Phase Behavior of Membranes Reconstituted from Dipentadecanoylphosphatidylcholine and the Mg^{2+}-Dependent, Ca^{2+}-Stimulated Adenosinetriphosphatase of Sarcoplasmic Reticulum: Evidence for a Disrupted Lipid Domain Surrounding Protein†

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ABSTRACT: A new method was used for reconstituting active sodium deoxycholate solubilized Ca^{2+}-ATPase of rabbit skeletal muscle sarcoplasmic reticulum. Removal of the detergent by dialysis at the pretransition temperature of the pure lipid (22 °C) favored the formation of sheet-like structures with a lipid and protein content close to that of the detergent-solubilized sample. Freeze–fracture electron micrographs revealed the Ca^{2+}-ATPase to be organized in rows corresponding to the typical banded pattern seen in low-temperature freeze–fracture micrographs of pure lipid bilayers. Incubation of the sheetlike structures at a temperature (38 °C) above the pure lipid main phase transition (33.5 °C) caused closure of the sheets into vesicles displaying homogeneous intramembranous particle distributions, at least for membranes containing less than 150 lipids per Ca^{2+}-ATPase. However, in membranes of higher lipid content, free lipid patches were seen both above and below the lipid phase transition. By use of high-sensitivity differential scanning calorimetry, three classes of excess heat capacity peaks were observed in the vesiculated samples. A broadened "free lipid" peak occurred for samples containing between 550 and 200 lipids per protein (T_m = 33.5 °C, as for the order–disorder transition in pure lipid vesicles). Between 200 and 150 lipids per Ca^{2+}-ATPase, a broad shoulder became apparent in the range of 29–32 °C. Below 150 lipids per Ca^{2+}-ATPase, a peak at 26–28 °C became increasingly prominent with lower lipid content. At a lipid to protein ratio of about 30, no peaks in heat capacity were observed. The temperature dependence of diphenylhexatriene fluorescence anisotropy revealed a similar pattern of membrane phase behavior, except that a phase transition was detected at 33.5 °C in all membranes studied. On the basis of these observations, we propose that the Ca^{2+}-ATPase is surrounded by a "lipid annulus" of motionally inhibited lipid molecules that do not contribute to a calorimetrically detectable phase transition. Beyond the annulus, "secondary domains" of disrupted lipid packing account for the peak at 26–28 °C and the 29–32 °C shoulders. At high lipid to protein ratios, the secondary domains coexist with protein-free, lipid-bilayer patches, which account for the peak at 33.5 °C.

Measurements of phospholipid phase transitions have provided a convenient tool for detecting the coexistence of structural or compositional lipid domains in both model and biological membranes. While one approach to this detection of domains has involved construction of phase diagrams (Shimshick & McConnell, 1973; Lentz et al., 1976a,b; Mabrey & Sturtevant, 1976), another is possible. The rationale behind this alternate approach is that phospholipids in structurally distinct domains should undergo different phase transitions whose thermodynamic characteristics will reflect the different structures of the coexisting domains. While the observation of multiple differential scanning calorimetric peaks does not require the coexistence of different phases in multicomponent lipid bilayers (Mabrey & Sturtevant, 1976), it must imply the presence of multiple domains in a membrane containing only a single phospholipid component. In this paper, we use this approach to detect and characterize membrane domains induced by the presence of the transmembrane, Mg^{2+}-dependent, Ca^{2+}-stimulated adenosinetriphosphatase (Ca^{2+}-ATPase)[1] protein of sarcoplasmic reticulum (SR).

We have chosen the SR membrane of skeletal muscle as a subject of study because of its relatively simple composition, with one protein, the Ca^{2+}-ATPase, accounting for up to 90% of the total membrane protein (Meissner, 1975). The active Ca^{2+}-ATPase has been reconstituted into membranes containing soybean phospholipid (Racker, 1972), endogenous SR lipids (Meissner & Fleischer, 1974; Wang et al., 1979), or synthetic phospholipid of well-defined composition (Hesketh et al., 1980; Konigsberg, 1982). However, with one recent exception (Andersen et al., 1983), it has proven difficult to obtain reconstituted membranes containing greater than about 200 lipids per Ca^{2+}-ATPase (Gomez-Fernández et al., 1980; Konigsberg, 1982). In the first part of this paper, we show that membranes containing 50–750 synthetic, saturated lipids

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[1] Abbreviations: SR, sarcoplasmic reticulum; Ca^{2+}-ATPase, Mg^{2+}-dependent, Ca^{2+}-stimulated adenosinetriphosphatase; $DC_{15}PC$, 1,2-dipentadecanoyl-3-*sn*-phosphatidylcholine; ESR, electron spin resonance; ^2H NMR, deuterium nuclear magnetic resonance; DPH, 1,6-diphenyl-1,3,5-hexatriene; DSC, differential scanning calorimetry; T_m, lipid phase transition temperature (order to disorder); Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; SDS, sodium dodecyl sulfate.

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per Ca^{2+}-ATPase can be reproducibly prepared at predetermined lipid to protein ratios by reconstitution below the main lipid phase transition of dipentadecanoylphosphatidylcholine ($DC_{15}PC$) bilayers. This phospholipid was chosen for these studies because of its convenient phase transition temperature (Parente & Lentz, 1984) and its ability to support phosphoenzyme formation and the ATPase activity of the Ca^{2+}-ATPase (Moore et al., 1981).

In the second part of this paper, we report the phase behavior of recombinant membranes over a wide range of lipid to protein ratios. These studies focus on the influence of the Ca^{2+}-ATPase on the molecular organization of phospholipid molecules in a limited domain surrounding the protein. In a previous paper (Lentz et al., 1983), we proposed a possible resolution to the apparent disparity between electron spin resonance (ESR) and deuterium nuclear magnetic resonance (^2H NMR) studies of intrinsic protein–lipid interactions in membranes. The former have been interpreted in terms of a motionally restricted lipid *annulus* or *boundary* adjacent to a protein (Jost et al., 1973; Hesketh et al., 1976), while the latter have indicated a more disordered lipid bilayer in the presence of such a protein (Rice et al., 1979; Jähnig, 1979). Our model proposed a motionally inhibited lipid annulus directly adjacent to the protein, coexisting with a secondary region of disrupted lipid order extending three to four lipid layers beyond the annular layer. Others have suggested an alternative picture involving a certain number of lipid molecules trapped between closely juxtaposed protein molecules (Gómez-Fernandez et al., 1979, 1980; Hoffmann et al., 1980), at least below the phospholipid phase transition. This eutectic complex is thought to coexist with pure lipid domains. In this paper, we present additional differential scanning calorimetric (DSC), freeze–fracture electron microscopy, and diphenylhexatriene (DPH) fluorescence data in support of our previously proposed picture of a disrupted secondary lipid layer surrounding the Ca^{2+}-ATPase.

Materials and Methods

Materials. All chemicals except glycerol were reagent grade or the highest commercially available purity. Glycerol was certified A.C.S. grade (Fisher Scientific). Detergents and poly(ethylene glycol) 6000 (PEG-6000) were as described by Moore et al. (1981). 1,2-Dipentadecanoyl-3-*sn*-phosphatidylcholine ($DC_{15}PC$) was purchased from Avanti Biochemical, Inc. (Birmingham, AL) and was found to be greater than 99% pure by thin-layer chromatography [Analtech GHL plates; chloroform/methanol/water (65/25/4 v/v/v); loading of 0.5 μmol].

