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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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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.

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[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.

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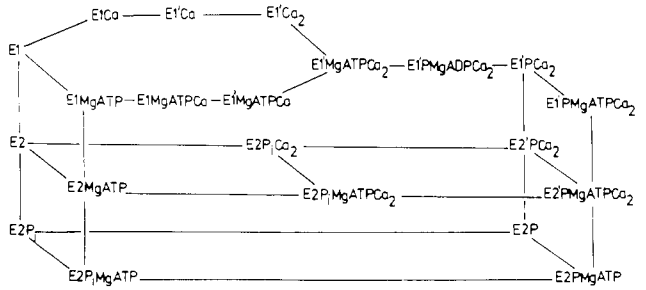
Scheme I: Simplified Model for the ATPase [See Gould et al. (1986)]



In order to understand the observed effects in molecular terms, it is necessary to relate changes in enzyme activity to specific conformational changes on the ATPase. This can only be achieved in terms of a complete kinetic model for the ATPase. Such a model has been presented elsewhere (Gould et al., 1986) and is shown in simplified form in Scheme I. The model follows closely that of de Meis and Vianna (1979) in postulating two conformational states for the ATPase, E1 and E2. The affinities for Ca^{2+} and MgATP are high in the E1 conformation but low in the E2 conformation, and the Ca^{2+} binding sites are exposed on the outer (cytoplasmic) side of the sarcoplasmic reticulum (SR) in E1 but exposed to the inside of the SR in E2. The binding of Ca^{2+} to both the E1 and E2 conformations and to the phosphorylated form E2P is cooperative, involving a conformation change on the ATPase (Gould et al., 1986). Following binding of Ca^{2+} and MgATP, the enzyme is phosphorylated and, after loss of MgADP, undergoes a conformation change to the E2 conformation from which Ca^{2+} is lost. Following hydrolytic cleavage of the phosphorylated intermediate, the enzyme recycles. Depending on experimental conditions, a number of steps in the sequence can be slow and partially rate determining, including the E2–E1 and E1'PCa_2–E2'PCa_2 transitions and dephosphorylation, the rates of these steps being modulated by MgATP. There is much evidence that the rate of the E2–E1 transition is increased by binding of MgATP, and the simulations presented in Gould et al. (1986) show that this effect can be produced by binding of MgATP to a single binding site on the ATPase, as shown in Scheme I. To fit the ATP dependence of ATPase activity, it is also necessary to postulate that MgATP binds to the phosphorylated enzyme E1'PCa_2 and that the rate of the E1'PMgATPCa_2–E2'PMgATPCa_2 transition is greater than that of the E1'PCa_2–E2'PCa_2 transition. Studies by Nakamoto and Inesi (1984) and Bishop et al. (1984) using the ATP analogue (trinitrocyclohexyldienylidine)adenosine triphosphate (TNP-ATP) have been interpreted in terms of binding of TNP-ATP to the ADP portion of the catalytic site following enzyme phosphorylation. Finally, there is also considerable evidence that the rate of dephosphorylation of E2P is also accelerated by the binding of MgATP (de Meis & de Mello, 1973; McIntosh & Boyer, 1983) and such a postulate is necessary to fit the experimental measurements of ATPase activity (Gould et al., 1986). Here it will be shown that effects of lipid environment on ATPase activity can be simulated in term of effects on these three steps.

Materials and Methods

Sarcoplasmic reticulum and purified $(Ca^{2+}-Mg^{2+})$-ATPase were prepared from female rabbit (New Zealand White) hind leg muscle as described in Froud et al. (1986). ATPase activity was determined by using a coupled enzyme assay in a medium, unless otherwise specified, containing 40 mM Hepes (pH 7.2), 5 mM $MgSO_4$, 2.1 mM ATP, 0.42 mM phosphoenolpyruvate, 0.15 mM NADH, pyruvate kinase (7.5 IU), and lactate dehydrogenase (18 IU) in a total volume of 2.5 mL, with $CaCl_2$ and EGTA added to give a saturating concentration of free Ca^{2+}. Buffers were brought to the appropriate pH by addition of KOH: at pH 7.2, the K^+ concentration was then 16 mM. The reaction was initiated by addition of an aliquot of a 25 mM $CaCl_2$ solution to a cuvette containing the ATPase and the other reagents. Concentrations of protein were estimated by using the extinction coefficient given by Hardwicke and Green (1974).

Dioleoylphosphatidylcholine (C_{18}-PC) was obtained from Lipid Products, and dimyristoleoyl- (C_{14}), dipalmitoleoyl- (C_{16}), dieicosenoyl- (C_{20}), dierucoyl- (C_{22}), and dinervonoyl- (C_{24}) phosphatidylcholines (PC) were from Avanti Polar Lipids. For lipid reconstitution, lipid (1 μmol) was mixed with buffer (40 μL; 50 mM potassium phosphate, 1 M KCl, and 0.25 M sucrose, pH 8) containing $MgSO_4$ (5 mM), ATP (6 mM), and potassium cholate (12 mg/mL) and sonicated to clarity in a bath sonicator (Megason). ATPase (0.125 mg) in a volume of 3–10 μL was then added and either incubated at 5 °C for 1 h (lipids of chain length C_{14}–C_{20}) or incubated at room temperature for 20 min followed by 40 min at 25 °C (lipids of chain length C_{22} and C_{24}). After incubation, samples were diluted with buffer (200 μL) and stored on ice until use. For ATPase assays, samples (12 μL, equivalent to 6 μg of ATPase) were added to the assay mixture (2.45 mL) described above. Assays in the presence of fatty acids were performed by addition of a solution of the fatty acid in methanol to the lipid-substituted ATPase in the assay mixture. Methyl oleate and oleyl alcohol were added to lipid before solution in the buffer containing potassium cholate. All samples were incubated in assay buffer for 10 min before initiation of the reaction by addition of Ca^{2+}.

