Effect of Lipid Membrane Structure on the Adenosine 5′-Triphosphate Hydrolyzing Activity of the Calcium-Stimulated Adenosinetriphosphatase of Sarcoplasmic Reticulum†

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ABSTRACT: An active Ca^{2+}-stimulated, Mg^{2+}-dependent adenosinetriphosphatase (Ca^{2+}-ATPase) isolated from rabbit skeletal muscle sarcoplasmic reticulum membranes has been incorporated into dilauroyl-, dimyristoyl-, dipentadecanoyl-, dipalmitoyl-, and palmitoyloleoylphosphatidylcholine bilayers by using a newly developed lipid-substitution procedure that replaces greater than 99% of the endogenous lipid. Freeze–fracture electron microscopy showed membranous vesicles of homogeneous size with symmetrically disposed fracture-face particles. Diphenylhexatriene fluorescence anisotropy was used to define the recombinant membrane phase behavior and revealed more than one transition in the membranes. Enzymatic analysis indicated that saturated phospholipid acyl chains inhibited both overall ATPase activity and Ca^{2+}-dependent phosphoenzyme formation below the main lipid phase transition temperature (T_m) of the lipid-replaced membranes. At temperatures above T_m, ATPase activity but not phosphoenzyme formation was critically dependent on acyl chain length and thus bilayer thickness. No ATPase activity was observed in dilauroylphosphatidylcholine bilayers. Use of the nonionic detergent dodecyloctaoxyethylene glycol monoether demonstrated that the absence of activity was not due to irreversible inactivation of the enzyme. Increased bilayer thickness resulted in increased levels of activity. An additional 2-fold rise in activity was observed when one of the saturated fatty acids in dipalmitoylphosphatidylcholine was replaced by oleic acid, whose acyl chain has a fully extended length comparable to that of palmitic acid. These results indicate that the Ca^{2+}-ATPase requires for optimal function a "fluid" membrane with a minimal bilayer thickness and containing unsaturated phospholipid acyl chains.

The Ca^{2+}-stimulated, Mg^{2+}-dependent ATPase (Ca^{2+}-ATPase)[1] of sarcoplasmic reticulum (SR) controls muscle relaxation through ATP-dependent uptake of calcium from the muscle myofibrillar space (Tada et al., 1978). The Ca^{2+}-ATPase is a major component of SR, accounting for about 90% of the total membrane protein (Meissner, 1975). A fluid, hydrophobic environment is believed to be required for full enzymatic activity. Early studies indicated that while full Ca^{2+}-ATPase activity required phospholipid, formation of the phosphoenzyme intermediate did not (Martonosi, 1969; Meissner & Fleischer, 1972). Recently, Dean & Tanford (1978) showed that the detergent-solubilized Ca^{2+}-ATPase can hydrolyze ATP in the presence of only 1–3 mol of phospholipid per mol of enzyme.

In order to determine the role of phospholipid acyl chain structure in Ca^{2+}-ATPase function, the enzyme has been incorporated previously into bilayer membranes composed of dioleoyl-, dipalmitoyl-, or dimyristoylphosphatidylcholine (Hesketh et al., 1976; Hidalgo et al., 1976; Nakamura et al., 1976). Studies with these lipid-replaced enzyme preparations led to the general conclusion that phospholipids with an ordered acyl chain configuration inhibited ATPase activity. The disordered acyl chain configuration occurring above the melting temperature (T_m) supported ATPase activity. The effect of a "rigid" phospholipid acyl chain environment on ATPase activity appeared to be mainly due to inhibition of enzyme conformational changes leading to calcium translocation and phosphoenzyme breakdown, while phosphoenzyme formation was thought to be affected to a lesser extent (Nakamura et al., 1976; Hidalgo et al., 1976). Finally, spectroscopic measurements have indicated not only that enzyme function was dependent on the physical state of the lipid but also that the enzyme itself perturbed the structure of the surrounding lipid (Hesketh et al., 1976; Hidalgo et al., 1976; Moore et al., 1978; Rice et al., 1979; Gomez-Fernández et al., 1980). In agreement with this observation, significant ATPase activity was measured in dipalmitoylphosphatidylcholine-replaced membranes at about 30 °C (Hesketh et al., 1976; Hidalgo et al., 1976), well below the phase transition temperature of the pure lipid (T_m 41 °C). Surprisingly, no ATPase activity was observed below the phase transition of dimyristoylphosphatidylcholine in membranes replaced with this synthetic lipid (Warren et al., 1974).

As summarized above, previous studies have emphasized that phospholipid acyl chain mobility is required for full ATPase activity. It seems likely, though, that other structural features of the membrane bilayer may be required for proper functioning of the Ca^{2+}-ATPase. It has been the purpose of the present study to investigate, in addition to the effects of the bilayer physical state, the effects of phospholipid acyl chain length (hence, bilayer thickness) and acyl chain unsaturation on the function of the Ca^{2+}-ATPase. We have approached

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[1] Abbreviations used: Ca^{2+}-ATPase, Ca^{2+}-stimulated, Mg^{2+}-dependent adenosinetriphosphatase; SR, sarcoplasmic reticulum; DLPC, dilauroylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; $DC_{15:0}PC$, dipentadecanoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; POPC, palmitoyloleoylphosphatidylcholine; $C_{12}E_8$, dodecyloctaoxyethylene glycol monoether; DPH, 1,6-diphenyl-1,3,5-hexatriene; PEG, poly(ethylene glycol); DTT, dithiothreitol; Tes, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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this task by developing a new lipid-replacement technique whereby the endogenous SR lipid has been replaced to equal or better than 98% by the synthetic phospholipids dilauroyl-, dimyristoyl-, dipentadecanoyl-, dipalmitoyl-, and palmitoyloleoylphosphatidylcholine (DLPC, DMPC, $DC_{15:0}PC$, DPPC, and POPC, respectively). A preliminary account of this work has been presented (Moore et al., 1981).