Isolation and Reconstitution of Ca^{2+}-ATPase. For this study, a new procedure was developed for incorporating the Ca^{2+}-ATPase into phospholipid bilayers of well-defined composition. Sarcoplasmic reticulum vesicles were isolated from rabbit skeletal muscle and endogenous lipid was replaced by synthetic $DC_{15}PC$ in the presence of deoxycholate as described previously (Moore et al., 1981). The lipid-replaced ATPase (typically 25–30 mg of protein) was taken up in an appropriate volume (typically 1–4 mL) of 0.01 M Tes, pH 7.5, 20% glycerol, 0.1 M KCl, 0.1 mM $CaCl_2$, 1 mM dithiothreitol, 10–13 mM $DC_{15}PC$, and 12 mM sodium deoxycholate to produce turbid suspensions that contained 50–750 phospholipids/Ca^{2+}-ATPase. If necessary, small aliquots (10–50 μL) of 0.2 M sodium deoxycholate were slowly added with gentle mixing, until the solution was clear (typically, about 0–10 μL for lipid-rich and about 150 μL for protein-rich samples). After incubation for 20 min at 35 °C, remaining unsolubilized material (5–20% of the protein) was removed by centrifugation for 40 min at 48 000 rpm in a Beckman 75 rotor. The supernatant (2–3 mL) was dialyzed either for 48–72 h at 22 °C (below T_m) or for 24–48 h at 35 °C (above T_m) against two 1-L changes of buffer (20% glycerol, 0.1 M KCl, 0.1 mM $CaCl_2$, 0.1 mM dithiothreitol, and 0.01 M Tes, pH 7.5). To obtain a preparation of uniform lipid and protein content, reconstituted samples were placed on top of a 10-mL linear 5–40% (w/w) sucrose gradient containing 20% glycerol, 0.1 M KCl, 0.1 mM $CaCl_2$, and 0.01 M Tes, pH 7.5, and centrifuged at 4 °C for 16 h at 33 000 rpm in a Beckman SW 41 rotor. Reconstituted samples contained less than 0.5 mol of PEG-6000 and 0.5 mol of deoxycholate per Ca^{2+}-ATPase (limits of radioactive tracer detection). Lipid and fatty acid methyl ester analysis revealed that endogenous SR phospholipid was replaced by $DC_{15}PC$ to an extent of 99% or more. The Ca^{2+}-ATPase accounted for greater than 90% of the protein of the reconstituted membranes, as judged from densitometric tracings of SDS–polyacrylamide gels.

Differential Scanning Calorimetry. Heat capacity profiles were obtained on lipid-replaced membranes with a high-sensitivity, differential scanning calorimeter (Tronac 750) built for us by Roger Hart of Hart Scientific (Orem, UT). This calorimeter is a modification of a design used previously by Suurkuusk et al. (1976) and is highly sensitive to changes in heat capacity, as required to detect the broad and subtle lipid phase transitions reported here. Data were corrected for calorimeter response and thermal lag by procedures tested in this laboratory with known standards (B. R. Lentz, K. W. Clubb, T. E. Jensen, and R. Hart, unpublished results). Ca^{2+}-ATPase-containing membranes used for calorimetry were suspended in 0.1 M KCl, 1 mM $MgCl_2$, 1 mM dithiothreitol, and 0.01 M Tes buffer (pH 7.5) containing 20 wt % glycerol, which was essential for maintenance of Ca^{2+}-ATPase activity during the 6–8 h required for heating and subsequent cooling scans at rates of ±15 °C/h. Faster scan rates (e.g., ±30 °C/h) produced hysteresis effects and therefore were not used.

Fluorescence Measurements. DPH was introduced into reconstituted membrane suspensions at 38 °C by injecting, with gently swirling, a small volume (1–4 μL) of 2 mM DPH dissolved in tetrahydrofuran. Lipid to DPH ratios were always in the range of 250–300/1. Samples containing dye were incubated at 38 °C for 1–2 h and then cooled to 4 °C and stored overnight at this temperature before performing first heating and then cooling scans at ±30 °C/h. Hysteresis between heating and cooling scans was undetectable in fluorescence measurements at this scan rate. Samples for fluorescence measurements were generally 0.1 mM in phospholipid and were prepared by diluting stock reconstituted membranes (9–15 mM in 20% glycerol buffer) to this concentration in 100 mM KCl. Of necessity, some loss of Ca^{2+}-ATPase activity was observed during the 3 h required for the fluorescence measurements. This loss of activity was accepted due to the inconvenience for fluorescence measurements of sclieren scattering and bubble entrapment associated with using the glycerol buffer. Controls showed the results obtained in the two buffers were identical. Fluorescence anisotropy measurements were made on a T-system SLM 4800 spectrofluorometer (SLM Instruments, Urbana, IL). Anisotropy measurements made at serial dilutions of membrane samples were used to correct for light scattering induced fluorescence depolarization (Lentz et al., 1979), although these corrections were in most cases unnecessary for the reconstituted membranes studied here. Details of the measurements as well as of the definition and calculation of "microviscosity activation energy" are given in the literature (Lentz et al., 1978, 1980).

As previously noted (Lentz et al., 1980), this parameter has both practical and historical advantages for the detection of lipid phase transitions but does not necessarily reflect a simple, macroscopic viscosity.

Freeze–Fracture Electron Microscopy. Samples were incubated between two thin copper sheets, rapidly frozen in a propane jet freezing device, and fractured by procedures similar to those previously reported (Lentz et al., 1980). Shadowing with Pt was at a 45° angle. The resultant replicas were viewed on a JEOL 100 CX microscope operated at 80 KV.

Other Methods. Ca^{2+}-ATPase activity was assayed as previously described (Moore et al., 1981) by monitoring H^+ release with the pH-sensitive dye phenol red or, equivalently, by direct determination of inorganic phosphorus (Moore et al., 1978; Goodwin et al., 1958). Protein was assayed by the method of Lowry et al. (1951), and lipid phosphorus was determined by a modification of the procedure suggested by Goodwin et al. (1958). Permeability of reconstituted vesicles to [^{3}H]inulin and [^{14}C]sucrose was determined as previously described (Young et al., 1981). Phospholipid fatty acid content was analyzed by gas–liquid partition chromatography as described by Moore et al. (1978).

Results

Characterization of Reconstituted Membranes. The deoxycholate-solubilized Ca^{2+}-ATPase was reconstituted into membranes containing 50–750 $DC_{15}PC$/ATPase via removal of the detergent by dialysis at temperatures either near (22 °C) the pretransition temperature (T_m, 20–22 °C) or slightly above (35 °C) the main phase transition temperature (T_m, 33.5–33.8 °C) of the pure lipid. The specific density of the reconstituted membranes fell between that of pure phospholipid (1.03 g/cm^3) and the lipid-free Ca^{2+}-ATPase (1.35 g/cm^3) (LeMaire et al., 1976). The homogeneity of the reconstituted membranes could, therefore, be judged by determining their buoyant density on sucrose gradients. Table I shows that the lipid content and homogeneity of the reconstituted membranes depended on the initial lipid and protein concentrations in the solubilized sample as well as on the dialysis temperature. In general, membranes of homogeneous composition were formed when Ca^{2+}-ATPase preparations were reconstituted at 22 °C, as indicated by the presence of only one narrow band on the gradients. Homogeneous membrane preparations with a composition close to that of the soluble sample were obtained in about four out of five experiments, for all initial lipid to protein ratios.

Increase of the dialysis temperature from 22 to 35 °C resulted in greater sample heterogeneity. At 35 °C, for lipid to protein ratios below 150–200, homogeneous membrane preparations (i.e., a single band on sucrose gradients) were obtained in about half of the experiments. Above a ratio of 150–200, we always observed at least two bands on the gradients. High lipid to protein ratio samples obtained in this way were often either quite heterogeneous, as judged by freeze–fracture electron microscopy, or devoid of Ca^{2+}-ATPase activity, or both.

