Fluorescence Quenching in Model Membranes. 3. Relationship between Calcium Adenosinetriphosphatase Enzyme Activity and the Affinity of the Protein for Phosphatidylcholines with Different Acyl Chain Characteristics†

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ABSTRACT: The dependence of function and lipid binding affinity of an integral transport protein on the fatty acyl chain characteristics of a membrane-forming phospholipid have been determined. When a newly developed fluorescence quenching technique [London, E., & Feigenson, G. W. (1981) *Biochemistry* (first paper of three in this issue); London, E., & Feigenson, G. W. (1981) *Biochemistry* (preceding paper in this issue)] is used for examining lipid–protein interactions in membranes, the Ca^{2+}ATPase from rabbit sarcoplasmic reticulum is found to bind with equal affinity a large variety of phosphatidylcholines used to reconstitute the protein into enzymatically active vesicles, regardless of fatty acyl chain length or details of unsaturation. In parallel with the lipid binding studies, we have measured the sensitivity of the catalytic activity of the Ca^{2+}ATPase to the fatty acyl chain characteristics of the phosphatidylcholine membranes in which the enzyme was reconstituted. The enzyme appears to be sensitive only to the effective fatty acyl chain length, which determines the thickness of the bilayer in which the protein is inserted and displays little sensitivity to such details of unsaturation as degree, position, and isomeric type. Both ATP hydrolyzing and Ca^{2+} transporting activities of the enzyme were similarly affected by bilayer thickness, and maximum activity was observed in membranes of intermediate thickness. These observations are reconciled in a number of possible models for the manner in which this integral protein interacts with membranes of varying thicknesses. A freeze–thaw method was used to reconstitute the Ca^{2+}ATPase, and the vesicles so obtained have been characterized by gel permeation chromatography, density gradient centrifugation, and electron microscopy (thin section). Convenient methods are described for (a) rapidly separating reconstituted Ca^{2+}ATPase from unincorporated protein simultaneously in a large number of small samples, giving good recovery of fractionated vesicles without significant dilution, and (b) measuring leakiness to Ca^{2+} of reconstituted vesicles. Additionally, the gel to liquid-crystal phase transition temperature and bilayer thickness have been determined respectively by differential thermal analysis and low-angle X-ray diffraction for some of the synthetic phosphatidylcholines, which range in chain length from 12 to 24 carbon atoms.

Convincing evidence has been obtained by a variety of techniques in support of the gross features of the current model for biological membranes as a dynamic mosaic of lipids and proteins (Singer & Nicholson, 1972; Nicholson, 1976; Keith & Snipes, 1977). However, some of the more detailed aspects of the structure and function of these limiting membranes have yet to be elucidated. A quantitative technique has recently been developed in this laboratory (London & Feigenson, 1981a,b) for examining one such aspect, namely, the apparent relative binding affinity of membrane proteins for different lipids. The principle of the method is that a spin-labeled phospholipid in contact with a protein fluorophor quenches fluorescence. The fluorescence can be recovered when the spin-labeled phospholipid is displaced from the protein by another lipid or small molecule.

Except in the case of some specialized tissues, most biological membranes are composed of a range of different lipid species with an even more varied complement of fatty acyl chains (van Deenan, 1965; Marai & Kuksis, 1973a,b). It is of interest to determine why such a heterogeneous assemblage of acyl groups is found in these membranes and if certain membrane proteins have a specific requirement for particular acyl chains. Using the Ca^{2+}ATPase (ATP phosphohydrolase, EC 3.6.1.3) from sarcoplasmic reticulum (SR)[1] as a model integral protein, we posed the following questions: (a) Is this membrane-bound protein sensitive, as reflected in its enzymatic activity, to the properties of the hydrocarbon region of the phospholipid bilayer in which it is inserted? (b) If this sensitivity exists, is it correlated with the affinity of the enzyme for these phospholipids which differ in the characteristics of their hydrocarbon region?

The Ca^{2+}ATPase is especially convenient for study because the purified protein is stable and can be readily obtained in large quantities and because much is known about its prop-

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[1] Abbreviations used: SR, sarcoplasmic reticulum; PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; T_t, gel to liquid-crystal phase transition temperature; BSA, bovine serum albumin; ATP, adenosine 5′-triphosphate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DTA, differential thermal analysis; d_i, internal diameter; (7,6)PC, 1-acyl-2-[2-(6-carboxyhexyl)-2-octyl-4,4-dimethyloxazolidinyl-3-oxy]glycero-3-phosphocholine; χ_A, mole fraction of total exogenous lipid which is lipid A; $K'_{B/A}$, the apparent relative binding constant of lipid B compared to lipid A; F, observed fluorescence value; F_0, unquenched fluorescence value; F_{min}, fluorescence value at $\chi_{(7,6)PC} = 1$; A_{750}, absorbance at 750 nm; d, the interlamellar repeat distance; m, the number of the diffraction order; θ, the Bragg angle; T_R, reduced temperature. The notation used for fatty acyl groups is that the first number denotes the number of carbon atoms, the number following the colon denotes the number of double bonds, the number(s) in parentheses denote(s) the position(s) of the double bond(s), and the letters t and c following the numbers in parentheses denote the isomeric nature of the double bonds as trans and cis, respectively.

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erties in membranes [see, for example, Davis & Inesi (1971); Martonosi, 1971, 1978; MacLennan & Holland, 1975; Dutton et al., 1976; Racker, 1977; Warren & Metcalfe, 1977; Dean & Tanford, 1978; Thomas & Hidalgo, 1978; Green et al., 1978; Meissner, 1978)]. Furthermore, the enzyme can be reconstituted by a variety of methods into vesicles of well-defined lipid composition (Miller & Racker, 1979; Zimniak & Racker, 1978; Racker et al., 1979; Warren et al., 1974). For the proposed series of experiments, we use the freeze–thaw method of reconstitution (Zimniak & Racker, 1978) since this procedure does not involve the use of detergents and gives enzymatically active vesicles with phosphatidylcholine (PC) as the only exogenous lipid. Our choice of PC as the examined phospholipid was influenced by the fact that (a) it is the predominant lipid in many biological membranes including SR (Sarzala et al., 1974; Marai & Kuksis, 1973a,b; Jain, 1972; Owens et al., 1972; Bruckdorfen & Graham, 1976), (b) the synthesis of PC's with different acyl chains is relatively straightforward (Robles & van den Berg, 1969), (c) the corresponding PC's have suitable phase transition temperatures (T_t; see below), and (d) PC is entirely compatible with the Ca^{2+}ATPase enzyme in the freeze–thaw reconstitution system.

So that the questions cited above could be answered, a number of different PC's were prepared which differed in acyl chain length and in the degree, isomeric type, and position of unsaturation. All enzyme activity and fluorescence quenching measurements were performed with the phospholipids in the liquid-crystal state. Accordingly, the choice of monounsaturated acyl groups was necessarily restricted to fatty acids with no more than 24 carbon atoms, beyond which the T_t of the corresponding PC's would be too high for reliable enzyme activity measurements to be made.

Experimental Procedures

Materials. Sepharose 4B, dimyristoyl-PC, distearoyl-PC, BSA (5% solution), BSA (fraction V powder), yeast hexokinase (type C-130), Na_2ATP, L-α-glycerophosphocholine (cadmium chloride complex, grade 1), Dowex 50W (8% cross-linked, 20–50 dry mesh), 1-*O*-*n*-octyl β-D-glucopyranoside (*n*-octyl glucoside), and cholic acid were from Sigma. Methanol-d_4, chloroform-*d*, hexadecane (spectrophotometric grade), and *N*,*N*′-dicyclohexylcarbodiimide were supplied by Aldrich Chemical Co., and dilauryl-PC and dipalmitoyl-PC were obtained from Calbiochem. Bio-Gel A-150m was from Bio-Rad, $^{45}Ca^{2+}$ from ICN, Blue Dextran 2000 from Pharmacia, and A23187 from Eli Lilly and Co. The following fatty acids were obtained from Nu Chek Prep: 11-dodecenoic, 12:1; 9-tetradecenoic (myristoleic) 14:1(9c); 9-*trans*-tetradecenoic (myristelaidic), 14:1(9t); 9-hexadecenoic (palmitoleic), 16:1(9c); 9-*trans*-hexadecenoic (palmitelaidic), 16:1(9t); 6-*trans*-octadecenoic (petroselaidic), 18:1(6t); 9-octadecenoic (oleic), 18:1(9c); 9-*trans*-octadecenoic (elaidic), 18:1(9t); 11-octadecenoic (vaccenic), 18:1(11c); 9,12-octadecadienoic (linoleic), 18:2(9c,12c); 11-eicosenoic, 20:1(11c); 13-*cis*-docosenoic (erucic), 22:1(13c); 13-*trans*-docosenoic (brassidic), 22:1(13t); and 15-tetracosenoic (nervonic), 24:1-(15c). Cholic acid was recrystallized from 70% (v/v) ethanol after filtering a hot 10% (w/v) solution in 95% (v/v) ethanol through Norit A activated charcoal (Matheson, Coleman and Bell). Egg PC was prepared from chicken egg yolks by the method of Singleton et al. (1965). Spin-labeled PC was synthesized according to the procedure of Boss et al. (1975) by using lysophosphatidylcholine from egg PC and the 2,2-dimethyloxazolidinyl-1-oxy derivative of 8-ketopalmitic acid synthesized as in Hubbell & McConnell (1971). Both egg PC and spin-labeled PC were pure by thin-layer chromatography as described below under Phosphatidylcholine Synthesis. [γ-^{32}P]ATP was synthesized by the method of Avron (1961) by using spinach leaf chloroplasts and was purified by the method of Cohn & Carter (1950). By use of the inorganic phosphate extraction procedure described under ATPase Assay, the purified [γ-^{32}P]ATP had 0.5%–1.5% of total counts as inorganic phosphate.