ATPase was labeled with fluorescein 5'-isothiocyanate (FITC) as described in Froud and Lee (1986). Briefly, to obtain a labeling ratio of FITC to ATPase of ca. 0.5:1, ATPase (0.6 mg) in buffer (35 μL; 50 mM potassium phosphate, 1 M KCl, and 0.25 M sucrose, pH 8) was incubated with FITC (2.5 nmol) added from a stock solution in dry dimethylformamide (5 mM). The reaction was left to stand at room temperature for 1 h and then lipid substituted as described above, except that following incubation samples were diluted into buffer containing 50 mM Tris and 0.2 M sucrose, pH 7.0. Fluorescence spectra were recorded on Spex Fluorolog and Perkin-Elmer MPF44A fluorometers. Labeled protein (15–20 μg) was added to buffer (2.5 mL; 50 mM Tris, 50 mM maleate, 5 mM $MgSO_4$, 100 mM KCl, and 100 μM EGTA) at the appropriate pH and left to equilibrate for 30 min at 30 °C, and the fluorescence intensity was then determined at the appropriate temperature. The response to Ca^{2+} was determined by addition of a stock solution to give a final total Ca^{2+} concentration of 400 μM. The response to vanadate ions was determined by addition of a stock solution of ammonium vanadate (100 mM) in KOH (100 mM) to a final concentration of 100 μM. Fluorescence spectra were recorded with excitation at 495 nm, fluorescence intensity being measured at 525 nm.

Equations for simulations of the steady-state kinetics of the ATPase were derived by using a version of the program Kinal of Cornish-Bowden (1977) modified to run on a microcomputer, the simulations also being performed on a microcomputer.

Results

ATPase Activities. As we prepare it, the $(Ca^{2+}-Mg^{2+})$-ATPase system has a lipid to protein ratio of 30 to 1. In the lipid titration technique, the native lipids around the ATPase

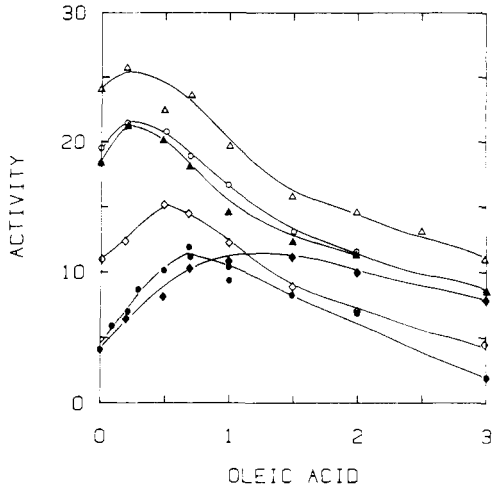


FIGURE 1: Effect of addition of oleic acid at the given molar ratios of oleic acid to phospholipid on the activity (IU per milligram) of the ATPase reconstituted with (●) C_{14}-PC, (○) C_{16}-PC, (△) C_{18}-PC, (▲) C_{20}-PC, (◊) C_{22}-PC, and (♦) C_{24}-PC. [MgATP] = 2.1 mM, pH 7.2, 37 °C.

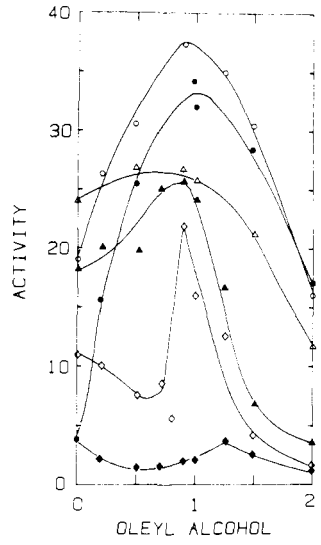


FIGURE 2: Effect of addition of oleyl alcohol at the given molar ratios of oleyl alcohol to phospholipid on the activity (IU per milligram) of the ATPase reconstituted with (●) C_{14}-PC, (○) C_{16}-PC, (△) C_{18}-PC, (▲) C_{20}-PC, (◊) C_{22}-PC, and (♦) C_{24}-PC. [MgATP] = 2.1 mM, pH 7.2, 37 °C.