Freeze–fracture electron microscopy has shown that vesicle-like structures are formed when membranes containing less than 150–200 lipid molecules/protein are reconstituted at 35 °C, i.e., at a temperature above the T_m of $DC_{15}PC$ (Moore et al., 1981). Both fracture faces revealed particles with an average diameter of 80 Å representing the Ca^{2+}-ATPase (MacLennan et al., 1971). On the other hand, samples containing varying lipid to protein ratios reconstituted at 22 °C, i.e., near the pretransition temperature of $DC_{15}PC$, formed large sheetlike structures (Figure 1a). The lipid phase had a banded morphology reminiscent of the $P_{\beta'}$ phase typical of pure phosphatidylcholine bilayers (Lentz et al., 1980), and the rowlike arrangement of particles, sometimes barely discernible in vesicles reconstituted at 35 °C (Moore et al., 1981), was dramatically evident (Figure 1a). Figure 1b,c shows membranes that were initially reconstituted at 22 °C but were then incubated near (Figure 1b, 33 °C) or above (Figure 1c, 38 °C) the main phase transition of $DC_{15}PC$ before being rapidly frozen and fractured. On incubation at 38 °C for 2.5 h, the sheet-like structures were completely replaced by vesicle-like structures, whereas at 33 °C only partial conversion into vesicle-like structures was observed after a similar incubation period. The 80-Å particles were more uniformly distributed within the lipid bilayer of the newly formed vesicles, as compared to the sheets (Figure 1). Samples incubated at 38 °C for more than 2.5 h showed no further changes in morphology.

Table I: Properties of Reconstituted Ca^{2+}-ATPase–$DC_{15}PC$ Membranes[a]

dialysis temp (°C)	lipid to protein ratio in sol prepn before dialysis	lipid to protein ratio in reconstituted membranes	Ca^{2+}-ATPase act. of reconstituted membranes [μmol (mg of Ca^{2+}-ATPase)$^{-1}$ min^{-1}]
22	~60	57	5.9
	95	118	5.1
	147	164	5.1
	225	246 + [152]	5.4
	381	378	4.8
	561	564	4.5
	724	760	ND
35	~60	54	3.8
	67	68 + [44]	5.4
	209	169	5.9
	381	216 + [278 + >1000]	4.1
	724	387 + 741 + [>1000]	ND
	946	500 + [302 + >1000]	4.0

[a] Molar lipid to protein ratios are given for the detergent-solubilized Ca^{2+}-ATPase preparation before dialysis and for the reconstituted membrane. The Ca^{2+}-ATPase was assumed to account for 90% of the protein and to have a M_r of 100 000. Values in brackets indicate sucrose gradient membrane fractions that generally contained less than 30% of the protein recovered from the gradients. Ca^{2+}-ATPase activity of the major sucrose gradient fraction is given. Lipid-rich fractions obtained after dialysis at 35 °C often demonstrated no activity. Activity was determined at 30 °C in the presence of the nonionic detergent dodecyloctaoxyethylene glycol monoether (Moore et al., 1981). For comparison, cholate-treated sarcoplasmic reticulum membrane had an ATP-hydrolysis rate of 5.6 mol of ATP (mg of Ca^{2+}-ATPase)$^{-1}$ min^{-1} at 30 °C. Representative results of a total of 25 reconstitution experiments are shown. ND indicates not determined.

Less extensive studies with dimyristoyl- and dipalmitoylphosphatidylcholine showed that our observations with $DC_{15}PC$ were extendable to these other saturated phospholipids when dialysis was carried out at temperatures comparably related to their phase transitions.

Ca^{2+}-ATPase–$DC_{15}PC$ membrane vesicles reconstituted at 35 °C trapped [^{3}H]inulin but not [^{14}C]sucrose (Moore et al., 1981). Permeability properties of membranes initially formed by low-temperature dialysis (Figure 1c) were assessed by incubating the newly dialyzed preparations (Figure 1a) for 3 h at 38 °C in the presence of [^{3}H]inulin and [^{14}C]sucrose. Despite their vesicle-like appearance, these membranes did not trap the two radioactive compounds, indicating that they were permeable to molecules with diameters of up to 30 Å.

Calorimetric Determinations of Membrane Phase Transitions. The phase behavior of homogeneous, vesiculated membrane samples (i.e., reconstituted at 22 °C and then incubated at 38 °C—see above) was determined by high-sen-

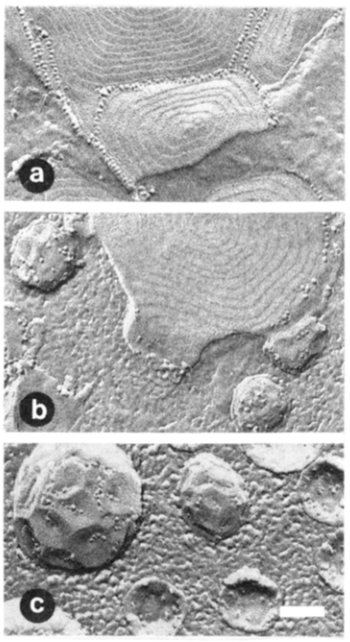

FIGURE 1: Electron micrographs of freeze–fracture replicas prepared from $DC_{15}PC$–Ca^{2+}-ATPase membranes. Samples were fractured and analyzed as previously described (Lentz et al., 1980). Platinum shading was at an angle of 45°. Micrographs are oriented so that the direction of shading was from the bottom of the page. The bar represents 100 nm. Samples reconstituted at 22 °C as described under Materials and Methods contained 300 phospholipids/ATPase and were present at a protein concentration of 5–10 mg/mL in 0.01 M Tes, pH 7.5, 20% glycerol, 0.1 M KCl, and 0.1 mM $CaCl_2$. In (a) the sample was stored at −70 °C following removal from the sucrose gradient and then warmed to 10 °C and incubated there for 3 h before being frozen at the temperature with the propane jet, quick-freezing method. At no time was the sample raised above the $DC_{15}PC$ main phase transition temperature (33.5 °C). In (b) and (c), samples were similarly stored but were incubated for 2 h at 33 °C and 2.5 h at 38 °C, respectively, before being cooled to 10 °C and quick-frozen. The ability of the sheet-like structures to vesiculate appeared to depend critically on the presence of a low level of residual sodium deoxycholate in the sample. Preparations dialyzed at 22 °C for 72–85 h were found not to close into vesicles unless 0.25 mol % sodium deoxycholate was added to the sample.