Phosphatidylcholine Synthesis. All PC's were synthesized by a modification of the method of Robles & van den Berg (1969) by using free L-α-glycerophosphocholine rather than the cadmium chloride salt. For a homogeneous reaction mixture, incubations were carried out in a glycerol bath at 90 °C, and the reaction was allowed to proceed for 5–7 days in the absence of light under reduced pressure. For purification of the PC, a crude suspension of the semisolid reaction mixture in a minimum of diethyl ether was applied directly to a preparative thin-layer plate (1000-μm layer thickness, Analtech silica gel GF) at approximately 50 mg of PC per plate. The chromatogram was developed in chloroform/methanol/acetone/acetic acid/water (10:2:4:2:1 v/v) and plates were dried for 30 min in a stream of nitrogen. The PC band was scraped from the plate in six fractions, and the lipid eluted from the gel with chloroform/methanol/water (10:10:1 v/v) (approximately 100 mL per 50 mg of PC). The solvent was removed by rotary evaporation at 40 °C by using ethanol to assist in the removal of the last traces of water and isobutyl alcohol to prevent foaming. The dry PC was dissolved in chloroform at 10–20 mg/mL and stored under nitrogen in the dark at −20 °C.

Purity of the newly synthesized PC's was >98% as judged by thin-layer chromatography in three solvent systems: chloroform/methanol/acetone/acetic acid/water (10:2:4:2:1 v/v), chloroform/methanol/ammonium hydroxide (concentrated) (65:25:5 v/v), and chloroform/methanol/water (65:25:4 v/v) (A) on acetone-washed precoated silica gel 60 F-254 plates (VWR Scientific) developed with 20% (w/v) ammonium sulfate spray followed by charring on a hot plate or (B) Adsorbosil 5-P plates (Applied Science Lab.) prerun in chloroform/methanol (10:1 v/v) and developed with Zinzadze reagent (Dittmer & Lester, 1964) followed by charring on a hot plate. For verification of the identity of the reaction product, the ^{1}H nuclear magnetic resonance spectrum of a 1 mM solution of dinervonoyl-PC was determined by using a Varian CFT-20 NMR spectrometer operated at 79.54 MHz.

Purification of Ca^{2+}ATPase. SR (R_1-washed fraction) was obtained from the white muscle of New Zealand white rabbits by the MacLennan procedure (1970). By use of the octyl glucoside solubilization method of Banerjee et al. (1979), purified Ca^{2+}ATPase was prepared from SR and stored at approximately 30 mg of protein/mL in 0.25 M sucrose and 10 mM Tris-HCl, pH 7.5, at −70 °C.

Preparation of Phospholipid Vesicles. Vesicles were prepared by transferring the lipid or lipid mixtures to glass tubes and evaporating organic solvent with nitrogen gas. For assistance in removal of the last traces of organic solvent, the lipid was redissolved in a minimum of diethyl ether and again taken to dryness with nitrogen gas. After water was added to give a final lipid concentration of 27 mM, the tube was sealed under nitrogen, and the phospholipid was then dispersed by mixing briefly on a Vortex mixer and sonicated to clarity in a bath sonicator (Laboratory Supplies Co., Inc.) at room temperature. In the case of lipids with T_t's exceeding room temperature, it was necessary to vortex and sonicate above this temperature (Bangham et al., 1974). Fresh vesicles were prepared daily and stored on ice under nitrogen.

Reconstitution. Purified Ca^{2+}ATPase was reconstituted into phospholipid vesicles by the freeze–thaw method of Kasahara & Hinkle (1977). Unless otherwise stated, the standard reconstitution mixture contained 8.1 mM PC vesicles, 0.2 M potassium oxalate, pH 7.5, and 0.5 mg of purified Ca^{2+}ATPase protein/mL in a total volume of 0.1 mL. The reconstitution mixture, contained in a small Pyrex tube, was frozen in liquid nitrogen for 60 s and allowed to thaw at room temperature for 10 min. After the suspension was mixed on a Vortex mixer, it was thawed for an additional 5 min at room temperature (unless otherwise noted) and immediately sonicated in a bath sonicator at room temperature for 40 s. Reconstitution with the higher melting lipids required that the final sonication step be performed above the T_t. Where possible, reconstituted vesicles were kept on ice. However, in the case of the higher melting phospholipids, it was necessary to store the reconstituted vesicles above their T_t to minimize loss of Ca^{2+} uptake activity.

In all experiments, control reconstituted vesicles were prepared which lacked enzyme.

Fractionation. Upon reconstitution by the freeze–thaw method outlined above, approximately 50% of the protein is incorporated into vesicles. Accordingly, the unreconstituted protein was resolved from vesicle-incorporated protein by either or two methods involving density gradient centrifugation.

(*A*) *Large-Scale Fractionation.* Sucrose gradients (3.5-mL total volume) were prepared in a stepwise manner with solutions containing 0.4 M sorbitol, 0.05 M Tris-HCl, pH 7.5, and 0.1–1.5 M sucrose in cellulose nitrate tubes ($^7/_{16}$ in. diameter × 2 $^3/_8$ in.). A continuous sucrose gradient formed after incubation at 4 °C for 12 h. The reconstitution mixture (1 mL) was layered on the gradient and centrifuged in an SW60 rotor at 55 000 rpm for 90 min at 4 °C. The gradients were dripped and 0.25-mL fractions collected for Ca^{2+} uptake, protein, phospholipid, and refractive index measurements.

(*B*) *Small-Scale Fractionation.* This procedure was developed because it allowed the simultaneous fractionation of a large number of small samples in a short period of time and gave good recovery of fractionated vesicles without significant dilution. The method involved layering up to 0.15 mL of reconstitution mixture on 0.25 mL of 0.3 M sucrose, 0.4 M sorbitol, and 50 mM Tris-HCl, pH 7.5, in 0.4-mL capacity polypropylene microcentrifuge tubes (Fisher Scientific Co.). After capping, the tubes are floated [see Murthy & Bharucha (1978)] in 20 mL of 0.5 M sucrose, contained in SW27 centrifuge tubes (31-mL capacity, Beckman Instruments, Inc.), and centrifuged for 30 min at 25 000 rpm in an SW27 rotor at a temperature above the T_t of the lipids. (Using less than 0.3-mL total volume per microcentrifuge tube will result in the collapse of the tubes under these conditions.) Each of the SW27 tubes can accomodate five microtubes, allowing a total of 30 samples to be fractionated at once. After the cap was removed, the microtube was clamped with pliers at the interface between the supporting sucrose cushion and the fractionated vesicles, effecting a clean separation of the two solutions. Suspended vesicles in the upper portion of the tube were harvested, and the walls of the tube were washed with 0.05 mL of 0.2 M potassium oxalate, pH 7.5, to ensure full recovery of the fractionated vesicles. These vesicles were stored above their T_t until all activity measurements were made. The free protein was present at the bottom of the tube as a hard translucent pellet which could be resuspended with difficulty after prolonged sonication.

The density of the supporting solution in the microcentrifuge tubes was chosen such that regardless of the type of phospholipid used the reconstituted vesicles floated while free protein sedimented at the temperature of centrifugation.

Ca^{2+} Uptake Assay. The standard assay mixture contained 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, (2–5) × 10^4 cpm of $^{45}Ca^{2+}$/nmol of Ca^{2+}, 1 mM NaATP, 0.05 M Tris-HCl, pH 7.5, 0.7 M Sorbitol, and reconstituted Ca^{2+}ATPase which provides 0.31 mM PC and 19.2 μg of Ca^{2+}ATPase protein/mL in a total volume of 0.25 mL. The reaction was initiated with reconstituted vesicles and stopped after 0.5 min at room temperature by passing 0.2 mL of the reaction mixture over a BSA-treated Dowex 50W-X8 (Tris form, pH 7.5) column prepared in a Pasteur pipet. Free Ca^{2+} was resolved from the reaction mixture by immediately washing the column with 3 mL of 0.25 M sucrose, and 1 mL of the eluant was counted in 10 mL of ACS (Amersham) liquid scintillation fluid in a Beckman LS-230 liquid scintillation counter.

The Dowex resin was pretreated with BSA by passing through the column 3 mL of a BSA solution at 3.3 mg/mL in 0.25 M sucrose.

ATPase Assay. Identical conditions were used for measuring Ca^{2+} uptake and ATPase activity with the exception that the ATPase assay mixture lacked $^{45}Ca^{2+}$ and contained [γ-^{32}P]ATP, 5 × 10^3 cpm/nmol ATP. The reaction was stopped with 0.05 mL of trichloroacetic acid (50% w/v), and 0.125-mL aliquots of the reaction mixture were transferred to tubes containing 1.05 mL of water/hydrochloric acid (concentrated)/5% (w/v) ammonium hydroxide (5:2:18 v/v) and 1.25 mL of isobutyl alcohol/toluene (1:1 v/v). This extraction medium was vigorously mixed on a Vortex mixer for exactly 20 s, and after the phases had separated, 0.5 mL of the upper organic phase was counted in 10 mL of ACS as above.