are displaced by incubating the ATPase in cholate with a 1000-fold excess of test lipid, followed by a 1200-fold dilution into buffer to dissociate the cholate from the lipid–protein complex (Warren et al., 1974). When assayed at 37 °C, saturating Ca^{2+} concentration, and an ATP concentration of 2.1 mM, the ATPase activity for the ATPase reconstituted with a series of phosphatidylcholines is found to be dependent on the fatty acyl chain length of the phosphatidylcholine, the optimal chain length being 18 carbons (Figures 1–3; Lee et al., 1986). In these experiments, all fatty acyl chains were monounsaturated, ensuring that the lipids were in the liquid-crystalline phase at the temperature of the experiment. We have found that ATPase activities measured for the purified ATPase at high (millimolar) concentrations of ATP tend to be variable between preparations, without any differences in purity being obvious on SDS gels. Most commonly, preparations give an ATPase activity of ca. 18 IU/mg at 37 °C, pH 7.2, a saturating concentration of Ca^{2+}, and an ATP concentration of 2.1 mM, but activities in the range 13–20 IU/mg are observed. Activities measured at low (micromolar)

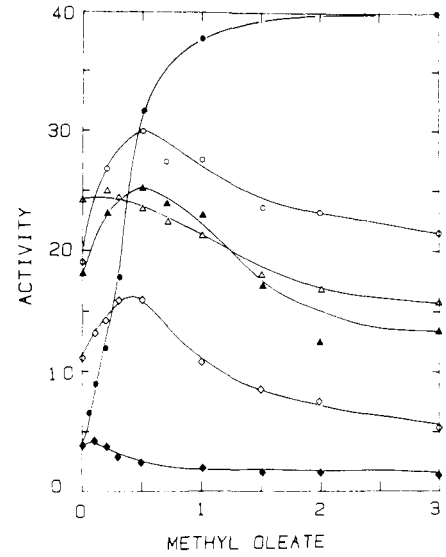


FIGURE 3: Effect of addition of methyl oleate at the given molar ratios of methyl oleate to phospholipid on the activity (IU per milligram) of the ATPase reconstituted with (●) C_{14}-PC, (○) C_{16}-PC, (△) C_{18}-PC, (▲) C_{20}-PC, (◊) C_{22}-PC, and (♦) C_{24}-PC. [MgATP] = 2.1 mM, pH 7.2, 37 °C.

concentrations of ATP are much more reproducible between preparations, showing that differences are not due to variable amounts of denatured ATPase. We have suggested that the variability could be due to differences in the degrees of stimulation of the $E1'PCa_2$–$E2'PCa_2$ transition by MgATP (Gould et al., 1986). Figure 1 shows the effect of the addition of oleic acid on the ATPase activity of the ATPase (original activity 18 IU/mg) reconstituted into a variety of phospholipid bilayers, measured at 2.1 mM ATP. Figures 2 and 3 show the effects of oleyl alcohol and methyl oleate on the reconstituted system: in these cases, because of their insolubility in water, oleyl alcohol and methyl oleate were mixed with lipid in cholate at the start of the reconstitution. Although the stimulation of activity by lower molar ratios of additive to lipid is reproducible, some variation was observed in the inhibition seen at higher molar ratios (thus, compare Figures 1 and 4). Figure 4 shows the effects of addition of 9-*cis*-tetradecenoic, 9-*cis*-hexadecenoic, 9-*cis*-octadecenoic, and 11-*cis*-eicosenoic acids on the activity of the ATPase reconstituted with C_{14}-PC (Figure 4A) and C_{18}-PC (Figure 4B), and Figure 5 illustrates the effects of oleylamine on the ATPase reconstituted with C_{18}-PC and C_{14}-PC. Elsewhere, we have presented a kinetic analysis of the ATP dependence of ATPase activity at 25 °C (Gould et al., 1986). We therefore also studied the effect of oleyl alcohol on the ATPase activity of the ATPase system reconstituted with a variety of phosphatidylcholines as a function of ATP concentration at 25 °C. Figure 6 compares the ATP dependence of activity for a low- and high-activity ATPase preparation at 25 °C and the effects of reconstitution with either C_{22}-PC or a mixture of C_{22}-PC and oleyl alcohol at a 1:1 molar ratio: ATPase activities for the low- and high-activity ATPase preparations before reconstitution were 13 and 18 IU/mg, respectively, at 2.1 mM ATP and 37 °C. Figure 7 compares ATPase activities for the ATPase reconstituted with C_{22}-PC and oleyl alcohol at molar ratios of 1:1 and 1.3:1. Figure 8 illustrates ATPase activities for the ATPase reconstituted with phosphatidylcholines with chain lengths between C_{14} and C_{24} and the effects of oleyl alcohol on these systems. It has been reported previously that decane increases the activity of the ATPase reconstituted with C_{14}-PC (Johannsson et al., 1981). Table I shows the effect of decane on this system in the presence of oleyl alcohol and oleic acid.

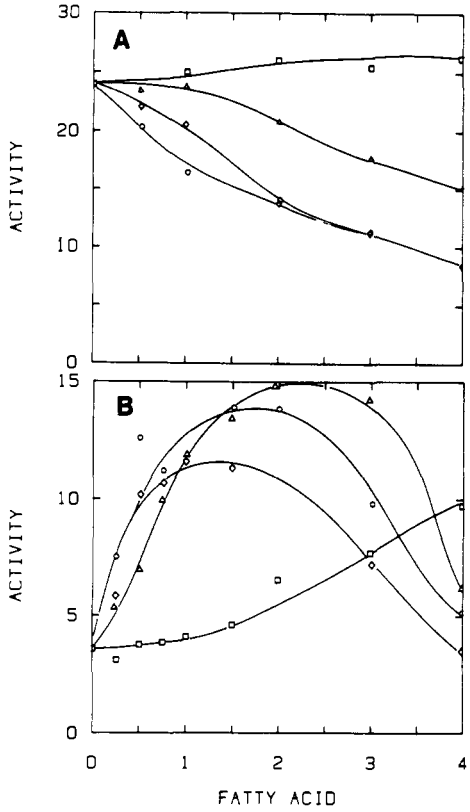


FIGURE 4: Effect of addition of fatty acids at the given molar ratios of fatty acid to phospholipid on the activity (IU per milligram) of the ATPase reconstituted with (A) C_{18}-PC and (B) C_{14}-PC: (□) myristoleic acid; (△) palmitoleic acid; (◇) oleic acid; (○) eicosenoic acid. [MgATP] = 2.1 mM, pH 7.2, 37 °C.

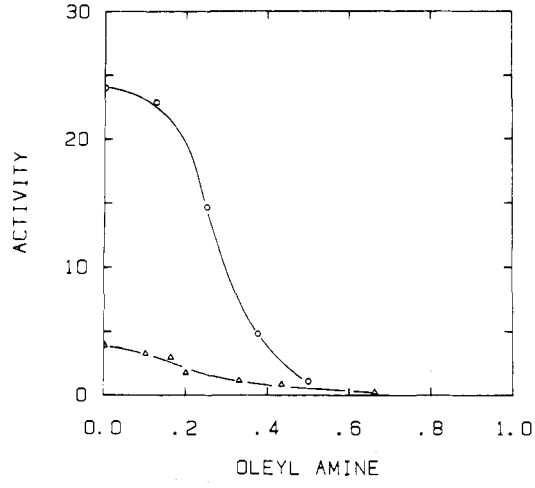


FIGURE 5: Effect of addition of oleylamine at the given molar ratios of oleylamine to phospholipid on the activity (IU per milligram) of the ATPase reconstituted with (△) C_{14}-PC or (○) C_{18}-PC. [MgATP] = 2.1 mM, pH 7.2, 37 °C.

Table I: Effect of Decane on the ATPase Activity of (C_{14}-PC) ATPase at 37 °C, 2 mM ATP, pH 7.2

additive	additive:lipid molar ratio	activities (IU/mg) molar ratio decane:lipid 0:1	1:1	1.3:1
none		3.7	35.2	36.2
oleyl alcohol	0.2:1	16.0	39.3	36.8
oleic acid	0.7:1	11.8	21.4	22.6
	1:1	10.5	18.5	20.6
	2:1	5.7	10.7	11.6

Fluorescein-Labeled ATPase. It has been shown that fluorescein isothiocyanate specifically labels the ATP binding

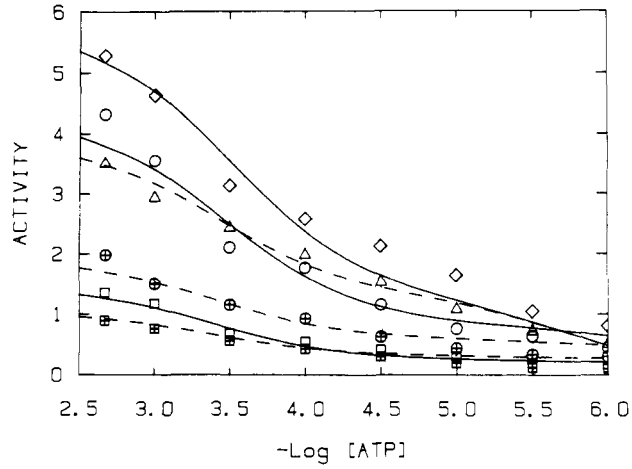


FIGURE 6: ATP concentration dependence of the activity (IU per milligram) of a low-activity preparation of the ATPase (△) and after reconstitution into C_{22}-PC in the absence (⊕) or presence (⊞) of oleyl alcohol at an alcohol:lipid molar ratio of 1:1 and for a high-activity preparation (◇) also after reconstitution into C_{22}-PC in the absence (○) or presence (□) of oleyl alcohol at a molar ratio of 1:1, pH 7.2, 25 °C. Lines represent simulations using the parameters in Table III.

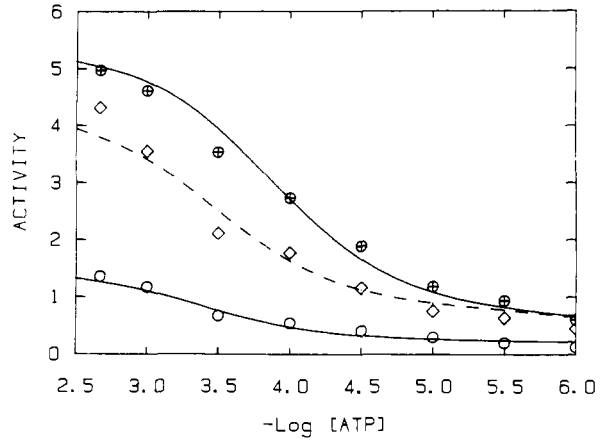


FIGURE 7: ATP concentration dependence of the activity (IU per milligram) of the ATPase reconstituted into C_{22}-PC alone (◇) or in the presence of oleyl alcohol at an alcohol:lipid molar ratio of 1:1 (○) or 1.3:1 (⊕), pH 7.2, 25 °C. Lines represent simulations using the parameters in Table III.

site of the ATPase (Pick & Karlish, 1980). In a series of papers, Pick has shown that addition of Ca^{2+} to the modified ATPase results in a small decrease in fluorescein fluorescence whereas addition of vanadate ions results in a small increase in fluorescence (Pick, 1982). Pick (1982) has suggested that the changes in fluorescein fluorescence follow only from changes in the E2/E1 equilibrium and that the ratio E1/E2 in the absence of Ca^{2+} or vanadate is given by

$$E1/E2 = b/a \quad (1)$$

where a is the Ca^{2+}-induced fluorescence quenching in the absence of vanadate and b is the vanadate-induced fluorescence enhancement. As discussed in Froud and Lee (1986), this analysis gives values for the E1/E2 equilibrium constant inconsistent with kinetic data, possibly because the fluorescence change on addition of vanadate does not simply reflect the change to the E2 conformation. However, we have shown that if it is assumed that the response to Ca^{2+} does correspond simply to changes in the E2–E1 equilibrium, then it is possible to determine the ratio E1/E2 under any set of conditions from the calcium response alone, as long as a value for the E1/E2 ratio is known under at least one set of conditions. It can be

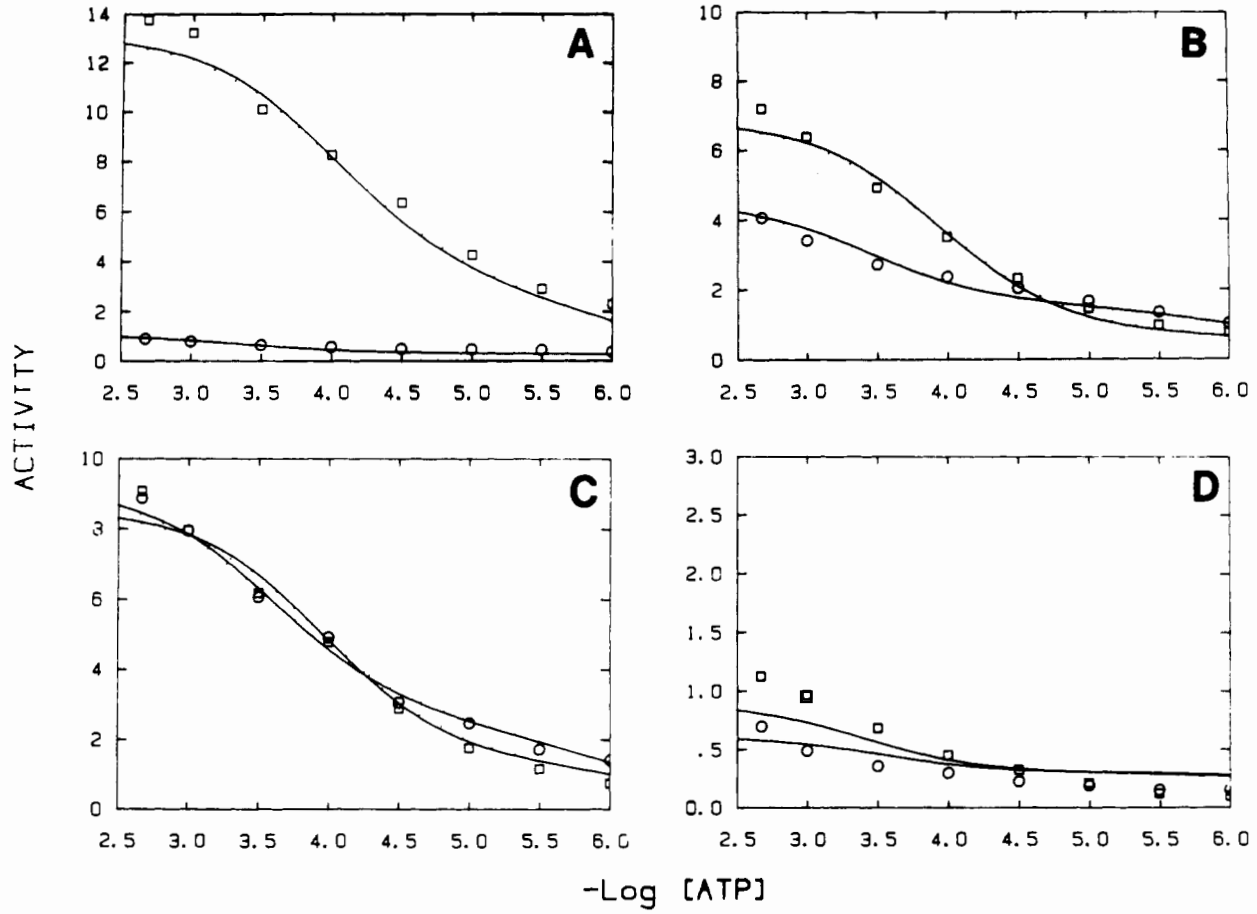


FIGURE 8: ATP concentration dependence of the activity (IU per milligram) of the ATPase reconstituted with phosphatidylcholines in the absence (○) or presence (□) of oleyl alcohol at an alcohol:lipid molar ratio of 1:1. (A) C_{14}-PC; (B) C_{18}-PC; (C) C_{20}-PC; (D) C_{24}-PC, pH 7.2, 25 °C. Lines represent simulations using the parameters in Table III.

readily shown that if under one set of conditions the calcium response and ratio E1/E2 are a_1 and x_1, respectively, and under another set of conditions are a_2 and x_2, respectively, then

$$x_2 = [x_1 a_1 + (a_1 - a_2)]/a_2 \quad (2)$$

On the basis of kinetic studies, it has been suggested that the value of E1/E2 for the native ATPase is 0.14 at pH 6 and 25 °C (Froud & Lee, 1986).

Table II shows the fluorescence response of fluorescein-labeled ATPase to the addition of calcium and vanadate, after reconstitution into various phospholipid systems. It is important in these experiments to be able to confirm that reconstitution is successful, and this is most easily done by measurement of ATPase activity and comparison with the data in Figures 1–3. Since fluorescein labeling of the ATPase inhibits ATPase activity, we labeled the ATPase at a molar ratio of FITC to ATPase of 0.4:1 so that the ATPase activity of the unmodified ATPase in the system could be measured: ATPase activity is linearly related to the fraction of ATPase unmodified by FITC, and we have shown that the fractional response of fluorescein fluorescence to the addition of Ca^{2+} and vanadate is independent of the extent of labeling up to an FITC:ATPase molar ratio of 1:1 (Froud & Lee, 1986). Values for the ratio E1/E2 were calculated from eq 2 with the results listed in Table II.

Discussion

In our reconstituted systems, the ATPase is present in unsealed membrane fragments so that ATPase activity is fully uncoupled from the accumulation of Ca^{2+} and the full ATPase activity is exhibited. Messineo et al. (1984) have shown that effects of fatty acids on sealed SR vesicles are complex, at least in part because of effects on Ca^{2+} sequestration: such effects will be absent in our system.

The activity of the (Ca^{2+}–Mg^{2+})-ATPase is markedly dependent on the structure of the surrounding phospholipid, with dioleoylphosphatidylcholine (C_{18}-PC) supporting maximal activity (Figures 1–3). For the ATPase reconstituted with a short-chain lipid such as C_{14}-PC or C_{16}-PC, addition of oleic acid, oleyl alcohol, or methyl oleate causes a large increase in activity, maximal activities in the presence of oleyl alcohol or methyl oleate being higher than observed for the ATPase reconstituted with C_{18}-PC. In contrast, for the ATPase reconstituted with the long-chain lipids C_{22}-PC and C_{24}-PC, little stimulation is seen on addition of the alkyl derivatives. At higher concentrations, these additives cause inhibition, particularly marked for oleic acid (Figure 1), and oleylamine only causes inhibition (Figure 5). The effect of oleyl alcohol on the activity of the ATPase reconstituted with C_{22}-PC is particularly complex, with a marked and sharp increase in activity at molar ratios of alcohol to lipid between 0.8:1 and 0.9:1, at 37 °C (Figure 2).

It has been suggested that the activities of membrane proteins depend on membrane "microviscosities" and that the lipid composition of the membrane affects the activities of membrane proteins through effects on microviscosity (Shinitzky, 1984). It has also been suggested that hydrophobic molecules that bind to the membrane can affect enzyme activities through effects on microviscosities (Shinitzky, 1984). It is hard to see how activity profiles of the type presented here could follow from effects on membrane microviscosity. Indeed, archetypal membrane "fluidizers" and "rigidifiers" are taken to be un-

Table II: Response of Fluorescein-Labeled ATPase to Calcium and Vanadate and Calculated E1/E2 Ratios at 20 °C

system	pH	% fluorescence response to Ca^{2+}	% fluorescence response to vanadate	E1/E2 ratio
native ATPase	6	−7.6	7.5	0.14[a]
	7	−5.6	11.0	0.5
	8	−3.0	10.5	1.9
C_{18}-PC ATPase	6	−7.3	7.7	0.2
	7	−6.1	10.3	0.4
	8	−3.0	10.5	1.9
C_{18}-PC ATPase + oleic acid, 2:1	6	−5.8	4.9	0.5
	7	−4.8	9.9	0.8
	8	−2.3	4.6	2.8
C_{18}-PC ATPase + oleic acid, 3:1	6	−5.3	4.4	0.6
	7	−5.2	9.3	0.7
C_{18}-PC ATPase + oleyl alcohol, 1:1	6	−6.1	6.5	0.4
	7	−4.5	11.4	0.9
	8	−3.0	10.3	1.9
C_{18}-PC ATPase + oleyl alcohol, 3:1	6	−4.0	5.0	1.1
	7	−5.2	10.0	0.9
	8	−2.2	8.8	2.9
C_{18}-PC ATPase + methyl oleate, 1:1	6	−7.0	7.5	0.2
	7	−6.0	11.3	0.4
	8	−4.1	11.1	1.1
C_{18}-PC ATPase + methyl oleate, 3:1	6	−5.5	5.3	0.6
	7	−5.0	12.4	0.7
	8	−3.6	12.0	1.4
C_{14}-PC ATPase	6	−3.1		1.8
	7	−1.4		5.2
C_{14}-PC ATPase + oleic acid, 0.5:1	7	−2.2	14.6	2.9
C_{14}-PC ATPase + oleic acid, 1:1	7	−2.9	12.5	3.2
C_{14}-PC ATPase + oleic acid, 3:1	7	−3.6	4.9	1.4
C_{14}-PC ATPase + methyl oleate, 0.05:1	7	−2.0	3.8	3.3
C_{14}-PC ATPase + methyl oleate, 0.1:1	7	−1.9	10.7	3.6
C_{14}-PC ATPase + methyl oleate, 0.2:1	7	−2.4	10.0	2.6
C_{14}-PC ATPase + methyl oleate, 0.25:1	7	−2.5	11.8	2.5
C_{14}-PC ATPase + methyl oleate, 0.5:1	7	−4.0	9.4	1.2
C_{14}-PC ATPase + methyl oleate, 1:1	7	−4.8	11.3	0.8
C_{14}-PC ATPase + methyl oleate, 3:1	7	−3.7	10.0	1.3
C_{18}-PC/C_{14}-PC, 1:1, − ATPase	7	−3.7	10.2	1.3
C_{18}-PC/C_{14}-PC, 1:4, − ATPase	7	−1.3	2.2	5.7

[a] Fixed: see text.

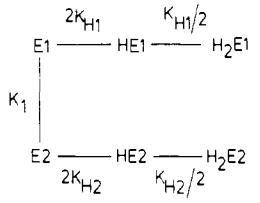
saturated fatty acids, such as oleic acid, and sterols, such as cholesterol or cholesterol hemisuccinate, respectively (Shinitzky, 1984), and yet fatty acids (Figures 1 and 4) and cholesterol and cholesterol hemisuccinate (Simmonds et al., 1982, 1984) all increase ATPase activity for the ATPase reconstituted with C_{14}-PC.

It is more likely that the conformational state of the ATPase is sensitive to the chemical structure of the surrounding phospholipids and that hydrophobic molecules affect activity by direct binding to the ATPase. In the preceding paper (Froud et al., 1986), we have interpreted binding of hydrophobic molecules to the ATPase in terms of binding to a homogeneous lipid annulus together with binding to a set of other sites on the ATPase from which phospholipids are excluded (referred to as nonannular sites). We have suggested that these nonannular sites could lie at protein–protein interfaces in ATPase dimers. From the dissociation constants for binding to lipid, to annular sites, and to nonannular sites given in Froud et al. (1986), it is possible to calculate the fraction of the annular and nonannular sites occupied by the hydrophobic additives under the conditions used for activity measurements. The binding constants for oleyl alcohol and methyl oleate are such that, under the conditions of these experiments, they will be essentially all bound to the membrane whereas at a 1:1 molar ratio of additive to lipid, for example, only 53% of oleylamine, 38% of oleic acid, and 2% of myristoleic acid will be bound. At a 1:1 molar ratio of additive to lipid, it can then be calculated that the fraction of annular sites occupied by oleylamine will be 0.31, by oleic acid 0.09, by myristoleic acid 0.03, by oleyl alcohol 0.08, and by methyl oleate less than 0.01. Binding to annular sites would be expected to cause a decrease in activity since it involves displacement of phospholipid from around the ATPase. Consistent with this interpretation are the observations that oleylamine causes inhibition in all systems (Figure 5), that oleic acid (Figure 1) and oleyl alcohol (Figure 2) cause inhibition at high concentrations, and that methyl oleate causes little inhibition (Figure 3). For the fatty acids, myristoleic acid causes no net inhibition, whereas palmitoleic acid causes inhibition, but at a higher concentration than required for oleic or eicosenoic acids (Figure 4), as expected from the increased strength of binding to lipid bilayers with increasing chain length (Table I; Froud et al., 1986) and as shown by the decreasing effective annular dissociation constants with increasing chain length (Table II; Froud et al., 1986).

Fluorescence quenching experiments also indicated binding of hydrophobic molecules to sites on the ATPase distinct from the annular sites (Froud et al., 1986). We have suggested previously that binding to these nonannular sites could explain the increases in activity seen for the ATPase reconstituted with C_{14}-PC (Simmonds et al., 1982, 1984). Effects of additives on the activity of the ATPase would then follow from the balance between stimulation following binding to nonannular sites and inhibition following binding to annular sites. For oleylamine, the relatively strong binding to annular sites and weak binding to nonannular sites (measured with respect to lipid-bound oleylamine; Table II; Froud et al., 1986) means that at a 1:1 molar ratio of oleylamine to lipid, the fractional occupation of annular and nonannular sites will be 0.31 and 0.05, respectively, and only inhibition of activity is seen (Figure 5). At the other extreme, for methyl oleate at a 1:1 molar ratio to lipid, the fractional occupations of annular and nonannular sites will be less than 0.01 and 0.2, respectively, and marked stimulation is observed with little inhibition (Figure 3). For the fatty acids at a 1:1 molar ratio to lipid, the fractional occupations of annular and nonannular sites change from 0.03 and 0.05, respectively, for myristoleic acid to 0.09 and 0.28, respectively, for oleic acid, explaining the more marked effects of the longer chain acid (Figure 4). The et al. (1981) have also found that the ability to reactivate delipidated ATPase increases with increasing chain length from C_{12} to C_{14}. For oleyl alcohol, the fractional occupations calculated from the parameters given in Froud et al. (1986) are comparable to those for oleic acid, although stimulation is more marked for oleyl alcohol than for oleic acid (Figure 1 and 2). This could reflect inaccuracies in the parameters for oleyl alcohol due to its limited water solubility (Froud et al., 1986). It also seems unlikely that the activity of the ATPase will depend solely on the fractional occupancy of sites on the ATPase and more likely that the structure of the additive will have some effect.

It has been suggested that the activity of the ATPase is dependent on the thickness of the membrane and that hy-

Scheme II: Effect of H^+ Binding on the E1/E2 Equilibrium



drophobic molecules such as alkanes could affect activity through changes in membrane thickness (Johannsson et al., 1981). An alternative explanation for the lesser effect of fatty acids than of oleyl alcohol or methyl oleate on the activity of the ATPase reconstituted with C_{14}-PC could therefore be that as well as any effects following from direct binding to the ATPase, the latter molecules cause an increase in thickness for the membrane whereas the fatty acids do not. This explanation is ruled out by the data in Table I which show that addition of decane to the ATPase reconstituted with C_{14}-PC in the presence of oleic acid does not increase activity to the level seen with oleyl alcohol and indeed that the activity seen in the presence of decane and oleic acid is less than that seen in the presence of decane alone, as expected if oleic acid is causing some inhibition.

Interpretation of the dependence on phospholipid acyl chain length of the effects on ATPase activity of these various alkyl derivatives requires more detailed consideration of the kinetics of the ATPase. In previous publications, we have presented a complete kinetic model for the (Ca^{2+}–Mg^{2+})-ATPase (Froud & Lee, 1986; Gould et al., 1986), and this is illustrated in simple form in Scheme I. The model postulates two conformations for the ATPase, E1 and E2, with the transition between the two conformations being the transport step. The E1 conformation has a higher affinity for MgATP than the E2 conformation: a consequence of this difference in affinities of the two conformations for MgATP is that rates of the transitions between the E1 and E2 conformations will be different from the corresponding rates for the transitions between E1MgATP and E2MgATP. It is also postulated that MgATP can bind in the nucleotide binding region of the phosphorylated forms of the enzymes E1′PCa_2 and E2′PCa_2 after dissociation of MgADP and that the rate of the transition E1′PMgATPCa_2–E2′PMgATPCa_2 is faster than the rate of the transition E1′PCa_2–E2′PCa_2. There are three relatively slow steps in the reaction sequence which, depending on the exact experimental conditions, can become partially rate determining, these steps being the E2–E1 and E1′PCa_2–E2′PCa_2 transitions and the rate of dephosphorylation: all three of these steps are modulated by ATP (Gould et al., 1986).

In the model (Scheme I), the steps involving transport of Ca^{2+} across the membrane are the transitions E1′PCa_2–E2′PCa_2 and E1′PMgATPCa_2–E2′PMgATPCa_2, and the steps returning empty Ca^{2+} binding sites to the outer face of the membrane are E2–E1 and E2MgATP–E1MgATP. It seems possible, therefore, that these steps will be sensitive to the membrane environment of the ATPase. We have shown elsewhere how the change in fluorescence intensity for the ATPase labeled with FITC following addition of Ca^{2+} can be used to determine the ratio E1/E2 (Froud & Lee, 1986). A change in this ratio would necessarily mean a change in the rate of either the transition E1 to E2 or the transition E2 to E1 or both.

We have found that the ratio E1/E2 is sensitive to pH and have interpreted the pH dependence in terms of Scheme II. For the native ATPase (Table II), the data can be fitted by assuming proton association constants $K_{H1} = 4.0 \times 10^6$ and

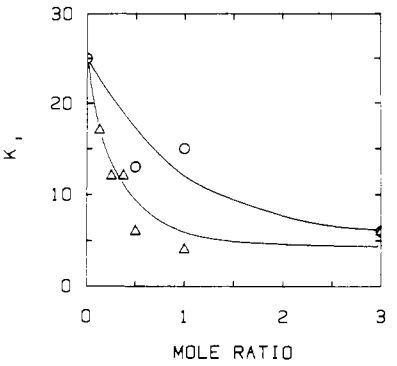


FIGURE 9: Effect of addition of oleic acid (○) or methyl oleate (△) at the given molar ratios of additives to phospholipids on the equilibrium constant K_1 describing the E1/E2 equilibrium for the ATPase reconstituted with C_{14}-PC. Values calculated as described in the text from the data given in Table II.

$K_{H2} = 2.0 \times 10^7$ and $K_1 = 2.5$ (Froud & Lee, 1986). