second, what is the relationship between ATPase activity and lipid melting characteristics in reconstituted systems? Third, does Ca^{2+}-ATPase partition into regions of specific chemical structure or physical order in a complex lipid environment? Each of these will be addressed in turn.

The CH_2 stretching frequencies in native sarcoplasmic reticulum occur between 2852.2 and 2853.4 cm^{-1}, over the experimental range of temperatures (Fig. 3). Compared with the frequencies for gel phase lipids [12,16,17], these observed frequencies indicate the existence of substantial disorder in the acyl chains, the extent of which con

tinually increases with increasing temperature. The data are consistent with Raman experiments of Lippert and co-workers [13,14], in which the skeletal optical branch of the spectrum (C-C stretching modes) indicated the presence of substantial numbers of gauche conformers. In addition, the current work shows that lipid order in purified sarcoplasmic reticulum is slightly increased at all temperatures from its lipid extract (Fig. 3). The current data are generally consistent with those of Fleischer and co-workers [2,9,10] in their ^{31}P-NMR and ESR studies of native and reconstituted sarcoplasmic reticulum. The ESR spectrum of a doxyl spin label (at the 5 position of the acyl chains) gave no indication of a signal referable to very slow motion, which might have been attributable to an immobilized lipid component. A slight increase in hyperfine splitting was noted as protein concentration in the vesicles was increased. This was attributed either to a small decrease in the disorder and/or motion of the lipid component in the presence of protein. The current Fourier transform infrared spectroscopy results support the former suggestion. The ^{31}P-NMR results demonstrate that the phospholipid head-group order, in contrast to that of the acyl chains, is decreased 10–15% in the presence of the Ca^{2+}-ATPase, as shown by a decrease in the chemical shift anisotropy. A detailed comparison of the Fourier transform infrared spectroscopy data with the ^{31}P-NMR is not appropriate, since the latter reflects head-group motions. Furthermore, with all three physical methods, the observed changes are small.

The current experiments address the role of lipid fluidity in determination of Ca^{2+}-ATPase function. Studies of the temperature dependence of ATPase activity in native sarcoplasmic reticulum have demonstrated a break in the Arrhenius plot at 15–20 °C [36–38]. There is no corresponding discontinuity in the temperature dependence of the C-H stretching frequency in native sarcoplasmic reticulum (Fig. 3) over the same range of temperature, indicating the absence of any cooperative lipid change of state. This observation is consistent with a suggestion of Hoffmann et al. [39] that protein conformation alterations may, in this instance, be responsible for the observed changes in the temperature dependence of the enzyme activity. Lippert and co-workers have presented some data from Raman scattering supporting the contention that changes in protein conformation do occur from 15 to 20 °C [13].

Further insight into the role of lipid fluidity as a determinant of enzyme function comes from the reconstitution experiments with DPPC as shown in Fig. 6. In these experiments, 95% of the endogenous lipid was replaced with DPPC at a lipid/protein ratio of about 30 : 1. As shown in Fig. 6, there is a large protein-induced perturbation in the lipid melting profile. The onset temperature for the phase transition occurs at 28 °C in the reconstituted system, while the completion of the melting event occurs at 41 °C. This is in contrast to the sharp melting of pure lipid at 41 °C. The melting characteristics of the lipid closely parallel the temperature dependence of ATPase activity, as shown for our preparations in Fig. 6. The temperature dependence of the ATPase activity is similar to published data [33]. Significant activity begins at 29 °C, in reasonable agreement with the Fourier transform infrared spectroscopy onset temperature for cooperative lipid melting. These results indicate that the protein requires a fluid lipid environment for function in accord with a suggestion of Hesketh et al. [33]. That this requirement may not be sufficient for the full expression of enzyme activity was demonstrated by Metcalfe and co-workers [40,41]. They showed that an optimal bilayer thickness exists, corresponding to an acyl chain length of 18 carbons.

A final area for which Fourier transform infrared spectroscopy is promising is its ability to examine the effect of protein on each lipid in a binary mixture, in situations where one of the lipids has its acyl chains perdeuterated. Chain perdeuteration is a relatively innocuous substitution [34], so that the current results for DOPC/DPPC-d_{62}/Ca^{2+}-ATPase can be extrapolated to fully proteated systems with confidence. The melting profiles for the proteated component are shown in Fig. 7 and for the deuterated component are shown in Fig. 8. The effect of protein on the DOPC is to induce a reduction of the CH_2 frequency at all temperatures, as was observed for the direct interaction of DOPC with Ca^{2+}-ATPase.

The effect of protein on the DPPC-d_{62} component is to increase both the onset temperature

(from 18 to 22°C) and the completion temperature (from 28 to 39°C) of melting.

A detailed interpretation of these results requires a model for the construction of phase diagrams from infrared frequency data. We have developed preliminary protocols along these lines [12] and a more detailed version will be published elsewhere. Our preliminary interpretation of the current experiments is that protein preferentially interacts with the more disordered component in the mixture, i.e., the DOPC. This results in an enrichment of bulk lipid in DPPC-d_{62} with concomitant higher temperatures for the melting of that component in the ternary system.

Calorimetric measurements (Jaworsky, M. and Mendelsohn, R., unpublished results) indicate regions of gel phase immiscibility for the DOPC/DPPC-d_{62} mixture. The Fourier transform infrared spectroscopy data suggest that domains of DPPC-d_{62} generally contain only small amounts of DOPC (as noted by the slight inflection in the DOPC component CH_2 frequency vs. temperature profiles at temperatures corresponding to the completion of the melt of the DPPC-d_{62} (Figs. 7 and 8)). Effective removal of the DOPC by protein (into DOPC-Ca^{2+}-ATPase domains, for example) would be expected to result in increased onset and completion temperatures for the melting of the DPPC-d_{62} component in the ternary system, as observed.

Our qualitative results for the effect of protein on this binary lipid mixture are in good accord with studies of London and Feigenson [42], who developed a technique using fluorescence quenching of spin-labeled phospholipids to measure relative binding constants of protein for two competing lipids. They concluded that at 13°C, the ratio of the binding constants of DOPC/Ca^{2+}-ATPase compared with DPPC/Ca^{2+}-ATPase was 2.5. We hope to use a combination of Fourier transform infrared spectroscopy with DSC to ascertain the same type of information without the use of probe molecules.

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