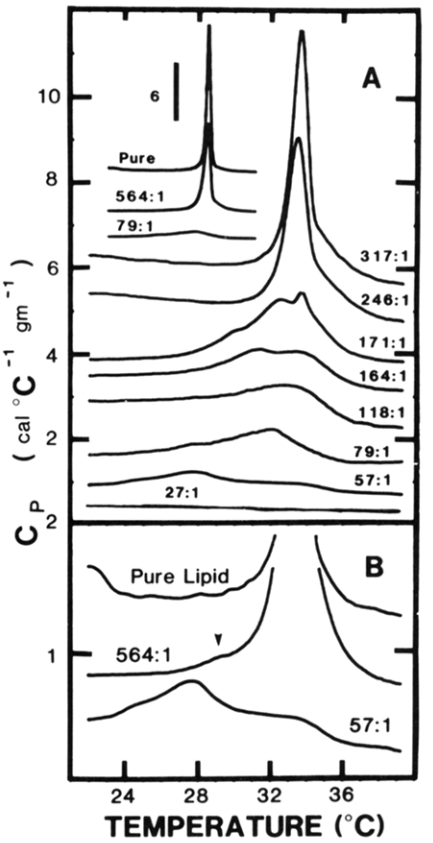

FIGURE 2: Temperature dependence of the heat capacity (C_P) for membranes containing indicated mole ratios of $DC_{15}PC$ to Ca^{2+}-ATPase (1 cal = 4.18 J). Data were obtained in heating scans at a rate of +15 °C/h. Samples contained between 0.5 and 2.5 μmol of lipid suspended in 0.1–0.2mL of glycerol-containing buffer (see Materials and Methods). The inset shows selected data sets plotted on a compressed scale (bar = 6 cal deg^{-1} g^{-1}) in order to illustrate the relation between a sample without protein ("pure") and those containing protein. The sample without protein (pure lipid) was prepared by slow dilution and dialysis of an octyl glucoside solubilized lipid suspension. (B) Temperature dependence of the heat capacity for three of the same data sets plotted in (A) but on a greatly expanded scale to emphasize subtle peaks below the main phase transition.

sitivity differential scanning calorimetry (DSC). DSC measurements of membrane heat capacity are presented in Figure 2 for membranes of different lipid to protein ratios. The data of Figure 2 were obtained in heating scans, but results were identical for cooling scans, at least for scan rates of ±15 °C/h. These data illustrate several facts about phosphatidylcholine phase behavior in membranes containing the Ca^{2+}-ATPase. First, as has been noted previously (Gómez-Fernández et al., 1980), the main phase transition is broadened with increasing protein content relative to membranes containing no protein. In our study, protein-free vesicles were prepared by detergent dialysis (Parente & Lentz, 1984) so as to be of comparable size to the roughly 1000-Å diameter reconstituted Ca^{2+}-ATPase vesicles (Figure 1c). Second, the main phase transition peak height decreased with decreasing lipid to protein ratio, until the peak corresponding to the pure lipid transition disappeared altogether below roughly 171/1 or 164/1. Third, significant peaks in heat capacity began to develop at temperatures below the pure lipid transition below lipid to protein ratios of 171/1. By a lipid to protein ratio of 79/1, these low-temperature events were the only ones observed. Even at high lipid to protein ratios, subtle low-temperature heat capacity bumps were observed as shoulders on the main pure lipid transition (e.g., see Figure 2B). Fourth, a pretransition was not consistently observed in heat capacity profiles of reconstituted membranes, even at high ratios of lipid to protein. In protein-free, large unilamellar vesicles, a pretransition of very low enthalpy (about 0.5 kcal/mol of lipid; Parente & Lentz, 1984) was just detectable (e.g., see the small peak at about 22 °C in the pure lipid data in Figure 2B). Finally, by a lipid to protein ratio of 27/1, no peaks in heat capacity could be observed above base-line noise (Figure 2A). Consistent with our less extensive earlier paper (Lentz et al., 1983), we interpret this in terms of an annulus of conformationally inhibited lipid surrounding the Ca^{2+}-ATPase. The subtle peaks in heat capacity especially evident at low lipid to protein ratios, therefore, are seen as due to a secondary region of disrupted lipid packing beyond the lipid annulus.

As a test of these interpretations, we integrated (21.5–38.5 °C) the heat capacity peaks in Figure 2 and plotted the resultant phase transition enthalpies per mole of *nonannular* lipid (ΔH^*) as a function of lipid to protein molecular ratio. For this, we assumed the number of dynamically exchangeable, conformationally inhibited annular lipids to be 29 per molecule of Ca^{2+}-ATPase, in agreement with our earlier estimate (Lentz et al., 1983), with recent ESR studies (Thomas et al., 1982; McIntyre et al., 1982), and with measurements of Ca^{2+}-ATPase activity as a function of lipid to protein ratio (Hesketh et al., 1976). Our enthalpy data are plotted in this way in Figure 3. At low lipid to protein ratio, a limiting value of

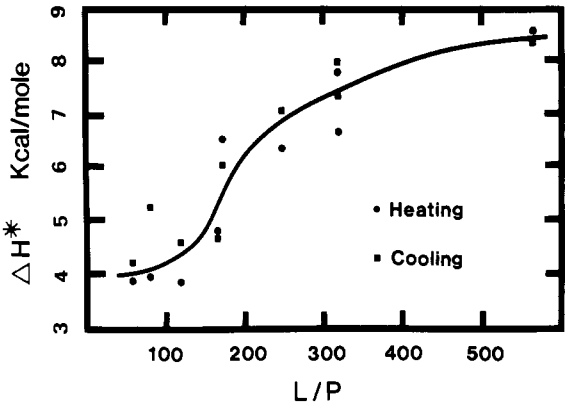

FIGURE 3: Dependence of the phospholipid phase transition enthalpy per mole of nonannular lipid (ΔH^*) on the ratio of phospholipid molecules per Ca^{2+}-ATPase molecule in reconstituted membranes. ΔH^* was calculated from the integrated enthalpies (ΔH) as $\Delta H^* = \Delta H/f$, where f is the fraction of nonannular lipid: f = (lipid to protein ratio − 29)/(lipid to protein ratio). Data are presented for both heating (●) and cooling (■) scans.

about 4 kcal/mol is obtained for the transition enthalpy in the disrupted secondary lipid region. At high lipid to protein ratio, a value of roughly 8.5 kcal/mol is approached, in agreement with the enthalpy measured for pure $DC_{15}PC$ liposomes without added protein (8.3–8.7 kcal/mol for large, multilamellar vesicles and 7.7–7.8 kcal/mol for large, unilamellar vesicles; Parente & Lentz, 1984). These results indicate that, below roughly 120 lipid molecules/Ca^{2+}-ATPase molecule, mainly protein–disrupted bilayer accounts for the nonannular lipid in our recombinant membranes, while above about 200–300 lipid molecules/Ca^{2+}-ATPase molecule the nonannular lipid appears dominated by the properties of protein–unaffected or "bulk" lipid bilayer.

Fluorescence Detection of Lipid Bilayer Order and Membrane Phase Transitions. In order to compare and contrast the pictures of membrane structure obtained by a macroscopic, thermodynamic approach (DSC) with a microscopic, spectroscopic method, we have further examined the phase behavior of our recombinant membranes by fluorescence depolarization of diphenylhexatriene (DPH). The temperature dependence of the DPH-derived microviscosity activation energy is plotted in Figure 4 for a number of recombinant membranes having different lipid to protein molecular ratios. While comparing these to the heat capacity profiles shown in Figure 2, several comments can be made. First, both the fluorometric and calorimetric data show the $DC_{15}PC$ main phase transition to be broadened by the presence of the Ca^{2+}-ATPase protein, although the fluorescence data emphasize the broadening at the base of the transition peak more than a change in the peak width at half-height. This apparent superposition of a sharp and broad activation energy peak is reminiscent of the behavior of DPH in cholesterol/dipalmitoylphosphatidylcholine multilamellar vesicles (Lentz et al., 1980). Second, the fluorescence data show the development of a low-temperature shoulder beside the main transition peak with increasing protein content, similar to the calorimetric data. However, unlike the calorimetric data, the fluorescence results show the major transition peak remaining at 33.5 °C, even for the most protein-rich membranes. Indeed, even the sample with only 27 lipid molecules/Ca^{2+}-ATPase molecule showed a flulorometrically detectable phase transition with the main peak at 33.5 °C, the position of the main transition peak in pure $DC_{15}PC$ large, unilamellar vesicles (Parente & Lentz, 1984). Finally, the protein-poor samples (e.g., 336:1 and 197:1 in Figure 4) consistently displayed a peak in microviscosity activation energy in the neighborhood of the pure lipid pretransition (about 20–22 °C). Due probably in part to a very low enthalpy, this transition was not consistently detectable in the calorimetric scans.

