Infrared Spectroscopic Study of a "Hard-To-Exchange" Phospholipid Population in the Vicinity of CaATPase†

Laurence Senak and Richard Mendelsohn*

Department of Chemistry, Newark College of Arts and Science, Rutgers University, 73 Warren Street, Newark, New Jersey 07102

Received February 1, 1993; Revised Manuscript Received April 8, 1993

ABSTRACT: CaATPase from rabbit sarcoplasmic reticulum (SR) has been isolated, purified, and reconstituted into unilamellar vesicles with 1,2-dipentadecanoylphosphatidylcholine (DPePC) and acyl-chain-perdeuterated 1,2-dipalmitoylphosphatidylcholine (DPPC-d_{62}). For total lipid:protein mole ratios between 25 and 70, a constant "hard-to-exchange" phospholipid population (HEPP) of 12 ± 4 native phospholipid molecules per protein monomer is observed, consistent with the studies of Bick et al. [(1991) *Arch. Biochem. Biophys. 286*, 346–352]. Thermotropic and conformational properties of the lipids in native SR and in the reconstituted systems were probed with FT-IR spectroscopy. The native SR phospholipids undergo a broad phase transition centered at about 1–2 °C and are thus disordered under physiological conditions. The thermotropic behavior of CH_2 wagging progressions characteristic of the palmitate chains differs from that of the total lipid population and is suggestive of membrane microheterogeneity. The individual thermotropic and conformational properties of the HEPP and the exogenous lipid in reconstituted vesicles containing CaATPase and DPPC-d_{62} were monitored. At temperatures below the onset of the gel–liquid-crystal phase transition, the HEPP possesses significant conformational disorder, and exhibits the monotonic introduction of gauche rotamers as the temperature is raised from −55 to 27 °C, in contrast to the exogenous lipid, which exhibits a constant high order over the same temperature range. Nevertheless, the HEPP undergoes a residual order–disorder phase transition with similar but not identical parameters (half-width, midpoint temperature) to the gel–liquid-crystal transition of the bulk (exogenous) lipid in the reconstituted systems. The CH_2 wagging progression intensities in systems containing CaATPase and HEPP reconstituted with DPePC reveal that the gel phases in these systems contain 1–1.5 gauche rotamers/chain more than their pure phospholipid counterparts. The biochemical and FT-IR results are compared with other experimental approaches in an attempt to produce a consistent description of the structure of phospholipids in the vicinity of membrane proteins.

The concept of a phospholipid population in the immediate vicinity of membrane proteins with physical characteristics that differ from those of the bulk phospholipid phase was introduced some 20 years ago for cytochrome *c* oxidase (Jost et al., 1973), and for CaATPase (Warren et al., 1974a,b), and has become an oft-quoted concept in descriptions of membrane structure. Yet direct demonstrations of the existence and determination of the physical properties of this putative lipid class have proven elusive, for several reasons. The main physical techniques used for studies of lipid conformational and motional characteristics, namely, electron spin resonance (ESR)[1] and fluorescence spectroscopies, require probe molecules whose spectroscopic properties are not easily interpreted in terms of the structural or motional characteristics of the phospholipids they monitor, whose membrane partitioning characteristics are mostly undetermined, and whose insertion into bilayers may produce unwanted local structural perturbations (Taylor & Smith, 1980). The general approach of most investigations has been to exchange as completely as possible the endogenous lipid population for a selected exogenous species. Spectroscopic signals used to monitor the lipid–protein interaction thus arise mostly from a single chemical species which nevertheless may exist in two (or more) physical environments. A complementary approach for the interpretation of spectroscopic data would entail the use of a nonperturbing technology, such as infrared (IR)[1] spectroscopy in the current work, that permits the separate detection of signals from different chemical species. If one population is known to be in the vicinity of the protein, then its individual physical properties may be deduced and compared with those of surrounding bulk lipid phase.

The biochemical feasibility of such an experiment was recently demonstrated by Bick et al. (1991), who reported that when CaATPase-containing vesicles from rabbit SR were stripped of phospholipids through the action of the detergent octaethylene glycol dodecyl ether, a phospholipid remnant (6–8 mol/mol of protein) enriched in unsaturated aminophospholipids was observed. This suggests a means for preparation of vesicles containing a "hard-to-exchange" phospholipid population (HEPP), presumably in close proximity to the protein, as well as an exogenous phospholipid species in a sample where they might be spectroscopically distinguished.

The current experiments report FT-IR studies of CaATPase in vesicles possessing HEPP as well as a bulk phase consisting of 1,2-dipentadecanoylphosphatidylcholine (DPePC) or acyl-chain-perdeuterated 1,2-dipalmitoylphosphatidylcholine (DPPC-d_{62}). The use of DPPC-d_{62} permits acquisition of separate signals (the CD_2 and CH_2 stretching modes) from the deuterated and proteated lipid components, respectively, of

† Supported by grants from the U.S. Public Health Service (GM-29864 to R.M.) and from NATO (CRG-910063, awarded to R.M., project coordinator).

* To whom correspondence should be addressed.

[1] Abbreviations: DPePC, 1,2-dipentadecanoylphosphatidylcholine; DPPC-d_{62}, acyl-chain-perdeuterated 1,2-dipalmitoylphosphatidylcholine; ESR, electron spin resonance; FT-IR, Fourier transform infrared; GC, gas chromatography; HEPP, hard-to-exchange phospholipid population; IR, infrared; NMR, nuclear magnetic resonance; SR, sarcoplasmic reticulum; T_m, gel–liquid-crystal phase transition temperature; TLC, thin-layer chromatography.

0006-2960/93/0432-6288$04.00/0

the reconstituted vesicles. Reconstitutions with fully proteated exogenous species have enabled us to detect IR markers (the CH_2 wagging progressions) that semiquantitatively characterize conformational order in (specifically) the DPePC. Taken together, these experiments have produced a description of the thermotropic and conformational properties of both the exogenous and the HEPP lipids.

