# Fluorescence Quenching in Model Membranes. 2. Determination of the Local Lipid Environment of the Calcium Adenosinetriphosphatase from Sarcoplasmic Reticulum†

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ABSTRACT: Fluorescence quenching by spin-labeled phospholipid is used to determine the affinities of different phospholipid species to an intrinsic membrane protein, the $Ca^{2+}$-ATPase of sacroplasmic reticulum. The phospholipids in contact with the $Ca^{2+}$ATPase are examined in a reconstituted sysem in which the enzyme is incorporated into a model membrane of defined phospholipid composition. The local phospholipid environment of the protein is considered to be governed at each phospholipid binding site by an equilibrium: lipid A + (lipid B – protein) ⇌ lipid B + (lipid A – protein). Phospholipid binding constants to the $Ca^{2+}$ATPase can be obtained from an analysis of fluorescence quenching data. The binding constants for a number of phospholipid species are nearly identical when the phospholipids are in the liquid-crystal state. However, temperature or $Ca^{2+}$-induced phase separation of phospholipid induces striking changes in the composition of the phospholipids in contact with the $Ca^{2+}$ATPase, relative to the overall composition of the membranes. The implications of these results with respect to the control of local phospholipid environment by intrinsic membrane proteins and the nature of the phospholipid binding sites on the proteins are discussed. General applicability of this type of fluorescence quenching study to the problem of lipid–protein interactions in membranes is considered, and this method is compared to other techniques.

The systematic investigation of lipid–protein interactions would benefit from measurements which have a sufficiently clear physical meaning that different experiments on a variety of lipid and protein systems could be compared quantitatively. There is now one account (Wolber & Hudson, 1979) which provides a theoretical basis for deriving lipid–protein binding constants in a membrane from fluorescence energy transfer experiments. Such binding constants would indeed have the sought-for physical meaning, but no such measurements have yet been reported.

Instead, lipid–protein interactions thus far have been treated qualitatively.[1] For example, electrostatic attraction of positively charged water-soluble proteins for negatively charged phospholipids has been characterized for the proteins cytochrome *c* (Vanderkooi et al., 1973; Birrell & Griffith, 1976), the synthetic polypeptide poly(L-lysine) (Hartmann & Galla, 1978), and a snake venom toxin (Dufourcq & Faucon, 1978). Release of membrane proteins into the aqueous medium by a specific phospholipase C suggests that protein–phosphatidylinositol (PI)[2] interaction occurs in some systems (Stein & Logan, 1965; Ikezawa et al., 1976; Low & Finean, 1977). Differential scanning calorimetry (DSC) has been used to compare the binding of different phospholipid species (Boggs et al., 1977a,b; Boggs & Moscarello, 1978). More recently, Brotherus et al. (1980) using electron spin resonance (ESR) found an electrostatic attraction between certain negatively charged molecules and a $Na^+,K^+$ATPase. The effect of the liquid-cyrstalline to gel transition upon the lipid composition of the environment of membrane proteins has been examined. Results indicate that many membrane proteins partition preferentially into the more fluid, and more disordered liquid crystalline phase, rather than the gel phase, when both phases coexist (Grant & McConnell, 1974; Kleeman & McConnell, 1976; Thilo et al., 1977; Gent & Ho, 1978).

In this paper, we describe the application of a fluorescence quenching technique to measure the relative binding constants of different lipids to the $Ca^{2+}$ATPase of sarcoplasmic reticulum incorporated into model membranes. These binding constants have a simple physical interpretation and can be used to deduce the local lipid composition in contact with the protein.

## Materials and Methods

The $Ca^{2+}$ATPase was purified by the method of MacLennan (1970). The specific ATPase activity of the purified protein was 15 μmol (min mg)$^{-1}$, and the activity was stable after storage at −70 °C. The purified $Ca^{2+}$ATPase, in contrast to the parent sarcoplasmic reticulum, was devoid of significant contamination by lower molecular weight proteins, as judged by Coomassie blue staining of $NaDodSO_4$–polyacrylamide gels (Weber & Osborn, 1969). The purified protein contained one higher molecular weight contaminant containing 10% of the protein stain on a gel with 30 μg of total protein. This band has about one-half the mobility of the $Ca^{2+}$ATPase and might simply represent incompletely dissociated $Ca^{2+}$ATPase.

(7,6)PA and (7,6)PG were synthesized from (7,6)PC by using phospholipase D by the same procedure developed for natural phospholipids (Yang et al., 1967). The product phospholipids were extracted into chloroform/methanol (2:1 v/v), acidified by shaking with 0.1 N HCl, and then purified

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[1] Recently Jost & Griffith (1980) described a method which utilizes electron spin resonance data to obtain lipid–protein binding constants.

[2] Abbreviations used: PI, diacyl-*sn*-glycero-3-phosphinositol; PC, diacyl-*sn*-glycero-3-phosphocholine; DLPC, 1,2-diauroyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; egg PC, diacyl-*sn*-glycero-3-phosphocholine from egg; (7,6)PA, 1-acyl-2-[2-(6-carboxyhexyl)-2-octyl-4,4-dimethyloxazolidinyl-3-oxy]glycero-3-phosphoric acid; (7,6)PC, 1-acyl-2-[2-(6-carboxyhexyl)-2-octyl-4,4-dimethyloxazolidinyl-3-oxy]glycero-3-phosphoglycerol.

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on preparative silica gel thin-layer chromatography (TLC) plates. The thin-layer chromatograms were first developed in chloroform/methanol/acetic acid/water (25:15:4:2 v/v). Final purification was performed by TLC in the solvent chloroform/methanol/concentrated ammonium hydroxide (65:25:5 v/v). Estimated purity was >98% as judged by analytical TLC. After prolonged storage in chloroform at −15 °C development of a small amount of phosphate-containing impurity, most likely lysolipid, was detected.

$^3$H-Labeled cholic acid (14 Ci/mmol) was obtained from New England Nuclear. Purity was determined by TLC in two systems: chloroform/acetone/acetic acid (70:10:10 v/v) and ethyl acetate/acetic acid/water (85:10:5 v/v). The $R_f$ in each system matched that for unlabeled cholate detected by charring. Cation-exchange resin AG-1X8 (50–100 mesh, $Cl^-$ form) was obtained from Bio-Rad. Other materials were obtained as described in the preceding paper in this issue.

The $Ca^{2+}$ATPase was incorporated into a new phospholipid population by a modification of the cholate dilution method (Racker et al., 1975), which is similar to the "lipid filtration" method used by other investigators (Warren et al., 1974b). The protocol, unless otherwise stated, was as follows: Aqueous phospholipid vesicles were flushed with $N_2$ for 1 min and sonicated to clarity (15 min to 1 h) in a sealed test tube by using a bath sonicator at about 35 °C (47 °C for samples containing DPPC). Aliquots of 12.5 mM sonicated spin-labeled and unlabeled phospholipid vesicles in 20 mM Tris-HCl, pH 7.5, were combined to give a total of 187 nmol of phospholipid in 15 μL. Two microliters of 10% cholate, pH 8.1 (w/v potassium salt), was added. Samples were incubated at room temperature for 30 min. Then 1.5 μL of 10 mg/mL $Ca^{2+}$ATPase protein was added, followed by a 90-min incubation at 4 °C. An aliquot of the sample (10–15 μL) was diluted 100-fold into 20 mM buffer, pH 7.5. Fluorescence was measured by using a Perkin-Elmer MPF-3 spectrofluorimeter at excitation wavelength 290 nm and emission wavelength 330 nm. Slits were set at nominal band-pass, 10 nm. ATPase activity was measured colorimetrically by a modification of the organic phosphomolybdate extraction method (Marsh, 1959).