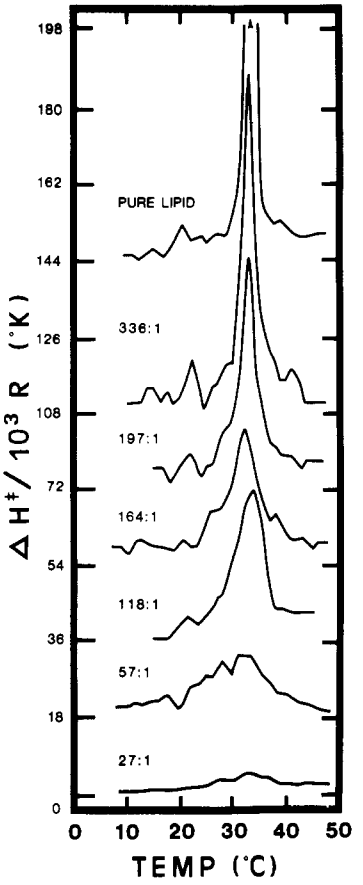

FIGURE 4: Temperature dependence of the DPH-derived microviscosity activation energy (ΔH^*) in $DC_{15}PC$ large, unilamellar vesicles ("pure lipid"; Parente & Lentz, 1984) or $DC_{15}PC/Ca^{2+}$-ATPase recombinant membranes (molar ratios indicated at the left of each plot). Data for heating scans are shown. Cooling scans were essentially identical, except that the lipid pretransition at roughly 22–25 °C was not so evident, as is typical for cooling scans (Lentz et al., 1980).

In addition to using DPH to monitor the phase transitions of our recombinant membranes, we detected variations in average membrane acyl chain order (Jähnig, 1979) with protein content by plotting DPH fluorescence anisotropy vs. lipid to protein ratio (Figure 5). In general, data obtained in both heating and cooling scans agreed well with each other and with data obtained at constant temperature and corrected for light scattering depolarization of fluoresence (Lentz et al., 1979). Below the phase transition (upper curve in Figure 5), the presence of increasing protein in the membrane caused a slight decrease in DPH fluorescence anisotropy, consistent with the previous measurements by Gómez-Fernández et al. (1980) on dipalmitoylphosphatidylcholine/Ca^{2+}-ATPase recombinant membranes. Above the phase transition (lower curve in Figure 5), the presence of the protein caused a dramatic increase in anisotropy at low lipid content, consistent with our observations on native SR membranes (Lentz et al., 1983). Gómez-Fernández et al. reported a qualitatively similar variation of anisotropy with protein and lipid content, although the increase in anisotropy with decreasing lipid content was not as dramatic as we have found (see Figure 5).

Morphological Changes Associated with Membrane Phase Transitions. Freeze–fracture electron micrographs were obtained routinely on all recombinant samples. These micrographs assured that only samples of clearly membranous vesicle

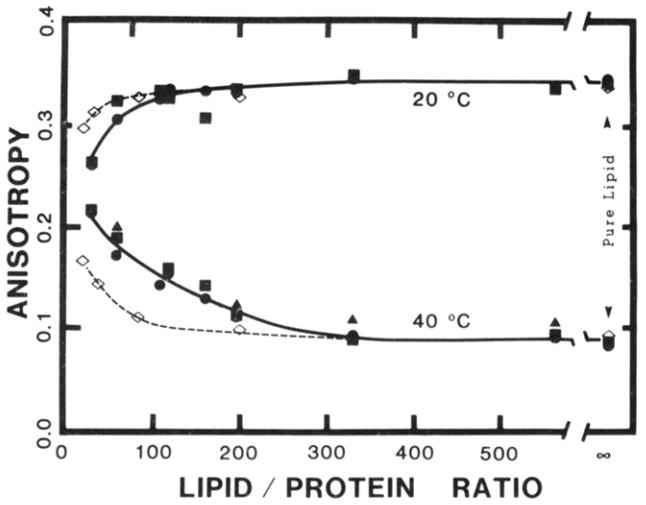

FIGURE 5: Variation of DPH fluorescence anisotropy with the molar ratio of lipid to protein in reconstituted $DC_{15}PC/Ca^{2+}$-ATPase membranes. Data were obtained from both heating (■) and cooling (●) scans as well as from isothermal experiments (▲) corrected for light scattering depolarization (Lentz et al., 1979) when these corrections were found to be significant. Data are reported for a temperature above (40 °C, lower curve) and below (20 °C, upper curve) the $DC_{15}PC$ main phase transition. For comparison, data for reconstituted dipalmitoylphosphatidylcholine/Ca^{2+}-ATPase membranes (◊) were extracted from Figure 5 of Gómez-Fernández et al. (1980). Points near the right ordinate (labeled ∞) are data obtained with pure $DC_{15}PC$ large, unilamellar vesicles (Parente & Lentz, 1984).

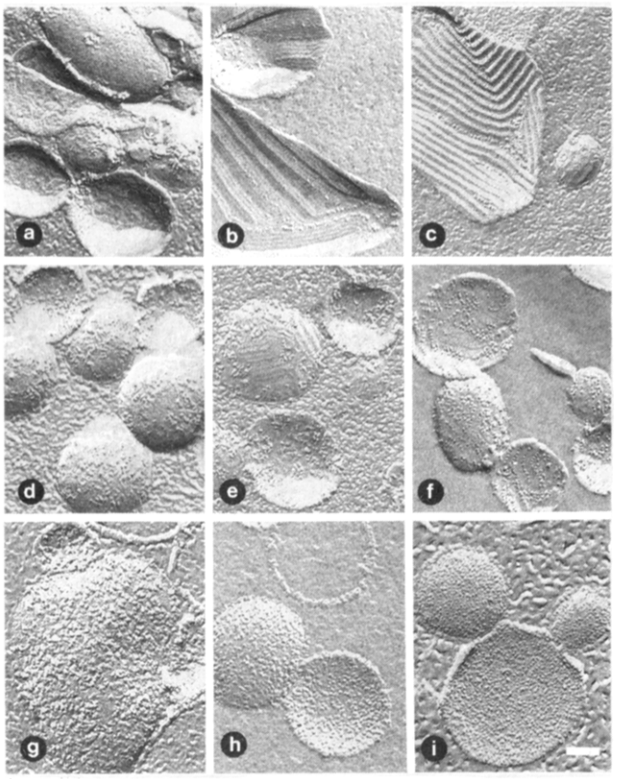

FIGURE 6: Electron micrographs (magnification 45000×) of freeze–fracture replicas obtained from recombinant $DC_{15}PC/Ca^{2+}$-ATPase membranes containing different molar ratios of lipid to protein and rapidly frozen from temperatures above (a, d, and g), within (b, e, and h), and below (c, f, and i) the $DC_{15}PC$ main phase transition. All membranes were reconstituted at 22 °C, incubated for 2.5 h at 38 °C, frozen during storage, and then preequilibrated at the indicated temperatures. Individual frames are for membranes containing 564 lipid molecules/protein molecule (a, 41 °C; b, 33.4 °C; c, 21.5 °C), 171 lipid molecules/protein molecule (d, 41 °C; e, 33.4 °C; f, 10 °C), and 79 lipid molecules/protein molecule (g, 41 °C; h, 27.5 °C; i, 10 °C). Fractured samples were platinum shadowed at an angle of 45°. Micrographs are shown with shadowing from below. The white bar in frame i represents 100 Å.

morphology were used for calorimetry or fluorescence experiments and further confirmed that such samples had a uniform distribution of intramembranous particles *between* vesicles. In order to determine the effects of temperature and lipid to protein ratio on the lateral distribution of intramembranous particles *within* the reconstituted membranes, samples were rapidly frozen from temperatures above, within, and below the calorimetrically detected phase transition. Altogether, five samples were examined in this way, and the results are illustrated in Figure 6. This figure and the rest of our micrographs showed that particle-rich patches appeared in lipid-rich samples (≥ ~160–170 lipid molecules/molecule of Ca^{2+}-ATPase). Surprisingly, the appearance of patches was not limited to temperatures within or below the membrane phase transition but appeared even in fluid-phase membranes (Figure 6a,d). Below or within the phase transition, morphologies consistent with the $P_{\beta'}$ phase were apparent in the protein-free patches (see banded or rivuletted patterns in the larger vesicles in Figure 6b,c; see facettes in the smaller vesicles of Figure 6e,f as well as Figure 1c). For vesicles preequilibrated at 38 °C and containing less than 160 lipid molecules/Ca^{2+}-ATPase molecule, no such protein-free patches were ever observed, above or below the phase transition (e.g., see Figure 6g,h,i).

Ca^{2+}-ATPase Activity. To assess irreversible loss of ATPase activity during reconstitution, the Ca^{2+}-stimulated, Mg^{2+}-dependent ATPase activity was assayed at 30 °C in the presence of 1.6 mM dodecyloctaoxyethylene glycol monoether, a nonionic detergent that fully supports sarcoplasmic reticulum Ca^{2+}-ATPase activity (Moore et al., 1981; Dean & Tanford, 1978). Preparations reconstituted at 22 °C, and then solubilized at 30 °C, displayed 80–100% of the ATP-hydrolyzing activity of solubilized, native SR membranes or of solubilized, cholate-treated SR membranes whose protein content was 90% Ca^{2+}-ATPase (cf. Table I). Slightly lower specific activities were observed for membranes reconstituted at 35 °C (Table I). These results demonstrate that the activity of the Ca^{2+}-ATPase was substantially maintained through the reconstitution procedure.

In order to test the relation of membrane phase transitions to Ca^{2+}-ATPase activity, the rate of ATP hydrolysis was also measured in the absence of detergent (i.e., for intact membrane vesicles) as a function of temperature in recombinant preparations ranging in lipid to protein ratio from 27:1 to 500:1. These data are recorded in Figure 7 and discussed below.

The reconstituted Ca^{2+}-ATPase preparations were not able to accumulate significant amounts of Ca^{2+} when assayed in the presence of 5 mM oxalate, a Ca^{2+}-precipitating agent. This need not mean that the Ca^{2+}-ATPase could not translocate Ca^{2+} but could be explained by the permeability properties of the vesicles. Because of their inability to trap sucrose or inulin (see above), it is quite unlikely that the vesicles containing the Ca^{2+}-ATPase could trap sufficient Ca^{2+} to precipitate the oxalate salt.

Discussion

Our principal aim in this study has been to determine the effect of the transmembrane Ca^{2+}-ATPase of sarcoplasmic reticulum on the structure of its surrounding lipid bilayer by recording the phase behavior of reconstituted membranes containing the Ca^{2+}-ATPase and a synthetic phosphatidylcholine having a conveniently located phase transition. Others have used a similar approach to address the influence of the Ca^{2+}-ATPase (Gómez-Fernández et al., 1979, 1980; Kleeman

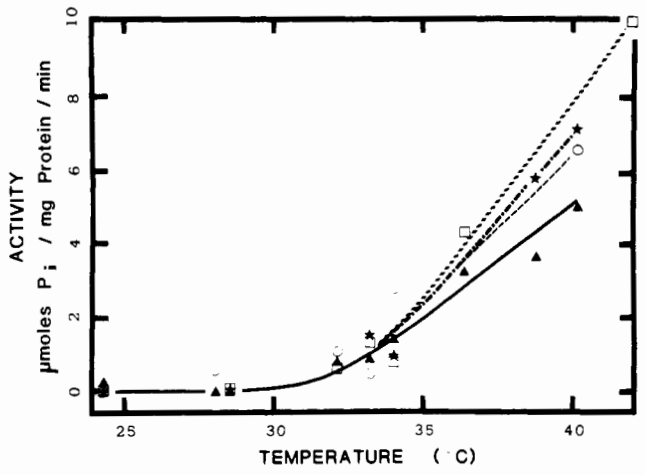

FIGURE 7: Temperature dependence of the Ca^{2+}-stimulated ATPase activity of reconstituted vesicles containing 40 (□), 80 (*), 164 (○), and 524 (▲) lipid molecules/molecule of protein. A sample containing 27 lipid molecules/protein molecule was also studied, but no activity could be detected in the absence of detergent at 24, 30, or 37 °C (data not shown). IU/mg of ATPase corresponding to micromoles of ATP hydrolyzed per milligram of ATPase per minute, assuming that the protein present in each sample was 90% Ca^{2+}-ATPase. Activities were corrected for partial irreversible loss of activity during the reconstitution procedure by comparing the activity of the sample at 30 °C solubilized in dodecyloctaoxyethylene glycol monoether to the activity of a partially delipidated SR membrane solubilized and assayed under the same conditions.

& McConnell, 1976) and other intrinsic membrane proteins (Heyn et al., 1981; Mendelsohn et al., 1980; Freier et al., 1983) on bilayer structure. In the case of calorimetric studies of protein–lipid recombinants, the most common observation has been of a single, slightly broadened heat capacity peak only slightly (if at all) shifted to lower temperature by the presence of the protein (Gómez-Fernández et al., 1979, 1980; van Zoelen et al., 1978; Petri et al., 1980; Freire et al., 1983). In general, observed decreases in transition enthalpy with increased membrane protein content have been interpreted in terms of the presence of either "boundary" or "trapped" lipid that was unable to undergo a phase transition. Trapped lipid was often considered as part of a eutectic protein–lipid complex existing below the phospholipid phase transition. In at least two calorimetric studies (Curatolo et al., 1977; Heyn et al., 1981), dual heat capacity peaks were observed, suggestive of multiple membrane domains, although these studies did not address the SR Ca^{2+}-ATPase.

Our calorimetric scans with Ca^{2+}-ATPase-recombinant membranes show a complex phase behavior with at least two types of heat capacity peaks associated with the main transition, in at least qualitative agreement with results on myelin proteolipid apoprotein (Curatolo et al., 1977) and bacteriorhodopsin (Heyn et al., 1981). However, our data disagree with those of Gómez-Fernández et al. (1979, 1980), who reported only a single heat capacity peak for Ca^{2+}-ATPase-recombinant membranes. At lipid to Ca^{2+}-ATPase molar ratios of less than 164/1 (which encompasses the ratio in the native SR membrane of about 100/1), we always observed a heat capacity peak at 26–28 °C (Figure 2). In the more lipid-rich samples (164/1 to 569/1), this peak coexisted with a peak at the bulk lipid phase transition temperature (33.5 °C). Our calorimetric results are supported by our observations obtained with the acyl chain order sensitive probe DPH (Figure 4), which also showed a low-temperature shoulder below the main phase transition.