Protein Determination. (a) The Lowry method (Lowry et al., 1951) was modified to allow rapid protein measurements to be carried out on dilute samples as follows: (i) Lowry reagents C and E were used at 5 times the recommended concentrations. (ii) Reagent E was added immediately after reagent C and the reaction mixture was incubated at room temperature for 30 min before reading A_{750} with BSA (fraction V) as the standard.

(b) A more convenient method for measuring protein in reconstituted samples took advantage of the intrinsic fluorescence properties of the Ca^{2+}ATPase. Light scattering problems were eliminated by making all measurements in 2% (w/v) potassium cholate, pH 7.5. Purified Ca^{2+}ATPase was used as the standard, and fluorescence was found to be linear with protein concentration up to at least 60 μg/mL. Fluorescence measurements were made in micro quartz cuvettes with either a home-built fluorimeter (described below) or a Perkin-Elmer MPF-3 fluorescence spectrophotometer with excitation at 287 nm, emission at 340 nm, and 8-nm slits.

Fluorescence Spectrophotometry. The freeze–thaw reconstituted vesicles range in size from 25- to 200-nm diameter. Under conditions where most of the protein fluorescence is quenched by spin-labeled phospholipid, it was not possible to obtain accurate fluorescence readings in a fluorimeter with poor stray light characteristics because scattered light from the large vesicles contributed significantly to the overall signal. For improvement of the accuracy of the measurements, a spectrofluorimeter was built with conventional 90° optics incorporating double monochromators with holographic gratings in both excitation and emission optics such that stray and scattered light was negligible. The fluorimeter was assembled with an ozone-free 450-W lamp, lamp power supply Model 8550-4, liquid filter Model 6123, and universal arc housing Model 6140 from Oriel Corp. The photomultiplier power

supply Model RQE-1601-5121 was from Northeastern Scientific, the picoammeter Model 414S from Keithley Instruments, Inc., and the chart recorder Model SR-205 from Heath Schlumberger. The DH-10 double monochromators, sample compartment, slits, scanning control modules Model 1020-SS, photomultiplier tube (1P28), and standard photomultiplier tube housing were obtained from Instruments SA, Inc.

Preliminary and comparative work was carried out with a Perkin-Elmer MPF-3 fluorescence spectrophotometer.

Vesicle Leakiness. Depending on the Ca^{2+} uptake activity of the reconstituted Ca^{2+}ATPase, measurements of the leakiness of reconstituted vesicles to $^{45}Ca^{2+}$ were made by using one or both of the following methods.

Method 1 (Active Loading). This method allowed leakiness to be determined under conditions similar to those used for measuring Ca^{2+} uptake in reconstituted vesicles. Briefly, the procedure involves actively loading the vesicles with $^{45}Ca^{2+}$ via the action of the Ca^{2+}ATPase, stopping active transport with an ATP-utilizing enzyme (hexokinase), and then following efflux of trapped Ca^{2+} from the vesicles as a function of time. The protocol used was as follows: To 1.75 mL of Ca^{2+} uptake medium (5 mM $MgCl_2$, 0.1 mM $CaCl_2$, (2–5) $\times 10^4$ cpm of $^{45}Ca^{2+}$/nmol of Ca^{2+}, 1 mM NaATP, 0.05 M Tris-HCl, pH 7.5, and 0.7 M sorbitol) was added 0.07 mL of reconstituted vesicles (8.1 mM PC, 0.2 mM potassium oxalate, pH 7.5, and ±0.5 mg of purified Ca^{2+}ATPase protein/mL), and loading was allowed to proceed at the temperature indicated for 20–30 min. Aliquots (0.15 mL) of the reaction mixture were taken at regular intervals for trapped Ca^{2+} determinations as described under Ca^{2+} Uptake Assay. The incubation medium was then split into two 0.7-mL fractions. To the first was added 0.03 mL of hexokinase mix (0.76 hexokinase unit/mL, 4 μg of protein/mL, 1.28 M ammonium sulfate, 0.1 mM Na_2EDTA, 1 mM potassium phosphate, pH 7, and 0.5 M fructose) and to the second, 0.03 mL of hexokinase blank (hexokinase mix without the enzyme), and trapped Ca^{2+} measurements were made at timed intervals as above.

Method 2 (Passive Loading). The passive loading technique was chosen for studying leakiness of vesicles in which Ca^{2+}-ATPase either was totally inactive or displayed very low levels of Ca^{2+} transport. In this case, the vesicles were passively loaded with $^{45}Ca^{2+}$ at the freeze–thaw reconstitution step, and then $^{45}Ca^{2+}$ efflux was followed by diluting vesicles into a $^{45}Ca^{2+}$-free medium. As a control to demonstrate that trapped Ca^{2+} in tight (nonleaky) vesicles is exchangeable with the external medium, the ionophore A23187 (Pressman, 1976) was added to unload the vesicles. The protocol used in these experiments is as follows: Vesicles were passively loaded by reconstituting in the presence of 12 mM $CaCl_2$ (2.5×10^4 cpm of $^{45}Ca^{2+}$/nmol of Ca^{2+}) without potassium oxalate as described under Reconstitution. The passively loaded vesicles (0.07 mL) were then added to 1.75 mL of dilution medium (Ca^{2+} uptake medium as described above without $^{45}Ca^{2+}$), and aliquots were taken at time intervals for trapped Ca^{2+} measurements. At the end of this incubation period, the medium was split into two 0.7-mL fractions and one treated with 8 μL of A23187 (1 mg/mL in absolute ethanol) and the other with 8 μL of absolute ethanol as a control. Again, aliquots were taken for trapped Ca^{2+} measurements at timed intervals after the addition of the ionophore.

Differential Thermal Analysis (DTA). Approximately 2 mg of phospholipid in chloroform was brought to dryness under a stream of N_2 and redissolved in a minimum of diethyl ether, and the solution was transferred to a small glass culture tube. The solvent was again evaporated under N_2, and 20 μL of N_2-saturated water was added to the dried lipid. After the sample was incubated for a few minutes above the T_t of the lipid, it was vortexed and the dispersion transferred to a glass capillary tube. Thermal analysis was performed on a home-built thermal analyzer (details to be presented elsewhere) at heating rates from 0.5 to 10 °C/min. Transition temperatures were reproducible to ±0.2 °C for any given phospholipid sample.

Bilayer Thickness. Reconstituted vesicles were prepared in 20 mM potassium oxalate, pH 7.5, for bilayer thickness measurements by low-angle X-ray scattering as described under Reconstitution. Under these conditions, the vesicles sedimented as a whitish paste when centrifuged for 6 h at 25000 rpm in an SW27 rotor as described under Fractionation (Method B). The loose pellet was transferred to the upper section of a thin-walled capillary tube (1-mm internal diameter, Blake Industries, Inc.) and spun to the bottom of the tube by centrifuging at 1000 rpm for 5 min at room temperature in a swinging-bucket clinical centrifuge, and the sample was then flame sealed in the capillary.

Cu Kα X-rays generated from a Rigaku rotating anode generator were filtered through a nickel filter and collimated by using the double-mirror optics (Franks, 1958) of a Searle X-ray diffraction camera. The diffraction pattern was recorded at temperatures from 2 to 60 °C by using a thermostated specimen holder. Sample-to-film distance was determined by using a hexadecane standard, and microdensitometry of the X-ray photographs was carried out on a Joyce Loebl Model IIIC microdensitometer.

Bilayer thickness was determined from the densitometer tracing by measuring the peak-to-peak distance of the first-order reflection by using the Bragg law, $2d = m\lambda/\sin\theta$, where d is the interlamellar repeat distance, m is the number of the diffraction order, λ is the wavelength of the incident beam (1.542 Å), and θ is the Bragg angle obtained from the relation $\tan 2\theta = x/y$ where x is half the distance between two maxima of the same order and y is the film-to-sample distance (5.8 cm). The thickness of the bilayer including the phosphocholine head-group zone is calculated as the difference between the interlamellar repeat distance and the width of the free water layer (22 Å) as described by Small (1967) and Janiak et al. (1976).

Gel Chromatography. Reconstituted vesicles were subjected to molecular sieve chromatography at 4 °C on Sepharose 4B and Bio-Gel A-150m columns as described by Huang (1969). The column was pretreated with 40 mg of partially sonicated egg PC vesicles in 1 mL eluant (0.2 M potassium oxalate, pH 7.5). Reconstituted vesicles at a concentration of 20 mg of lipid/mL and 0.5 mg of protein/mL in eluant were fractionated on the column operating at a head pressure of 15 cm of eluant. Linear flow rate was 6.5 mL/(cm^2 min), volumetric flow rate was 0.23 mL/min, and fractions of 1.5 mL were collected for protein and phospholipid determination. Phospholipid was determined by the method of London & Feigenson (1978). Protein was measured as described above under Protein Determination (Method B).