MATERIALS AND METHODS

Phospholipids obtained from Avanti Polar Lipids Inc. (Birmingham, AL) were of stated purities 99% or greater, and were used without further purification. Recrystallized deoxycholate was obtained from Boehringer Mannheim Corp. (Indianapolis, IN), and ^{14}C-labeled deoxycholate was purchased from California Bionuclear Corp. (Los Angeles, CA). Both detergents were of stated purity >99% and were used without further purification. Solvents were of the highest quality commercially available, and water was doubly distilled.

Sample Preparation. Purified sarcoplasmic reticulum was isolated from the skeletal muscle of albino rabbits as described previously (Mendelsohn et al., 1984a). Endogenous lipids were exchanged via the deoxycholate-mediated exchange protocol of Warren et al. (1974a), later modified by Hidalgo et al. (1976). Briefly, CaATPase from purified SR is solubilized in DOC-enriched lipid vesicles of DPPC-d_{62} or DPePC which have been heated to 40 °C and sonicated to clarity. Weight ratios (protein/deoxycholate/lipid) used to obtain the desired complexes were 1:0.5:2 and 1:0.5:1 for ~50:1 and 25:1 lipid:protein mole ratio reconstitutions, respectively. These conditions resulted in consistent results for all preparations. Several attempts to reduce the extent of the HEPP either by resuspending preformed complexes with additional exogenous lipid or by altering the detergent failed. The complexes were allowed to incubate at 37 °C for 30–45 min with occasional vortexing and sonication. Samples were layered on a discontinuous sucrose density gradient (15/50%) and centrifuged for 20 h at 125000*g* in a Beckmann TL-100 benchtop ultracentrifuge equipped with a TLS-55 swinging-bucket rotor. The protein-containing lipid vesicles were removed from the interface and centrifuged for 1 h at 75000*g*. The resulting pellet was then homogenized and suspended in a sucrose/Tris buffer and frozen at –40 °C until further use. For IR spectroscopy, 0.5–1 mL of lipid/protein complex is pelleted at 400000*g* for 1 h in 2 mL of double-distilled water and applied to the cell windows. Pure phospholipid vesicles were prepared by dispersing the desired phospholipid in double-distilled water in sealed ampules (4:1 w/w H_2O:lipid ratio) at temperatures well above the T_m. Accuracy in lipid:protein ratios is estimated as ±5%.

Biochemical Characterization. Protein concentrations were determined by the method of Lowry et al. (1952), and lipid levels were determined by the phosphorus assay of Chen et al. (1956). Enzyme activity measurements were accomplished with the coupled enzyme assay of Warren et al. (1974b). Residual detergent was determined by incorporation of ^{14}C-labeled deoxycholate into the reconstitution experiment. Lipids for thin-layer (TLC) and gas chromatographic (GC) analysis were extracted by the method of Bligh and Dyer (1959). For GC studies, the extracts were trans-esterified in acidic methanol and the methyl esters extracted with diethyl ether. A Hewlett-Packard HP 5890 GC equipped with a Supelco SP2380 30-cm capillary column was run with a thermal gradient program increasing from 150 to 275 °C at a rate of 5 °C/min. The lipid extracts were used directly for one- and two-dimensional TLC studies using a procedure of Horrocks (1968), as modified by Bick et al. (1991). Under this approach, various classes of phospholipid head groups may be isolated on Silica Gel 60 TLC plates utilizing a mobile phase of 70:30:8:1 chloroform/methanol/water/ammonium hydroxide. Plasmalogenic lipids may be directly identified by exposing a plate previously exposed in one dimension to concentrated sulfuric acid fumes and chromatographing in a second dimension using the above-mentioned mobile phase, following verification that no residual acid remains behind. The plasmalogens are not hydrolyzed under these conditions. Both dimensions are visualized by plate exposure to iodine vapor.

Electron microscopy was accomplished using a Philips Model CM10 electron microscope to examine samples, negatively stained with uranyl acetate, on a charged grid. Laser light-scattering measurements to determine vesicle size distribution were carried out in the laboratory of Professor R. Moss.

FT-IR Spectroscopy. For FT-IR thermotropic studies, the samples were contained in a thermostated transmission cell (Harrick Scientific, Ossining, NY) between two CaF_2 windows separated by a Teflon spacer of 6-μm thickness. The samples were examined at temperatures both above and below 0 °C. Temperature control above 0 °C was achieved with a circulating water bath (Haake, Inc.). Below 0 °C, the cell was contained in a dewar (Beckman Instruments) which was temperature-controlled through the boil-off of liquid N_2. Temperatures were monitored with a thermocouple (Physitemp Instruments, Inc., Clifton, NJ) placed in the cell jacket. Spectra were acquired on a Digilab FTS-40 spectrometer equipped with a TGS detector at 4-cm^{-1} resolution under N_2 purge by co-addition of 256 interferograms. These were apodized with a triangular function and Fourier-transformed with one level of zero-filling to yield data encoded every 2 cm^{-1}. For measurement of CH_2 and CD_2 stretching frequencies, the spectrum of water (or ice for spectra obtained below 0 °C) at the same path length and temperature as the sample was subtracted from all sample spectra. Following base-line correction, frequencies were measured (uncertainty 0.1 cm^{-1}) with a center of gravity algorithm. For quantitation of the CH_2 wagging mode progression intensities in the region 1300–1150 cm^{-1}, subtraction of the underlying PO_2^- symmetric stretching band was accomplished through the subtraction of a spectrum recorded at high enough temperature to ensure lipid disorder, without protein denaturation. Subtraction factors were chosen by maximizing the band heights of the progression and by choosing a consistent shape for the base line of the residual contour of wagging progression components of a given sample. Integration of the resultant wagging progression bands was accomplished by transferring subtracted spectra to an off-line microcomputer employing software generously provided by D. Moffatt of the National Research Council of Canada. The same points chosen for base-line flattening were used for integration. Progression intensities (estimated precision, 3%) were calculated by ratioing the area of band of interest to the area of the underlying phosphate band.

