Binding of Long-Chain Alkyl Derivatives to Lipid Bilayers and to (Ca^{2+}–Mg^{2+})-ATPase†

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Received February 19, 1986; Revised Manuscript Received August 13, 1986

ABSTRACT: Binding constants for myristoleic, palmitoleic, palmitic, oleic, and eicosanoic acids and oleyl- and stearylamine to lipid bilayers have been determined by using microelectrophoresis. Quenching of the fluorescence of the hydrophobic tryptophan analogue *N*-palmitoyl-L-tryptophan *n*-hexyl ester incorporated into lipid bilayers by oleic acid and oleylamine and their brominated derivatives is interpreted in terms of unlimited binding to the bilayers. The tryptophan fluorescence of the (Ca^{2+}–Mg^{2+})-ATPase purified from sarcoplasmic reticulum is quenched when reconstituted into bilayers of 1,2-bis(9,10-dibromostearoyl)-phosphatidylcholine (BRPC). Addition of fatty acids, oleylamine, oleyl alcohol, and methyl oleate to the ATPase reconstituted with BRPC reduces the quenching caused by BRPC, indicating binding of these molecules at the lipid–protein interface (annular sites). The charged molecules bind more strongly at the annular sites than do the uncharged molecules. Additional quenching of BRPC–ATPase by brominated derivatives of these molecules indicates binding at sites distinct from the lipid–protein interface, with binding constants similar to those for binding at annular sites, except for oleylamine. Quenching of tryptophan fluorescence of the ATPase by fatty acids and oleylamine suggests that ca. 50% of the tryptophan residues of the ATPase are located close to the lipid–water interface of the membrane.

The most obviously unique feature of intrinsic membrane proteins is that at least part of their surface must be hydrophobic to interact with the hydrophobic fatty acyl chain region of the phospholipid bilayer. It is important to understand the nature of this surface, the nature of the phospholipid–protein interaction, and the dependence, if any, of protein function on the chemical structure and physical state of the surrounding, annular phospholipids. It is known that the lipid composition of all biological membranes is highly complex, and it has often been suggested that this complexity is essential for the proper functioning of the membrane, possibly because of a fine sensitivity of the activity of intrinsic membrane proteins to membrane fluidity. However, it can equally well be argued that the complexity of phospholipid composition demonstrates that, within definable limits, the exact composition of the membrane is unimportant so that no precise control of composition is necessary to give a fully functional membrane—hence its lack of a simple, unique composition (Lee, 1985; Lee et al., 1986). The most fruitful approach to these problems seems to be through the use of highly simplified, reconstituted membrane systems containing single species of protein and lipid: one of the membrane proteins studied most extensively in this way is the (Ca^{2+}–Mg^{2+})-ATPase that can be purified from muscle sarcoplasmic reticulum. It has been shown that the ATPase is only active when the surrounding phospholipid is in the liquid-crystalline phase (Warren et al., 1974a) but that the exact fluidity in the liquid-crystalline phase is unimportant (East et al., 1984). Similar conclusions can be drawn for rhodopsin (Lee et al., 1986; Baldwin & Hubbell, 1985) and the erythrocyte hexose transporter (Carruthers & Melchior, 1984). The activity of the ATPase, however, is sensitive to the chemical structure of the surrounding phospholipid, with dioleoylphosphatidylcholine supporting maximal activity and phospholipids containing either shorter or longer fatty acyl chains or different head groups supporting lower activities (Warren et al., 1974a; Johannsson et al., 1981; Caffrey & Feigenson, 1981; East & Lee, 1982; Lee, 1985). Short-chain phospholipids have also been shown to support low activities for rhodopsin (Lee et al., 1986; Baldwin & Hubbell, 1985) and the erythrocyte hexose transporter (Carruthers & Melchior, 1984).

The specificity of the lipid–protein interactions of the (Ca^{2+}–Mg^{2+})-ATPase has been studied by using a variety of spectroscopic techniques. It has been shown that when the ATPase is reconstituted into bilayers containing brominated phospholipid, the fluorescence of the tryptophan residues of the ATPase is quenched as a result of binding of the brominated phospholipid at the lipid–protein interface. Addition of a nonbrominated phospholipid that can bind at the lipid–protein surface will result in an increase in fluorescence intensity due to displacement of brominated phospholipid from the annular surface of the ATPase. The fluorescence quenching observed in these systems can be fitted to a model assuming a homogeneous lipid annulus around the ATPase, with little selectivity between phospholipids, as long as the phospholipids are in the liquid–crystalline phase (East & Lee, 1982). Thus, relative binding constants to the ATPase of phosphatidylcholines with fatty acyl chains of lengths between C14 and C24 or with methyl-branched fatty acyl chains and of phosphatidylethanolamines and phosphatidylserines differ by no more than a factor of 2 (East & Lee, 1982; Froud et al., 1986a). These results, therefore, suggest that phospholipids interact with a nonspecific, hydrophobic surface of the ATPase and do not bind to specific binding sites on the ATPase. This, of course, is consistent with the idea that the membrane-penetrant portion of the ATPase is composed of a number of α-helical segments (Brandl et al., 1986). Such a surface will clearly be heterogeneous, both because of the molecular "roughness" of the amino acid residues making up the hydrophobic α-helices and because of the presence of charged

† This work was supported by AFRC, SERC, Wellcome Trust, and Smith, Kline and French Foundation. J.M.E. is a Wellcome Trust Lecturer.

0006-2960/86/0425-7535$01.50/0

amino acid residues close to the lipid–water interface of the membrane which could affect interactions with the charged head groups of the phospholipids. A full description of binding to such a heterogeneous surface is an impossibly difficult task. However, as long as the surface is not too heterogeneous, binding can reasonably be described in terms of a discrete number of binding "sites" of equal affinity, where "sites" is used in the same sense as it has been used in lattice theories of liquids [see East et al. (1985)].

A number of other experiments are also consistent with a largely homogeneous lipid annulus for the ATPase. First, fluorescence quenching in mixtures of unlabeled and spin-labeled phospholipids fits to a nonspecific, homogeneous lipid annulus (London & Feigenson, 1981). Second, fluorescence quenching in mixtures of dioleoyl- and dibromomyristoylphosphatidylcholine and in mixtures of dibromostearoyl- and dimyristoleoylphosphatidylcholine gives the same relative phospholipid binding constants, a result arguing against any marked heterogeneity in the binding of these lipids (F. Michelangeli and A. G. Lee, unpublished observations). Third, the rate of exchange of spin-labeled phospholipids into and out of the annular shell around the ATPase has been shown to be fast, consistent with a weak lipid–protein interaction and a homogeneous lipid annulus (East et al., 1985). Fourth, when the ATPase is purified from the original sarcoplasmic reticulum using cholate or deoxycholate as detergent, the molar ratio of lipid to protein is reduced to 30:1 from the ratio of ca. 90:1 typical of the native membrane, and yet the lipid composition of the purified ATPase is identical with that of the native sarcoplasmic reticulum (Warren et al., 1974b): if the ATPase exhibited strong binding to any particular species of lipid in the original membrane, then this species would be expected to become relatively enriched on lipid depletion. Lastly, it is found that changes in activity of the ATPase occur gradually with changes in the phospholipid composition of the membrane and very major changes in the phospholipid composition of the membrane are required to bring about significant changes in activity, as expected for nonspecific lipid–protein interactions (Lee, 1985).

Binding at the lipid–protein interface is, however, not totally without specificity. Thus, binding of phospholipids in the gel phase is relatively weak compared to binding of lipids in the liquid-crystalline phase (East & Lee, 1982). This can most probably be understood in terms of a weak van der Waals interaction between rigid, all-trans fatty acyl chains and a molecularly rough protein surface. Again, however, the data are consistent with a homogeneous lipid annulus, in which the affinity of all the annular sites around the ATPase is reduced for gel-phase phospholipid. Further, addition of cholesterol to the ATPase reconstituted with brominated phospholipid results in no increase in fluorescence intensity, suggesting that it also can only bind weakly at the lipid–protein interface of the ATPase (Simmonds et al., 1982). This relative exclusion could be a consequence of the rigidity of the cholesterol molecule although the observed stronger binding of cholesterol hemisuccinate (Simmonds et al., 1984) suggests that the polar group might also be important. Silvius et al. (1984) have, however, used electron spin resonance (ESR)[1] techniques to study the binding of a spin-labeled sterol to the ATPase and come to different conclusions: this will be discussed later. In order to understand the importance of polar groups in phospholipid–protein interactions, we report here on the ability of a variety of long-chain alkyl derivatives to bind at the lipid–protein interface of the ATPase, displacing phospholipid.

Recent studies have also emphasized the importance of protein–protein interactions in membrane structure. Thus, bacteriorhodopsin exists in the purple membrane of *Halobacterium halobium* as a trimer (Henderson & Unwin, 1985), and the (Ca^{2+}–Mg^{2+})-ATPase probably exists as a dimer (Hymel et al., 1984) which, under some conditions, can aggregate into long linear arrays (Dux & Martonosi, 1983). These protein–protein interactions must reflect the balance between association of the hydrophilic and hydrophobic portions of a protein molecule with other molecules of proteins and with phospholipids. If, as seems likely, protein–protein interactions involve a hydrophobic interaction between α-helices, then it is possible that long-chain alkyl derivatives will also be able to bind at protein–protein interfaces. Evidence for binding at protein–protein interfaces in the ATPase has been presented, based on fluorescence quenching methods (Lee et al., 1982; Simmonds et al., 1982). Thus, although addition of cholesterol to the ATPase reconstituted with brominated phospholipid results in no change in fluorescence intensity, indicating that cholesterol cannot bind at the lipid–protein interface of the ATPase, addition of brominated derivatives of cholesterol and other sterols to the ATPase reconstituted with nonbrominated phospholipids results in fluorescence quenching, and addition of these brominated sterols to the ATPase reconstituted with brominated phospholipid results in further quenching, beyond that seen with the brominated phospholipid alone. Taken together, these experiments indicate that the sterols can bind to sites on the ATPase at which phospholipids do not bind: we refer to these sites as nonannular sites and have suggested that they could occur at protein–protein interfaces in dimers of the ATPase (Simmonds et al., 1982, 1984). Experiments with fluorescence probes containing the dansyl group have also been interpreted in terms of binding at the lipid–protein interface (annular sites) and at protein–protein interfaces (nonannular sites) (Lee et al., 1982, 1983). Here we further characterize this binding.

Materials and Methods

Egg yolk phosphatidylcholine and dioleoylphosphatidylcholine (DOPC) were from Lipid Products. Molecules were brominated in chloroform. Products were characterized by NMR and thin-layer chromatography. The hydrophobic tryptophan analogue *N*-palmitoyl-L-tryptophan *n*-hexyl ester (NPTH) was prepared by esterification of tryptophan with thionyl chloride in hexanol followed by coupling to palmitic acid with *N*,*N*′-dicyclohexylcarbodiimide.

(Ca^{2+}–Mg^{2+})-ATPase was purified from hind leg muscle of rabbit as described in East and Lee (1982). The final preparation contained 30 lipid molecules per ATPase, assuming a protein molecular weight of 110000. Lipid substitutions were performed essentially as in East and Lee (1982). Typically, 1 μmol of lipid was sonicated to clarity in buffer (40 μL; 50 mM potassium phosphate, 1 M KCl, and 0.25 M sucrose, pH 8.0) containing 5 mM $MgSO_4$, 5 mM ATP, and 12 mg/mL potassium cholate. ATPase (0.125 mg in a volume of 3–10 μL) was then added and the sample left for 1 h at 5 °C to equilibrate or, for long-chain phospholipids, incubated for 20 min at room temperature followed by 40 min at 5 °C. After equilibration, samples were diluted with 200 μL of buffer and stored on ice until assay. For assay of ATPase activity,

[1] Abbreviations: NPTH, *N*-palmitoyl-L-tryptophan *n*-hexyl ester; BRPC, 1,2-bis(9,10-dibromostearoyl)phosphatidylcholine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DOPC, dioleoylphosphatidylcholine; ESR, electron spin resonance; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

12 μL of the enzyme mixture was diluted into 2.45 mL of assay mixture as described in East and Lee (1982). Methyl oleate and oleyl alcohol were added to the lipids in cholate solution before addition of ATPase whereas fatty acids and amines were added from methanol solution directly to the reconstituted ATPase after dilution into the assay mixture.

Fluorescence measurements were made in buffer (40 mM Hepes, 100 mM NaCl, and 1 mM EGTA, pH 7.2) by using either Spex Fluorolog or Perkin-Elmer MPF44A fluorometers. Fluorescence was excited at 285 nm and detected at 340 nm. For the ATPase experiments, the ATPase concentration was 0.065 μM, and the lipid concentrations were 51 μM. For the experiments with NPTH, NPTH was incorporated into liposomes of DOPC at a NPTH:DOPC molar ratio of 1:30 and a DOPC concentration of 51 μM. Protein was estimated by using the extinction coefficient given by Hardwicke and Green (1974).

Measurements of electrophoretic mobility were made on a Rank Brothers Mark 1 microelectrophoresis apparatus. Care was taken to focus at the stationary layer. The buffer used for these measurements was 10 mM Tris-HCl, 10 mM NaCl, and 0.1 mM EDTA.

Analysis of Binding to Lipid. The results of the microelectrophoresis experiments were analyzed as described elsewhere (Rooney & Lee, 1983) in terms of a dissociation constant for binding of the uncharged form of the fatty acid or amine to the membrane (K_d), the maximum number of molecules adsorbed per unit area (σ^{max}), the intrinsic shift in pK on binding to the membrane (ΔpK), and areas occupied in the membrane surface by the molecules of phospholipid and fatty acid or amine. Binding of Na^+ to bound anions or Cl^- to bound cations was considered as in Rooney and Lee (1983). Calculated curves of the ζ potential (ζ) vs. concentration were compared visually to the experimental data: variations in the fitted values by more than 10% from the given values result in an obviously worse fit.

In a medium of high ionic strength, where charge effects can be ignored, binding to phospholipid bilayers can be described by an effective dissociation constant, K_d^{eff}:

$$K_d^{eff} = [\text{lipid}]^{free}[D]^{free}/[D]^{bnd} \quad (1)$$

where $[D]^{free}$ and $[D]^{bnd}$ are, respectively, the total free and membrane-bound concentrations of additive and $[\text{lipid}]^{free}$ is the number of unoccupied binding sites in the phospholipid bilayer. Values of $[D]^{free}$ and $[D]^{bnd}$ were calculated from the full binding equations given in Rooney et al. (1983), using the dissociation constants K_d obtained from the electrophoresis experiments and given in Table I. From these values, K_d^{eff} was calculated by using eq 1. For unlimited binding (simple partition), $[\text{lipid}]^{free}$ is set equal to the total phospholipid concentration. It should be emphasized that binding of charged molecules can only be approximated by eq 1 under conditions where the charge on the membranes is small (typically less than 10 mV). Under other conditions, the full expressions derived above must be used, and fits to eq 1 will be nonlinear: attempts to describe binding of charged molecules to membranes under conditions where the membrane potential is expected to be large in terms of eq 1 lead to the suggestion of two classes of binding site within the membrane (Kubo & Hostetler, 1985).

Results

Interactions with Phospholipid Bilayers. In a previous paper (Rooney et al., 1983), we have shown how measurements of electrophoretic mobility can be used to determine binding constants for fatty acids to phospholipid bilayers. We chose

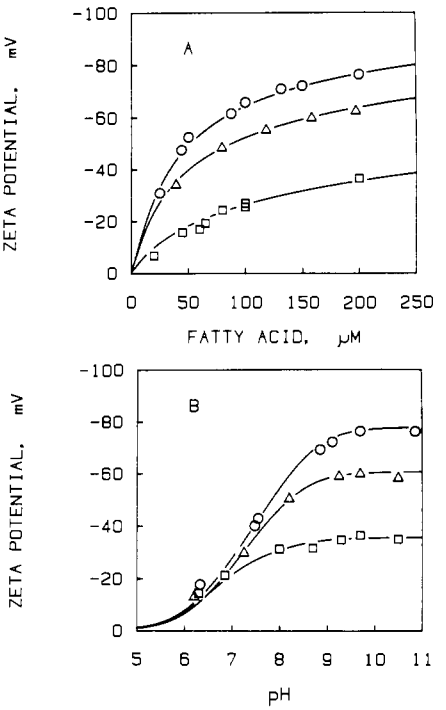

FIGURE 1: (A) ζ potentials of liposomes of egg phosphatidylcholine (580 μM) as a function of the concentration of added fatty acid at pH 9.7 in 10 mM Tris-HCl, 10 mM NaCl, and 0.1 mM EDTA at 25 °C. Symbols represent means of at least 10 mobility measurements, and curves represent the predicted variation of ζ potential with the parameters in Table I. (○) Eicosanoic acid; (△) palmitoleic acid; (□) myristoleic acid. (B) As in (A) but as a function of pH in the presence of eicosanoic acid [200 μM (○)], palmitoleic acid [160 μM (△)], and myristoleic acid [200 μM (□)].

conditions under which only a fraction of the fatty acid will be bound to the liposomes and then measured electrophoretic mobilities as a function of fatty acid concentration at fixed pH and as a function of pH at fixed fatty acid concentration. Figure 1A shows ζ potentials for liposomes of egg yolk phosphatidylcholine as a function of the concentration of fatty acid at pH 9.7, and Figure 1B shows data at a fixed fatty acid concentration as a function of pH. The data were analyzed as described previously (Rooney et al., 1983; Rooney & Lee, 1983). The pK of the fatty acids in aqueous solution was taken as 5 (Spink, 1963), the area per molecule of fatty acid as 20 Å^2 (Cadenhead et al., 1975), and the area per lipid molecule as 60 Å^2 (Levine, 1973).

As shown previously, it is not possible to determine separately σ^{max} and the dissociation constant K_d from this type of experiment, although the quotient σ^{max}/K_d is well-defined. Since there is evidence for changes in the properties of lipid bilayers beyond a molar ratio of 2:1 fatty acid/phospholipid (Schullery et al., 1981), we have calculated dissociation constants at $\sigma^{max} = 1/50$ Å^2 (corresponding to a 2:1 fatty acid/lipid stoichiometry) and at $\sigma^{max} = 1/20$ Å^2 (corresponding to unlimited binding). The data were then fitted to the equations in Rooney et al. (1983) to give K_d, the dissociation constant for binding of the uncharged form, and ΔpK, the shift in pK value on binding. The association constant, K_a, between Na^+ and the bound anionic form of the fatty acids was taken as 0.6 M^{-1}, the value determined by Eisenberg et al. (1979) for the binding of Na^+ to phosphatidylserine. The values giving the best fit to the data are listed in Table I.

Similar experiments were performed with alkylamines, with the results obtained in Table I, assuming a pK for the amines in bulk solution of 10.64 (Gowland & Schmid, 1969). Binding of Cl^- to the bound cationic form of the amine was assumed

Table I: Binding Constants to Egg Phosphatidylcholine Calculated from Electrophoresis Data (at 25 °C)

compound	ΔpK^a	K_d (μM)^b σ^{max} = 1/50 Å^2	K_d (μM)^b σ^{max} = 1/20 Å^2	K_d^{eff} (μM)^c
myristoleic acid	2.4	5.0	12.5	530
palmitoleic acid	2.4	0.5	1.25	65
palmitic acid	2.5	0.2	0.5	28
oleic acid	2.4	0.2	0.5	17
eicosenoic acid	2.4	0.15	0.38	17
oleylamine	−0.5	0.2^d	0.5^d	9
stearylamine	−0.5	0.2^d	0.5^d	9
hexadecyltrimethylammonium bromide		72.0	180.0	170

^{a} ΔpK is the shift in pK for the fatty acid or amine on binding to the membrane. ^{b} K_d is the dissociation constant for binding of the uncharged form of the fatty acid or amine to the membrane. ^{c} Calculated from eq 1 as described in the text at pH 7.2, [NaCl] = 0.1 M, and lipid concentration = 25 μM. ^{d} Upper limit.

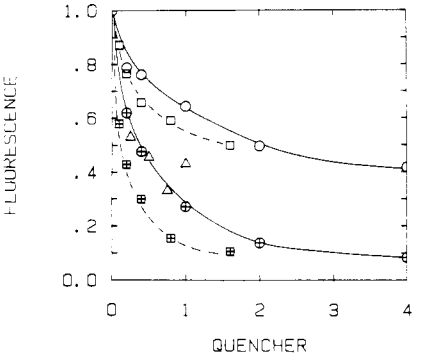

FIGURE 2: Quenching of fluorescence of NPTH in liposomes of dioleoylphosphatidylcholine at a lipid concentration of 51 μM as a function of the molar ratio of additive to lipid: (○) oleic acid; (⊕) 9,10-dibromostearic acid; (□) oleylamine; (⊞) 9,10-dibromostearylamine; (△) 9,10-dibromostearyl alcohol.

with an association constant, K_a, of 2.0 M^{-1} (Rooney & Lee, 1983). For oleylamine and stearylamine, binding was so strong that only an upper limit on the dissociation constant for binding could be obtained.

Because the electrophoresis experiments cannot provide information about any possible saturation of binding to lipids, we also studied binding using a fluorescence quenching method. On incorporation of the hydrophobic tryptophan analogue NPTH into lipid bilayers, there is a marked increase in fluorescence intensity and a shift of the spectrum to lower wavelengths compared to spectra in methanol, consistent with incorporation of the probe into a hydrophobic environment within the lipid bilayer (Moules et al., 1982). As shown in Figure 2, addition of oleic acid, 9,10-dibromostearic acid, oleylamine, or 9,10-dibromostearoylamine to a suspension of liposomes containing NPTH causes quenching of fluorescence, the effect of the brominated analogue being more marked than that of the nonbrominated parent molecule. Stern–Volmer plots of $F_0/F - 1$ against the total molar ratio of brominated fatty acid or amine to lipid are markedly nonlinear (not shown). However, if it is assumed that the brominated and nonbrominated molecules have equal lipid binding constants, then it is possible to use the binding constants derived from the microelectrophoresis experiments to calculate from the total molar ratio of additive to lipid the molar ratio of additive to lipid in the membrane: Figure 3 shows that a Stern–Volmer plot of $F_0/F - 1$ against bound molar ratio of 9,10-dibromostearic acid or 9,10-dibromostearoylamine is linear, assuming unlimited binding to the membrane (simple partition). It is also shown that the plots become nonlinear if it is assumed

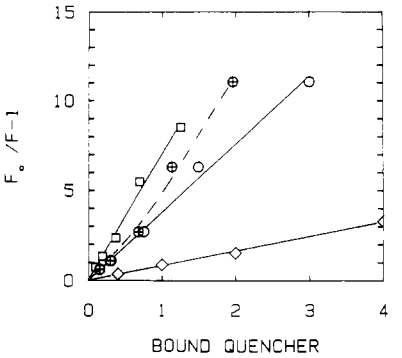

FIGURE 3: Stern–Volmer plot of $F_0/F - 1$ against the molar ratio of bound additive to lipid, calculated as described in the text, for the quenching of fluorescence of NPTH in liposomes of dioleoylphosphatidylcholine (51 μM). F_0 is the fluorescence in the presence of the given concentration of quencher. Solid lines were calculated by assuming unlimited binding to the membrane for (□) 9,10-dibromostearylamine, (○) 9,10-dibromostearic acid, and (◇) 9,10-dibromomethylstearate. Broken line (⊕) was for 9,10-dibromostearic acid with binding limited to a molar ratio of fatty acid to lipid of 2:1.

that binding is limited to a 2:1 molar ratio of additive to lipid. Similarly, Stern–Volmer plots for quenching by oleic acid and oleylamine are linear when plotted as a function of molar ratio of bound additive to lipid, calculated on the basis of unlimited binding. Methyl oleate causes no quenching of fluorescence, whereas methyl 9,10-dibromostearate does quench, with a linear Stern–Volmer plot when plotted against total molar ratio of additive to lipid, suggesting that the ester is so hydrophobic that under these conditions it is virtually all bound to the membrane. 9,10-Dibromostearoyl alcohol causes less quenching than the corresponding ester, and at molar ratios of alcohol to lipid greater than 1:1, the samples become very turbid, suggesting limited binding (not shown).

Interaction with the (Ca^{2+}–Mg^{2+})-ATPase. In previous papers (East & Lee, 1982; Simmonds et al., 1982, 1984), we have shown that quenching of the fluorescence of tryptophan residues in the ATPase by brominated compounds can be used to measure binding constants to the ATPase. When the ATPase is reconstituted into mixtures of normal lipids and the brominated lipid prepared by bromination of dioleoylphosphatidylcholine (BRPC), the tryptophan fluorescence intensity depends on the mole fraction of brominated lipid in the mixture according to

$$F' = F/F_0 = 0.4 + 0.6(1 - f)^{1.6} \quad (2)$$

where F_0 is the fluorescence intensity in the absence of brominated lipid and F is the fluorescence intensity when the fraction of annular sites on the ATPase occupied by BRPC is f.

If the ATPase is reconstituted into a mixture of, for example, BRPC and fatty acid, then binding of fatty acid at the lipid–protein interface will result in displacement of BRPC according to a series of reactions of the type

$$PL^* + L \rightarrow PL + L^*$$

where L represents fatty acid and L* BRPC. Such displacement reactions can be described by an equilibrium constant

$$K_d^a = \frac{[L][PL^*]}{[PL][L^*]} \quad (3)$$

where square brackets represent concentrations in moles per liter of the aqueous phase. If the concentration of lipid L* is in large excess over protein sites (so that the displaced

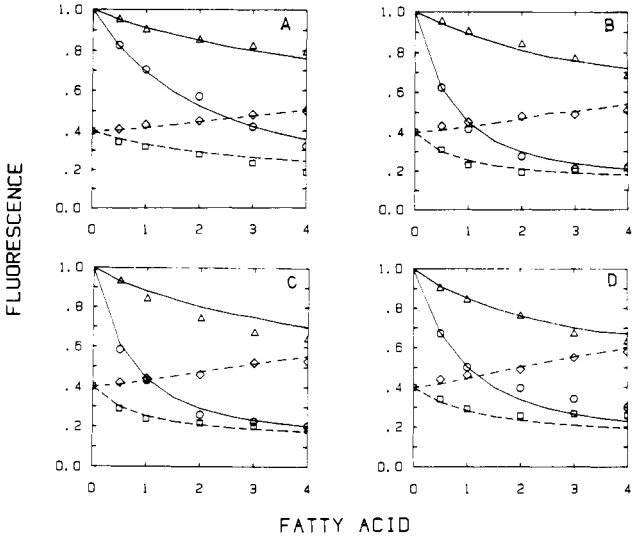

FIGURE 4: Effects on fluorescence intensities of addition of fatty acids to ATPase reconstituted with DOPC (△) and BRPC (◇) and of brominated fatty acids to ATPase reconstituted with DOPC (○) and BRPC (□). The ATPase concentration was 0.065 μM, and the lipid to protein molar ratio was 790:1. The data for the fatty acid–BRPC–ATPase system (◇) were normalized with respect to the fluorescence intensities for an equal concentration of fatty acid added to the DOPC–ATPase system (△). Lines are theoretical calculations (see text). (A) Myristoleic acid; (B) palmitoleic acid; (C) oleic acid; (D) eicosenoic acid.

phospholipid causes no significant change in phospholipid concentration), then the equation can be simplified to

$$K_d^a = \frac{[PL^*]}{[PL]}\chi_L \quad (4)$$

where χ_L is the molar ratio of fatty acid L to lipid in the lipid bilayer. The concentration of fatty acid bound to the lipid bilayer can be calculated in terms of aqueous concentrations, in the case of simple partition, by

$$[L] = \frac{[lipid][L]^{free}}{[lipid] + K_d^l} \quad (5)$$

where $[L]^{free}$ is the concentration of fatty acid free in the aqueous solution, [lipid] is the lipid concentration, and K_d^l is the dissociation constant for binding to lipid. If the annular binding sites are assumed to be identical and independent, then the fraction of sites occupied by BRPC, f in eq 2, can be expressed, using eq 4, as

$$f = K_d^a/[\chi_L(1 + K_d^a/\chi_L)] \quad (6)$$

In the above analysis, we have assumed that the binding of one molecule of fatty acid at the annulus will result in the displacement of one molecule of phospholipid. However, if the area of the lipid–protein interface is to be maintained, this seems rather unlikely, and a more reasonable model would be one in which two molecules of acid are required to displace one molecule of phospholipid. This situation has been analyzed in detail elsewhere (Lee, 1983) where it has been shown that, for the fluorescence quenching experiments, the problem can be overcome by expressing the molar ratio of fatty acid in the membrane on a chain basis, that is, by putting

$$\chi_L = [L]/2[L^*] \quad (7)$$

As shown in Figure 4 and elsewhere (Simmonds et al., 1982), addition of fatty acids to the ATPase reconstituted into bilayers of dioleoylphosphatidylcholine (DOPC) results in fluorescence quenching. The effect of fatty acids on the fluorescence intensity of the ATPase reconstituted with BRPC is, therefore, expressed as the ratio of the fluorescence intensity observed for the fatty acid–BRPC–ATPase system to that observed for the fatty acid–DOPC–ATPase system. Addition of amines to the DOPC–ATPase system also caused quenching (data not shown) and is accounted for in the same way, but addition of oleyl alcohol or methyl oleate causes no change in fluorescence. The fluorescence quenching curves for the BRPC–ATPase system are then analyzed in terms of the equations derived above to give the relative annular binding constants listed in Table II.

Table II: Binding Constants to Annular and Nonannular Sites Derived from Fluorescence Quenching Experiments

	dissociation constants			
	annular sites		nonannular sites	
	chain molar ratio units	effective binding constant (μM)[a]	molar ratio units	effective binding constant (μM)[b]
myristoleic acid	0.35	370	0.4	210
palmitoleic acid	1.3	170	0.6	40
oleic acid	2.0	70	1.0	17
eicosenoic acid	1.5	50	1.5	25
oleyl alcohol	3.0–5.0	4[c]	3.0–2.5	2[c]
methyl oleate	10[d]	8[c]	4	2[c]
oleylamine	0.6	11	10	90
cholesterol[e]	5.0[e]		1.2	

[a] Calculated as $2K_d^{eff}$(lipid) × K_d(annular). [b] Calculated as K_d^{eff}(lipid) × K_d(nonannular). [c] Calculated by using estimated values of K_d(lipid) (see text). [d] Lower limit. [e] Recalculated from the data in Simmonds et al. (1982), with a dissociation constant for binding at annular sites expressed in molar ratio units.

We have shown that addition of a variety of other brominated hydrophobic molecules to BRPC–ATPase causes further fluorescence quenching, attributed to binding at nonannular sites on the ATPase (Simmonds et al., 1982, 1984). The total fluorescence quenching in these systems is best described by

$$F = 0.15 + 0.25(1 - f_{na})^{1.6} + 0.6(1 - f_a)^{1.6}(1 - f_{na})^{1.6} \quad (8)$$

where f_a is the fraction of annular sites occupied by BRPC or brominated additive and f_{na} is the fraction of nonannular sites occupied by the brominated molecule (Simmonds et al., 1982, 1984). The fractional occupation of nonannular sites is calculated in terms of the molar ratio of additive L in the membrane to lipid by an equation analogous to eq 4:

$$f_{na} = \chi_L/(K_d^{na} + \chi_L) \quad (9)$$

where K_d^{na} is the dissociation constant for binding at the nonannular sites and χ_L is the normal molar ratio of L to L* (that is, not expressed on a fatty acyl chain basis). The coefficient of 1.6 linking occupancy of annular sites to fluorescence quenching was established by measuring the fluorescence intensity for the ATPase reconstituted into mixtures of DOPC and BRPC, assuming equal binding of DOPC and BRPC to annular sites (East & Lee, 1982). There is no equivalent experiment to establish the corresponding coefficient for the nonannular sites. For simplicity, we assume the same coefficient of 1.6 for the nonannular sites as for the annular sites, and we find that this value gives a good fit to the fluorescence quenching curves of Figures 4 and 5. For comparison, a fluorescence quenching coefficient of 1 gives less curved plots, with the value of K_d^{na} giving the best fit, being about 2 times higher than that obtained with a coefficient of 1.6. The set of equations describing binding of L to lipid (eq 5) and to the annular (eq 4) and nonannular (eq 9) binding sites was solved numerically by the Bolzano method

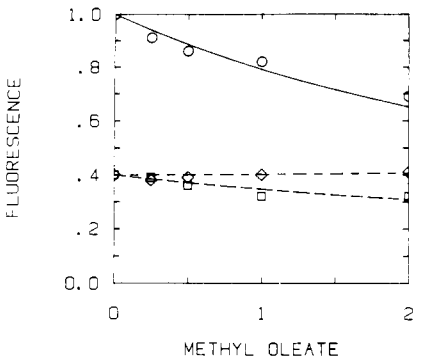

FIGURE 5: Effect on fluorescence intensities of the addition of methyl oleate to the ATPase reconstituted with BRPC (◊) and of brominated methyl oleate to ATPase reconstituted with DOPC (○) and BRPC (□). The ATPase concentration was 0.065 μM, and the lipid to protein ratio was 790:1. Lines are theoretical calculations (see text).

(McCormick & Salvadori, 1964) and used to calculate fluorescence quenching (eq 8) as a function of the added concentration of L. As shown in Figures 4 and 5, the values for K_d^{na} obtained by analysis of the addition of brominated molecules to the BRPC–ATPase system and the values for K_d^{a} obtained by analysis of the addition of nonbrominated molecules to the BRPC–ATPase system allowed the calculation of fluorescence quenching following the addition of brominated molecules to the DOPC–ATPase system, and the good agreement with the experimental data served as a check on these calculated binding parameters.

For oleyl alcohol and methyl oleate, it was necessary to estimate binding constants to lipid bilayers. An effective binding constant of 0.7 μM was estimated for oleyl alcohol based on extrapolation of the partition data of Jain and Wray (1978) for shorter chain alcohols. The effective binding constant of methyl oleate was set equal to that calculated for the uncharged form of oleic acid, 0.4 μM. Under the conditions of these experiments, the exact values of the lipid binding constants are unimportant since essentially all the oleyl alcohol or methyl oleate will be bound. Little increase in fluorescence is seen on addition of methyl oleate to the BRPC–ATPase system (Figure 5) so that binding to annular sites must be weak: a lower limit to the dissociation constant of 10 can be estimated (Table II). For oleyl alcohol, agreement between the simulations and the experimental data is poor at molar ratios of oleyl alcohol to lipid greater than 1:1. Since at these higher molar ratios, samples were turbid, limited binding seems likely, as observed for simple lipid bilayers. As shown in Table I, a range of values of the binding constant gives a reasonable fit to the data at molar ratios of oleyl alcohol to lipid less than 1:1.

Discussion

An understanding of the structure and function of membrane proteins requires an understanding of the nature of the lipid–protein interaction. Measurements of the relative binding constants of a variety of phospholipids to the (Ca^{2+}–Mg^{2+})-ATPase have shown that binding constants are relatively insensitive to the chemical structure of the phospholipids (London & Feigenson, 1981; East & Lee, 1982), suggesting that the phospholipids are interacting with a generalized hydrophobic surface on the ATPase rather than with distinct binding sites. This is also consistent with the measured fast rate of exchange of phospholipids between the surface of the ATPase and the bulk phospholipid component of the membrane (East et al., 1984). To the extent that measurements have been made on other membrane proteins, nonselective binding of at least most phospholipids to membrane proteins seems to be a general phenomenon (Watts et al., 1979; Esmann et al., 1985; Lee et al., 1986).

Amino acid sequences have been reported for a number of membrane proteins (Eisenberg et al., 1984), and all contain possible hydrophobic α-helical sequences that could span the fatty acyl chain region of the membrane, these possible α-helical regions often being located between clusters of charged amino acids. For the (Ca^{2+}–Mg^{2+})-ATPase, for example, the first two possible α-helical regions are surrounded by regions containing many negatively charged amino acids, whereas for other possible membrane-spanning regions there is less evidence for any particular concentration of charged residues (MacLennan et al., 1985; Brandl et al., 1986). There is now much evidence that the ATPase occurs as a dimer within the membrane (Hymel et al., 1984) and freeze–fracture electron microscopy is consistent with the dimer involving protein–protein contact of the hydrophobic domains of the protein within the hydrophobic core of the membrane (Wang et al., 1979). It seems likely that such protein–protein contact will involve hydrophobic sequences not surrounded by a high density of charged residues, since, unless there was pairing of charged residues, extensive charge repulsion would prevent dimer formation. For the trimer of bacteriorhodopsin in particular, protein–protein interaction is unlikely to depend significantly on charge–charge interactions, since the trimer is formed in solutions of high ionic strength (Henderson & Unwin, 1975). The observation that the ATPase can be solubilized in detergent in a monomeric and fully active form depleted of phospholipid (Kosk-Kosicka et al., 1983; Yamamoto et al., 1984; Andersen et al., 1985) suggests that detergents can bind both at the normal lipid–protein interface (annular sites) and at the sites involved in protein–protein contact within the membrane (nonannular sites), again suggesting that the interactions at the annular and nonannular sites are predominantly hydrophobic.

Our approach to the study of the lipid–protein and protein–protein interactions in the ATPase system has been to study the quenching of the tryptophan fluorescence of the ATPase resulting from the binding of brominated phospholipids and brominated alkyl derivatives. In order to analyze the quenching data, it is necessary to adopt a simple model. We have assumed a homogeneous lipid annulus with equal fluorescence quenching resulting from binding of a brominated phospholipid anywhere on the lipid–protein surface of the ATPase. The fairly even distribution of tryptophan residues throughout the proposed α-helical transmembrane sequences of the ATPase (MacLennan et al., 1985; Brandl et al., 1986) together with the expected fluorescence energy transfer between tryptophan residues makes this assumption reasonable (London & Feigenson, 1981). Similarly, we have assumed a homogeneous set of nonannular binding sites, binding at any of these sites again resulting in equal quenching. The fact that the experimental data fit this simple model suggests that the majority of the binding sites conform to the model: the presence of a small number of highly specific binding sites (with binding to these sites causing little quenching), together with a larger number of less specific sites, cannot be ruled out, but we have no evidence that requires their inclusion. The strongest evidence for the model we propose comes from the observations that whereas a molecule such as methyl oleate causes little change in fluorescence for the BRPC–ATPase system, addition of methyl 9,10-dibromostearate to the DOPC–ATPase system does result in quenching and addition to the BRPC–ATPase system results in additional quenching.

These observations cannot readily be interpreted in terms of changes in quenching following from conformational changes on the ATPase or from changes in packing density at the lipid–protein interface of the ATPase: they are, however, readily explicable in terms of binding of the ester at sites on the ATPase from which phospholipids are excluded.

A complication in any study of binding to the ATPase system is the extensive binding of hydrophobic molecules to the lipid–bilayer portion of the membrane. We have shown that binding constants for charged molecules to lipid bilayers can be established conveniently using the technique of microelectrophoresis. Although this technique alone cannot give both the number of binding sites and the affinity of the sites, it can be used to calculate bound concentrations from free concentrations over a concentration range far from saturation of the lipid binding sites. In order to study the saturation behavior of the bilayer, we measured fluorescence quenching caused by brominated fatty acids and amines. Stern–Volmer plots of fluorescence quenching of the hydrophobic tryptophan analogue NPTH incorporated into lipid bilayers are curved when plotted against the total concentration of additive but become linear when plotted against the bound concentration of additive, calculated by assuming unlimited binding (Figure 3). Stern–Volmer plots are also curved when plotted against the bound concentration of additive calculated by assuming saturation of the lipid phase at a molar ratio of additive to lipid of 2:1. The most obvious explanation of these findings is that fatty acids and amines can simply partition into the bilayers under these conditions, with no saturation of the bilayer. For the fatty acids, there is no indication of a discontinuity at a 2:1 molar ratio of fatty acid to lipid of the type apparent in the experiments of Schullery et al. (1981) under very different conditions. Consistent with unlimited binding is the observation that unsaturated fatty acids at high pH will themselves form bilayer vesicles (Gebicki & Hicks, 1968). The data (Figure 2) also suggest that methyl 9,10-dibromostearate partitions into the bilayer but the relatively low level of fluorescence quenching suggests that it could phase separate within the bilayer, as suggested for cholesterol esters (Grover et al., 1979). For oleyl alcohol, sample turbidity increased markedly beyond a 1:1 molar ratio of alcohol to lipid, suggesting that the aqueous solubility limit of the alcohol had been exceeded.

Assuming unlimited binding of fatty acids to the lipid bilayer, the binding constants given in Table I can be derived from the electrophoresis data. As expected, binding increases with increasing chain length, but without a uniform incremental increase. Similar observations have been made in measurements of fatty acid partition between water and heptane (Goodman, 1958) and attributed to aggregation and micelle formation by the fatty acids in water (Smith & Tanford, 1973). The binding of palmitic acid is slightly stronger than that of palmitoleic acid (Table I), consistent with the higher critical micelle concentration observed for unsaturated fatty acids compared to the corresponding saturated fatty acids (Klevens, 1953). The binding constants that we measure are about 6 times higher than those estimated by Pjura et al. (1984) using hygroscopic desorption.

Having derived binding constants for these hydrophobic molecules to lipid bilayers, it is possible to study their binding to the (Ca^{2+}–Mg^{2+})-ATPase in reconstituted membrane systems. As in previous studies (East & Lee, 1982; Simmonds et al., 1982, 1984), we have shown that binding of hydrophobic molecules at annular sites can be quantitated from the increase in fluorescence intensity that follows displacement of BRPC and that binding at nonannular sites can be quantitated from the fluorescence quenching caused by addition of brominated molecules.

As described elsewhere (Lee, 1983), it seems likely that when a fatty acid or other single-chain molecule binds at the lipid–protein interface of the ATPase, the area of the interface will be largely conserved, so that two fatty acid molecules would be required to fully displace one molecule of phospholipid. Binding then cannot be described in terms of a simple equation such as eq 3 because of statistical factors: the appropriate binding equations are given in Lee (1983). However, except at very low mole fractions of fatty acid, binding curves calculated by using the full binding expression including statistical factors are identical with those calculated by using the simple binding equation if the mole ratio of fatty acid is expressed on a chain basis (eq 7) rather than on the normal mole basis. Binding constants for the lipid–protein interface of the ATPase calculated in this way are listed in Table II. If it is assumed that one fatty acid molecule is able to fully displace one molecule of phospholipid from the lipid annulus, then the binding constants listed in Table II should be doubled, with the mole fraction of fatty acid being calculated on a normal mole basis (Lee, 1983).

It is clear that binding constants for molecules at the lipid–protein interface depend very markedly on the polar group of the molecule, so that the neutral alcohol and ester bind considerably less well than the corresponding charged acid or amine (Table II). This suggests that the ability to bind at the lipid–protein interface is related to miscibility with phospholipids. The charged fatty acids and amines seem to have unlimited miscibility with phospholipid (Figure 3), although there is evidence that, at least under some conditions, phospholipids may no longer adopt a bilayer phase at high molar ratios of fatty acid (Schullery et al., 1981). Unlike the fatty acids, esters appear to have limited miscibility with phospholipids (Figure 3; Grover et al., 1979; Hamilton et al., 1984), and the data for oleyl alcohol also suggest limited miscibility, but in this case, binding is probably limited by aqueous solubility [see Jones & Lee (1985)]. The case of cholesterol is particularly interesting. Although cholesterol can bind to lipid bilayers up to a cholesterol/phospholipid stoichiometry of 1:1, it is thought that the structure adopted is not a simple random mixture but rather that a relatively ordered array of cholesterol/phospholipid "complexes" is formed (Rogers et al., 1979; Presti et al., 1982). Cholesterol binds relatively weakly at the lipid–protein interface The charged derivative cholesterol hemisuccinate has, however, been shown to partition into lipid bilayers beyond a 1:1 sterol:phospholipid stoichiometry and has been shown to bind more strongly at the lipid–protein interface of the ATPase (Simmonds et al., 1984).

Binding of fatty acids to the lipid–protein interface appears to increase in strength with decreasing chain length, when expressed in terms of the concentration of fatty acid bound to the membrane, but the 4–6-fold change in the relative binding constant from myristoleic acid to eicosanoic acid (Table II) should be compared to the 30-fold weaker binding of myristoleic acid to lipid bilayers than eicosenoic acid (Table I).

Further information about the structure of the membrane comes from the observation of quenching by fatty acids and alkylamines and a variety of brominated derivatives. Addition of simple fatty acids or amines to bilayers containing the hydrophobic tryptophan analogue NPTH causes marked quenching of fluorescence (Figure 2). Such quenching in membrane systems does not seem to have been reported before,

although quenching of fluorescence of tryptophan and other indoles in solution by proton-donating groups such as $-NH_3^+$ and COOH has been observed and attributed to formation of an encounter complex between the quencher and the excited indole followed by transfer of an electron from the excited indole to the quencher or proton donation to the excited indole ring (Ricci & Nesta, 1976; Creed, 1984): all the suggested quenching mechanisms involve close contact between the indole ring and the quencher. Interestingly, fatty acids and amines also cause marked quenching of the tryptophan fluorescence of the ATPase (Figure 4). Since the carboxyl and amine groups of these compounds are likely to be anchored at the membrane–water interface, this would suggest that a number of the tryptophan residues in the ATPase are also located close to this interface. This agrees with structural predictions based on the amino acid sequence of the ATPase (MacLennan et al., 1985; N. M. Green, personal communication). Of the 13 tryptophans, 5 (38%) are located at the ends of the predicted α-helical membrane-spanning sequences. This is also consistent with experiments with water-soluble quenchers. We find that KI at 0.6 M is able to quench fluorescence by 33% (I. K. Moules and A. G. Lee, unpublished experiments), and Ludi et al. (1985) and Shinitzky and Rivnay (1977) found that ca. 50% of the fluorescence intensity can be quenched by water-soluble quenchers. Further, the tryptophan residues quenched by KI are also accessible for quenching from the lipid phase since, for example, the hydrophobic quencher $C_2Cl_4Br_2$ at 0.05 mM can quench 67% of the fluorescence, but addition of KI (0.6 M) to the ATPase quenched with $C_2Cl_4Br_2$ causes only 3% extra quenching.

The maximum level of quenching that we have observed with hydrophobic quenchers is 85% (see Figures 4 and 5), consistent with the proposed location of two tryptophan residues (15% of the total) in hydrophilic regions of the ATPase (MacLennan et al., 1985). The results obtained with KI would then suggest that these two tryptophans are buried and inaccessible to the external medium.

We found that it was possible to simulate the observed quenching caused by fatty acids and amines in terms of the binding constants for the lipid–protein interface derived from their ability to displace brominated lipid (Table II). For the fatty acids, the quenching data fitted the equation:

$$F = 0.5 + 0.5(1 - f_a)^{1.6}$$

where f_a is the fraction of annular sites occupied by fatty acid (Figure 4). For oleylamine, the quenching data fitted the equation:

$$F = 0.6 + 0.4(1 - f_a)^{1.6}$$

where f_a is the fraction of annular sites occupied by oleylamine (data not shown). The fraction of tryptophan fluorescence quenchable by these molecules was close to the fraction of tryptophans located at the ends of the postulated α-helical segments. It was not found possible to fit the data assuming that quenching followed from binding both at annular and at nonannular sites.

The presence of binding sites on the ATPase for hydrophobic molecules, distinct from the annular sites, has been deduced from quenching studies with brominated sterols (Simmonds et al., 1982, 1984) and from fluorescence titrations with dansylundecanoic acid (Lee et al., 1982). For the fluorescence quenching studies, these nonannular sites are defined operationally as sites to which hydrophobic molecules can bind but from which phospholipids are excluded. We have no direct evidence for the location of these sites, but an attractive possibility is that they lie at protein–protein interfaces in ATPase dimers. From the fluorescence quenching results presented here, binding constants at the nonannular sites have been calculated (Table II) and are, in general, similar to those calculated for binding to annular sites, except for the markedly weaker binding of oleylamine. The relatively nonspecific nature of binding at the nonannular sites could explain the ability of detergents such as $C_{12}E_8$ and Triton X-100 to solubilize the ATPase in monomeric form (Dean & Gray, 1983; McIntosh & Davidson, 1984; Martin et al., 1984) where presumably detergent molecules occupy the sites on the ATPase previously involved in protein–protein interaction. These studies suggest that protein–protein interactions for the ATPase are relatively nonspecific.

Silvius et al. (1984) have used ESR techniques to study the binding of a spin-labeled analogue of cholesterol to the ATPase and have reached very different conclusions from ours about the nature of the interactions in this system (Simmonds et al., 1982, 1984). In their experiments (Silvius et al., 1984), a spin-labeled cholesterol analogue was incorporated into membranes containing the ATPase and phospholipid at molar ratios of phospholipid to ATPase varying between 103:1 and 37:1, and the resulting, composite, ESR spectra were analyzed to give free:bound cholesterol ratios (y) at given molar ratios of phospholipid to ATPase (x). A linear plot of y against x then gave an intercept on the x axis, assumed to be equal to the number of binding sites on the ATPase for cholesterol, and a slope from which a relative binding constant could be calculated. Their analysis gave a number of binding sites for cholesterol equal to 22, with a relative binding constant of 0.65 (Silvius et al., 1984), clearly in disagreement with our interpretation in terms of a small number of nonannular binding sites for cholesterol (Simmonds et al., 1982, 1984). However, for the reasons now given, we do not believe that their data are in fact inconsistent with our model. To understand the argument, it is necessary to appreciate the very different protocols used by Silvius et al. (1984) for their ESR experiments and by us for fluorescence quenching experiments. In our experiments, the molar ratio of phospholipid to ATPase is kept constant and high (790:1) so that essentially all the phospholipid will be in the bulk phase with only a very small fraction (ca. 3%) of the total phospholipid being annular phospholipid. In the ESR experiments, however, the molar ratio of phospholipid to ATPase is varied and is low, so that the annular phospholipid constitutes a significant fraction of the total phospholipid. If, as we have suggested (Simmonds et al., 1982,1984), cholesterol is excluded from the annulus around the ATPase, then the fraction of phospholipid which is annular phospholipid needs to be accounted for in the analysis of the ESR data. From our ESR studies (East et al., 1985), it can be estimated that the fraction of the total lipid that is annular lipid varies from 0.45 at a total phospholipid:ATPase ratio of 37:1 to 0.19 at a total phospholipid:ATPase ratio of 103:1. If it is assumed that cholesterol is excluded from the annular lipid shell, then rather than plotting y against the total phospholipid:ATPase ratio, it would seem to be more appropriate to plot y against the (total − annular phospholipid):ATPase ratio. We find that whereas a linear least-squares fit of the data of Silvius et al. (1984) of y against the total phospholipid:ATPase ratio gives a number of binding sites (N) of 26 (with a relative binding constant $K_{av} = 0.46$), a fit of y against the (total − annular phospholipid):ATPase ratio gives an N value of 11 with $K_{av} = 1.1$. Further, as described elsewhere (East et al., 1985), the method of analysis used by Silvius et al. (1984) is only truly valid for protein molecules effectively isolated within the membrane, and this

will clearly not be the case at low molar ratios of phospholipid/ATPase where the possibility of random protein–protein contact has to be taken into account. Of the seven data points obtained by Silvius et al. (1984), four were obtained at a molar ratio of phospholipid to ATPase greater than 60:1, where such random contacts can be considered unlikely (East et al., 1985). A linear least-squares fit of x against y for these four data points gives a number of binding sites $N = 5.8$ with $K_{av} = 2.9$. Unfortunately, the error on these points is such that the range of possible N values is from a negative number to 25. We conclude, therefore, that the error in spectral deconvolution of ESR spectra (East et al., 1985) and the problems of data interpretation described above are such that the ESR data of Silvius et al. (1984) cannot be considered to be inconsistent with our suggestion for a small number of binding sites for cholesterol on the ATPase, with cholesterol being excluded from the lipid annulus around the ATPase (Simmonds et al., 1982, 1984).

In previous papers, we have suggested that binding of hydrophobic molecules at annular sites will, in general, lead to a decrease in ATPase activity, whereas binding at the non-annular sites can increase activity, depending on the occupancy of the annular sites (Simmonds et al., 1982, 1984). In the following paper (Froud et al., 1986b), we consider the effects on ATPase activity in more detail.

Registry No. DOPC, 10015-85-7; ATPase, 9000-83-3; (*Z*)-$HO_2C(CH_2)_7CH{=}CHBu$, 544-64-9; (*Z*)-$HO_2C(CH_2)_7CH{=}CH(CH_2)_5Me$, 373-49-9; $Me(CH_2)_{14}CO_2H$, 57-10-3; (*Z*)-$HO_2C(CH_2)_7CH{=}CH(CH_2)_7Me$, 112-80-1; (*Z*)-$NH_2(CH_2)_8CH{=}CH(CH_2)_7Me$, 112-90-3; $NH_2(CH_2)_{17}Me$, 124-30-1; $Me(CH_2)_{15}NMe_3^+Br^-$, 57-09-0; (*Z*)-$HO(CH_2)_8CH{=}CH(CH_2)_7Me$, 143-28-2; (*Z*)-$MeO_2C(CH_2)_7CH{=}CH(CH_2)_7Me$, 112-62-9; $NH_2(CH_2)_8(CHBr)_2(CH_2)_7Me$, 104531-68-2; $HO_2C(CH_2)_7(CHBr)_2(CH_2)_7Me$, 19117-94-3; $MeO_2C(CH_2)_7(CHBr)_2(CH_2)_7Me$, 25456-04-6; $HO(CH_2)_8(CHBr)_2(CH_2)_7Me$, 62285-01-2; eicosenoic acid, 26764-41-0; cholesterol, 57-88-5.

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Effects of Lipids and Long-Chain Alkyl Derivatives on the Activity of (Ca^{2+}-Mg^{2+})-ATPase†

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Received February 19, 1986; Revised Manuscript Received August 12, 1986

ABSTRACT: The ATPase activity of the (Ca^{2+}-Mg^{2+})-ATPase reconstituted into bilayers of phosphatidylcholines depends on the fatty acyl chain length of the phospholipids. It is shown that the fluorescence response to Ca^{2+} of the ATPase modified with fluorescein isothiocyanate is also dependent on phospholipid structure and is interpreted in terms of a change in the equilibrium between two forms of the ATPase, E1 and E2. A kinetic scheme for the ATPase is presented in which ATPase activity is markedly dependent on the rate of the transition between two phosphorylated forms of the ATPase, E1′PCa_2 and E2′PCa_2, and it is postulated that changing the phospholipid structure changes this rate. The rate of dephosphorylation of the ATPase and the ATP dependence of the E1′PCa_2–E2′PCa_2 transition are also lipid dependent. Binding of oleyl alcohol causes large, lipid-dependent changes in ATPase activity, and these are interpreted in terms of changes in the rates of these same steps. Oleylamine, which has been shown to bind more strongly at annular sites than at nonannular sites, inhibits ATPase activity irrespective of lipid structure, whereas fatty acids, which bind less strongly at annular sites, only inhibit at high concentrations. Methyl oleate, which binds more strongly at nonannular sites than at annular sites, causes marked stimulation for the ATPase reconstituted with short-chain lipids.

Membrane proteins function in an environment defined in part by the lipid component of the membrane, and it is therefore important to understand the effects of lipid structure on the activities and conformations of membrane proteins. These interactions can be studied in reconstituted systems, consisting of single species of lipid and protein. We have shown that for the (Ca^{2+}-Mg^{2+})-ATPase purified from sarcoplasmic reticulum, the fluidity of the surrounding membrane has little affect on ATPase activity (East et al., 1984) as long as the lipid is in the liquid-crystalline phase (Warren et al., 1974). However, the activity of the ATPase is markedly dependent on the chemical structure of the surrounding phospholipid, with dioleoylphosphatidylcholine supporting optimal activity and lipids with other head groups or fatty acyl chains supporting lower activity (Lee et al., 1986). In apparent contrast, it has been shown that the activity of the ATPase is high when dissolved in a detergent such as dodecyl octaethylene glycol monoether ($C_{12}E_8$),[1] largely in the absence of phospholipid (Dean & Tanford, 1978; Moller et al., 1980; Kosk-Kosicka et al., 1983), from which it has been concluded that the phospholipid environment of the ATPase is relatively unimportant (Tanford, 1984).

One approach to the study of the interaction between a membrane protein and its surrounding phospholipid is to study the binding of hydrophobic molecules to the membrane protein. In the preceding paper (Froud et al., 1986), we showed how quenching of tryptophan fluorescence caused by molecules containing bromine could be used to determine binding to the ATPase. Here we study the effects of binding on the activity of the ATPase reconstituted into bilayers of defined phospholipid composition.

† This work was supported by AFRC, SERC, and Wellcome Trust. J.M.E. is a Wellcome Trust Lecturer.

[1] Abbreviations: $C_{12}E_8$, dodecyl octaethylene glycol monoether; C_{14}-PC, bis(9-*cis*-tetradecenoyl)phosphatidylcholine; C_{16}-PC, bis(9-*cis*-hexadecenoyl)phosphatidylcholine; C_{18}-PC, bis(9-*cis*-octadecenoyl)-phosphatidylcholine; C_{20}-PC, bis(11-*cis*-eicosenoyl)phosphatidylcholine; C_{22}-PC, bis(13-*cis*-docosenoyl)phosphatidylcholine; C_{24}-PC, bis(15-*cis*-tetracosenoyl)phosphatidylcholine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SR, sarcoplasmic reticulum; FITC, fluorescein 5′-isothiocyanate; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate.

0006-2960/86/0425-7544$01.50/0