The decrease of phase transition enthalpy with increasing protein content observed in recombinant membranes has traditionally been interpreted to indicate the number of lipid molecules in a calorimetrically undetectable lipid annulus (Gómez-Fernández et al., 1979, 1980; van Zoelen et al., 1978; Curatolo et al., 1977; Petri et al., 1980; Freire et al., 1983). Such an interpretation inherently assumes that all lipids in a recombinant membrane display their bulk phase behavior, except those lipids associated with a boundary (annular) lipid domain adjacent to protein (or, alternatively, a trapped lipid domain in protein–lipid eutectic mixtures). Our results are clearly inconsistent with this assumption (Figure 2). Indeed, when we plot our data to test this assumption, the low enthalpy approached at low lipid content (Figure 3) suggests that lipid bilayer organization just beyond the lipid annulus is disrupted by the presence of Ca^{2+}-ATPase, as we have previously proposed (Lentz et al., 1982, 1983).

Both our calorimetric and fluorometric observations offer evidence for the existence of free-lipid regions at high lipid to Ca^{2+}-ATPase molar ratios. Sharp heat capacity or microviscosity activation energy peaks at 33.5 °C (see Figures 2 and 4), characteristic of a pure $DC_{15}PC$ membrane phase transition, were observed at protein to lipid ratios greater than 164/1. In addition, although the phospholipid pretransition, characteristic of a pure lipid bilayer, was difficult to observe calorimetrically, it was consistently detected fluorometrically in samples containing greater than 164 lipid molecules/Ca^{2+}-ATPase molecule. Finally, the variation of phospholipid phase transition enthalpy with membrane lipid content (Figure 3) offers additional evidence for free or bulk lipid patches in lipid-rich recombinant membranes, since the enthalpy approached at high lipid content is that of a bulk lipid bilayer.

For all recombinant membranes containing less than 164 lipid molecules/Ca^{2+}-ATPase molecule, we observed no evidence of lipid patch formation, either above (Figure 6g) or below (Figure 6i) the lipid phase transition. Our procedures would have revealed patches having dimensions of minimally 75–100-Å diameter (containing roughly 75–130 lipid molecules). This observation rules out the coexistence of protein–lipid eutectic complexes and free lipid patches (Gómez-Fernández et al., 1980; Hoffman et al., 1980) in this composition range. At lipid to Ca^{2+}-ATPase molar ratios ≥164/1, however, our recombinant membranes did demonstrate lipid patch formation in freeze–fracture electron micrographs. Surprisingly, this was observed both above and below the phospholipid phase transition (Figure 6a,d and Figure 6c,f), in disagreement with the reports of Kleeman & McConnell (1976) and Hoffman et al. (1980), who both reported homogeneously distributed intramembranous particles above the phase transition and free lipid patch formation below the phase transition. It should be noted that we have used a rapid, propane jet freezing technique (Lentz et al., 1980) to reduce the possibility of freezing artifacts associated with conventional freezing procedures. By contrast, the high-temperature micrographs of Kleeman and McConnell and Hoffman et al. show "wormy" morphology (a common freezing artifact in fluid phase membranes) that makes it difficult to distinguish intramembranous particle distributions.

We interpret the sum of our results in terms of the model shown in Figure 8. In lipid-rich membranes, at least three types of bilayer lateral domains coexist. Boundary lipid consists of a layer of about 30 exchangeable but conformationally inhibited lipid molecules directly adjacent to the Ca^{2+}-ATPase molecule. The secondary, disrupted lipid layer is viewed as containing roughly 130–170 lipid molecules/Ca^{2+}-ATPase whose packing arrangement in the bilayer is disrupted due to the presence of the relatively large Ca^{2+}-

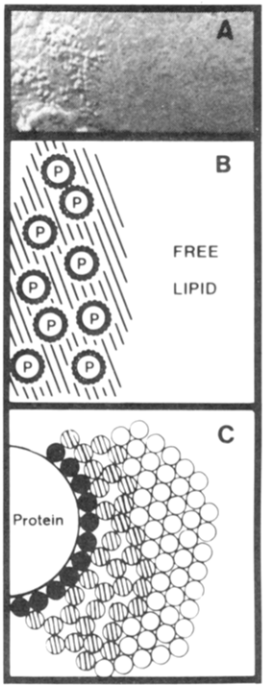

FIGURE 8: Schematic drawing (surface views) of the arrangement of different lipid domains in our recombinant membranes. Frame A is a high-magnification view (68000×) of a small section of one membrane from the sample recorded at low magnification in Figure 6a. This high-magnification view shows clusters of intramembranous particles on the left of the field coexisting with pure lipid regions on the right. Frame B depicts the lateral arrangement of protein molecules and lipid domains seen in frame A and illustrates the coexistence of free lipid and protein-affected secondary lipid domains (striped) presumed in our high-lipid recombinant membranes. Finally, frame C illustrates the disrupted lipid packing in the neighborhood of an individual Ca^{2+}-ATPase molecule near the interface between a protein cluster and a free lipid domain. In this illustration, annular lipid is shown as shaded circles, disrupted secondary lipid as striped circles, and free lipid as open circles.

ATPase molecule. Presumably, the disrupted layer would not be uniform in its properties in membranes of different lipid to protein ratios. This would account for the breadth and variety of heat capacity contributions on the low-temperature side (and even to some extent on the high-temperature side) of the sharp, bulk lipid phase transition peaks in Figure 2. Consistent with this picture, strong evidence exists from oriented sample electron spin resonance studies (Setaka et al., 1979) that lipid molecules around the Ca^{2+}-ATPase protein are considerably disoriented relative to a pure lipid bilayer, even in fluid-phase membranes. In addition, recent infrared spectroscopic measurements on Ca^{2+}-ATPase recombinant membranes detected a lowered phase transition, which was interpreted in terms of "disordered" lipid between protein molecules (Cortijo et al., 1982). Finally, nonuniform lipid packing within the disrupted layer can offer an explanation for the disparate observations of intrinsic protein induced lipid order and disorder as reported by ESR and ^2H NMR, respectively (Hesketh et al., 1976; Rice et al., 1979).

The concept of a disrupted secondary lipid layer may apply to other transmembrane proteins. Thus, measurements of glycophorin lateral diffusion (Vas et al., 1981) and bacteriorhodopsin rotational diffusion and recombinant phase behavior (Heyn et al., 1981) both show evidence for a lowered phase transition in a disordered lipid environment induced by the presence of the protein. Also, Mendelsohn et al. (1981) have interpreted Raman and infrared spectroscopic data obtained with glycophorin recombinant membranes in terms of lipid conformation disorder associated with the presence of the protein. Finally, our proposal of disrupted lipid packing in the secondary domains offers a plausible explanation for the greatly enhanced permeability of biomembranes or protein-reconstituted membranes (Caruthers & Melchoir, 1982) relative to pure lipid bilayer membranes.

At high lipid to Ca^{2+}-ATPase molar ratios, our model proposes that patches of Ca^{2+}-ATPase molecules surrounded by boundary and disrupted lipid regions coexist with patches of bulk or free lipid bilayer, as shown in Figure 8. The observation of free lipid patches in our high lipid content recombinants (Figure 6a and Figure 8A) indicates that Ca^{2+}-ATPase molecules prefer to interact with other Ca^{2+}-ATPase molecules rather than distribute randomly through the lipid bilayer. The disappearance of patching in membranes approaching the lipid to protein ratio of the native SR membrane (approximately 100/1) suggests that lipid molecules and the Ca^{2+}-ATPase preferentially pack in roughly the proportions found in the native SR membrane. This could result from weak interactions between Ca^{2+}-ATPase molecules, which, in extreme cases, might result in formation of oligomers, as has been argued (Scales & Inesi, 1976). Alternatively, it could reflect a thermodynamically favorable arrangement in which the unfavorable interface between disrupted and free lipid domains is minimized. In either case, our results suggest a new view of the nature of protein–lipid interactions in SR membranes. It now seems inappropriate to consider the Ca^{2+}-ATPase molecules with their associated boundary lipid as being separated by regions of normal lipid bilayer. The lipid and protein mutually interact to produce a unique cooperative structure responsible for the permeability and other properties of the SR membrane.