Electron Microscopy. Freeze–thaw reconstituted egg PC vesicles were prepared at 8.1 mM phospholipid in 0.02 M potassium oxalate, pH 7.5. The suspensions were then placed in 0.4-mL capacity polypropylene microcentrifuge tubes and centrifuged at 25000 rpm in an SW27 rotor at 4 °C for 30 min as described above under Small Scale Fractionation. The pellet was subsequently embedded in Epon-Anasolete epoxy resin, sectioned, and positively stained with uranyl acetate and

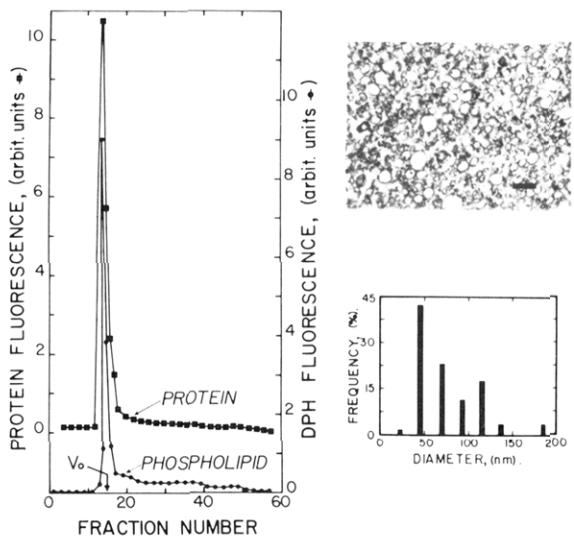

FIGURE 1: Characterization of egg PC freeze–thaw vesicles by fractionation on Sepharose 4B (left) and electron microscopic examination of thin sections (right). Conditions under which gel filtration was performed and the methods used in analysing fractions for protein and phospholipid are described under Experimental Procedures. The void volume as determined with Blue Dextran 2000 is indicated by V_0. The bar in the electron micrograph represents 230 nm, and below the micrograph is the corresponding frequency distribution of particle diameters.

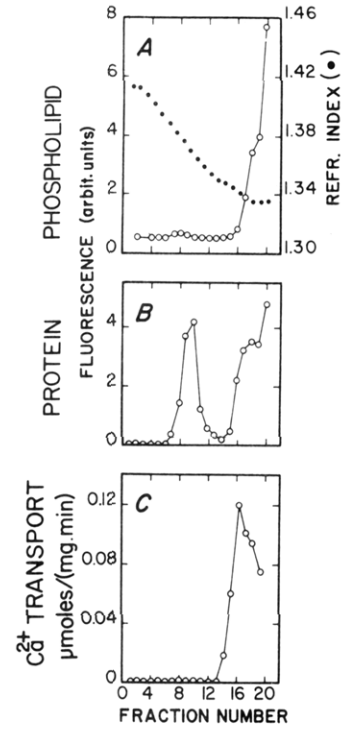

FIGURE 2: Sucrose density gradient fractionation of freeze–thaw reconstituted Ca^{2+}ATPase vesicles prepared with egg PC. Details of the fractionation method and analysis of fractions for protein, phospholipid, refractive index, and Ca^{2+} transporting activity are described under Experimental Procedures. Under standard fractionation conditions, unincorporated protein bands between fractions 7 and 11.

lead citrate (Telford & Matsumura, 1970). Grids were viewed in an Allied Electronics Industries Electron Microscope, Model EM6B, operating at 60 kV.

Results

Characterization of the Freeze–Thaw Reconstitution System. Vesicles reconstituted by the freeze–thaw procedure had not been well characterized, and it was necessary to do this before using these vesicles in the proposed series of experiments.

The freeze–thaw method involves a brief sonication step producing nonuniformly sized vesicles (see below). We were concerned about the possibility that the Ca^{2+}ATPase protein was selectively incorporating into vesicles of a particular size, as in the case of cytochrome oxidase (Eytan & Broza, 1978). When analyzed by gel chromatography using Sepharose 4B columns, the elution profiles indicated that this was not the case (Figure 1). Identical results were obtained with agarose A-150m columns (data not shown). In either case, the elution profiles show that approximately half of the phospholipid and protein eluted in the void volume fraction. This suggests that the vesicles range in diameter from 25 nm to greater than 150 nm (Eytan & Broza, 1978; Rhoden & Goldin, 1979) and was confirmed by electron microscopic examinations of thin sections (Figure 1). The electron micrographs show that 75% of the vesicles have diameters less than 100 nm while the remaining 25% lie in the 100–200-nm range. Given that the number of phospholipids per vesicle is described by $0.093\ d_i^2$, where d_i is the internal diameter of a vesicle [see Enoch & Strittmatter (1979) for dimensions used], it is estimated that phospholipid mass is approximately evenly distributed between large (100–200 nm) and small (25–100 nm) vesicles. Another interesting feature revealed in these electron micrographs is that the vesicles are unilamellar, a result which greatly simplifies interpretation of enzymatic activity measurements.

Since the freeze–thaw method does not involve the use of detergents, lipids and protein do not form a homogeneous mixture prior to reconstitution. The question then arises as to the fraction of protein which is actually incorporated into the vesicle membrane during reconstitution. When the reconstitution mixture was subjected to density gradient centrifugation, it was found that under standard conditions 50% of the protein becomes incorporated (Figure 2). This result, that about half of the protein is not incorporated into vesicles, presented a distinct problem in attempting to use these freeze–thaw vesicles for the type of experiments planned. For example, if the free protein fraction did not equilibrate fully with exogenous lipid and/or behaved differently from incorporated protein to the various phospholipid combinations used, both enzyme activity and fluorescence quenching measurements could be rendered uninterpretable. It was deemed necessary therefore to either (a) alter the method of reconstitution to secure 100% incorporation or (b) separate free protein from the reconstitution mixture.

Various approaches were used to get the Ca^{2+}ATPase enzyme to reconstitute fully. For example, the length and speed of the thawing step was varied, the concentration of phospholipid and protein (at fixed phospholipid/protein ratio) was changed, and the entire freeze–thaw procedure was repeated many times on the one sample, all to no avail. However, almost complete reconstitution was observed when the phospholipid-to-protein molar ratio exeeded 5000:1 or when the protein was reconstituted incrementally (one-third to one-fourth of the total protein reconstituted at any given time and the reconstitution procedure repeated 3 or 4 times). Although it was possible to obtain high percentage incorporation, neither of the latter two methods were satisfactory for experiments where large numbers of samples were to be prepared in a relatively short period of time with a minimum of exogenous phospholipid. Accordingly, the other alternative, that of separating unincorporated protein from the reconstitution mixture, was used. To this end, a microfractionation method

Table I: Dependence of the Thermotropic Phase Transition Temperature (T_t), Bilayer Thickness, Leakiness to Ca^{2+}, and Enzymatic Activity of the Ca^{2+}ATPase on the Fatty Acyl Chain Characteristics of the Phosphatidylcholines Used To Reconstitute the Protein

phosphatidylcholine type		T_t (°C)[a]	bilayer thickness (nm)[a]	trap Ca^{2+} when loaded[b] actively	trap Ca^{2+} when loaded[b] passively	Ca^{2+} transport [μmol/(mg min)][c]	ATP hydrolysis [μmol/(mg min)][c]	Ca^{2+}/ATP[d]
lauric	12:0	0	3.6	–	±	0	0	
myristic	14:0	24	4.1					
palmitic	16:0	42	4.3					
stearic	18:0	54	4.7					
dodecenoic	12:1 (11)		3.6	–	–	0	0	
myristoleic	14:1 (9c)		3.8	+	+	0.07	0.14	0.50
palmitoleic	16:1 (9c)		4.3			0.31	0.72	0.43
oleic	18:1 (9c)		4.3			0.46	1.22	0.38
vaceenic	18:1 (11c)		4.1			0.44	1.34	0.32
eicosenoic	20:1 (11c)		4.6			0.56	0.85	0.66
erucic	22:1 (13c)	11	5.0	+		0.68	1.30	0.52
nervonic	24:1 (15c)	24	5.3	+		0.18	0.60	0.30
myristelaidic	14:1 (9t)		3.7	+	+	0.03	0.04	0.75
palmitelaidic	16:1 (9t)		4.1	+	+	0.17	0.84	0.20
elaidic	18:1 (9t)	11	4.4	+	+	0.40	1.12	0.36
petroselaidic	18:1 (6t)	23	4.5			0.26	0.71	0.37
brassidic	22:1 (13t)	41	4.2					
linoleic	18:2 (9c, 12c)		4.2			0.52	1.34	0.39
egg			4.3	+	+	0.45	1.45	0.31
(7,6)PC			4.2			0.62	0.99	0.63

[a] Details of the methods used to measure T_t and bilayer thickness are described under Experimental Procedures. [b] The inability of vesicles to trap measurable amounts of Ca^{2+} is denoted by (–), trapping Ca^{2+} as depicted in Figure 3 is denoted by (+), and (±) denotes very leaky vesicles (half-time for unloading ~5 min). Leakiness of passively loaded dierucoyl- and dinervonoyl-PC vesicles was not measured. [c] Values reported represent the average of duplicate determinations. [d] The Ca^{2+}/ATP ratio is an index of the efficiency of the transport process. See text for details.

was developed as described under Experimental Procedures and proved particularly convenient for these experiments.

Having characterized the freeze–thaw reconstituted vesicles, it was now possible to examine (a) the effect of varying such properties of the PC hydrocarbon region as fatty acyl chain length and degree, position, and isomeric type of unsaturation on the Ca^{2+} transporting and ATP hydrolyzing activities of the reconstituted enzyme and (b) the relationship of such effects on enzymatic activity to the affinity of the enzyme for the different PC species.