RESULTS

Biochemical Characterization of SR and CaATPase/Phospholipid Complexes. The major phospholipid classes in the SR as well as the acyl chain compositions of the native and reconstituted preparations as determined from TLC and GC analysis are summarized in Tables I and II. The results for native SR are in accord with prior studies (Hidalgo et al., 1976). The PC fraction is enriched in palmitate (16:0) and linoleate (18:2) and depleted in myristate (14:0) and arachidonate (20:4) relative to the phosphatidylethanolamine fraction. The major chain lengths within the phosphatidylserine

Table I: Fatty Acid Constituents of Native Sarcoplasmic Reticulum

	phospholipid[a] species		
chain length	PC	PE	PS+PI
14:0	3.1	10.4	19.7
16:0	46.5	31.6	21.6
18:0	12.3	24.2	*b*
18:1	16.2	15.5	58.7
18:2	21.9	11.0	*b*
20:4	*b*	7.5	*b*

[a] In separate experiments (Vrbjar et al., 1992), the relative amounts of PC, PE, and PS+PI were found to be 70:15:9. [b] Less than 1% of these chain lengths.

Table II: Biochemical Characterization of Lipid/Protein Complexes

		acyl chain length percentages							
lipid/HEPP/CaATPase[a]	activity (IU)[b]	14:0	15:0	16:0	16:0 (d_{62})	18:0	18:1	18:2	20:4
DPePC									
13:11:1	2.5	1.4	54.0	13.7		10.0	11.5	9.3	
34:15:1	3.0	1.8	69.0	12.1		9.2	2.9	5.2	
DPPC-d_{62}									
51:15:1	1.2	5.3		10.0	77.0	4.7		3.1	
15:15:1	1.0			34.0	48.0	6.5	6.5	4.3	
SR									
80:1[c]	6.0	4.8		28.0		7.7	13.0	31.5	14.9

[a] Molar ratios of the indicated lipid/HEPP/CaATPase. [b] International units. [c] Total lipid:protein molar ratio in native SR.

and phosphatidylinositol fractions are myristate, palmitate, and oleate (18:1).

The purification protocols used in the current work produced essentially detergent-free (1 detergent molecule/550 protein molecules) complexes. The vesicle size distribution as determined from light scattering seemed to follow Poisson statistics and peaked at a diameter of 1700 Å. Atypical small unilamellar vesicles (about 650–750 Å in diameter) are shown in the electron micrographs in Figure 1A while a typical fairly large unilamellar vesicle (2000 Å in diameter) is shown in Figure 1B. Less than 0.1% of the total mass was in the form of vesicles less than 750 Å in diameter. Aggregated regions of large variable dimensions were occasionally observed in the electron micrographs; these may result from the electron microscopy sample preparation protocols, since few particles of greater than 4000 Å were noted in the light-scattering experiments.

The biochemical evidence for the existence of a constant unexchangeable population of endogenous phospholipids in a series of reconstituted samples is given in Figure 2. As the total lipid:protein molar ratio is increased from 25:1 to 80:1, the HEPP averages 12 ± 4 molecules per protein monomer, independent (slope statistically indistinguishable from zero) of the amount of exogenous lipid incorporated. This result is to be compared with the recent work of Bick et al. (1991), who solubilized ("stripped") the lipids from SR vesicles with the nonionic detergent octaethylene glycol dodecyl ether. They observed 7 ± 1 phospholipids/protein monomer, in good accord with the current work, considering the very different characteristics of the detergents used. The HEPP consisted of a high proportion of 16:0 acyl chains with substantial amounts of 18:0, 18:1, and 18:2, in ratios generally similar to those of the native SR, as can be seen by a comparison of Tables I and II. In addition, a significant population of plasmalogen was noted. This class had previously been detected and subject to quantitative analysis (Bick et al., 1991).

FT-IR Studies of Native SR and Lipid Extracts. The temperature dependence of the CH_2 symmetric stretching

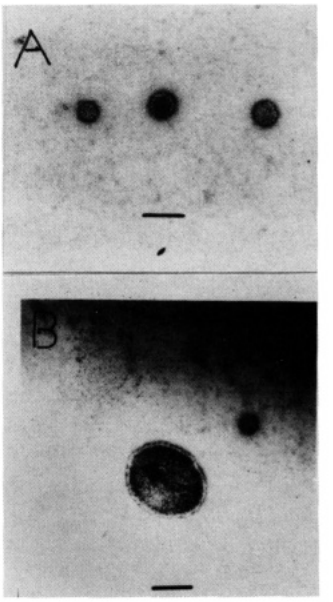

FIGURE 1: Typical electron micrographs of vesicles found in reconstituted protein/lipid complexes of CaATPase/DPePC prepared as described under Materials and Methods. Magnification was 36000×. (A) Three atypical unilamellar vesicles with diameters of 600–700 Å. (B) Two vesicles with diameters of 600 and 2000 Å, respectively. The larger is quite typical. The peak in the size distribution as determined from light scattering is about 1700 Å. Less than 0.1% of vesicle mass is in the form of vesicles smaller than 700 Å. The bar on each micrograph represents 1000 Å.

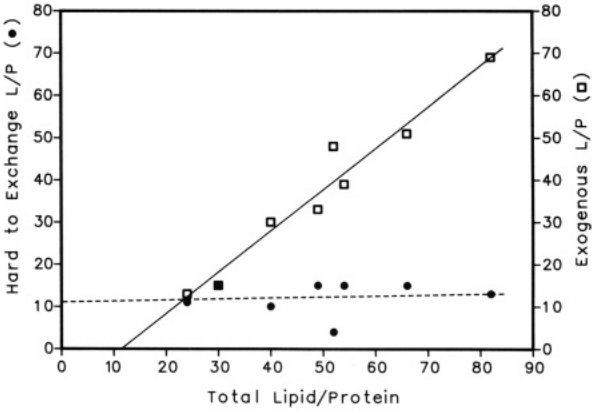

FIGURE 2: Exogenous phospholipid:protein ratios (□) and "hard-to-exchange" phospholipid population (HEPP):protein ratios (●) are plotted against total phospholipid:protein molar ratios. The HEPP/protein remains at 12 ± 4 mol of lipid/mol of protein independent of added exogenous lipid levels.

frequency near 2850 cm^{-1} has been widely used as a qualitative index of phospholipid conformational order (Mendelsohn & Mantsch, 1986). Band positions are known to increase 2–5 cm^{-1} as a result of gauche rotamer formation (Snyder et al., 1982) during the gel–liquid-crystal phase transition, although detailed relationships between the frequency increase and the precise number of gauche bonds have not been ascertained. IR melting curves in Figure 3 display this parameter for purified SR and its lipid extract over the temperature range of −50 to +40 °C. The measured frequency is an average (weighted by the number of CH_2 groups) of all the acyl chains present.