Our data suggest a substantial sensitivity of the ATP-hydrolyzing activity of SR Ca^{2+}-ATPase to the structure and organization of the surrounding lipid bilayer. Reference to Figure 7 demonstrates that ATP-hydrolyzing activity for all samples increased significantly between 32 and 34 °C. Comparison with the heat capacity profiles of Figure 2 reveals that this temperature range corresponds to the main phase transition in pure $DC_{15}PC$ large, unilamellar vesicles. This was true even for activity profiles of samples of low lipid content (i.e., 40:1 and 80:1 in Figure 7), for which there should be no protein-unaffected lipid to undergo the pure $DC_{15}PC$ phase transition. This curious observation suggests that annular lipid directly adjacent to the protein undergoes a noncooperative (hence, undetectable by DSC) conformational change at about 32–34 °C and that the protein is sensitive to this change in annular lipid structure. In support of this hypothesis, our DPH experiments gave evidence of a change in acyl chain order in this temperature range even in a sample presumably containing only annular lipids (see 27:1 data in Figure 4).

The data in Figure 7 further demonstrate that ATP-hydrolyzing activity increased more dramatically with increasing temperature and reached somewhat higher levels at low lipid to protein ratio than at high lipid to protein ratio. This appears to conflict with a previous report that very low lipid content (<30 lipids/Ca^{2+}-ATPase) inhibited ATPase activity (Hesketh et al., 1976). However, there is no real conflict between these observations, since the latter related to disruption of the boundary lipid layer while, for our results, the annular lipid layer presumably remained intact for all samples containing greater than 27 lipid molecules/protein molecule. Indeed, our failure to observe activity in a 27:1 sample agrees with the report of Hesketh et al. While an unambiguous interpretation of our observation of slightly

higher activities at low lipid to protein ratios is not possible, it could be that enhanced lipid packing disorder (anticipated in membranes of high protein content) favors whatever enzyme conformational changes are required for ATP-hydrolyzing activity. If so, it would appear that the SR Ca^{2+}-ATPase primarily requires conformational freedom of annular lipids to display activity but is also sensitive to the organization of lipid molecules beyond the annular layer.

A crucial element in our ability to carry out this project was development of a reconstitution procedure capable of reproducibly and reliably producing recombinant membranes over a wide range of protein and lipid contents. This we did by reconstitution at the pretransition temperature of the synthetic lipid rather than above the main transition temperature (see Table I). It seems appropriate to comment on a possible mechanism to explain the success of this method. The Ca^{2+}-ATPase is present in a low aggregation state and coordinates with about 40 phospholipid and 50 deoxycholate molecules when native sarcoplasmic reticulum is solubilized with limiting amounts of detergent (Meissner, 1977). Removal of detergent by dialysis results in the formation of Ca^{2+}-ATPase–lipid aggregates that associate with increasing amounts of phospholipids as more detergent is removed. When the aggregates contain 80–100 phospholipids/ATPase, they trap [^{3}H]inulin (Meissner, 1977) and maximally accumulate Ca^{2+} in the presence of ATP (Wang et al., 1979; Meissner, 1977), indicating that enclosed compartments have been reformed.

Extending these earlier observations to the present study, it would appear that, above the T_m of $DC_{15}PC$, the Ca^{2+}-ATPase can recombine with limiting amounts of lipid ($\sim$150–200/ATPase) to form vesicular structures. These vesicles appear to be unable to accept additional amounts of phospholipid as the detergent is removed, causing the remaining lipid to form a heterogeneous population of lipid vesicles with a low Ca^{2+}-ATPase protein content and lacking ATPase activity. Similar restraints do not seem to apply to membranes reconstituted near the pretransition temperature of the lipid. The sheet-like structures formed at the lower temperature contain exposed bilayer ends that apparently permit limitless incorporation of additional phospholipid molecules as the detergent is removed by dialysis. In agreement with this interpretation, reconstitution of pure lipid vesicles below the T_m by sodium deoxycholate removal leads to formation of huge, unilamellar vesicles 1–2 orders of magnitude larger than the vesicles obtained by similar treatment above the T_m (R. A. Parente and B. R. Lentz, unpublished results). It seems likely that the bilayer curvature required for vesicle formation is inconsistent with the pleated $P_{\beta'}$ structure, thereby preventing early closure of bilayers to form small vesicles. Once the constraint of the $P_{\beta'}$ structure is removed by incubation above the main phase transition, the presence of a very small amount (about 0.25 mol %) of sodium deoxycholate seems necessary to catalyze the fusion events necessary for vesicle closure. Thus, homogeneous Ca^{2+}-ATPase preparations with a high lipid content can be achieved under our conditions only below the T_m of the lipid.

Registry No. $DC_{15}PC$, 3355-27-9; ATPase, 9000-83-3.

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Interaction of Gentamicin and Spermine with Bilayer Membranes Containing Negatively Charged Phospholipids†

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ABSTRACT: We measured the electrophoretic mobility of multilamellar phospholipid vesicles, the ^{31}P NMR spectra of both sonicated and multilamellar vesicles, and the conductance of planar bilayer membranes to study the binding of spermine and gentamicin to membranes. Spermine and gentamicin do not bind significantly to the zwitterionic lipid phosphatidylcholine. We measured the concentrations of gentamicin and spermine that reverse the charge on vesicles formed from a mixture of phosphatidylcholine and either phosphatidylserine or phosphatidylinositol. From these measurements, we determined that the intrinsic association constants of the cations with these negative lipids are all about 10 M^{-1}. This value is orders of magnitude lower than the apparent binding constants reported in the literature by other groups because the negative electrostatic surface potential of the membranes and the resultant accumulation of these cations in the aqueous diffuse double layer adjacent to the membranes have not been explicitly considered in previous studies. Our main conclusion is that the Gouy-Chapman-Stern theory of the aqueous diffuse double layer can describe surprisingly well the interaction of gentamicin and spermine with bilayer membranes formed in a 0.1 M NaCl solution if the negative phospholipids constitute <50% of the membrane. Thus, the theory should be useful for describing the interactions of these cations with the bilayer component of biological membranes, which typically contain <50% negative lipids. For example, our results support the suggestion of Sastrasinh et al. [Sastrasinh, M., Krauss, T. C., Weinberg, J. M., & Humes, H. D. (1982) *J. Pharmacol. Exp. Ther. 222*, 350-358] that phosphatidylinositol is the major binding site for gentamicin in renal brush border membranes.

The interaction of gentamicin and spermine with membranes is of interest for several reasons. Gentamicin is an aminoglycoside antibiotic used to treat infections caused by Gram-negative bacteria; it interferes with protein synthesis in susceptible microorganisms. The clinical utility of gentamicin and the related aminoglycoside antibiotics is limited by their nephrotoxic and ototoxic effects [e.g., see Sande & Mandell (1980)], and several investigators have suggested that these toxic effects are related to the interaction of the antibiotics with negatively charged phospholipids in biological membranes (Sastrasinh et al., 1982; Laurent et al., 1982; Brasseur et al., 1984). Spermine is produced within cells and may affect a number of different membrane functions [e.g., see Ballas et al. (1983), Schuber et al. (1983), Hong et al. (1983), and Koenig et al. (1983a,b)].

The molecular structures of gentamicin (Cooper et al., 1971; Daniels, 1975) and spermine are illustrated in Figure 1: both spermine and gentamicin have about 3.5 positive charges at pH 7.4 (Josepovitz et al., 1982). Several groups have studied

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