The size of the reconstituted vesicles was determined by chromatography on a 30 × 1.5 cm Sepharose 4B column. The sample was prepared by the addition of 22 μL of 10% cholate (w/v), pH 7.5, to 0.25 mL of 25 mM sonicated (7,6)PC and incubated for 30 min at room temperature. A total of 20 μL of 10 mg/mL $Ca^{2+}$ATPase was added, and the sample was incubated 30 min more at 4 °C and then diluted to 20 mL. After the sample was concentrated to about 1 mL with an Amicon ultrafilter by using an XM 100A filter, 0.75 mL of the sample was loaded onto the Sepharose 4B column. Fractions of 0.88 mL were collected. Optical density at 265 nm was measured to detect lipid. Protein was assayed by intrinsic fluorescence after the addition of cholate (to 1.66%, w/v) to each fraction. Cholate is added to abolish fluorescence quenching of bound (7,6)PC (London & Feigenson, 1978a). For comparison, a sample of 0.25 mL of 16 mM egg PC was chromatographed on the Sepharose 4B column. In this experiment, lipid was detected by enhancement of the fluorescence of DPH added to each fraction (London & Feigenson, 1978b).

The fluorescence for samples reconstituted with (7,6)PC was measured as described in the preceding paper (London & Feigenson, 1981). Samples containing $Ca^{2+}$ATPase were never incubated at greater than 47 °C, and exposure to temperatures >37 °C was limited to a few seconds at most, during which time there was no loss in ATPase activity. $^1$H NMR spectra were obtained on a Varian CFT-20 NMR spectrometer operating at 79.54 MHz.

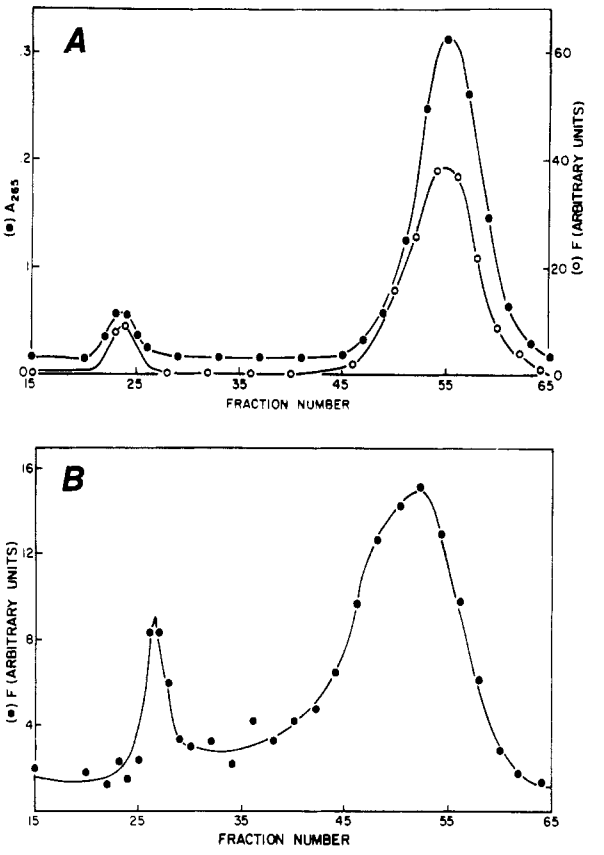


FIGURE 1: Sepharose 4B chromatography of model membranes. (A) $Ca^{2+}$ATPase incorporated into (7,6)PC membranes by cholate dilution. (B) Sonicated egg PC. Void volume of column was near fraction 25. For other experimental details, see Materials and Methods.

## Results

*Physical and Chemical Characterization of Model Membranes.* The $Ca^{2+}$ATPase-containing vesicles formed by the cholate dilution procedure were characterized as to size, protein location, residual cholate concentration, and enzyme activity. The size distribution of the reconstituted membranes, i.e., with incorporated $Ca^{2+}$ATPase, is shown in Figure 1, as monitored by Sepharose 4B chromatography. The protein and the (7,6)PC are seen to be associated. About 95% of the $Ca^{2+}$-ATPase is incorporated into the small particles that contain (7,6)PC. In contrast to the reconstituted sample, purified $Ca^{2+}$ATPase particles elute in the void volume. Thus it appears that the reconstituted membranes are in the form of small unilamellar bilayer vesicles. Additional evidence that cholate dilution produces bilayered membranes comes from the observation that DPPC–cholate dilution vesicles undergo the liquid-crystal to gel transition, as monitored by diphenylhexatriene polarization, although at 4 °C higher than sonicated DPPC vesicles (unpublished observations). Although $Ca^{2+}$ transport has been detected after cholate dilution by using a crude phospholipid mixture (Racker et al., 1975), we have not detected $Ca^{2+}$ transport in the phosphatidylcholine vesicles used in this work. This may be due to residual cholate in the vesicles (see below) which affects permeability of the bilayer to multivalent cations (Castellino & Violand, 1979). Fluorescence quenching studies using a detergent-free (freeze–thaw) system that does reconstitute $Ca^{2+}$ transport in phosphatidylcholines, including (7,6)PC, give fluorescence quenching results identical with those observed by using cholate

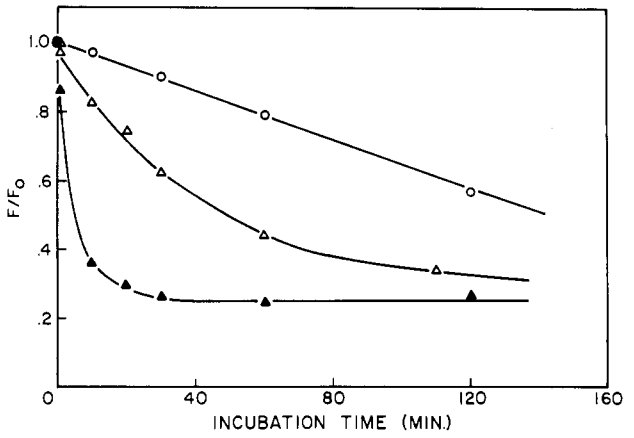


FIGURE 2: Effect of time of incubation of $Ca^{2+}$ATPase with cholate and (7,6)PC, prior to dilution, upon protein fluorescence after dilution. Incubation in 0.56% cholate at 4 °C (○). Incubation in 0.5% cholate at 23 °C (△). Incubation in 1.0% cholate at 23 °C (▲). At time zero, protein was added to cholate + (7,6)PC. $F/F_0$ is the ratio of fluorescence after a period of incubation to that at time zero.

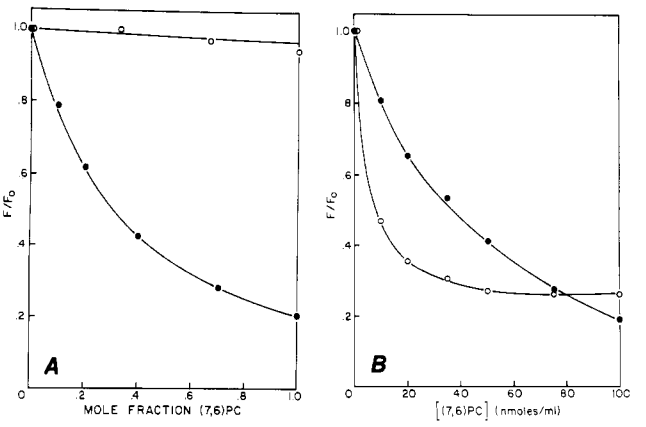


FIGURE 3: Effect of (7,6)PC upon the fluorescence of the $Ca^{2+}$ATPase and yeast hexokinase at 23 °C. (A) $Ca^{2+}$ATPase in (7,6)PC/egg PC (●). Yeast hexokinase in (7,6)PC/egg PC (○). Samples were prepared by cholate dilution and contained final concentrations of 8.1 μg/mL protein, 0.1 mM exogenous phospholipid, and 0.01% cholate (w/v), in 20 mM Tris-HCl, pH 7.5 (see Materials and Methods). The abscissa is the mole fraction of (7,6)PC in the membranes (see text), and the ordinate is the ratio of fluorescence in the presence ($F$) and absence ($F_0$) of (7,6)PC. (B) $Ca^{2+}$ATPase in (7,6)PC alone (○) and together with egg PC (●). The abscissa refers to the concentration of (7,6)PC in 20 mM Tris-HCl, pH 7.5. In the samples with egg PC, total lipid concentration was 100 nmol/mL. Other conditions as in (A).

dilution (Caffrey & Feigenson, 1981).