The increased frequency of the CH_2 stretching vibration in the whole SR at all studied temperatures compared with the extracted lipids reflects protein-induced disorder in the lipid acyl chains. A broad phase transition centered at about 1–2 °C is observed in each case (the midpoint temperature is perhaps 1 °C higher for the native SR, but this increase is difficult to ascertain with any certainty). As shown below,

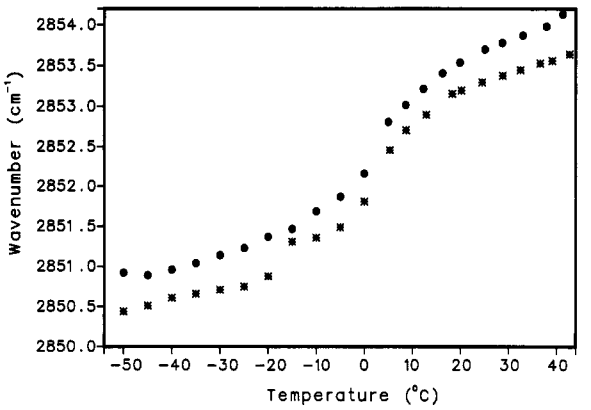

FIGURE 3: Temperature-induced variation for the CH_2 symmetric stretching mode of purified SR (●) and its lipid extracts (*).

Table III: CH_2 Wagging Frequencies in Complexes and Model Systems

k	DPPC	DPePC	SR	DPePC/HEPP/CaATPase
1	1199.8	1201.7	1197.5	1201.4
2	1221.0	1224.9	1221.8	1226.0
3	1244.2	1250.0	1245.2	1250.2
4	1265.4	1273.1	1266.4	1272.4

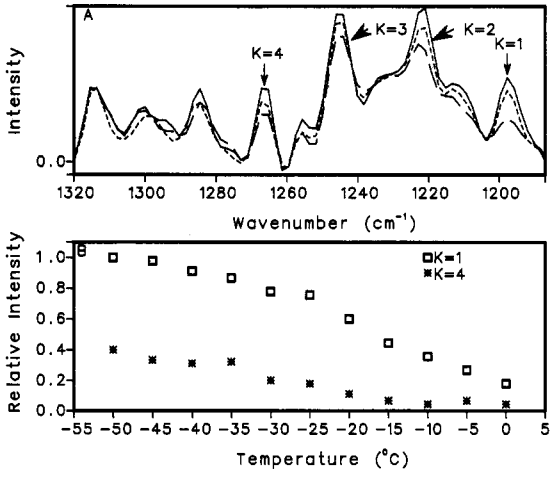

FIGURE 4: (A) Second-derivative FT-IR spectra of purified SR at −50 °C (—), −30 °C (- - -), and −10 °C (– –). Labeled are $k = 1$ through $k = 4$ CH_2 wagging progression bands. (B) Temperature-induced variation of the $k = 1$ (□) and $k = 4$ (*) CH_2 wagging progression band intensities in purified SR, normalized to the underlying PC_4^{2-} band. These intensities were derived from difference spectra, as discussed under Materials and Methods section.

chain heterogeneity and domain formation contribute to the transition breadth, which is not markedly affected by protein.

This laboratory has recently introduced a more quantitative parameter for defining conformational order in phospholipid acyl chains (Senak et al., 1992; Chia & Mendelsohn, 1992). The CH_2 wagging progressions of adjacent methylene units in the trans conformation couple to produce a pattern characteristic of the number of CH_2 groups (Snyder, 1960; Snyder & Schachtschneider, 1967). Relevant frequencies are listed in Table III. The loss of conformational order in native SR at low temperatures may thus be further examined. Second-derivative data are the best means of displaying the progression for the wagging region (1175–1380 cm^{-1}), and these are shown in Figure 4A. The most prominent feature is the series of bands whose frequencies precisely match the $k = 1$ through $k = 4$ modes of palmitate (16:0) chains (Table III), a major component of native SR (Table I). As the progression intensity vanishes in a fashion that parallels the

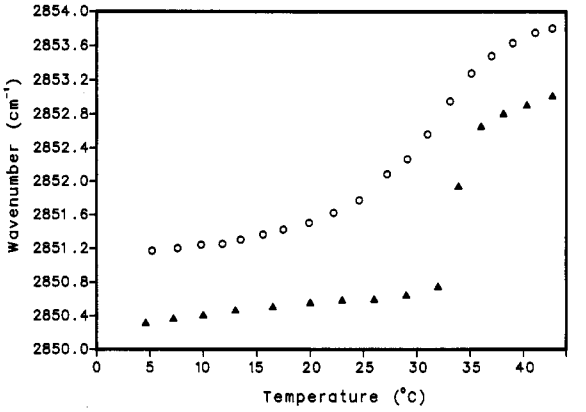

FIGURE 5: Temperature-induced variation in the CH_2 symmetric stretching mode of the 46:6:1 DPePC/HEPP/protein complex (○) and DPePC vesicles in the absence of protein (▲).

introduction of conformational disorder into the chains, the current experiment provides a snapshot of the melting of this particular lipid component within a complex mixture. Weaker bands in the spectra at 1212 and 1254 cm^{-1} (Figure 4A) may correspond to the $k = 1$ and 2 components of the oleate progression. Species with higher levels of unsaturation (e.g., 18:2) do not give rise to a well-behaved CH_2 wagging progression, as has been discussed elsewhere (Chia & Mendelsohn, 1992). For accurate determination of changes in progression band intensities, second-derivative spectra were felt to be unreliable. Thus, the temperature dependence of the $k = 1$ and $k = 4$ palmitate band intensities (obtained not from derivative spectra but from subtraction protocols as discussed under Materials and Methods) is seen in Figure 4B. These features were chosen for their relative isolation in the spectra, thus minimizing the use of data reduction protocols which degrade signal-to-noise levels.