Materials and Methods

Materials

All materials used were reagent grade. Deoxycholic acid was from Sigma (St. Louis, MO) and was recrystallized from ethanol before use (Meissner et al., 1973). Dodecyloctaoxyethylene glycol monoether ($C_{12}E_8$) was obtained from Nikko Chemicals Co. (Tokyo, Japan) and was recrystallized from hexane before use. Poly(ethylene glycol)-6000 (PEG-6000) was from Tridom-Fluka Chemicals, Inc. (Hauppague, NY). [^{14}C]PEG-6000 was obtained from Amersham Searle Radiochemicals (Arlington Heights, IL), and [^{14}C]deoxycholic acid, [^{3}H]inulin, and [^{14}C]sucrose were from New England Nuclear (Boston, MA). L-α-Dipalmitoylphosphatidylcholine was purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). L-α-Dilauroyl-, L-α-dimyristoyl-, and L-α-palmitoyloleoylphosphatidylcholines were purchased from Biochemisches Labor (Bern, Switzerland). L-α-Dipentadecanoylphosphatidylcholine was synthesized according to the method of Robles & van den Berg (1969). All phospholipids were tested for purity by thin-layer chromatography on Quantum Q5W silica gel plates (developed with $CHCl_3$/MeOH/H_2O, 65/25/4 v/v/v) and repurified when necessary to assure better than 98% purity (Lentz et al., 1976).

Methods

Preparation of Lipid–Deoxycholate Resuspension Buffer. Phospholipids used for reconstitution experiments were lyophilized for 24 h out of benzene/chloroform (4:1) under high vacuum. Normally, 34–40 μmol of phospholipid was used for each reconstitution experiment. To the dried lipid sample was added 5.5 mL of a buffer containing 20% glycerol, 0.1 M KCl, 0.1 mM $CaCl_2$, 5.7 mM deoxycholate, 1 mM DTT, and 0.01 M Tes, pH 7.5 (*lipid–deoxycholate resuspension buffer*). The suspension generally became optically clear when sonicated at 1–2 °C above the phospholipid gel to liquid-crystalline transition temperature in a bath-type sonifier for 20 min.

Lipid Replacement and Reconstitution of Membrane Vesicles Containing the Ca^{2+}-ATPase. Intermediate density sarcoplasmic reticulum (SR) vesicles were isolated from rabbit skeletal muscle and were characterized as described previously (Meissner, 1975). The vesicles (4.5 mg of protein/mL) were treated with 6.6 mM cholate (Meissner et al., 1973; Moore et al., 1978) to obtain membranes with a lipid/ATPase ratio of 53/1 on a molar basis. The partially delipidated membranes were stored in 0.3 M sucrose, 50 mM KCl, and 0.5 mM Hepes, pH 7.5, at −65 °C before further use.

The lipid/ATPase ratio was further lowered to 7–9/1 on a molar basis according to a modification of the procedure of Dean & Tanford (1978). All steps were carried out at 0–4 °C unless otherwise indicated. SR vesicles (48 mg of protein) were delipidated in 120 mL of a buffer containing 20% glycerol, 0.1 M KCl, 1 mM $MgCl_2$, 1 mM DTT, 24 mM deoxycholate, 19 mM PEG-6000, and 0.01 M Tes, pH 7.5 (*PEG buffer*). The mixture was sonicated in a bath sonifier at 4 °C for 15 min to facilitate precipitation and delipidation of the Ca^{2+}-ATPase. During sonication, the mixture was removed at 5-min intervals and placed on ice for 30 s before resuming sonication. After sonication, the mixture was centrifuged for 30 min at 30000 rpm (70000*g*) in a Beckman 42.1 rotor. The white pellets were resuspended in a total volume of 6–7 mL of the PEG buffer. To the resuspended pellets was added 0.5 mL of the lipid–deoxycholate resuspension buffer to permit a small-scale exchange between the exogenous and endogenous lipid pools. The mixture was incubated for 10 min at 1–2 °C above the phospholipid T_m, diluted to 50 mL with PEG buffer, and allowed to precipitate at 0 °C for 10 min. The sample was centrifuged for 40 min at 33000 rpm (85000*g*) in a Beckman 42.1 rotor. Loosely associated PEG-6000 was removed from protein by resuspension of the pellet in 50 mL of 20% glycerol, 0.1 M KCl, 0.1 mM $CaCl_2$, 1 mM DTT, and 0.01 M Tes, pH 7.5, and centrifugation for 40 min at 33000 rpm in a Beckman 42.1 rotor.

The washed pellet was resuspended in approximately 5 mL of the lipid–deoxycholate resuspension buffer. In most cases, the solution was turbid after resuspension of the pellets; therefore, small aliquots of 0.15 M deoxycholate were added until a sharp change in the turbidity was noted. Usually, 100–150 μL was needed. The mixture was incubated for 20 min at 22 °C (DLPC, POPC, or SR lipid) or at 1–2 °C above the phospholipid T_m, followed by centrifugation for 40 min at 40000 rpm (105000*g*) in a Beckman 75 rotor to remove material that was not solubilized. The clear supernatant was transferred into two separate dialysis bags, and the solution was dialyzed for 24–30 h at 22 °C or above the lipid T_m against two 1-L changes of dialysis buffer (20% glycerol, 0.1 M KCl, 0.1 mM $CaCl_2$, 1 mM DTT, and 0.01 M Tes, pH 7.5).

The reconstituted samples were applied to 10-mL continuous sucrose gradients, composed of 5–40% sucrose in 20% glycerol, 0.1 M KCl, 0.1 mM $CaCl_2$, and 0.01 M Tes, pH 7.5. The gradients were centrifuged at 4 °C for 5–6 h at 37000 rpm (169000*g*) in a Beckman SW 41 rotor. The major band was removed with a Pasteur pipet, diluted with 3 volumes of water, and concentrated by centrifugation for 40 min at 40000 rpm (105000*g*) in a Beckman Ti 75 rotor. The pellet was resuspended at a protein concentration of 5–10 mg/mL in buffer containing 20% glycerol, 0.1 M KCl, 0.1 mM $CaCl_2$, and 0.01 M Tes, pH 7.5, frozen on dry ice, and stored at −65 °C. Ca^{2+}-ATPase activity of the vesicles was stable for at least 3 months when stored under these conditions.

Freeze–Fracture Electron Microscopy. Samples for freeze–fracture electron microscopy were prepared by using the propane-jet, quick-freezing method and fractured as reported by Lentz et al. (1980). The microscope used was a JEOL Model 100-CX. Samples frozen from below the membrane phase transition temperature were equilibrated for at least 12 h before being jet frozen.

Detection of Membrane Phase Transitions. The phase behavior of reconstituted Ca^{2+}-ATPase vesicles was monitored by using the fluorescent, hydrophobic membrane probe 1,6-diphenyl-1,3,5-hexatriene (DPH). Details of the fluorescence measurements are described elsewhere (Lentz et al., 1978). The "microviscosity" was calculated from the anisotropy and the estimated fluorescence lifetime of DPH (Shinitzky et al., 1971; Lentz et al., 1976). The derivative of the natural logarithm of microviscosity with respect to reciprocal temperature is referred to as the "microviscosity activation energy" (Lentz et al., 1978) and is expected to be constant with temperature for a system not undergoing a phase separation or phase transition (Lentz et al., 1976). This quantity was obtained numerically by a stepwise quadratic fit of the microviscosity data. DPH fluorescence anisotropy was recorded in heating scans at scan rates of 18 °C/h by using a T-system SLM 4000 fluorometer (SLM Instruments, Urbana, IL).

Table I: Properties of Lipid-Ca^{2+}-ATPase Recombinant

property	DLPC	DMPC	$DC_{15:0}PC$	DPPC	POPC	SR lipid
lipid/Ca^{2+}-ATPase (mol/mol)	207	198	96	195	74	96
% replacement	>99	>99	98	>99	99	
% Ca^{2+}-ATPase activity in $C_{12}E_8$ at 30 °C[a]	95	81	78	46	100	82
sample homogeneity as judged by freeze-fracture	homogeneous	homogeneous	homogeneous	heterogeneous	homogeneous	heterogeneous
temp of main phase transition peaks (°C)						
pure lipid	7	23.8	33.8	40.6	~0	<0
lipid-Ca^{2+}-ATPase recombinant		20.2 + 23.6	33.5	40.2		
$\Delta T_{1/2}$ of main phase transition (°C)						
pure lipid		1.6	1.8	1.6		
lipid-Ca^{2+}-ATPase recombinant		8.4	5.6	5.5		
temp range of main phase transition, T_L-T_U (°C)						
pure lipid		23-30	30-37	37-42		
lipid-Ca^{2+}-ATPase recombinant		15-30	23-37	31-44		
temp for Ca^{2+}-ATPase activity > 0.25 μmol (mg of ATPase)$^{-1}$ min^{-1}, $T_{0.25}$ (°C)		29	31	34	7	7
temp difference between beginnings of ATPase activity and phase transition, $T_{0.25}$-T_L (°C)		14	8	3		
activation energy of Ca^{2+}-ATPase activity from 32-46 °C (kcal/mol)		29	25	34	15	16

[a] 100% ATPase corresponds to an ATP hydrolysis rate of 5.6 ± 1.0 μmol (mg of Ca^{2+}-ATPase)$^{-1}$ min^{-1} by partially delipidated membranes in the presence of 1.86 mM $C_{12}E_8$ at 30 °C. Activities in Figures 3 and 4 have been corrected for irreversible loss of activity by using the percent of full activity recorded.

Enzymatic Assays. Ca^{2+}-ATPase activity was determined by monitoring H^+ released into the assay medium as a result of ATP hydrolysis by using the pH-sensitive dye phenol red. Sample and reference cuvettes contained 3 mL of a buffer containing 0.1 M KCl, 5 mM $MgCl_2$, 10 μM $CaCl_2$, 2.5 mM Hepes, pH 7.3, and phenol red (OD = 1 at 559 nm). The sample cuvette contained, in addition, 1.5 mM ATP. The reaction was initiated by the addition of 10–60 μg of protein. A thermocouple was placed in the sample cuvette before and after each measurement to measure temperature. In control experiments, it was found that direct measurement of inorganic phosphate release (Moore et al., 1978) yielded essentially the same activities at all temperatures.

Phosphoenzyme activity measurements were performed as described previously (Moore et al., 1978), except that membranes containing saturated phospholipids were preincubated for 5 min at or above the peak of the main lipid phase transition, T_m. The additional preincubation step was necessary to obtain low ^{32}P-labeled phosphoenzyme levels in the presence of 1 mM EGTA.

Protein was assayed by the method of Lowry et al. (1951), and phosphorus was determined as indicated previously (Meissner et al., 1973). Phospholipid analysis was by gas–liquid chromatography (Moore et al., 1978). Permeability of reconstituted vesicles to [^3H]inulin and [^{14}C]sucrose was determined as previously described (Young et al., 1981).

Results

Composition of Lipid-Replaced and Reconstituted Membranes. Twofold delipidation of sarcoplasmic reticulum (SR) membranes, as outlined under Materials and Methods, produced a sample that consistently contained 7.7 ± 1.1 phospholipids/ATPase. The yields of protein obtained after the first and second delipidation steps were 43% and 26%, respectively. Subjecting the sample to a third cycle of delipidation by using the PEG precipitation technique did not result in a significantly lower lipid/protein ratio but reduced the yield to 17%. For this reason, membranes were delipidated only twice prior to lipid exchange and relipidation with synthetic lipids. After reconstitution, membranes were placed on a sucrose gradient to obtain Ca^{2+}-ATPase preparations with a defined lipid/protein ratio. In the case of the saturated phospholipids, one major band (containing 70–90% of protein placed on the gradient) was obtained after centrifugation. More disperse bands were observed for POPC-replaced and SR phospholipid containing membranes, suggesting that these membranes had a more heterogeneous lipid–Ca^{2+}-ATPase composition.

Thin-layer chromatography at high lipid loading (Lentz et al., 1976) revealed the presence of only one phospholipid component in extracts of reconstituted membranes (not shown). Gas–liquid chromatographic analysis of the fatty acid methyl esters prepared from the extracted phospholipids indicated that endogenous SR phospholipids were replaced by the synthetic phospholipids to an extent of ≥98% and that the extent of phospholipid replacement was independent of the lipid used (Table I). Measurement of [^{14}C]PEG-6000 and [^{14}C]deoxycholate radioactivity showed that reconstituted membrane vesicles contained less than 0.1 mol of PEG-6000/mol of ATPase and no more than 2.0 mol of deoxycholate/mol of ATPase (limits of detection). Densitometer tracings of sodium dodecyl sulfate–polyacrylamide gels indicated that the major band previously identified as the Ca^{2+}-ATPase (Meissner et al., 1973) contained approximately 90% of the protein (not shown). Thus, the lipid-replacement procedure described in this study provided us with a purified Ca^{2+}-ATPase preparation enriched in a synthetic phospholipid and containing low levels of endogenous phospholipid and detergent contamination. To further emphasize the utility of the procedure, we note that only 30–40 μmol of synthetic phospholipid per 50 mg of protein was required to replace better than 98% of the SR phospholipid.

Morphology of Lipid-Replaced Membranes. The morphology of the reconstituted membranes containing saturated and unsaturated phospholipids was investigated by freeze–fracture electron microscopy (Figure 1). Within each preparation, vesicles of fairly homogeneous size were formed with DLPC-, DMPC-, $DC_{15:0}PC$-, and POPC-replaced membranes.

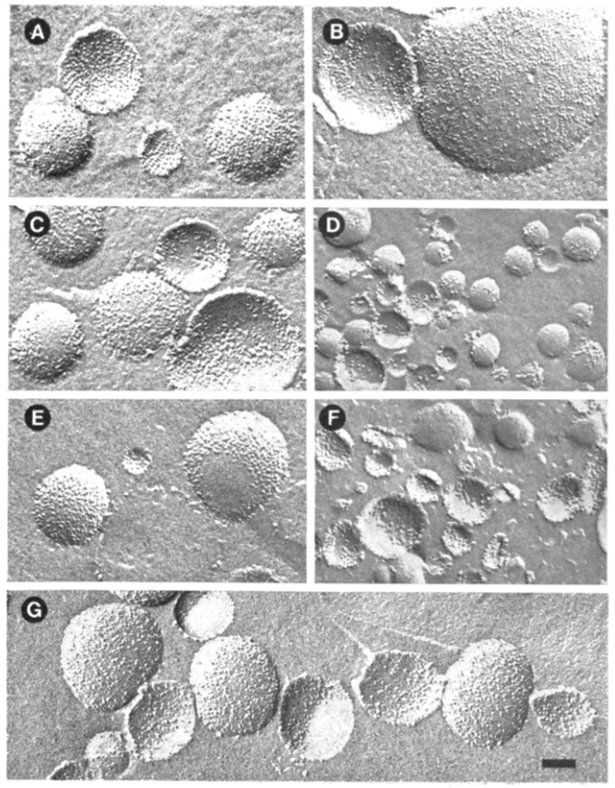

FIGURE 1: Electron micrographs of freeze–fracture replicas prepared from lipid–Ca^{2+}-ATPase recombinants and native SR vesicles. Samples listed in Table I were quick-frozen as described under Materials and Methods. Platinum shading was from below. Magnification 45 000×. Bar indicates 100 nm. (A) $DC_{15:0}PC$–Ca^{2+}-ATPase recombinants frozen from 0 °C; (B) DLPC–Ca^{2+}-ATPase recombinants frozen from 0 °C; (C) same as (A) but frozen from 35 °C; (D) SR phospholipid–Ca^{2+}-ATPase recombinants frozen from 0 °C; (E) POPC–Ca^{2+}-ATPase recombinants frozen from 0 °C; (F) native SR vesicles frozen from 0 °C; (G) DMPC–Ca^{2+}-ATPase recombinants frozen from 0 °C.

Vesicles prepared with DPPC were somewhat more heterogeneous in size with diameters ranging from 0.05 to 0.2 μm (not shown). Storage of the vesicles at −65 °C with subsequent thawing did not affect vesicle morphology.

Fracture faces of all reconstituted membranes revealed particles with an average diameter of about 80 Å. According to previous reports, the 80-Å particles correspond to the Ca^{2+}-ATPase (Deamer & Baskin, 1969; MacLennan et al., 1971; Packer et al., 1974). A significant difference between native and reconstituted membranes was, however, that native vesicles had an asymmetric distribution of particles between convex and concave fracture faces (Deamer & Baskin, 1969), while both fracture faces of the lipid-replaced membranes contained nearly equal amounts of particles (Figure 1). Thus, in agreement with previous reports (Packer et al., 1974; Meissner, 1978; Wang et al., 1979), the Ca^{2+}-ATPase protein had lost its typically asymmetric arrangement in reconstituted membranes.

The distribution of the particles in the fracture faces differed, depending on whether the vesicles were frozen from below or above the phase transition temperature of the phospholipids. Figure 1A,G shows that membranes prepared with $DC_{15:0}PC$ ($C_{15:0}$ acyl chains, T_m 33 °C) and DMPC ($C_{14:0}$ acyl chains, T_m 24 °C), respectively, and equilibrated at 0 °C, contained particles arranged in a fairly uniform manner with some indication of a rowlike pattern. Membranes reconstituted with DLPC ($C_{12:0}$ acyl chains, T_m ~7 °C) or POPC ($C_{16:0}$; $C_{18:1}$ acyl chains, T_m ~0 °C) had, at 0 °C, particles often arranged in a connected network (DLPC, Figure 1B) or a patchlike pattern (POPC, Figure 1E), indicating a possible protein–lipid phase separation. This could be due to the fact that 0 °C is in the range of the phase transition of both POPC and DLPC. In support of the hypothesis of a protein–lipid phase separation, a similar pattern was observed in Figure 1C for $DC_{15:0}PC$ membranes (T_m 33 °C) at 35 °C, which is within the transition of the phospholipid in these membranes (see below). Protein-rich patches seen in the present study within mixed phase regions of DLPC, $DC_{15:0}PC$, and POPC (Figure 1B,C,E) have been previously reported for DMPC and DPPC recombinants quenched from below the T_m (Kleeman & McConnell, 1976; Gomez-Fernández et al., 1980). Alignment of particles in rows at temperatures well below the main phase transition (Figure 1A) has not been reported previously. However, the exact distribution of particles is likely affected by the rate of freezing. In this regard, it may be noted that freezing of our samples was much more rapid than can be accomplished by the procedures used in previous studies. On the other hand, at temperatures near and below T_m, appreciably longer incubation times may be required in order to reach equilibrium than those used in the present or previous studies (Lentz et al., 1980).

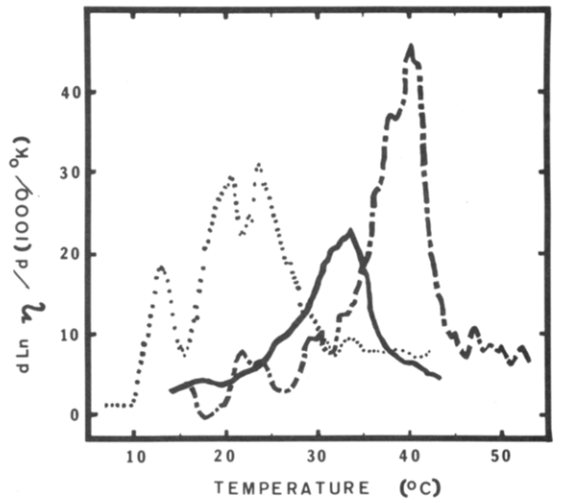

FIGURE 2: Temperature dependence of the DPH-derived microviscosity activation energy for DMPC– (···), $DC_{15:0}PC$– (—), and DPPC– (-·-) Ca^{2+}-ATPase recombinants. Samples analyzed are those listed in Table I.

Phase Behavior of Ca^{2+}-ATPase Reconstituted Vesicles. The phase transitions of Ca^{2+}-ATPase membranes replaced with three synthetic saturated phosphatidylcholines are illustrated in plots of the DPH microviscosity activation energy shown in Figure 2. Temperatures and ranges of the phase transition peaks are summarized in Table I and are contrasted with those obtained for phospholipid bilayers free of protein. Phase transition data for DLPC-replaced membranes were not recorded since these showed no Ca^{2+}-ATPase activity. Several features of these data are noteworthy. First, all three replaced membranes show a major phase transition peak at a temperature only 0.2–0.4 °C lower than the peak temperatures observed in multilamellar vesicles composed of the pure lipid. Second, the major phase transition was considerably broadened by the presence of the protein for all three lipids. Third, of the three lipid-replaced membranes, only the DMPC recombinant showed a clearly discernible peak corresponding to the pretransition peak of pure DMPC multilamellar vesicles (Lentz et al., 1978). Fourth, for DMPC-replaced membranes, a second peak was observed at a temperature about 2–3 °C below the pure lipid transition. In the case of $DC_{15:0}PC$- and DPPC-replaced membranes, a low-temperature shoulder but

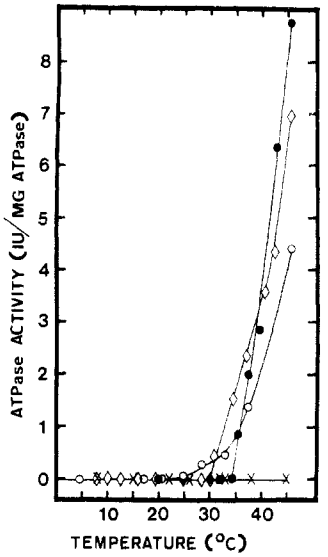

FIGURE 3: Temperature dependence of Ca^{2+}-ATPase activity of membranes containing saturated phospholipids. IU/mg of ATPase corresponds to micromoles of ATP hydrolyzed per milligram of ATPase per minute. Data for DLPC- (×), DMPC- (○), $DC_{15:0}PC$- (◊) and DPPC- (●) replaced membranes are presented. Activities shown were corrected for partial irreversible loss of activity during delipidation and relipidation (cf. Table I). Each point represents the average of three to five determinations of a single preparation (see Table I), SE = ±10%.

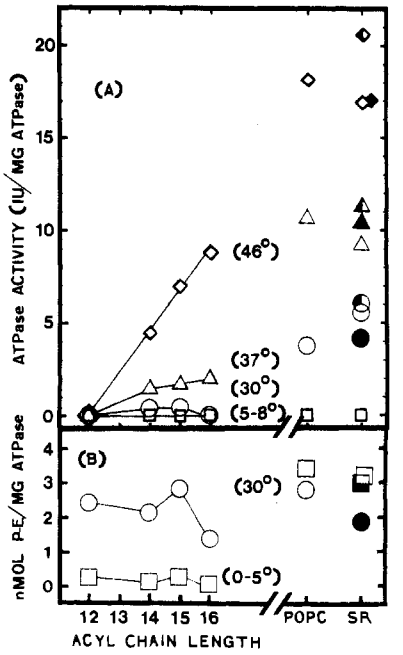

FIGURE 4: Dependence of (A) Ca^{2+}-ATPase and (B) ^{32}P-labeled phosphoenzyme levels on phospholipid acyl chain length (left of break) and unsaturation. ATPase activities at 5–8 (□), 30 (○), 37 (△), and 46 °C (◊) are as described in Figure 3. ^{32}P-labeled phosphoenzyme levels represented the average of triplicate determinations (SE = ±20% or less) at 0–5 (□) and 30 °C (○) of samples listed in Table I. ^{32}P-labeled phosphoenzyme levels were not corrected for partial irreversible loss of Ca^{2+}-ATPase activity during lipid replacement. SR phospholipid replaced membranes (empty symbols), delipidated membranes (53 lipids/ATPase) (filled symbols), and native SR vesicles (half-filled symbols) were assayed in the presence of the ionophore A23187 (2 μg/mL).

no second peak was observed. Coexistence of two peaks or a peak and shoulder would be in accord with a two-phase system undergoing independent phase transitions. It might be that these two transitions reflect "annular" and bulk-lipid regions (Hesketh et al., 1976) or protein-rich and bulk-lipid domains (Gomez-Fernández et al., 1980). An alternative explanation is that the two transitions are due to sample heterogeneity, although freeze–fracture electron micrographs did not reveal particle-poor vesicles in numbers greater than ~5% of the total vesicle number.

Ca^{2+}-ATPase Activity. Due to the well-known enzyme-denaturing effects of detergents, it was important to establish that loss of activity was due to the lipid environment and not to irreversible inactivation of the enzyme. Therefore, all membranes were assayed at 30 °C for ATP-hydrolyzing activity in the presence of 1.86 mM $C_{12}E_8$, a nonionic detergent reported to fully support Ca^{2+}-ATPase activity (Dean & Tanford, 1978). We have found 30 °C to be the highest practical temperature for standardizing activity measurements since the enzyme lost activity during the course of the assay when maintained at more than a few degrees above 30 °C in the $C_{12}E_8$-solubilized form. The data of Table I suggest that, with the exception of DPPC, the enzyme remained nearly fully active during delipidation and incorporation into saturated and unsaturated phospholipids. Loss of about 50% of the activity in DPPC-replaced membranes was probably due to the fact that this sample was kept for prolonged times at a high temperature (43 °C) during the reconstitution procedure. In Figures 3 and 4A, we have accounted for the partial irreversible loss of ATPase function by normalizing measured data (see Table I).

Figure 3 illustrates the temperature dependence of Ca^{2+}-ATPase activity of membranes containing saturated phospholipids with acyl chain lengths of 12 (DLPC), 14 (DMPC), 15 ($DC_{15:0}PC$), and 16 (DPPC) carbons. Different activity profiles were obtained for the four membranes. The most striking finding was that no Ca^{2+}-ATPase activity could be detected in DLPC-replaced membranes despite the fact that this sample showed nearly 100% activity when assayed in the presence of the detergent $C_{12}E_8$ (cf. Table I). In DMPC-replaced membranes, a gradual increase in activity was noted above 24 °C, indicating that these membranes could support ATPase activity above the two main phase transition peaks observed for these membranes (20.2 and 23.6 °C, Table I, Figure 2). In contrast, $DC_{15:0}PC$- and DPPC-replaced membranes displayed significant ATPase activity [>0.25 μmol (mg of protein)$^{-1}$ min^{-1}] at temperatures below the main phase transition peaks of these two membranes (T_m 33.5 °C for $DC_{15:0}PC$–Ca^{2+}-ATPase and T_m 40.2 °C for DPPC–Ca^{2+}-ATPase recombinants). For all three synthetic lipid-replaced membranes, ATPase activity markedly increased at temperatures above the onset of the phase transition.

Figure 4A compares the ATPase activities of membranes containing acyl chains of varying length and degree of unsaturation at 5, 30, 37, and 46 °C. At 5 °C, none of the samples displayed appreciable ATPase activity, regardless of whether the Ca^{2+}-ATPase was embedded in a bilayer present in a gel or liquid-crystalline phase. At 46 °C, our data (Figure 2) indicate that all of the lipids were present in a liquid-crystalline phase. Nevertheless, significant differences in ATPase activity in the replaced membranes were noted at this temperature. In membranes with saturated acyl chains, the activity increased with an increase in the acyl chain length of the phospholipid. In addition, a nearly 2-fold rise in ATPase activity was observed when one of the saturated acyl chains in DPPC was replaced by oleic acid, an acyl chain containing one double bond and 18 carbon atoms but having a fully extended length comparable to that of palmitic acid (Seelig & Seelig, 1977). No additional significant rise in activity was obtained when the Ca^{2+}-ATPase was present in the more highly unsaturated native phospholipid environment of sarcoplasmic reticulum.

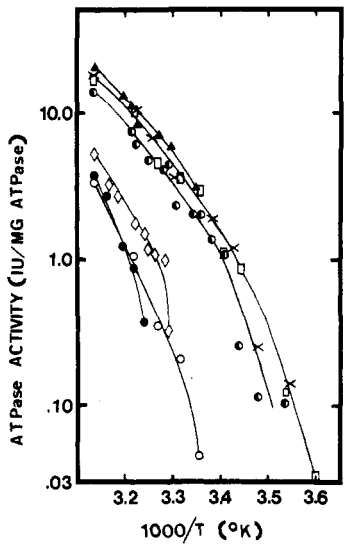

FIGURE 5: Arrhenius representation of the temperature dependence of Ca^{2+}-ATPase activity. ATPase activity data for DMPC (○), $DC_{15:0}PC$ (◇), DPPC (●), POPC (×), SR phospholipid replaced membranes (◐), delipidated SR vesicles (53 lipids/ATPase) (□), and native SR vesicles (▲) are described in Figure 3 except that activities were not corrected. ATPase activities of native vesicles were measured in the presence of the ionophore A23187 (2 μg/mL). Lipid-replaced membranes containing the amounts of lipid indicated in Table I were assayed in the absence of the ionophore since in preliminary trials essentially the same activities were measured in the absence and presence of the ionophore.

A similar behavior was observed at 37 °C, a temperature at which the lipids, with the exception of DPPC, were present in the liquid-crystalline phase. Data at 30 °C have been included in Figure 4A to permit comparison of ATPase activities and phosphoenzyme levels (see below and Figure 4B). Together, the data of Figures 2, 3, and 4A demonstrate that the ATP hydrolyzing activity of the Ca^{2+}-ATPase was dependent on both the acyl chain length and the structure of the lipid surrounding the enzyme.

In Figure 5, the temperature dependence of Ca^{2+}-ATPase activity of lipid-replaced membranes is presented in the form of Arrhenius plots. As previously observed for native (Inesi et al., 1973; Hidalgo et al., 1976; Moore et al., 1978) and lipid-replaced (Hidalgo et al., 1976; Nakamura et al., 1976; Hesketh et al., 1976) membranes, the data could not be well fitted by a straight line over the entire temperature range investigated for each lipid-replaced Ca^{2+}-ATPase preparation. Table I summarizes the activation energies that were associated with the high-temperature (32–46 °C) portions of the Arrhenius plots of Figure 5. Reference to Figure 2 demonstrates that DMPC recombinants existed in a fluid phase while $DC_{15:0}PC$ and DPPC recombinants were undergoing an order to disorder phase separation in this temperature range. As indicated by the steeper slopes, Ca^{2+}-ATPase activation energies for membranes containing the saturated phospholipids DMPC, $DC_{15:0}PC$, or DPPC were appreciably higher than those containing unsaturated acyl chains. A direct comparison of activation energies is possible since previous studies with membranes containing different amounts of SR phospholipid showed that the Ca^{2+}-ATPase activation energies at temperatures above 20 °C were relatively insensitive to differences in lipid content (Moore et al., 1978). A comparison of activation energies at temperatures below 20 °C was not possible because of insignificant ATPase activity in membranes with saturated phospholipids.

^{32}P-Labeled Phosphoenzyme Intermediate. The ability of the lipid-replaced membranes to form a ^{32}P-labeled phosphoenzyme intermediate was determined at 0 and 30 °C by using [γ-^{32}P]ATP in the presence of Ca^{2+} (Figure 4B). At 0 °C, low levels of Ca^{2+}-dependent ^{32}P-labeled phosphoenzyme intermediate (less than 0.2 nmol/mg of protein) were formed by Ca^{2+}-ATPase preparations containing phospholipids with saturated acyl chains. By contrast, POPC–Ca^{2+}-ATPase recombinants containing a phospholipid with one unsaturated acyl chain had a phosphoenzyme level comparable to that of the control sample containing SR phospholipid. ^{32}P-labeled phosphoenzyme levels in DLPC-, DMPC-, and $DC_{15:0}PC$-replaced membranes increased to levels near those observed in POPC-replaced or SR phospholipid replaced membranes when the assay temperature was raised to 30 °C (Figure 4B) or when samples were assayed at 0 °C in the presence of the detergent $C_{12}E_8$ (not shown). Especially striking was that DLPC-replaced membranes were able to form a ^{32}P-labeled phosphoenzyme intermediate at 30 °C despite the fact that these membranes displayed no ATPase activity at this temperature. DPPC-replaced membranes displayed varying levels of ^{32}P-labeled phosphoenzyme intermediate (1.5 ± 1.0 nmol of ^{32}P/mg of protein) when assayed at 30 °C. The wide variation in the data was likely due to the fact that this membrane was assayed near the onset of its main lipid phase transition (Table I). In agreement with this interpretation, a more consistent ^{32}P-labeled phosphoenzyme level (3 ± 0.5 nmol of ^{32}P/mg of protein) was determined at 40 °C.

Ca^{2+} Loading Activity. The lipid-replaced membranes, including those containing SR phospholipid, possessed no significant Ca^{2+} loading activity when assayed under optimal conditions, i.e., in the presence of the Ca^{2+}-precipitating agent oxalate. The reason for the inability of the vesicles to accumulate Ca^{2+} is not clear at present. It may be that "leaky" vesicles were re-formed during membrane reconstitution (Young et al., 1981). In agreement with this interpretation, direct measurement of the permeability barrier (Young et al., 1981) indicated that $DC_{15:0}PC$– and POPC–Ca^{2+}-ATPase recombinants did not trap [^{14}C]sucrose, even though these recombinants enclosed a [^{3}H]inulin space of 2–3 μL/mg of protein. Another possible explanation for the lack of Ca^{2+} loading activity would be that phosphatidylethanolamine (Knowles et al., 1976; Bennett et al., 1978) may be required or that a "coupling" factor may have been removed during the delipidation–relipidation procedure.

Discussion

The present study was undertaken to investigate the effect of lipid acyl chain structure on the activity of the Ca^{2+}-ATPase of sarcoplasmic reticulum. Toward this end, we developed a new lipid-substitution procedure that could replace greater than 98% of the endogenous SR lipid. Freeze–fracture electron microscopy was used to assess overall membrane morphology as well as to determine the disposition of the Ca^{2+}-ATPase protein in the reconstituted bilayers. The effect of the protein itself on acyl chain order was probed by diphenylhexatriene fluorescence. Finally, we measured Ca^{2+}-ATPase activity in the presence of the nonionic detergent $C_{12}E_8$ in order to control for irreversible loss of enzyme activity during delipidation and reconstitution.

Effect of Lipid Bilayer Physical State on Ca^{2+}-ATPase Activity. The relationship between the lipid bilayer physical state and Ca^{2+}-ATPase activity has been investigated previously in membranes reconstituted by the procedure of Warren et al. (1974) mainly with DPPC (Hesketh et al., 1976; Nakamura et al., 1976; Hidalgo et al., 1976). Two reports have agreed in detecting essentially no activity in these recombinants below 30 °C (Hesketh et al., 1976; Nakamura et al., 1976)

while the third reported activity but an altered Arrhenius activation energy below this temperature (Hidalgo et al., 1976). The observation of significant activity below 30 °C was likely due to appreciable amounts of endogenous SR lipid reported in DPPC-replaced membranes (Hidalgo et al., 1976). We have found also that a single replacement by the Warren procedure was insufficient to obtain the low levels of endogenous lipid contamination reported here (Table I). It may be noted that fully replaced membranes prepared by the Warren procedure displayed in one carefully documented report (Nakamura et al., 1976) a lower ATPase activity than that obtained here. The reasons for the lower activity are not clear since samples were not assayed for irreversible loss of activity during sample preparation. Similarly, the morphology of the lipid-replaced membranes used in previous enzyme studies is not known, except that Nakamura et al. (1976) used negative-staining electron microscopy to show that vesicle-like structures were formed with DPPC. We have extended these previous reports to include studies of additional synthetic saturated phosphatidylcholine species (DLPC, DMPC, and $DC_{15:0}PC$) combined with the Ca^{2+}-ATPase by a new procedure that maintains nearly native ATPase activity.

The observation of ATPase activity in DPPC-replaced membranes beginning at about 30 °C has been interpreted by Hesketh et al. (1976) in terms of a lipid annulus surrounding the Ca^{2+}-ATPase enzyme. Hesketh et al. concluded from electron-spin resonance spectra of 5-doxylstearic acid and 2,2,6,6-tetramethylpiperidinyl-1-oxy (Tempo) that the perturbation of the DPPC phase transition did not extend beyond the lipid annulus. Within the context of this conclusion, Hesketh et al. interpreted the "anomalous" observation of ATPase activity below the pure DPPC phase transition (41 °C) as reflecting the lack of a normal phase transition in the annular lipid. They concluded that a well-defined annulus or "boundary" of protein-affected lipid insulated the Ca^{2+}-ATPase from the effects of phase changes in the surrounding bulk bilayer. Our results do not support the conclusions of Hesketh et al. and offer a simpler explanation for the activity and phase behavior of DPPC–Ca^{2+}-ATPase recombinants. First, our DPH fluorescence results (Figure 2) agree with the electron-spin resonance data of Hesketh et al. but not with their contention that the DPPC phase transition was unaffected by the presence of the Ca^{2+}-ATPase. Second, comparison of the phase and activity behavior of DMPC–, $DC_{15:0}PC$–, and DPPC–Ca^{2+}-ATPase recombinants indicates that the DPPC recombinant is not anomalous. With increasing acyl chain length, the temperature at which appreciable ATPase activity could be measured ($T_{0.25}$ in Table I) correlated increasingly more closely with the lower delimiting temperature of the lipid phase transition (T_L in Table I). Thus, the quantity $T_{0.25} - T_L$ decreased from 14 °C for DMPC to 8 °C for $DC_{15:0}PC$ to 3 °C for DPPC membranes (see Table I). Apparently, a more disordered acyl chain configuration is required to relieve the constraints imposed on the ATPase by a suboptimal bilayer thickness, as will be discussed below.

Effect of Acyl Chain Length and Unsaturation on Ca^{2+}-ATPase Activity. The effect of phospholipid acyl chain length on Ca^{2+}-ATPase has been recently studied by Johannsson et al. (1981) and Caffrey & Feigenson (1981), using phosphatidylcholines with unsaturated fatty acyl chains. In agreement with the present study, overall ATPase activity was found to be suppressed by decreased lipid acyl chain length. Nonetheless, there are differences in methodology between the present study and these recent reports that are worth mentioning. Johannsson et al. (1981) reported the Ca^{2+}-ATPase activity of protein–lipid–sodium cholate complexes of undefined morphology and composition. Without morphological evidence to verify that the Ca^{2+}-ATPase was incorporated into a bilayer-like phase, it remains unclear whether loss of activity observed by these authors was due to suboptimal bilayer thickness or to loss of bilayer structure in general. For this reason, it is difficult to compare the study of Johannsson et al. (1981) to the work reported here. Caffrey & Feigenson (1981), on the other hand, incorporated the Ca^{2+}-ATPase into complexes containing about 1500 synthetic lipids and 60–80 native lipids per Ca^{2+}-ATPase. Despite the nonphysiological lipid/protein ratio and the contamination by native lipids, the recombinant complexes were clearly shown to be membranous vesicles. In qualitative agreement with our results, these authors reported essentially no ATPase or Ca^{2+} loading activity in membranes containing $C_{12:1}$ or $C_{12:0}$ fatty acids and maximal activity in membranes with $C_{18:1}$ fatty acids. However, because no test was made for irreversible loss of activity, it is impossible to compare the exact dependence of activity on chain length with that obtained here.

In the present study, we have used saturated phospholipids of different acyl chain length to investigate the effect of bilayer thickness on Ca^{2+}-ATPase activity at temperatures above the lipid phase transition. We found that the Ca^{2+}-ATPase requires for function a membrane with a minimal bilayer thickness. The most convincing evidence for this point came from the observation that DLPC recombinants exhibited no measurable ATPase activity over the entire temperature range studied. Yet, freeze–fracture electron microscopy showed that membrane vesicles were formed containing the Ca^{2+}-ATPase (Figure 1B). Also, full activity was observed when this sample was assayed in the presence of $C_{12}E_8$. Increase in bilayer thickness obtained by incorporating the enzyme into DMPC, $DC_{15:0}PC$, and DPPC membranes resulted in increased ATPase activity (Figure 4A).

An effect of acyl chain unsaturation was indicated by our observation that Ca^{2+}-ATPase activity was increased 2-fold when the enzyme was incorporated into POPC rather than DPPC membranes. Since both lipids have the same effective chain length, it would appear that the Ca^{2+}-ATPase required for optimal function not only a minimal bilayer thickness but, in addition, an environment that is more "fluid" than can be provided by saturated acyl chains. Alternatively, it is possible that the observed increase in activity indicated a partial requirement for acyl chains containing one or more double bonds. No further stimulation in ATPase activity was noted when the enzyme was reconstituted into its native lipid environment (Figure 4). Thus, the Ca^{2+}-ATPase does not require for full activity either the high degree of unsaturation of the native lipids or any particular lipid head group other than choline.

^{32}P-Labeled Phosphoenzyme Intermediate. Studies with partially delipidated (Martonosi, 1969; Meissner & Fleischer, 1972) and DPPC-replaced membranes (Nakamura et al., 1976; Hidalgo et al., 1976) have suggested that one or more of the steps leading to the breakdown of the phosphoenzyme intermediate is the step kinetically sensitive to lipid environment. Phosphoenzyme formation was thought to be affected to a lesser degree by a change in lipid environment. The present study shows that, at 0–5 °C, no ^{32}P-labeled phosphoenzyme intermediate is formed in membranes containing phospholipids with saturated acyl chains. Fluctuating levels were observed near the onset of the lipid main phase transition, whereas normal high levels of ^{32}P-labeled phosphoenzyme intermediate were measured at or above the peak temperature of the main lipid phase transition. Thus, our data suggest that

phosphoenzyme formation, like Ca^{2+}-ATPase activity, is sensitive to the bilayer physical state. This compares with reports of partially lipid-replaced DPPC membranes where ^{32}P-labeled phosphoenzyme formation was found to be somewhat affected by the lipid bilayer physical state but less than overall Ca^{2+}-ATPase activity (Nakmura et al., 1976; Hidalgo et al., 1976). Our observation of low phosphoenzyme levels in all saturated recombinants at 0 °C does agree with the report of Nakamura et al. (1976) for fully replaced DPPC–Ca^{2+}-ATPase recombinants. The effect of the lipid phase transition on phosphoenzyme formation in fully replaced DPPC membranes was not studied by these investigators. In contrast to its dependence on physical bilayer state, phosphoenzyme formation appears to be relatively insensitive to acyl chain length. In this regard, a remarkable finding was that DLPC–Ca^{2+}-ATPase recombinants were able to form a ^{32}P-labeled phosphoenzyme intermediate at 30 °C even though no ATPase activity was observed at this temperature.

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