Samples made by cholate dilution contain a final total concentration of 0.01% w/v cholate. Experiments were performed to determine the amount of cholate bound to the vesicles. Bound cholate was measured by using the method of Hummel & Dreyer (1962) by chromatography of 0.1 mg/mL DOPC sonicated in the presence of 0.01% w/v [$^3$H]cholate on a 9 × 1.1 cm Sephadex G-50 column (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 0.02% $NaN_3$). The results indicate that under the conditions of cholate dilution the DOPC vesicles contain 7% cholate by weight. As determined by a similar experiment, DMPC vesicles contain 2% cholate. These results are in agreement with those for similar systems (Warren et al., 1974a,b). In a sample of DOPC vesicles with 0.01% w/v cholate present in the sample but not in the column buffer, only 0.5–1% by weight of the vesicles is cholate, indicating that most of the cholate dissociates from the vesicles on a time scale of minutes.

*Basic Properties of Fluorescence Quenching.* It is important to determine the reconstitution conditions which ensure equilibration of the exogenous and endogenous pools of lipid around the protein. As shown in Figure 2, the fluorescence quenching, $F/F_0$, reaches an equilibrium value of 0.2 with (7,6)PC as the only exogenous lipid. This figure shows the time and cholate concentration dependence of the establishment of this equilibrium. In this system, the rate-limiting step in the development of quenching appears to be the interaction of cholate with $Ca^{2+}$ATPase. This conclusion is based upon the independence of quenching development on lipid–cholate preincubation time and the strong dependence of quenching development upon protein–cholate preincubation time (data not shown).

For measurement of the effect of residual cholate on fluorescence quenching, reconstituted samples of $Ca^{2+}$ATPase with various mixtures of egg PC and (7,6)PC were treated to remove cholate. Samples were chromatographed on 4-cm Pasteur pipet columns of the anion-exchange resin AG-1X8. About 96% of the total cholate could be removed by these columns. After passage through the resin, the samples were incubated long enough for the bound cholate to dissociate from the vesicles, and then fluorescence quenching was measured. There was no change in fluorescence quenching upon removal of cholate.

Experiments described below are performed to determine whether the fluorescence quenching of the $Ca^{2+}$ATPase would respond to changes in the lipid environment of the protein in a predictable manner. In these experiments, the lipid-to-protein ratio is held constant at 1200:1 (mol/mol). The mole fraction of spin-labeled lipid in the total exogenous lipid is not corrected for endogenous lipid (4 mol % of the total). This phospholipid is mostly PC (64%), with some PE (20%) and PI (9%) and smaller amounts of other phospholipids (Owens et al., 1972). There is no evidence that this endogenous lipid is required for activity or is tightly bound to the $Ca^{2+}$ATPase since the ATPase activity of the dilipidated enzyme can be restored even by detergent (Dean & Tanford, 1978), and it has been shown that endogenous lipid equilibrates with exogenous lipid and can be totally replaced by exogenous lipid by using the cholate reconstitution method of Warren et al. (1974a).

Figure 3A shows a simple control experiment wherein the $Ca^{2+}$ATPase is quenched when reconstituted into model membranes containing (7,6)PC whereas the soluble, non-membrane-bound enzyme yeast hexokinase is not. As the fraction of (7,6)PC in the membranes is increased, the fluorescence quenching of the $Ca^{2+}$ATPase is increased. This reflects the greater amount of (7,6)PC bound around the $Ca^{2+}$ATPase. When the $Ca^{2+}$ATPase is reconstituted with pure (7,6)PC, there is still about 20% residual fluorescence. This may be due to a pool of inaccessible or partially accessible tryptophanyl groups, which are too far from the (7,6)PC to be effectively quenched, as has been observed in acrylamide quenching of soluble proteins (Eftink & Ghiron, 1976). This inaccessible pool could arise because the $Ca^{2+}$ATPase protrudes out of the membrane into an aqueous environment (MacLennan & Holland, 1976).

Figure 3B illustrates the effect of reconstitution with (7,6)PC alone compared to reconstitution with a phospholipid mixture containing (7,6)PC. A given amount of (7,6)PC in two samples results in less quenching when reconstituted together with egg PC than alone, because the egg PC competes with the (7,6)PC for binding to the protein. This result shows that as the mole fraction of (7,6)PC is increased in a (7,6)-PC/egg PC mixture it is the increase in mole fraction of (7,6)PC that results in increased fluorescence quenching, not the increase in the absolute amount of (7,6)PC. This figure

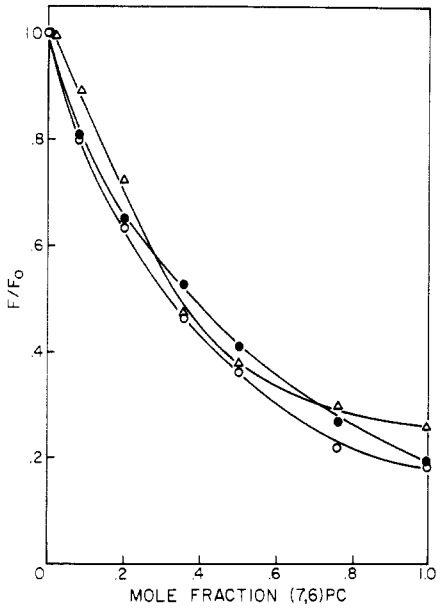


FIGURE 4: Fluorescence quenching of $Ca^{2+}$ATPase by (7,6)PC in the presence of different liquid-crystalline PC species. (7,6)PC/egg PC at 23 °C (●), (7,6)PC/DOPC at 23 °C (○), and (7,6)PC/DMPC at 37 °C (△). Other experimental conditions as in Figure 3A.

also shows that above 50 nmol/mL (7,6)PC, which gives a ratio of exogenous to endogenous phospholipid of 9:1, quenching is maximal so that above this ratio the endogenous phospholipid associated with the purified $Ca^{2+}$ATPase has no appreciable effect on fluorescence quenching. We note that the fluorescence measured for samples reconstituted with 100 nmol/mL of (7,6)PC was the same for both curves shown in Figure 3B. The reason that the values of $F/F_0$ are different at this point is that in the absence of added phospholipid cholate decreases the $Ca^{2+}$ATPase fluorescence ($F_0$). This effect may be related to the denaturation of the $Ca^{2+}$ATPase observed in bile salts and to phospholipid-dependent protection from denaturation by bile salts (le Maire et al., 1976a).

One other example of a simple response of fluorescence quenching to a change in lipid environment is the abolition of quenching by the addition of a high concentration of cholate to $Ca^{2+}$ATPase reconstituted in (7,6)PC (London & Feigenson, 1978a). At high cholate concentrations, the detergent dissociates the (7,6)PC from the $Ca^{2+}$ATPase, and the original fluorescence is restored.