Three trends in these data are observed: First, there exists a region of stable constant intensity from −95 to −55 °C (not shown on Figure 4B) followed by a gradual diminution of intensity between −55 and about −30 °C and a final more rapid loss of intensity with a vanishing of the progression (especially easily noted for the $k = 1$ component) near 0 °C.

FT-IR Studies of DPePC/CaATPase Complexes. The CH_2 symmetric stretching modes from the entire phospholipid population in a 46:6:1 DPePC/HEPP/CaATPase complex and pure DPePC vesicles are plotted as a function of temperature in Figure 5. The melting profiles exhibit trends consistent with previous results for DPPC/CaATPase and DMPC/glycophorin complexes (Mendelsohn et al., 1981, 1984b), namely, a broadened transition range, lowered onset and midpoints of melting, and a general shift toward higher frequency over the entire temperature range upon addition of protein. All these are consistent with protein-induced bilayer disordering. The sharp transition noted for pure DPePC at 33–34 °C is in good agreement with the gel–liquid-crystal transition as previously observed (Moore et al., 1981).

Additional evidence for the disordering effect of CaATPase on the exogenous component comes from the wagging progression of DPePC for the control and two complexes (DPePC/HEPP/CaATPase; 13:11:1 and 46:4:1) at 5 °C as shown in Figure 6. The pronounced band progression features as labeled are clearly evident for the pure phospholipid but are significantly diminished in intensity in spectra of the lipid/protein complexes. Quantitative aspects of the conformational disordering in the complexes and controls as monitored through intensity loss in the $k = 4$ component of the methylene wagging progressions may be seen in Figure 7A,B. The relationship

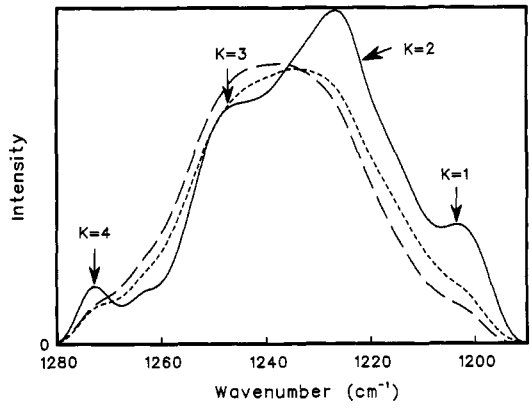

FIGURE 6: FT-IR spectra at 5 °C of DPePC/HEPP/CaATPase, 13:11:1 (- - -) and 46:6:1 (– –), complexes as well as pure DPePC vesicles (—) in the region 1280–1180 cm^{-1}. The k = 1 through k = 4 CH_2 wagging progression bands are labeled.

between intensity loss and the loss of all-trans conformational order has been discussed elsewhere (Senak et al., 1992): Briefly, it is assumed that the intensity of the sharp progression components arises solely from the all-trans chain conformation. It is also assumed that the intensity at the lowest temperature studied is characteristic of the all-trans conformation. The loss in intensity due to disorder is thus given by

$$I_{rel} = p^n \quad (1)$$

where I_{rel} is the progression component intensity relative to that at the lowest temperature studied, n is the number of CH_2 groups, and p is the probability of a trans bond. The probability of a gauche bond is therefore $1-p$, and the number of gauche bonds in the chain is $n(1-p)$. For example, for a C_{15} acyl chain (13 CH_2 groups), a factor of 7 intensity diminution is produced by p = 0.86 in eq 1, which in turn leads to a calculated number of 1.8 gauche bonds/chain. The numbers of gauche bonds thus calculated are also indicated on the ordinate scales in Figure 7. Note that the measurements are unreliable above 30 °C, as the introduction of substantial disorder (more than about 2.5 gauche bonds/chain) renders the sharp progression bands extremely weak. The constancy of the progression intensity in DPePC at low temperatures (Figure 7A) indicates the occurrence of a fully ordered state, which is thus suitable as a reference intensity for the calculations.

FT-IR Studies of DPPC-d_{62}/CaATPase Complexes. To examine the physical state of the HEPP in enzymatically active preparations, DPPC-d_{62} was used as the exogenous lipid. Under these conditions, the CD_2 stretching vibrations provide (Mendelsohn & Mantsch, 1986) a useful probe of acyl chain order in the exogenous component, while the CH_2 stretching modes of the HEPP monitor that population exclusively. In Figure 8B, the CD_2 antisymmetric stretching mode for a DPPC-d_{62} control, as well as a 53:13:1 DPPC-d_{62}/HEPP/CaATPase, is plotted as a function of temperature over the range –55 to 45 °C. The behavior of the exogenous lipid phase in the complexes is similar to that for the DPePC/CaATPase system, and shows a broadened phase transition with a T_m 2–3 °C lower than the control sample. At low temperatures (–55 to 0 °C), the CD_2 frequency is constant and low, consistent with an ordered acyl chain state. Nevertheless, the acyl chains are more disordered than in pure DPPC-d_{62}, as can be seen by the ~1-cm^{-1} reduction in the CD_2 symmetric stretching frequency in the latter at all temperatures from –55 to 30 °C. We note that this observation pertains directly to the exogenous lipid whereas in the studies