Ca^{2+} Uptake and ATPase Activities of the Reconstituted Ca^{2+}ATPase. Ca^{2+}ATPase, when reconstituted into vesicles which are not leaky to Ca^{2+}, displays an ATP hydrolyzing activity which is tightly coupled to Ca^{2+} transport. The enzyme was reconstituted into vesicles prepared from different PC species, and both Ca^{2+} uptake and ATPase activities were determined. All activity measurements were made at temperatures above the T_t of the phospholipids used in reconstituting the enzyme as (a) reported in the literature (van Dijck et al., 1976; Mabrey & Sturtevant, 1976), (b) estimated by comparison with the T_t of a series of dioctadecenoyl-PC configuration isomers (Barton & Gunstone, 1975), or (c) determined by DTA (Table I). This meant that in the case of all but two phospholipids activity measurements could be carried out at room temperature. The exceptions were dipetroselaidyl-PC and dinervonoyl-PC (T_t = 22.5 and 24 °C, respectively) where measurements were made at 31 °C and enzyme activities were corrected to room temperature by multiplying by the ratio of the enzymatic activity in egg PC at room temperature to that at 31 °C. The results, presented in Table I, show that both the Ca^{2+}-transporting and the ATPase activities of the enzyme are sensitive to changes in the fatty acyl chain length of the phospholipid used to reconstitute the protein, with little sensitivity to details of unsaturation. Maximum activity is observed with PC's of intermediate chain length, and activity is attenuated as the length of the alkyl chains increase or decrease beyond the optimum range.

Both enzymatic activities of the Ca^{2+}ATPase display a similar dependence on the acyl chain characteristics of the PC's used to reconstitute the protein (Table I). The overall efficiency of the transport process in the reconstituted vesicles is reflected in the molar ratio of Ca^{2+} uptake and ATP hydrolysis (Ca^{2+}/ATP) calculated from initial reaction velocities. The ratios observed in this series of experiments varied nonsystematically from 0.20 to 0.75, with an average value of 0.44 ± 0.16 for all PC species giving active vesicles. It is difficult to make useful comparisons between efficiencies observed in this work and those reported in the literature since measured values markedly depend on the phospholipid-to-protein ratio, the method of reconstitution, the type of purified Ca^{2+}ATPase and phospholipids used, and whether reconstituted vesicles were fractionated or not (Martonosi, 1971; Racker & Eytan, 1973, 1975; Knowles et al., 1976; Warren & Metcalfe, 1977). The present results suggest that the enzyme activities remain *coupled* to the same extent regardless of the characteristics of the hydrocarbon region of the PC bilayer in which the protein is inserted. In contrast, enzyme activity *level* is probably reflecting the sensitivity of this transport protein to the properties of the vesicle membrane as determined by the fatty acyl chains of the PC's used to reconstitute the enzyme. Accordingly, the depressed Ca^{2+} transporting rates in PC's with either long or short alkyl chains are unlikely to be an artifact introduced as a result of these phospholipids giving rise to vesicles that are leaky to Ca^{2+}, because if the attenuated Ca^{2+} transporting activities were simply due to vesicle leakiness it is expected that the system would become uncoupled, giving rise to elevated ATP hydrolyzing rates. This is not observed; i.e., the two enzymatic activities vary in parallel and remain coupled to approximately the same extent (Table I). However, since the literature has no information regarding the ability of these particular PC species to form sealed vesicles, for completeness, the leakiness to Ca^{2+} of these reconstituted vesicles was determined (Figure 3, Table I). With the exception of dilauryl-PC and didodecenoyl-PC, all phospholipids examined gave vesicles which are impermeable to Ca^{2+} on the

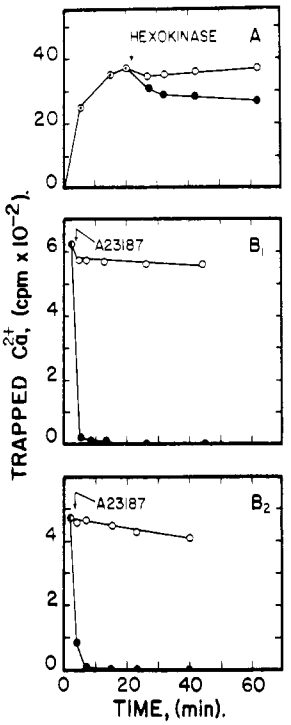

FIGURE 3: Measurement of the leakiness to Ca^{2+} of egg PC freeze–thaw vesicles loaded (A) actively and (B) passively with $^{45}Ca^{2+}$. Passively loaded vesicles were prepared (B_1) with and (B_2) without the Ca^{2+}ATPase enzyme. Details of the methods involved are described under Experimental Procedures. In (A), vesicles were actively loaded with $^{45}Ca^{2+}$ (⊙) and efflux from these vesicles was monitored following treatment with a hexokinase (●) or a hexokinase blank (○) solution. In (B), vesicles were passively loaded as described under Experimental Procedures, and efflux was measured following treatment of the vesicles with A23187 (●) or ethanol (○) as a control.

time scale of the Ca^{2+} uptake assay (0.5 min). It is concluded, therefore, that the activity of the enzyme in vesicles prepared from phospholipids which trap Ca^{2+} reflects a sensitivity of the enzyme to the hydrocarbon region of the lipid as distinct from an indirect leakiness effect. In the case of the two shortest chained PC's, both lipids produce vesicles (as judged by gel permeation chromatography; data not shown) which are extremely leaky to Ca^{2+}. From this result alone, it is not possible to determine whether the inability to transport Ca^{2+} is due to the unique hydrocarbon properties of these lipids or to the fact that such vesicles are leaky. However, by the reasoning presented above regarding the tight coupling of the two activities of the enzyme, we conclude that for all lipids tested enzymatic activity level reflects a direct effect of the PC acyl chain characteristics on the functioning of the Ca^{2+}ATPase enzyme.

The observed dependence of enzymatic activity on the fatty acyl chain properties of the PC's used to reconstitute the Ca^{2+}ATPase suggests a possible link between activity of the enzyme and bilayer thickness, as determined by the number of carbons in the fatty acyl chains (Table I). Accordingly, bilayer thickness of vesicles prepared from the individual PC species which ranged in chain length from 12 to 24 carbon atoms was measured by using low-angle X-ray diffraction (Table I). When enzymatic activity is now plotted as a function of measured bilayer thickness, the profile shown in Figure 4 is obtained. This result suggests that the Ca^{2+}ATPase is maximally active in vesicles having a suitable range of bilayer thickness, and only large deviations from this optimum thickness range are sensed by the enzyme and are reflected in an attenuated activity. We note that the drop in enzymatic activities in the membranes of greatest thickness was reproducible in three sets of experiments.

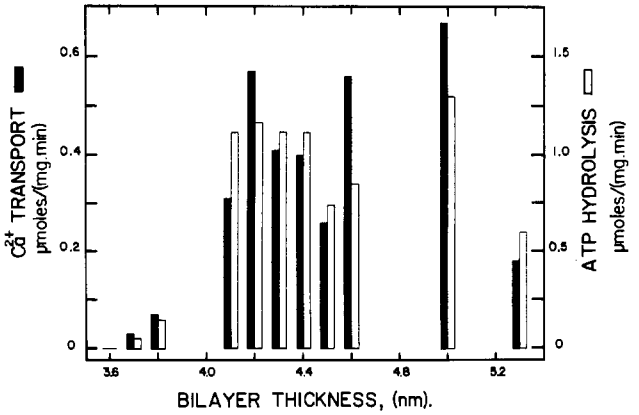

FIGURE 4: Dependence of the ATP-hydrolyzing and Ca^{2+}-transporting activities of the Ca^{2+}ATPase enzyme on bilayer thickness of vesicles in which the protein is reconstituted.

Binding Affinity Measurements. A method has been developed in this laboratory whereby the relative binding affinity of an integral membrane protein for different lipid components of the membrane can be determined. The method is a fluorescence quenching technique which involves reconstituting the protein of interest into membranes containing varying proportions of a spin-labeled phospholipid together with a second lipid, hereafter referred to as the test lipid, holding the total amount of phospholipid in the system constant. The intrinsic fluorescence F of the protein is monitored as a function of the mole fraction of spin-labeled phospholipid $\chi_{(7,6)PC}$ with the first test lipid, lipid A, and the experiment is repeated with a second test lipid, lipid B, generating two fluorescence quenching curves. The fluorescence quenching data are treated somewhat differently from data in the preceding papers (London & Feigenson, 1981a,b) in order to distribute experimental error in a more realistic way over the entire course of the experiment. That is, in order not to require the initial part of each quenching curve to begin with an identical unquenched fluorescence value $F_0 = 1$ (which has the effect of artificially scaling the rest of the data), the raw data are plotted as $(F - F_{min})/(F_0 - F_{min})$, i.e., the fractional change in fluorescence vs. $\chi_{(7,6)PC}$ where F_{min} represents the residual fluorescence at $\chi_{(7,6)PC} = 1$. Fluorescence quenching data obtained for the Ca^{2+}ATPase enzyme reconstituted in a variety of PC species by using (7,6)PC as the quenching phospholipid are presented in Figure 5. As described in the preceding papers, points of equal fractional quenching on these curves represent the mole fraction of (7,6)PC which must be added to the system to have identical numbers of (7,6)PC molecules in contact with the protein in the membrane. A quantitative measure of the relative binding constant of one test phospholipid compared to another can be obtained from an analysis of the quenching curves. This simply involves determining the χ_A and χ_B, giving equivalent levels of residual fluorescence as the $\chi_{(7,6)PC}$ is changed, and plotting χ_B^{-1} vs. χ_A^{-1} according to eq 1. The slope of the line in this double-reciprocal plot gives the requisite quantity $K'_{B/A}$, the apparent relative binding constant of lipid B compared to lipid A.

$$\chi_B^{-1} = \chi_A^{-1}(K'_{B/A}) + (1 - K'_{B/A}) \quad (1)$$

Double-reciprocal plots of the quenching data for the Ca^{2+}ATPase enzyme in Figure 5 are shown in Figure 6 wherein all PC binding is compared to dioleoyl-PC as the reference lipid. The slopes of the lines were determined by

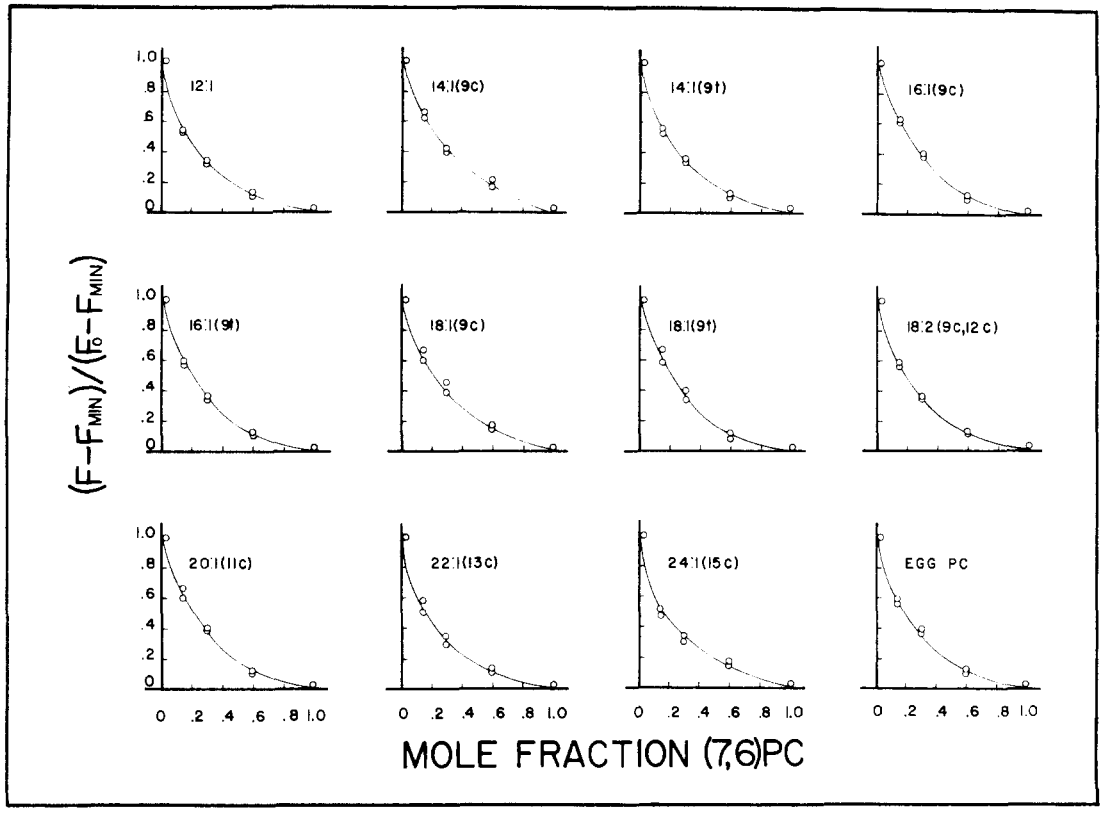

FIGURE 5: Quenching of Ca^{2+}ATPase fluorescence by (7,6)PC in reconstituted freeze–thaw vesicles prepared with different PC species. Fatty acyl chain length and unsaturation characteristics of the PC's used are given in the upper right-hand corner of each curve. Methods used to obtain the fluorescence quenching curves are described under Experimental Procedures and Binding Affinity Measurements under Results. In these curves, we are plotting the fractional change in fluorescence vs. the mole fraction of quenching lipid, (7,6)PC, in the exogenous PC fraction.

linear regression analysis and are shown on each graph in Figure 6. It can be seen that the data fit the linear regression line very well (coefficient of determination, $r^2 \geq 0.98$). The mean value of the $K'_{B/A}$ for the different lipids is 1.07, with a standard deviation of 0.18. Thus, the Ca^{2+}ATPase, when reconstituted into PC's with widely varying acyl chain characteristics, displays no measurable preferential binding of one PC species compared to any other.

Discussion

We have determined the sensitivity of the catalytic properties of an integral membrane protein, Ca^{2+}ATPase from SR, to the fatty acyl chain characteristics of the lipids used to reconstitute it into enzymatically active vesicles. The enzyme appears to be sensitive only to the effective fatty acyl chain length, which determines the thickness of the bilayer in which the protein is inserted, and displays little sensitivity to details of unsaturation in the fatty acyl chains. Along with the enzymatic activity studies, we have investigated the nature of the interaction between the Ca^{2+}ATPase protein and the lipids used to provide vesicles with bilayers of varying hydrocarbon region thicknesses. The enzyme does not preferentially bind any of the lipids tested, regardless of fatty acyl chain length or details of unsaturation.

The binding affinity and enzymatic activity results, taken together, suggest a number of models for the manner in which this integral protein interacts with membranes of different thicknesses (Figure 7). For any of these models, the fluorescence quenching results can be accounted for by some appropriate, but as yet unknown, arrangement of fluorophors in the protein. Accordingly, the only criteria which must be satisfied by the models are (a) that the observed dependence of enzymatic activity on PC acyl chain length must be reasonable and (b) that energetically expensive changes incurred as a result of changing bilayer thickness are not allowed since the experimentally determined relative binding constants for all the PC's tested are approximately unity.

Model A. In this model, the protein spans the membrane and is anchored to just one surface of the bilayer. The head group of the lipid in the other half of the bilayer, with which the protein does not interact strongly, is free to position itself at any point on the surface of the protein, depending on the hydrocarbon chain length. Accordingly, most of the lipid–protein interaction energy is confined to one surface of the bilayer and is thus relatively insensitive to changes in bilayer thickness.

Concerning the dependence of enzymatic activity on the width of the hydrocarbon region of the membrane, the model suggests that activity is maximized when the protein is inserted in a bilayer of intermediate thickness. However, in membranes that are either too thin or too thick, conformational changes in the protein and/or aggregation in the plane of the membrane brought about by these unfavorable environments might be expected to result in a lowered enzymatic activity. Since it appears that the enzyme cannot catalyze ATP hydrolysis without net movement of Ca^{2+} through the active enzyme species, i.e., the two activities remain coupled to the same extent in this system, then a perturbation that affects Ca^{2+} transport should similarily affect ATP hydrolyzing activity and vice versa. In view of the proposed model, therefore, the observed enzymatic activity profile as a function of bilayer thickness seems reasonable.

Model B. In this case, the protein spans the membrane regardless of bilayer thickness by adjusting its orientation and/or conformation. Thus, in membranes of intermediate thickness, enzymatic activity is greatest. However, at the

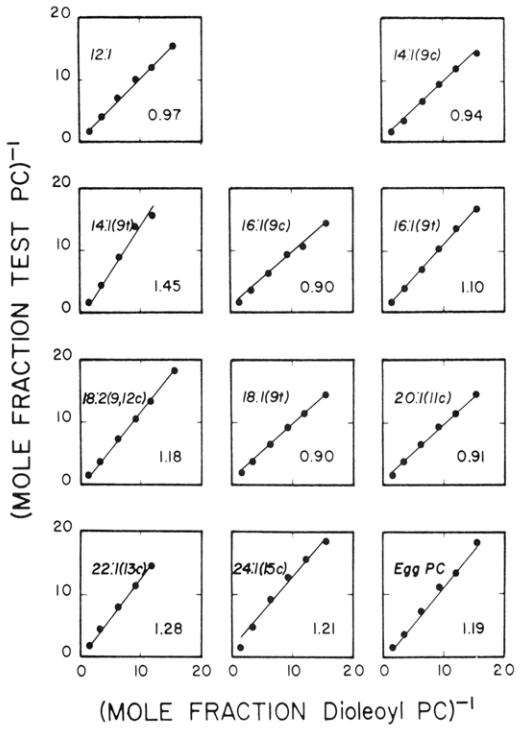

FIGURE 6: Double-reciprocal plots of the fluorescence quenching data presented in Figure 5 according to eq 1 in the text with dioleoyl-PC as the reference lipid. The lines drawn were obtained by linear regression analysis. PC identity and the apparent relative binding constant are given in the upper left- and lower right-hand corners of each curve, respectively. For all PC species examined, the coefficient of determination r^2 obtained from linear regression analysis was $0.98 \leq r^2 \leq 1$.

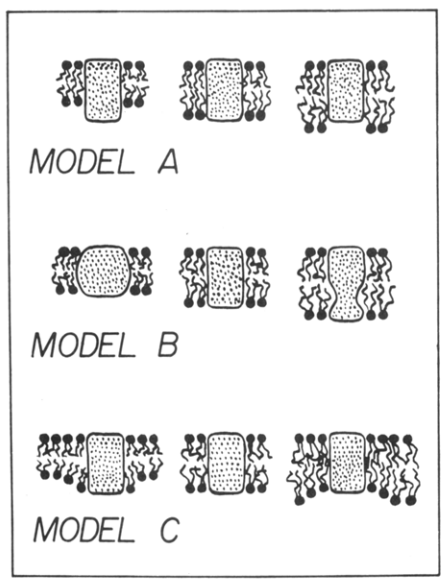

FIGURE 7: Schematic illustration of the proposed manner in which the Ca^{2+}ATPase protein interacts with membranes of varying thicknesses. In model A, the protein interacts strongly with just one surface of the bilayer. Model B has the protein accommodating to bilayer thickness by changing its conformation. In model C the protein spans the membrane by locally deforming it when bilayer thickness is not optimal.

extremes of membrane thickness, the adopted conformation/orientation does not support maximum activity.

Little is known about the nature of the energetics of possible conformational or orientational changes which proteins might undergo as a result of changes in bilayer thickness. It is difficult, therefore, to determine how realistic this model might

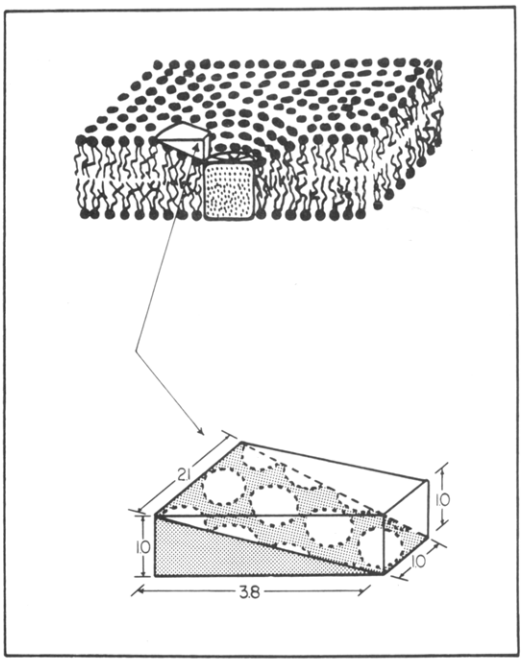

FIGURE 8: Schematic illustration of how the Ca^{2+}ATPase protein locally deforms a thick membrane. The region of the bilayer deformed for a single phospholipid binding site is magnified, and the dimensions are given in nanometers. In this model, it is assumed that (a) the Ca^{2+}ATPase enzyme has 30 phospholipid binding sites on its surface (Hesketh et al., 1976; Warren et al., 1975), (b) the perturbation of nearby lipids decays linearly and extends to a maximum of four lipid shells from the protein [cf. Knowles et al. (1979)], and (c) for simplicity the protein dimples just one surface of the bilayer. See text for details.

be. However, given that the enzyme does not preferentially bind any of the lipids tested, the energy involved in these changes must be small, i.e., $\leq kT$ on a per lipid binding site basis, where k is the Boltzmann constant and T = 295 K.

Model C. Here, the protein is considered to deform the lipid bilayer when the thickness of the membrane is not optimal. Such a model has been proposed to explain anesthetic potency (Ashcroft et al., 1977; Haydon et al., 1977) and the variation in ionophoric activity of gramicidin A in bilayers of different thicknesses (Hladky & Haydon, 1972; Veatch et al., 1975). For an explanation of the observed activity effects under conditions where the bilayer is strongly deformed, an ill-defined stress on the protein would be postulated to attenuate enzymatic activity.

With regard to model C, two types of calculations are described in order to estimate the energy involved in deforming the membrane to accommodate the protein. This energy of deformation can then be used to calculate a predicted $K'_{B/A}$ for comparison with the experimentally observed value and evaluation of the proposed model. We assume in all cases that the bilayer is essentially incompressible (volumetric modulus of compressibility = 10^{10}–10^{11} dyn/cm^2; Evans & Hochmuth, 1978) such that, regardless of the type of deformation involved, volume is conserved. Furthermore, the work done in deforming the bilayer is that which is involved in creating hydrocarbon–water interface and in lowering the entropy of the lipid. Both calculations apply to membranes which would *dimple* locally around the protein. As yet, we have no suitable way to estimate the energetics of the deformation process in thin membranes which would *thicken* locally to accommodate the protein.

The first calculation models the dimpling around the protein as an elastic deformation of the membrane (Figures 7 and 8). Since it is the *relative* binding constant which is of interest, we calculate the *relative* deformation energy, and a simple way

of doing this is to assume that the deformation of the bilayer by the protein is zero for a system of intermediate chain-length lipid such as dioleoyl-PC or (7,6)PC. The calculation is made for the largest difference in chain length, 10 Å, which is observed when the relative binding of dinervonoyl-PC and dioleoyl-PC is compared (Table I). Given this assumption and those presented in the legend to Figure 8, the bilayer volume deformed per phospholipid binding site upon inserting the protein into a dinervonoyl-PC membrane is estimated to be 2.6×10^3 Å^3. Assuming an elastic thickness modulus of compressibility of 10^8 dyn/cm^2 (Evans & Hochmuth, 1978) or 2.4×10^{-24} cal/Å^3 implies that per site the enegy of deformation is 6.4×10^{-21} cal or 3.76×10^3 cal/(mole site). If we consider this to be the major contribution to the free energy of the exchange reaction

$$B + PA \rightleftharpoons A + PB$$

where A and B represent dioleoyl- and dinervonoyl-PC, respectively, and P a phospholipid binding site on the protein, then $\Delta G'_{B/A}$ for the exchange is 3.76×10^3 cal/(mole sites) and $K'_{B/A} = \exp(-\Delta G'_{B/A}/RT) = \exp(-6.38) = 0.002$ at room temperature. When a more conservative estimate of 3 is used for the number of lipid shells perturbed by the protein, the deformed volume per site is 2×10^3 Å, corresponding to a $K'_{B/A}$ of 0.007. Since the experimentally observed $K'_{B/A}$ value is unity, it seems highly unlikely that this model, wherein the bilayer dimples upon insertion of the protein, correctly describes the physical situation.

Another way of looking at the dimpling process is to consider a local thinning of the membrane around the protein as involving interdigitation of the phospholipid alkyl chains from opposite sides of the bilayer (cf. Taylor & Haydon, 1966). Given the volumetric incompressibility of the membrane, upon thinning, the surface area per phospholipid molecule increases. This process is expected to be energetically expensive, however, because the formation of additional hydrocarbon–water interface necessarily increases interfacial free energy (White, 1974). We have estimated the energy required for such an interdigitation process as applied to the Ca^{2+}ATPase system (see Figure 9 for details) to be 3.2 kcal/(mole sites), assuming that the free energy change upon transfer of a CH_2 group from pure liquid hydrocarbon to an aqueous solution is approximately 800 cal/mol at 25 °C (Tanford, 1973). By the reasoning presented above, this value of 3.2 kcal/mol gives a $K'_{B/A}$ of 0.004 at room temperature. This value is sufficiently different from the experimentally observed value of unity to allow us to discount interdigitation as a likely event.

On the basis of conductivity measurements carried out with gramicidin in black lipid membranes, Veatch et al. (1975) have estimated the free energy involved in a local dimpling of the membrane by the ionophore to be approximately 4.5 kcal/mol. Unfortunately, a comparison of this value with the calculations presented above for the reconstituted Ca^{2+}ATPase system is not very meaningful because the systems are so different; e.g., the gramicidin work was carried out by using planar bilayers prepared from monoacylglycerides and alkane solvents.

In summary, the calculations on bilayer deformation energy for model C for both the interdigitation and the elastic modulus methods, lead to values of $K'_{B/A}$, for lipids differing in chain length by 10 Å, which are sufficiently different from the observed value of unity as to make these physical pictures unlikely. However, we feel that the purpose of these calculations is to explore certain physical possibilities, and the quantitative nature of the result is somewhat arbitrary, being based upon the necessity of choosing particular geometric models and specific values for the elastic modulus and for the free energy change for the transfer of a methylene group from hydrocarbon to water.

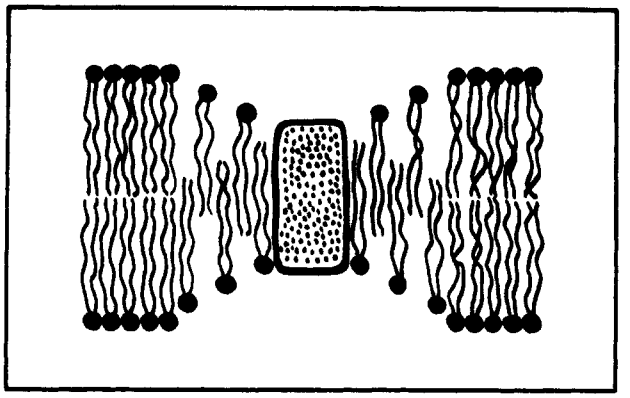

FIGURE 9: Schematic illustration of local dimpling of the membrane around the Ca^{2+}ATPase protein involving interdigitation of the PC acyl chains from opposite sides of the bilayer. To calculate the energy required for interdigitation, we are assuming (a) the dimensions used in Figure 8, (b) that interdigitation occurs in the first three boundary lipid layers, and (c) that the extent of penetration of the aqueous phase into the hydrocarbon region of the membrane decreases linearly with distance from the lipid–protein interface with exposure of 2, 1, and 0.5 CH_2 groups per acyl chain for the first three shells, respectively. From Figure 8, there are 1, 1.25, and 1.5 phospholipids per binding site, respectively, in the first three boundary shells of the protein. Since there are two acyl chains per phospholipid, this gives a total of eight CH_2 groups exposed to the aqueous phase per binding site. However, as depicted in this figure, it is expected that only half of the surface of the CH_2 group would be available for interaction with water, reducing the number of exposed CH_2 groups to four per binding site.

The problem of lipid–protein interactions in membranes and the possibility of a preferential adsorption among lipids with different acyl chains have been examined theoretically by Wolfe (1979). The preferential binding of one species of lipid from a mixed bilayer is opposed by the increase in the entropy of mixing term for the free energy of the system. If the composition of the boundary layer and the bulk lipid are to differ, then the entropy of demixing must be countered by differences among the various molecular species in the amount of internal free energy lost or gained upon adsorption to the protein. Using the mean field theory of Marčelja (1976), Wolfe (1979) and Wolfe & Bates (1980) calculated that this change in internal free energy (the free energy per molecule not including mixing entropy) is always small and only weakly dependent on temperature. Thus, the transition temperature difference of the component lipid species is not expected, on these theoretical grounds, to produce a preferential adsorption of one species where molecular packing constraints do not influence lipid segregation [see Israelachvili et al. (1976); Israelachvili, 1977; Carnie et al., 1979]. Our experimental findings that the Ca^{2+}ATPase protein does not preferentially bind lipids with widely varying transition temperatures are consistent with the theoretical calculations of Wolfe but at variance with the implicit assumptions used by Owicki et al. (1978). We note, however, that the theoretical calculations described above are based on the assignment to boundary lipid of an order parameter intermediate between that of a gel phase and a liquid-crystal phase lipid. Accordingly, it is possible that this partial freezing model is not applicable if the adsorption (binding) process is not at all related to a liquid-crystal to gel phase transition.

We have measured the $K'_{B/A}$ for each test PC species over the entire $\chi_{(7,6)PC}$ range studied and found that it does not differ significantly from unity regardless of whether spin-labeled PC ($\chi_{(7,6)PC} > 0.5$) or test PC ($\chi_{(7,6)PC} < 0.5$) is the predominant

lipid. This observation suggests ideal mixing in all proportions of the test and spin-labeled PC's and also implies that the relative binding affinity of the enzyme is not influenced by the composition and, in particular, the thickness of the bilayer in which it is inserted. We emphasize that it is the *relative* binding constant that is invariant since we have no information on the *absolute* binding constant for any of the PC's examined. However, regardless of the particular PC's used, it is possible that for any given $\chi_{(7,6)PC}$ the absolute affinity of the protein for either lipid species is identical but that this absolute affinity might change as the $\chi_{(7,6)PC}$ changes. In other words, the *absolute* affinity of the protein for lipids could vary, for example, as a function of membrane thickness, even though the apparent *relative* affinity is insensitive to membrane thickness. This problem of the absolute affinity of protein for lipid cannot, in principle, be answered with the fluorescence quenching method since this method can only provide relative binding constants.

The T_t of the higher melting lipids was determined by differential thermal analysis and, in all cases, experiments were performed with the component lipids in the liquid-crystalline phase. The most extreme phospholipid combination used in this study involves mixtures of didodecenoyl-PC or dinervonoyl-PC with (7,6)PC, giving a maximum chain-length difference of six carbon atoms. In this regard, it is worthwhile noting that the binary system, didodecanoyl-PC/distearoyl-PC, exhibits monotectic phase behavior (Mabrey & Sturtevant, 1976; Ladbrooke & Chapman, 1969) in that immiscibility is observed in the gel state only. At all temperatures above the liquidus line, these two lipids, which also differ in chain length by six carbon atoms, are miscible in all proportions. By comparison with this series of saturated PC's, therefore, it seems reasonable to assume that the test and spin-labeled PC's are miscible in all proportions in the liquid-crystal phase under present experimental conditions. The fact that identical fluorescence quenching curves were obtained with PC's of intermediate as well as of long and short chain length lends support to this supposition.

Our results demonstrate that the Ca^{2+}ATPase enzyme is maximally active when inserted into bilayers of suitable thickness and that deviations from this broad optimum result in lowered enzymatic activity. Provided that the component lipids are in the liquid-crystalline state, the enzyme appears to be relatively insensitive to the isomeric type, number, and position of the double bonds in the acyl chains. We have not been able to monitor the activity of the enzyme in membranes thicker than those of dinervonoyl-PC because of the unavailability of the appropriate fatty acids and the fact that the T_t of the corresponding PC's would be too high for reliable activity measurements to be made.

It is difficult to compare the results of the present investigation with other studies of membrane proteins involving acyl chain differences because of the disparate methodologies and materials used. For example, in one case, the protein (Ca^{2+}ATPase from SR) was delipidated by using phospholipase digestion followed by albumin washes, and reactivation of ATPase activity was examined with free fatty acids of different chain lengths (The & Hasselbach, 1973). In other systems, the reconstitution methods involve the use of detergents, and activity measurements were made with some of the phospholipids at or below their T_t (Warren et al., 1974; Vik & Capaldi, 1977; Warren & Metcalfe, 1977). Boggs & Moscarello (1978) have examined the interaction of lipophilin, a proteolipid fraction from myelin, with PC's of different acyl chain length by using differential scanning calorimetry. The hydrophobic protein was reconstituted into lipid vesicles by dialysis in the absence of detergent. On the assumption that boundary and bulk lipid are in slow exchange in this system, these results indicate that phospholipid binding does not depend on acyl chain length, in agreement with our observations on the Ca^{2+}ATPase enzyme.

Throughout this discussion we have proposed that the enzymatic activity of the Ca^{2+}ATPase reconstituted into vesicles prepared from PC's with varying acyl chain characteristics is sensitive to bilayer thickness as determined by the hydrocarbon chain length of the phospholipids comprising the membrane. It is possible, however, that the enzyme is responding not to bilayer thickness but to some other property of the membrane which changes as the chain length and unsaturation characteristics of the hydrocarbon region change. Since all activity measurements were made above the T_t of the host phospholipid, the phase of the lipid is unlikely to be a variable. One property which might be important is the so-called "microviscosity" of the hydrocarbon region of the membrane, which could change with the reduced temperature T_R, i.e., the number of degrees above the T_t at which activity measurements are made, divided by T_t ($T_R = (T - T_t)/T_t$). However, microviscosity is unlikely to be an important variable in these experiments since the activity of the enzyme in membranes of intermediate thickness does not vary much whereas the T_R of the phospholipids in these membranes does vary significantly, ranging from 0 to 0.3 in the case of dipetroselaidoyl- and dipalmitoleoyl-PC, respectively.

While the phospholipid binding studies reported here show that the Ca^{2+}ATPase protein displays no acyl chain binding specificity, it is necessary to point out that the PC's examined in this series of experiments had the same fatty acyl groups at both sn_1 and sn_2 positions. It cannot be excluded that a preference exists for the more common mixed phospholipid species (Marai & Kuksis, 1973a,b; Madeira & Antunes-Madeira, 1976), and experiments are in progress to examine this question.

The Ca^{2+}ATPase enzyme was reconstituted by the freeze–thaw method rather than by some other procedure primarily because this method does not involve the use of detergents. The presence of residual detergent in the membrane may act in some way to randomize the distribution of lipids about the enzyme (Changeux, 1974), thus masking any real preferential adsorption to the surface of the protein. It is possible, although highly unlikely considering the phospholipid/protein ratio used for reconstitution, that the trace amount of octyl glucoside carried over with the Ca^{2+}ATPase from the enzyme purification step influences the observed lack of acyl chain binding specificity.

The Ca^{2+}ATPase protein is reconstituted in the presence of a vast excess of phospholipid (molar ratio 1600/1), and the enzyme itself contributes less than 5 mol % of the total endogenous lipid.[2] It is a concern as to whether this endogenous pool of lipid exchanges with the exogenous phospholipid. While an exact determination of the extent of exchange has not been made, the following evidence suggests that exchange is complete. First, virtually identical fluorescence quenching curves are obtained by the cholate dilution [see London & Feigenson (1981b)] and freeze–thaw methods of reconstitution when compared for a given phospholipid species. Since cholate

[2] We have since observed that enzymatically active Ca^{2+}ATPase delipidated [following the method of Dean & Tanford (1978)] to the extent of ≤6 mol of phoshorus per mol of protein behaves identically with the nondelipidated protein described above with regard to fluorescence quenching in (7,6)PC/egg PC.

has been shown to completely equilibrate lipids around the Ca^{2+}ATPase enzyme (Warren et al., 1974), it seems likely that upon reconstitution by either method both endogenous and exogenous lipids become fully mixed. Second, a preferential binding of endogenous lipid to the exclusion of spin-labeled and other test phospholipids should be evident in the fluorescence quenching curves. In such an event, the loss of fluorescence per unit increase in the $\chi_{(7,6)PC}$ would be expected to be much less sharp than is experimentally observed. In addition, the fact that didodecenoyl-PC completely eliminates ATPase activity while the unreconstituted enzyme exhibits a significant rate of ATP hydrolysis suggests that the two lipid pools are mixed.

In summary, we have measured the sensitivity of the catalytic activity of an integral membrane protein, Ca^{2+}ATPase, to the fatty acyl chain characteristics of the lipid used to reconstitute it into enzymatically active vesicles. Regardless of the degree, position, and isomeric type of unsaturation in these acyl chains, the enzyme appears to be sensitive only to the effective fatty acyl chain length (which determines bilayer thickness). In membranes of intermediate thickness, the enzyme is maximally active. However, when inserted in a membrane that is either too thin or too thick, enzymatic activity is depressed. In parallel with the enzymatic activity studies, we have used a fluorescence quenching technique for examining lipid–protein interactions in membranes and have shown that the enzyme does not preferentially bind any of the lipids tested regardless of fatty acyl chain length or details of unsaturation. Both of these observations have been reconciled in a number of possible models for the manner in which this integral protein interacts with membranes of varying thicknesses. In certain instances it has been possible to evaluate the proposed model, and, from estimates of the energies involved, some of the models appear to be unrealistic. This is the first systematic study of the dependence of function and binding affinity of an integral protein on the fatty acyl chain characteristics of a membrane-forming phospholipid. Consequently, the generality of these observations cannot be ascertained until similar information is obtained on other membrane-bound proteins.

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