*Binding of Various Phosphatidylcholines: Effect of Temperature-Induced Phase Separations.* A series of studies was undertaken to compare binding of different phospholipids to the $Ca^{2+}$ATPase. Figure 4 shows fluorescence quenching vs. mole fraction of spin-labeled phospholipid for $Ca^{2+}$ATPase reconstituted with (7,6)PC/egg PC at 23 °C, (7,6)PC/DOPC at 23 °C, and (7,6)PC/DMPC at 37 °C. There are no significant differences between the curves, which indicates that the environment of the protein, in terms of the number of (7,6)PC molecules bound, is the same for any particular mole fraction of (7,6)PC in the membranes. In other words, the nonquenching lipids egg PC, DOPC, and DMPC compete with (7,6)PC for binding to the protein to the same degree, implying that they bind with equal strength relative to (7,6)PC.

With regard to ATP hydrolyzing activity of the $Ca^{2+}$ATPase in the different lipids examined, values ranged from 2–4 μmol (min mg)$^{-1}$ for (7,6)PC to 10–13 μmol (min mg)$^{-1}$ for DOPC. These ATPase values are within the range observed for the $Ca^{2+}$ATPase reconstituted with various saturated and unsaturated PC species (Warren et al., 1974b; Nakamura et al., 1976; Bennett et al., 1978a).

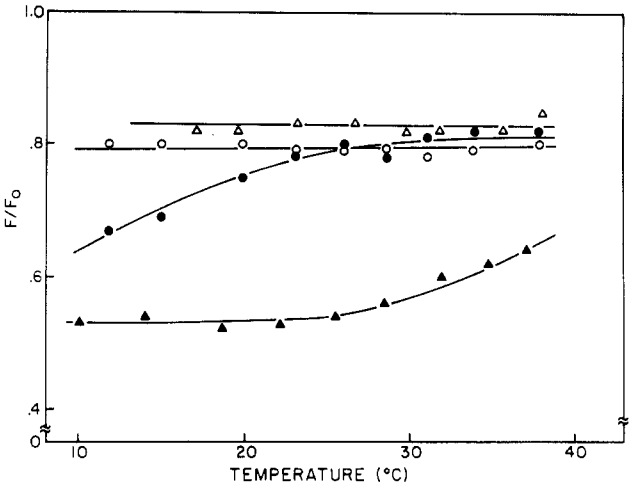


FIGURE 5: Temperature dependence of fluorescence quenching of $Ca^{2+}$ATPase in samples containing 10 mol % (7,6)PC. (7,6)PC/egg PC (△), (7,6)PC/DOPC (○), (7,6)PC/DMPC (●), and (7,6)PC/DPPC (▲). Temperature was increased at <1 °C min$^{-1}$ during each experiment. Other experimental conditions as in Figure 3A.

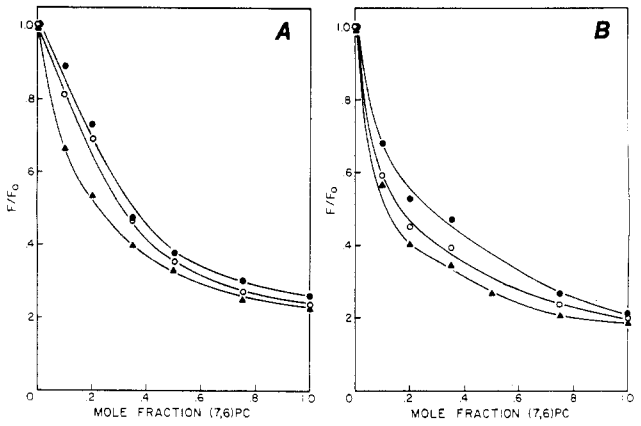


FIGURE 6: Fluorescence quenching of $Ca^{2+}$ATPase at different temperatures in the presence of standard PC species. (A) In (7,6)-PC/DMPC at 13 °C (▲), 23 °C (○), and 37 °C (●). (B) In (7,6)PC/DPPC at 13 °C (▲), 23 °C (○), and 37 °C (●). Other conditions as in Figure 3A.

The temperature dependence of fluorescence quenching is shown in Figure 5. The fluorescence quenching in (7,6)-PC/egg PC, (7,6)PC/DOPC, and (7,6)PC/DMPC when all lipids are in the liquid-crystalline phase is equal and temperature independent. However, when temperature is reduced below the phase transition temperature of the nonquenching lipid, fluorescence quenching is increased in (7,6)PC/DMPC and in (7,6)PC/DPPC. Although the curves are incomplete, it appears that a transition corresponding to the liquid-crystal to gel phase transitions of DMPC and DPPC is reflected in the fluorescence quenching. As discussed elsewhere (London & Feigenson, 1978a), these curves for cholate dilution vesicles are somewhat broader and displaced to lower temperatures compared to the curves for multilayer dispersions of the lipids.

Temperature-dependent changes in fluorescence quenching can be seen clearly when $F/F_0$ is plotted against mole fraction (7,6)PC as shown in Figure 6. Over almost the entire range of composition there is increased fluorescence quenching of $Ca^{2+}$ATPase in (7,6)PC/DPPC and (7,6)PC/DMPC at low temperatures relative to (7,6)PC/egg PC. The effect is more marked in (7,6)PC/DPPC, which reflects the higher transition temperature as illustrated in Figure 5. The stronger fluorescence quenching observed at the lower temperatures in (7,6)PC/DMPC and (7,6)PC/DPPC in Figures 5 and 6

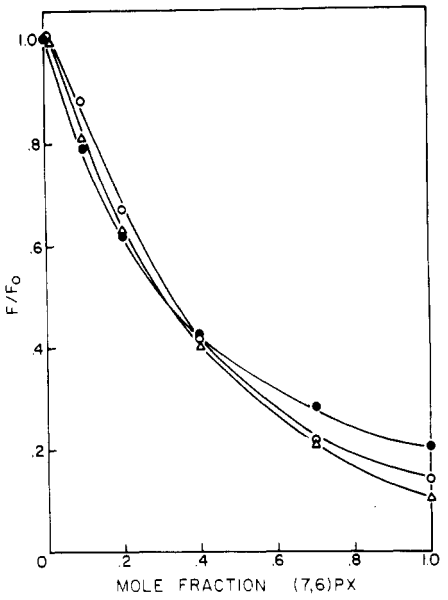


FIGURE 7: Fluorescence quenching of $Ca^{2+}$ATPase at 23 °C by spin-labeled phospholipids with different polar moieties. (7,6)PC/egg PC (●), (7,6)PA/egg PC (○), and (7,6)PG/egg PC (△). Other experimental conditions as in Figure 3A.

reflects higher amounts of (7,6)PC bound to the $Ca^{2+}$ATPase. Thus it appears as if DMPC and DPPC in the gel phase are not competing as effectively with (7,6)PC as egg PC and DOPC for binding to the protein. In other words, at low temperature, DMPC and DPPC bind to the $Ca^{2+}$ATPase more weakly than do egg PC or DOPC. This apparent difference in "binding" is simply a consequence of the temperature-induced lateral phase separation (see Discussion).

*Binding of Phospholipids with Different Polar Head Groups: Electrostatic and Divalent Cation Effects.* In Figure 7, the fluorescence quenching of $Ca^{2+}$ATPase by phospholipids with different head groups is compared. Note that the type of spin-labeled phospholipid is different in these experiments rather than the type of unlabeled phospholipid. PC is a zwitterion at neutral pH while PG and PA carry approximately a single negative charge. $^1H$ NMR studies analogous to those reported for (7,6)PC (London & Feigenson, 1981) indicate that at and above 23 °C and near neutral pH sonicated liposomes of (7,6)PA and (7,6)PG as well as (7,6)PC are in the liquid-crystal rather than the gel state. The fluorescence quenching curves in Figure 7 are all of similar shape, which indicates that binding is not affected by the difference in head-group structure or charge among these phospholipids. There is 5–10% more quenching in the samples reconstituted in pure (7,6)PA or (7,6)PG than in (7,6)PC, which may or may not represent a significant effect.