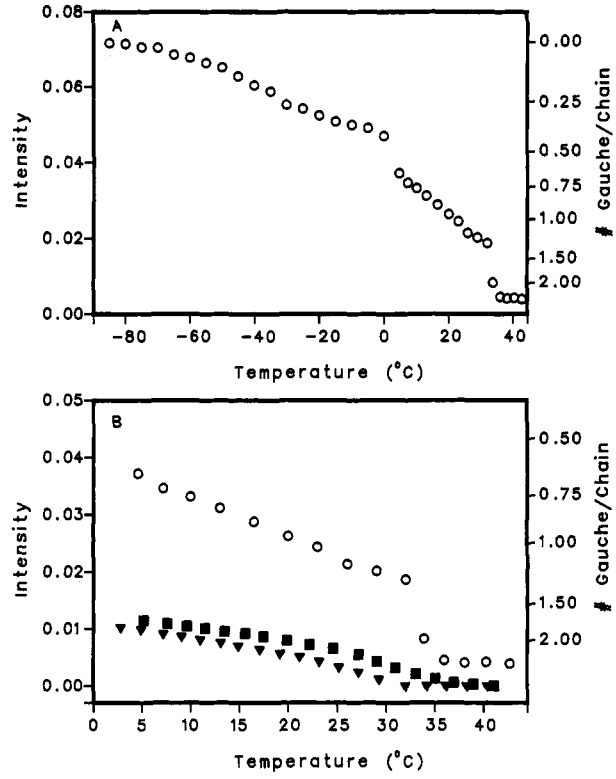

FIGURE 7: (A) Temperature dependence of the k = 4 CH_2 wagging progression band intensity for DPePC (○) over the full range of temperatures used in the current work. The intensity is nearly constant below –70 °C, indicating nearly complete all-trans conformational order. (B) Temperature dependence of the k = 4 CH_2 wagging progression band intensity for DPePC/HEPP/CaATPase, 13:11:1 (▼) and 46:6:1 (■), complexes as well as for DPePC (○). Indicated on the left and right ordinates respectively are the intensity of this band relative to the underlying PO_4^{2-} band, and the number of gauche bonds present.

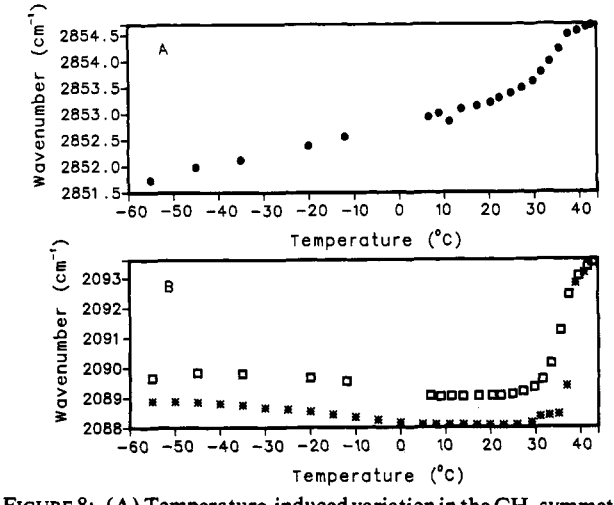

FIGURE 8: (A) Temperature-induced variation in the CH_2 symmetric stretching mode of the "hard-to-exchange" phospholipid population (HEPP) of a DPPC-d_{62}/HEPP/CaATPase 53:13:1 complex (●). (B) Temperature dependence of the CD_2 symmetric stretching mode of the DPPC-d_{62} component (□) of the DPPC-d_{62}/HEPP/CaATPase 53:13:1 complex and pure DPPC-d_{62} vesicles (*).

with DPePC, the C–H stretching mode monitored both exogenous and HEPP lipids.

The temperature-induced variation in the HEPP CH_2 symmetric stretching frequency in the 15:15:1 DPPC-d_{62}/HEPP/CaATPase complex is shown in Figure 8A. The CH_2 stretching frequency of 2851.7 cm^{-1} at –55 °C is increased from 2850.8 cm^{-1} in native SR (Figure 1), indicative of more

disorder in the HEPP. As the temperature is raised from −55 to 27 °C, a monotonic increase from 2851.7 to 2853.5 cm^{-1} is observed. This reflects substantial noncooperative introduction of gauche rotamers into the HEPP fraction. The incomplete disorder is revealed by the occurrence of a more cooperative melting process over the temperature range 27–37 °C with a midpoint of 32–33 °C. Above 37 °C, the CH_2 frequency is that of a fully disordered state. Thus, the behavior of the HEPP differs substantially from the exogenous component prior to the main chain melting event.

DISCUSSION

Notable lack of consensus exists in the literature concerning the physical properties of lipid molecules in the vicinity of membrane proteins. The wide range of interpretations extant is sampled below. Warren et al. (1974a,b) experimentally determined the minimum number of phospholipid molecules (about 20–30) required to sustain the activity of CaATPase. From the viewpoint of lipid dynamics, the original ESR experiments sampling the lipids in the vicinity of cytochrome *c* oxidase (Jost et al., 1973) were interpreted in terms of a motionally restricted annulus, and this type of interpretation has persisted [for a review, see Marsh (1985)]. From the perspective of lipid conformation, ^2H NMR experiments (Rice et al., 1979; Paddy et al., 1981; Jahnig, 1979) indicated slight protein-induced disordering of lipid. Lentz et al. (1983, 1985), interpreting a combination of scanning calorimetry and fluorescence data, proposed a model in which a motionally inhibited lipid annulus coexisted with a disrupted lipid class that extended three to four layers beyond the annulus. Additional interpretations have focused on lipid molecules trapped between closely apposed proteins (Davoust et al., 1980). No consistent picture has emerged in spite of attempted reconciliations based on time-scale differences between experimental technologies.

The assumption underlying the current analysis is the obvious one, namely, that the HEPP *de facto* constitutes a lipid class preferentially in the vicinity of CaATPase. The data in Table I reveal at most a weak preferential selection of chain lengths, a result in good accord with Caffrey and Feigenson (1981), who examined the exchange characteristics of a series of PC's for the environment around CaATPase, and perhaps the slight preference for non-PC lipid classes (including plasmalogens) as emphasized by Bick et al. (1991). Although nothing specific about the rates of exchange between this class and the bulk lipids is implied or can be deduced from the IR measurements, the current approach offers the advantage that it reports directly either qualitatively (CH_2 or CD_2 stretching frequencies) or semiquantitatively (CH_2 wagging progression intensity) on the conformational states of both the exogenous and endogenous lipid fractions.

The CH_2 stretching data for the purified SR vesicles (Figures 3 and 4) provide base-line values for their interpretation in the HEPP. The melting curves (either in the presence or in the absence of protein) reveal a phase transition that occurs over a ~25 °C temperature range centered near +1–2 °C. Under physiological conditions, the IR data indicate that most of the lipid is disordered. Protein induces a generalized conformational disordering of the lipid population, as revealed by increased CH_2 stretching frequencies at all temperatures.

The presence of the all-trans conformation at temperatures below 0 °C is revealed through the palmitate CH_2 wagging progressions in Figure 4A. As previously noted (Senak et al., 1992), a small amount of disorder is sufficient to drastically reduce the intensity of the progression bands. The temperature dependence of the progression components (Figure 4B) reveals that the intensity persists until close to 0 °C, well beyond the onset of the phase transition for the entire lipid population as monitored from the CH_2 stretching frequencies. The altered thermotropic behavior of the palmitate compared with the entire lipid population (Figure 3) suggests the presence of microheterogeneity (phase-separated domains) in the SR membranes. The broadened phase transition detected via the CH_2 stretching vibrations (Figure 3) is the summation of the melting of these domains.

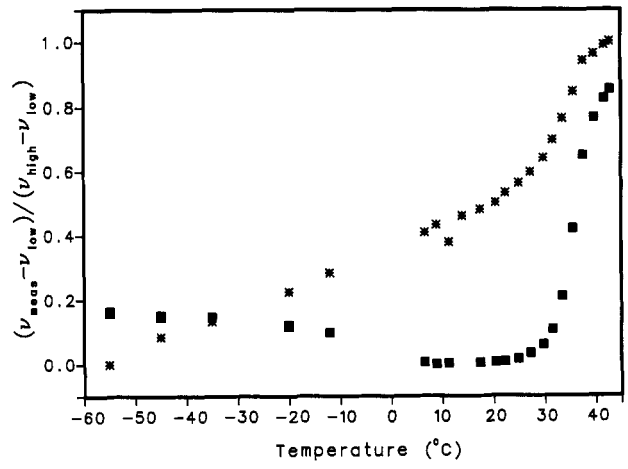

FIGURE 9: Variation of the CH_2 symmetric stretch of the HEPP component (*) and the CD_2 symmetric stretch of the DPPC-d_{62} (■) component of the DPPC-d_{62}/HEPP/CaATPase 53:13:1 complex as a fraction of the total shift in frequency throughout the thermal transitions of the respective species.

For CaATPase reconstituted with DPePC (Figures 5–7), the presence of a single exogenous lipid (along with the HEPP) permits the characteristics of its sharp phase transition to be used as an indicator of CaATPase-induced perturbations of that species. Addition of protein produces the usual responses, namely, reduced transition cooperativity, lowered midpoint temperature, and a general disordering of the lipid. The CH_2 stretching data again reflect the average conformational state of the entire phospholipid population. The melting of the DPePC component alone is revealed from the $k = 4$ component of the wagging progression. The intensity for the pure lipid (Figure 7) drops precipitously at T_m, consistent with the loss of all-trans conformational order. Introduction of protein reduces the progression intensity at 5 °C by a factor of about 4. According to the model in eq 1, this corresponds to the presence of an additional 1–1.5 gauche bonds/chain in the presence of protein at 5 °C, compared with pure DPePC vesicles at the same temperature. Independent of the quantitative model, all data consistently reveal a protein-induced conformational disordering of the exogenous lipid component, in accord with the concept of Lentz et al. of a poorly packed (and therefore presumably conformationally disordered) secondary lipid domain (Lentz et al., 1983, 1985).

The observed HEPP (12 ± 4 mol/mol of protein) is insufficient to constitute a so-called "lipid annulus". Direct insight into the conformation of the HEPP fraction is gained from studies of CaATPase/HEPP/DPPC-d_{62} complexes. In Figure 9, the actual frequencies have been replaced by a factor, $(\nu_{measured} - \nu_{low})/(\nu_{high} - \nu_{low})$, which converts the ordinate scale into a fraction of the change available to the frequency parameter, thus placing the CH_2- and CD_2-derived melting data on a comparable basis. Substantially different behavior is evident for the exogenous DPPC-d_{62} component compared with the HEPP. The DPPC-d_{62} undergoes most of its available range of frequency shift at the (broadened) transition without

the continuous introduction of gauche rotamers prior to that point, while 50% of the change in the HEPP CH_2 stretching frequency occurs noncooperatively prior to its residual melting event. At temperatures between 0 and 20 °C, the CD_2 symmetric stretching frequency is approximately constant at a position (2089 cm^{-1}) appropriate for a mostly ordered conformation (Mendelsohn & Mantsch, 1986). A discontinuity of 0.7 cm^{-1} in the temperature/frequency plot is noted at 0 °C (Figure 9) which may be related to technical difficulties in the subtraction of the solvent association band (2000–2300 cm^{-1}) that sharpens considerably upon freezing and may be very dependent on solute structure. Alternatively, the discontinuity may be related to hydration properties of the head group that propagate to the acyl chains in an ice environment. No shift in this frequency is seen between 5 and 25 °C. The transition characteristics (range 30–40 °C, midpoint 35 °C) are broadened and slightly reduced in temperature from those of pure DPPC-d_{62} which has T_m = 37 °C and a transition width of 5–6 °C.

The thermotropic properties of the HEPP differ from those of DPPC-d_{62}. Compared with the CH_2 frequency for native SR at −55 °C (2850.5 cm^{-1}), the HEPP (2851.7 cm^{-1}) possesses substantially more conformational disorder. The absence of a wagging progression from the latter at low temperatures is consistent with this interpretation. From −55 °C until the onset of the cooperative melting process at 27 °C, there is the progressive, noncooperative introduction of gauche rotamers into the chains so that at the onset of the residual phase transition the frequency is already at the rather high value of 2853.5 cm^{-1}. A quantitative estimate of the disorder required to produce this frequency shift is unavailable, although simulations (Dluhy et al., 1983) with a two-state model indicate that at least 50–70% fractional disorder is needed to produce a 1-cm^{-1} frequency increase. Thus, the observed 1.8-cm^{-1} increase prior to the thermotropic melting event implies substantial disorder in the HEPP, in marked contrast to the DPPC-d_{62} component.

A reasonable structural model for both the HEPP and exogenous lipid phase behavior and conformation requires the former at low temperatures to conform to the invaginations at the surface of CaATPase, through the noncooperative formation of gauche rotamers. This disordering continues until the onset of the more cooperative melt and probably reflects poor acyl chain packing (i.e., a mismatch between the protein and lipid surfaces that propagates from the protein–lipid interface). The presence of gauche rotamers presumably produces more favorable lipid–protein interactions which impede the exchange of the HEPP for the exogenous species, and result in the reduced rates of motion as detected by ESR spectroscopy.

The ability of the already disordered HEPP to undergo a transition (albeit with a reduced range of frequency increase) is somewhat surprising. Two factors may be relevant. First, it is likely that exchange rates between HEPP and DPPC-d_{62} are increased with temperature, possibly to the point where there is sufficient HEPP in the bulk phase to mix with the exogenous fraction and hence to undergo a transition. Another (not necessarily mutually exclusive) possibility is that the HEPP is noncovalently weakly bound to the protein via lipid interfacial and polar regions. This would permit some conformational flexibility in the chains, possibly enhanced toward their methyl terminals. These regions of the chains could "sense" the DPPC-d_{62} molecules in their immediate environment and respond partially to the phase transition of the exogenous species.

ACKNOWLEDGMENT

Dr. N. Franks and Dr. W. Lieb (Department of Physics, Imperial College, London), collaborators on NATO Grant CRG-910063, are thanked for their insights and discussions.

REFERENCES

Bick, R. J., Youker, K. A., Pownall, H. J., Van Winkle, W. B., & Entman, M. L. (1991) *Arch. Biochem. Biophys. 286*, 346–352.

Bligh, E. C., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911–917.

Caffrey, M., & Feigenson, G. W. (1981) *Biochemistry 20*, 1949–1961.

Chen, P. S., Toribara, T. Y., & Warner, H. (1956) *Anal. Chem. 28*, 1756–1758.

Chia, N.-C., & Mendelsohn, R. (1992) *J. Phys. Chem. 96*, 10543–10547.

Davoust, J., Bienvenue, A., Fellmann, P., & Devaux, P. F. (1980) *Biochim. Biophys. Acta 596*, 28–42.

Dluhy, R. A., Mendelsohn, R., Casal, H. L., & Mantsch, H. H. (1983) *Biochemistry 22*, 1170–1177.

Hidalgo, C., Ikemoto, N., & Gergely, J. (1976) *J. Biol. Chem. 251*, 4224–4232.

Horrocks, L. A. (1968) *J. Lipid Res. 9*, 469–472.

Jahnig, F. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6361–6365.

Jost, P. C., Griffith, O. H., Capaldi, R. A., & Vanderkooi, G. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 480–484.

Lentz, B. R., Clubb, K. W., Barrow, D. A., & Meissner, G. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2917–2921.

Lentz, B. R., Clubb, K. W., Alford, D. R., Hochli, M., & Meissner, G. (1985) *Biochemistry 24*, 433–442.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Marsh, D. (1985) *Progress in Protein–Lipid Interactions* (Watts, A. W., & De Pont, J. J. H. H. M., Eds.) Vol. 1, pp 143–172, Elsevier, Amsterdam.

Mendelsohn, R., & Mantsch, H. H. (1986) *Progress in Protein–Lipid Interactions* (Watts, A. W., & De Pont, J. J. H. H. M., Eds.) Vol. 2, 103–146, Elsevier, Amsterdam.

Mendelsohn, R., Dluhy, R., Taraschi, T., Cameron, D. G., & Mantsch, H. H. (1981) *Biochemistry 20*, 6699–6706.

Mendelsohn, R., Brauner, J. W., Faines, L., Mantsch, H. H., & Dluhy, R. A. (1984a) *Biochim. Biophys. Acta 774*, 237–246.

Mendelsohn, R., Anderle, G., Jaworsky, M., Mantsch, H. H., & Dluhy, R. A. (1984b) *Biochim. Biophys. Acta 775*, 215–224.

Metcalfe, L. D., & Schmitz, A. (1961) *Anal. Chem. 33*, 363.

Moore, B. M., Lentz, B. R., Hoechli, M., & Meissner, G. (1981) *Biochemistry 20*, 6810–6817.

Paddy, M. R., Dahlquist, F. R., Davis, J. H., & Bloom, M. (1981) *Biochemistry 20*, 3152–3162.

Rice, D. M., Meadows, M. D., Scheiman, A. O., Goni, F. M., Gomez-Fernandez, J. C., Moscarello, M. A., Chapman, D., & Oldfield, E. (1979) *Biochemistry 18*, 5893–5903.

Senak, L., Moore, D., & Mendelsohn, R. (1992) *J. Phys. Chem. 96*, 2749–2754.

Snyder, R. G. (1960) *J. Mol. Spectrosc. 4*, 411–434.

Snyder, R. G., & Schachtschneider, J. H. (1963) *Spectrochim. Acta 19*, 85–116.

Snyder, R. G., Strauss, H., & Elliger, C. A. (1982) *J. Phys. Chem. 86*, 5145–5150.

Taylor, M. O., & Smith, I. C. P. (1980) *Biochim. Biophys. Acta 599*, 140–149.

Vrbjar, N., Kean, K. T., Szabo, A., Senak, L., Mendelsohn, R., & Keough, K. M. W. (1992) *Biochim. Biophys. Acta 1107*, 1–10.

Warren, G. B., Toon, P. A., Birdsall, N. J. M., Lee, A. G., & Metcalfe, J. C. (1974a) *Biochemistry 13*, 5501–5507.

Warren, G. B., Toon, P. A., Birdsall, N. J. M., Lee, A. G., & Metcalfe, J. C. (1974b) *Proc. Natl. Acad. Sci. U.S.A. 71*, 622–626.