The $Ca^{2+}$ATPase is a negatively charged protein at physiological pH with p$I$ = 5.9 (Melgunev & Akimove, 1980), but the distribution of protein charges near the lipids is not known, and thus it is not possible to predict whether negatively charged lipid should be repelled relative to lipid with no net charge. The effect of a change in electrostatic forces on the association of lipid with $Ca^{2+}$ATPase can be investigated by varying the state of ionization of the protein. When pH is changed from 7.5 to 5.9, the $Ca^{2+}$ATPase loses its net negative charge. Figure 8A shows the effect of lowering pH to 5.6 on the quenching by (7,6)PG. In this pH range, there is no change in ionization of PG (measured in detergent dispersion; E. Phizicky and G. Feigenson, unpublished results). The (7,6)PG is bound more tightly at the lower pH, suggesting that the charge on the protein affects binding. By comparison, since

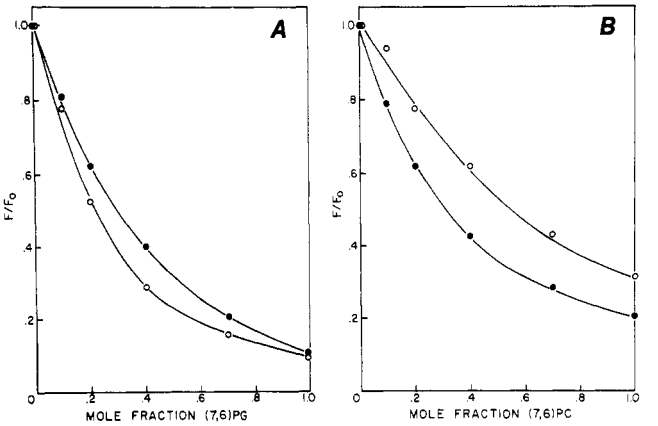


FIGURE 8: Effect of pH change upon fluorescence quenching of $Ca^{2+}$ATPase. (A) In (7,6)PG/egg PC at pH 7.5 (●) and pH 5.6 (○). (B) In (7,6)PC/egg PC at pH 7.5 (●) and at pH 5.6 (○). Other conditions as in Figure 3A.

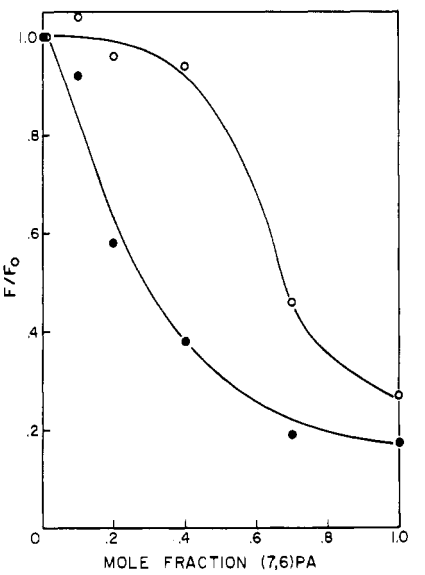


FIGURE 9: Effect of $Ca^{2+}$ addition upon fluorescence quenching of $Ca^{2+}$ATPase in (7,6)PA/egg PC. Without $Ca^{2+}$ (●); with 10 mM $CaCl_2$ (○). Other conditions as in Figure 3A.

PC remains zwitterionic over the pH range examined, it was expected that pH should not alter the binding of (7,6)PC to the protein. However, in Figure 8B, we see an apparent reduction of (7,6)PC binding at lower pH. This probably results from cholate protonation (p$K_a$ = 6.4; Merck Index, 1976) and consequent partitioning of cholic acid into the bilayer, thus diluting (7,6)PC and thereby lowering the quenching. In the absence of this cholate effect, the enhanced binding of (7,6)PG observed at lower pH would be expected to be more pronounced than is seen in Figure 8A.

The effect of the addition of $Ca^{2+}$ on quenching in (7,6)-PA/egg PC is shown in Figure 9. There is a dramatic decrease in quenching when 10 mM $CaCl_2$ is added. This effect is seen with as little as 10 mol % (7,6)PA. There was no significant decrease in quenching when $Ca^{2+}$ was added to the $Ca^{2+}$ATPase reconstituted with egg PC alone, and thus the reduction in quenching does not represent a direct interaction of the $Ca^{2+}$ATPase with the $Ca^{2+}$. This is in agreement with previous work which shows fluorescence changes of only a few percent when $Ca^{2+}$ is added to sarcoplasmic reticulum (Dupont, 1976). The quenching decrease is a result of $Ca^{2+}$-induced lateral phase separation of a (7,6)PA phase which excludes the $Ca^{2+}$ATPase. It should be noted that in these samples $Ca^{2+}$ was added after the reconstitution step, and it

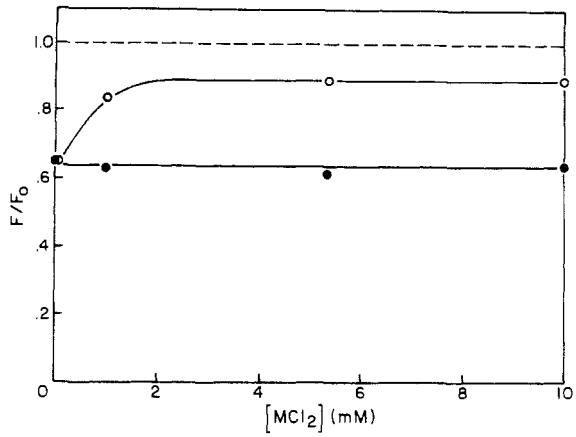


FIGURE 10: Effect of divalent cations upon fluorescence quenching of $Ca^{2+}$ATPase in 20 mol % (7,6)PA/egg PC. In the presence of $MgCl_2$ (●); in the presence of $CaCl_2$ (○). Other conditions as in Figure 3A.

is not certain that $Ca^{2+}$ was able to cross the membranes to bind to (7,6)PA on both sides of the bilayer.

The effect of $Ca^{2+}$ and $Mg^{2+}$ at various concentrations upon fluorescence quenching in 20 mol % (7,6)PA/egg PC is illustrated in Figure 10. In this experiment, divalent cations were present during the cholate dilution step rather than added after vesicle formation. Thus, divalent cations are on both inner and outer faces of the bilayer vesicles. In the presence of $Ca^{2+}$, but not $Mg^{2+}$, there is reduced fluorescence quenching. This effect on the fluorescence quenching probably results from the lateral phase separation which is induced by $Ca^{2+}$ but not by $Mg^{2+}$ in this concentration range (Ito & Ohnishi, 1974; Jacobson & Papahadjopoulos, 1975). Above 1 mM, the $Ca^{2+}$ effect reaches a plateau value. This corresponds to a concentration of $Ca^{2+}$ at which the PA is predominantly in the form of $Ca^{2+}$/PA complexes (Barton, 1968). The association of $Mg^{2+}$ and PA is strong enough so that at 10 mM $Mg^{2+}$ PA is in the form of a $Mg^{2+}$ complex (Barton, 1968), although some $Mg^{2+}$ will be in the ionic diffuse double layer around the vesicles. Therefore, the absence of an effect on the fluorescence quenching implies that a $Mg^{2+}$/PA complex can bind to $Ca^{2+}$ATPase with the same affinity as can uncomplexed PA.

We observe that changes in the lipid environment of the protein caused by $Ca^{2+}$ or temperature-induced phase separation, as reflected by quenching changes, occur as fast as we can make the measurements. This implies that the exchange rate between bound and free lipids is faster than seconds.

*Protein-Lipid Binding Constants: Model Calculations and Experimental Results.* The apparent binding constant to a membrane protein of lipid B relative to lipid A, $K'_{B/A}$, can be calculated from the curve of fluorescence quenching vs. mole fraction of spin-labeled lipid. The basis of this calculation is considered in the Discussion and is derived in detail in the Appendix. Figure 11A shows a number of hypothetical fluorescence quenching curves for different experiments in which the $Ca^{2+}$ATPase is reconstituted with one spin-labeled lipid and one unlabeled lipid. The dashed curve was calculated from the equation $F/F_0 = 0.8(1 - [(7,6)PC])^2 + 0.2$ (see preceding paper in this issue). Because the fluorescence quenching curves are more complicated than simple binding isotherms (see Discussion), the relative binding constant of spin-labeled to unlabeled lipid cannot be obtained directly from a single fluorescence quenching curve. Instead, the data from two sets of experiments (i.e., two fluorescence quenching curves) are compared. First, consider the case in which each of two experiments involves a different unlabeled lipid but the

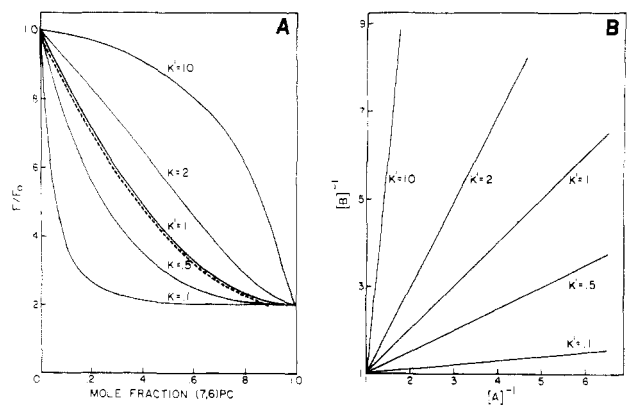


FIGURE 11: Hypothetical fluorescence quenching curves and corresponding double-reciprocal plots. (A) Hypothetical fluorescence quenching curves of $Ca^{2+}$ATPase in (7,6)PC/lipid B (—) and in (7,6)PC/lipid A (- - -) for different values of $K'_{B/A}$. (B) Double-reciprocal plot analysis of fluorescence quenching for different values of $K'_{B/A}$. See text for details.

same spin-labeled lipid. In this case, the results of the two experiments are combined in order to eliminate the binding of the spin-labeled lipid. The dashed curve in Figure 11A represents the hypothetical result of a first set of experiments in which model membranes are composed of lipid A, (7,6)PC, and protein. The solid curves represent possible fluorescence quenching results for a second set of experiments in which model membranes are composed of lipid B, (7,6)PC, and protein. If lipid B and lipid A bind to the protein with equal strengths, then they will compete with (7,6)PC for binding with equal effectiveness, and the fluorescence quenching in the two sets of experiments will lie along the same curve, and thus the fluorescence quenching in samples of lipid B, (7,6)PC, and protein will lie along the solid curve $K'_{B/A} = 1$. If lipid B can bind to protein 10 times more strongly than can lipid A, then lipid B would compete with (7,6)PC 10 times more strongly than would lipid A, and the fluorescence quenching in samples of lipid B would lie along the curve $K'_{B/A} = 10$. Similarly, if lipid B binds to protein 10 times more weakly than does lipid A, fluorescence quenching in samples containing lipid B would lie along the curve $K'_{B/A} = 0.1$. The analysis of two sets of experiments in which lipid A and lipid B are spin-labeled lipids and in which there is one unlabeled lipid is analogous. The effect of the nitroxide upon binding is canceled in both types of experiment, so that perturbation of binding by the presence of the nitroxide group is not an important consideration.

The binding constant is calculated from the fluorescence quenching curves by using a double-reciprocol plot as shown in Figure 11B. A straight line is drawn corresponding to eq A12 (derived in Appendix):

$$[\text{lipid B}]^{-1} = [\text{lipid A}]^{-1}K'_{B/A} + (1 - K'_{B/A})$$

The slope of the double-reciprocal plot is then the relative binding constant for the two lipid species (see Discussion).

A summary of the relative binding constants obtained from the various fluorescence quenching experiments described in this paper is shown in Figure 12. In the liquid-crystal state at pH 7.5, the relative binding constants are approximately equal within 25% for several lipids. At lower temperatures, DMPC and DPPC bind to the $Ca^{2+}$ATPase more weakly than do DOPC, egg PC, or DMPC at 37 °C. At pH 5.6, more PG binds to the $Ca^{2+}$ATPase than binds at pH 7.5. $Ca^{2+}$–PA complexes bind to the protein much more weakly than does PA alone. The physical basis for these effects as well as the assumptions in their analysis is considered in the following section.

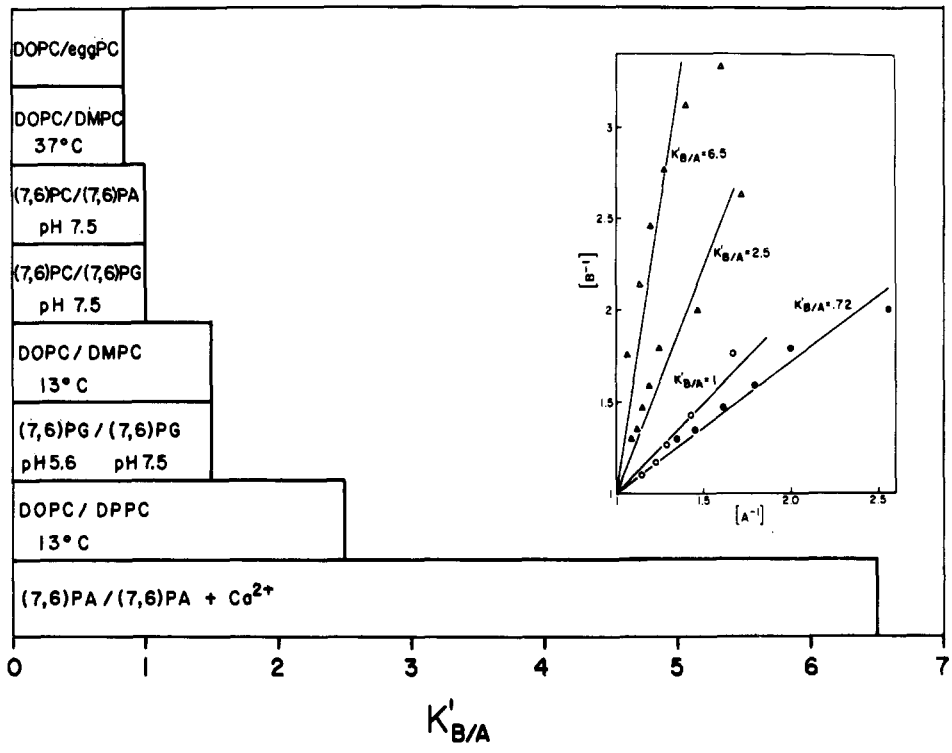


FIGURE 12: Summary of relative apparent binding constant, $K'_{B/A}$, of different phospholipids to the $Ca^{2+}$ATPase. (Insert) Experimental analyses of fluorescence quenching data by double-reciprocal plots. Analysis for $K'_{B/A}$ where B/A is DOPC/egg PC at 23 °C (●), (7,6)PC/(7,6)PA at 23 °C (○), DOPC/DPPC at 13 °C (▲), and (7,6)PA/(7,6)PA + $Ca^{2+}$ at 23 °C (△). See text for details.

## Discussion

Lipid binding to a membrane protein occurs at many sites. Although this situation represents multiple equilibria, if, following Tanford (1961), we use a simple model in which the binding sites are identical and independent, then the relative binding constant at a particular site on a membrane protein is defined for the equilibrium governing competitive binding:

$$A + BP \rightleftharpoons B + AP$$

where A represents lipid A, B represents lipid B, and P is a lipid binding site on the protein.

The relative binding constant $K_{B/A}$ is defined

$$K_{B/A} = A_A A_{BP} / A_B A_{AP} \quad (1)$$

where $A$ = chemical activity. This relative binding constant is the ratio of the two absolute binding constants of lipid to protein. A value of $K_{B/A} = 1$ implies that lipids B and A have equal free energy differences for transfer from the bulk bilayer to the protein surface. However, the value of $K_{B/A}$ does not address the problem of the absolute affinity of lipid for protein. Because, in many cases, an intrinsic membrane protein is surrounded by lipid, i.e., there is always a lipid molecule at each location around the protein, the relative binding constant $K_{B/A}$ is the physically meaningful parameter. Notice that although the absolute binding affinity of a membrane protein for lipid must be very high in order for every site to be occupied by lipid, there is no reason a priori that any relative binding constant should be strong or weak.

The expression for the binding constant can be rewritten in terms of concentration (eq 2); the concentration of a species

$$K_{B/A} = \frac{A_A A_{BP}}{A_B A_{AP}} = \frac{\gamma_A \gamma_{BP}}{\gamma_B \gamma_{AP}} \frac{[A]}{[B]} \frac{[BP]}{[AP]} \quad (2)$$

is expressed in terms of mole fraction in the membrane, AP and BP represent lipid species which are in contact with protein, and $\gamma$ is the activity coefficient. Since the activity coefficients are not readily determined, the simple ratio of concentrations given by $K'_{B/A}$ is most convenient to use. This is now designated as an *apparent* binding constant to show that it contains the activity coefficient information. The theoretical basis and experimental method for determining $K'_{B/A}$ from fluorescence quenching data are described under Results and Appendix.

*Assumptions and Limitations of Fluorescence Quenching Analysis.* Calculations of the binding constants for different lipids from fluorescence quenching experiments depend on assumptions which must be made explicit. A fluorescence quenching curve is not simply a plot of lipid binding to protein and therefore cannot be directly analyzed for the relative binding strength of spin-labeled lipid compared to unlabeled lipid (see preceding paper in this issue). This complexity arises because there is overlap in the fluorescent domains quenched by spin-labeled lipids bound at different sites on the protein. For calculation of a binding constant, it is assumed that equal $F/F_0$ values on two fluorescence quenching curves reflect equal numbers of bound spin-labeled lipids, although the actual value of this number of bound spin-labeled lipids is unknown. This allows the combination of the results of two fluorescence quenching experiments to obtain one relative binding constant. Furthermore, it is assumed that all of the lipids have the same surface area so that the total number of potential binding sites is not a variable.

The binding data are treated as if there is a large excess of free phospholipid over protein-bound phospholipid. In the reconstituted samples, the ratio of total phospholipid to protein is 10 μmol/mg or about 1200 lipid molecules/protein molecule. If there are about 30 sites for binding phospholipid on the protein (Hesketh et al., 1976; Bennett et al., 1978a,b) then there are 40 total phospholipids/site, assuming protein is not too heterogeneously distributed in the vesicles.

In cases of phase separation, it is assumed that the protein is excluded from the gel phase. There is some support for this approximation (Kleeman & McConnell, 1976; Chapman et al., 1979).

Finally, a complication can arise from protein–protein interactions. An oligomer could be less exposed to lipid and therefore less susceptible to quenching than a monomer, and so oligomer/monomer equilibria could affect fluorescence quenching. There is evidence that the $Ca^{2+}$ATPase exists as an oligomer under some conditions, but the oligomeric state of the active protein in the reconstituted systems is not yet known (le Maire et al., 1976a,b; Pick & Racker, 1979).

*Influence of Molecular Details of Protein Fluorescence on Quenching.* An important factor which influences the fluorescence quenching curve is the disposition of the fluorescent groups of a protein. The simplest model is one in which fluorophors are evenly distributed throughout the protein. There will be fluorescent residues that are either partly or totally inaccessible to fluorescence quenching (Eftink & Ghiron, 1976). A more realistic distribution might be of fluorophor groups which are randomly distributed in the protein, giving local density fluctuations of fluorophors. Another factor which can influence the apparent distribution of fluorophors in a membrane protein is the existence of energy-transfer networks among tyrosines and tryptophans (Steinberg, 1971). The topology of the protein will determine the fraction of fluorescent groups accessible to quenching.

Since 80% of the fluorescence of the $Ca^{2+}$ATPase is quenched in model membranes of almost pure (7,6)PC it must be that a large majority of the fluorescence protein groups are accessible to quencher. Fluorescence can originate from some or all of the 18–20 tryptophans of the $Ca^{2+}$ATPase (Thorley-Lawson & Green, 1975). Quenching can also occur by (7,6)PC contact with some of the (approximately) 20 tyrosine groups (MacLennan et al., 1971), including the deactivation of the excited states of tyrosines which do not fluoresce but which efficiently transfer their excitation energy to tryptophan groups. Thus it is likely that the fluorescence contains contributions from many sites on the protein.

In general, there could be an error in $K'_{B/A}$ caused by different distributions of fluorophors at the various binding sites. Regions with a high density of fluorophors would be more strongly represented in the binding constant than regions with few fluorophors. On the other hand, if fluorescent residues randomly populate parts of the protein with various values of $K'_{B/A}$, the effects due to nonuniform fluorescent groups will tend to be averaged out. In addition, the distribution of fluorescent groups can be disregarded if the nature of the forces binding phospholipid to protein are such that the value of $K'_{B/A}$ is the same at all sites. If $K'_{B/A}$ varied significantly among the sites, the double-reciprocal plots would be curved. Instead, from the double-reciprocal plots of Figure 12 (insert), it appears that one value of $K'_{B/A}$ can describe the lipid environment of the $Ca^{2+}$ATPase satisfactorily in a given lipid mixture.

Another factor which must be considered is the effect which changes in conformation of a protein have upon the fluorescence quenching. Without prior knowledge of the complete three-dimensional structure of each membrane protein, all these factors influencing fluorescence quenching cannot be evaluated exactly. However, from the above arguments, it is very likely that the fluorescence quenching process gives information about binding of phospholipids to much of the protein surface in contact with the phospholipid bilayer.

An important question concerns the sensitivity of fluorescence quenching to a difference in phospholipid binding at a limited number of sites. Since about 30 phospholipids bind around the protein and there is a maximum of 80% quenching, a single-bound spin-labeled phospholipid can quench only a few percent of the fluorescence. The detection of a single very specific binding site among a large number of nonspecific sites would be difficult. Furthermore, if there are no fluorescent groups at a specific binding site, there would be no observable fluorescence quenching response. A modification of the quenching method can detect such a site. The strategy would be to use a probe that quenches fluorescence via a long-range energy-transfer mechanism. Wolber & Hudson (1979) have recently given the theoretical background for this approach.

*Binding of Lipids to the $Ca^{2+}$ATPase.* With the above considerations in mind, we can turn to the interpretation of the binding constants derived from fluorescence quenching and shown in Figure 12. There is no evidence for a significant specificity of $Ca^{2+}$ATPase for saturated (DMPC) or unsaturated (DOPC, egg PC) phosphatidylcholines in the liquid-crystal state. A detailed study by Caffrey & Feigenson (1981) shows equal apparent relative binding constants for numerous different phosphatidylcholines. Insensitivity of binding to fatty acyl structure means that complications arising because the spin-labeled phospholipid used in this study is a mixture of species, with one fatty acyl chain either 16 or 18 carbons in length, can be neglected.

The temperature dependence of fluorescence quenching has been analyzed in terms of the $K'_{B/A}$ for the phospholipids. For the $Ca^{2+}$ATPase, this is useful because the $K'_{B/A}$ can be used to describe the composition of phospholipids in contact with the protein, even though the binding changes at lower temperatures could be explained in terms of changes in phospholipid structure rather than changes in the intrinsic affinity of protein for lipid.

Electrostatic forces can affect the binding of lipid to membrane proteins (see introduction). Lipid binding to $Ca^{2+}$ATPase appears to be influenced by changes in electrostatic interactions (see Figure 8). However, pH-induced changes in fluorescence quenching must be approached with caution, as ionization of all species present and the resultant changes in partition between aqueous and lipid phases must be considered. These effects are further complicated by the differences between bulk and local pH and ion concentrations arising from the electrostatic double layer (MacDonald et al., 1976; Träuble et al., 1976; Vaz et al., 1978b). Studies using detergent-free or nonionic detergent reconstitution methods may prove particularly useful for these experiments.

The most dramatic changes in lipid binding are induced by $Ca^{2+}$-dependent phase separations. When it was first observed that $Ca^{2+}$ could induce lateral phase separation of lipid mixtures containing a charged phospholipid, it was suggested that such phase separations could affect the environment of membrane proteins (Ohnishi & Ito, 1974). The fluorescence quenching experiments reported here directly confirm that such changes in lipid environment can occur. These phase separations can occur at relatively low concentrations of $Ca^{2+}$ and at low mole fractions of negatively charged lipid (0.05–0.1). $Ca^{2+}$ concentration in the sarcoplasmic reticular lumen can reach $\sim$1 mM (Sandow, 1970), and negatively charged phospholipids comprise a mole fraction of $\sim$0.1 of the sarcoplasmic reticular lipids (Owens et al., 1972). Further characterization of the conditions necessary for $Ca^{2+}$-induced phase separation should help to charify the physiological role, if any, of this process.

## Summary

These studies demonstrate the application of fluorescence quenching methods to the determination of the phospholipid composition in contact with intrinsic membrane proteins. Fluorescence quenching studies are particularly attractive for

several reasons. Only a small amount of phospholipid and protein are required for a fluorescence quenching experiment. The method is nondestructive, and additional properties of the sample may be measured, such as enzymatic activity. The fluorescence quenching method detects binding directly, and, since only the protein-bound phospholipid quenches fluorescence, there is no background signal except from light scattering from free phospholipid, unlike other physical methods such as ESR, NMR, and calorimetry. These other methods require that the boundary lipid be in a distinctive physical state whereas fluorescence quenching simply depends upon lipid contact with the protein.

The formalism developed here treats the lipid–protein interaction in terms of relative binding constants. These are apparent binding constants because the thermodynamic activity of the lipids is not known and indeed must change significantly for lipids undergoing lateral phase separations.

When considering whether an "annulus" of bound lipid exists, one really wants to know whether those lipids in contact with protein differ from free lipids with respect to (1) lipid composition or (2) local motion and order. Fluorescence quenching indicates that at neutral pH, for the bulk of the binding sites, the $Ca^{2+}$ATPase does not selectively bind phospholipids but that negative phospholipids bind somewhat more tightly as the protein becomes more positively charged. Selective binding at a very few sites is not ruled out by these experiments.

## Appendix

Consider reconstituted membranes consisting of protein, lipid A (an unlabeled lipid), and lipid N (a nitroxide spin-labeled phospholipid). The binding of lipid at each site on the protein is governed by the equilibrium

$$N + AP \rightleftharpoons A + NP \tag{A1}$$

where A ≡ a molecule of lipid A, N ≡ a molecule of lipid N, and P ≡ a lipid binding site on the protein. The equilibrium constant for this exchange is given by eq A2 where $A_A$ =

$$K_{N/A} = \frac{A_A A_{NP}}{A_N A_{AP}} = \frac{\gamma_A[A]\gamma_{NP}[NP]}{\gamma_N[N]\gamma_{AP}[AP]} \tag{A2}$$

chemical activity of lipid A, [A] = concentration of lipid A in mole fraction units, and $\gamma$ = activity coefficient.

The equilibrium expression may be rearranged as

$$K_{N/A}\frac{\gamma_N\gamma_{AP}}{\gamma_A\gamma_{NP}} = \frac{[A][NP]}{[N][AP]} = K'_{N/A} \tag{A3}$$

with $K'_{N/A}$ defined as an apparent relative binding constant. Equations A4–A7 follow from the definition of concentrations

$$[A] + [AP] = [A_T] \tag{A4}$$

$$[N] + [NP] = [N_T] \tag{A5}$$

$$[A_T] + [N_T] = 1 \tag{A6}$$

$$[AP] + [NP] = [P_T] \tag{A7}$$

where subscript T denotes total concentration of a species in the sample. Notice that in mole fraction units the total concentration of phospholipid is 1 because under experimental conditions lipid is employed in great excess over protein binding sites. Under such conditions, eq A3 can be rewritten as

$$K'_{N/A} = \frac{[A_T][NP]}{[N_T][AP]} \tag{A8}$$

Now consider a similar system of $Ca^{2+}$ATPase reconstituted with ordinary lipid B and lipid N in which equations analogous to eq A1–A8 apply. Combining the expressions for the two reconstituted systems:

$$\frac{K'_{N/A}}{K'_{N/B}} = \frac{[A_T][NP]/([N_T][AP])}{[B_T][NP]^\dagger/([N_T]^\dagger[BP])} \tag{A9}$$

The dagger has been added to the spin-label phospholipid terms to distinguish the concentration of the spin-labeled species in the lipid B system from those in the lipid A system.

Now consider the two complete fluorescence quenching profiles for the binary mixtures lipid A/lipid N and lipid B/lipid N as shown in Figure 11A. Let the same concentration of protein and therefore of binding sites on protein $[P_T]$ be used in each profile. At points of equal $F/F_0$, we assume that equal numbers of lipid N are bound, i.e., $[NP] = [NP]^\dagger$. It is then also true that [AP] = [BP], and eq A9 simplifies to eq A10. The left-hand side of eq A10 gives the ratio of the

$$\frac{K'_{N/A}}{K'_{N/B}} = K'_{B/A} = \frac{[A_T][N_T]^\dagger}{[N_T][B_T]} \tag{A10}$$

competition of lipid A binding vs. lipid N compared to lipid B binding vs. lipid N.

Substituting from eq A6 and analogous equation for lipid B into equation A10:

$$K'_{B/A} = \frac{[A_T][1 - B_T]}{[1 - A_T][B_T]} \tag{A11}$$

Thus $K'_{B/A}$ may be calculated from the fluorescence quenching curves at a point of equal fluorescence quenching ($F/F_0$) by substituting the value of the overall concentration of unlabeled lipid in each of the two samples into the right-hand side of equation A11. A linearized form of equation A11 may be derived by rearrangement to give eq A12. The slope of a plot

$$\frac{1}{[B_T]} = \frac{1}{[A_T]}K'_{B/A} + (1 - K'_{B/A}) \tag{A12}$$

of $1/[B_T]$ vs. $1/[A_T]$ is $K'_{B/A}$, the desired parameter.

The values of $A_T$ and $B_T$ are computed for each pair of fluorescence quenching curves (Figure 11A) at several values of $F/F_0$ to yield the coordinate pair $1/A_T$, $1/B_T$, which is plotted on the double-reciprocal graph shown in Figure 11B.

Once $K'_{B/A}$ is known, the amount of each lipid bound to a protein in a membrane of lipid A, lipid B, and protein can be calculated from A8.

$$K'_{B/A} = \frac{[B][AP]}{[A][BP]}$$

Since [A] and [B] are approximately the bulk concentration in the membrane of each of these species, the ratio [AP]/[BP] can be immediately calculated. If the number of lipid binding sites on the protein is known, the exact number of bound lipid A and bound lipid B molecules can be calculated from eq A8 and A13. In cases of phase separation, the cancellation of

$$[AP] + [BP] = [P_T] \tag{A13}$$

activity coefficients implied in the left-hand side of eq A10 may not be justified. Hence, $K'_{N/A}/K'_{N/B}$ rather than $K'_{B/A}$ is derived from the fluorescence quenching analysis. The problem is discussed in detail elsewhere (London, 1980).

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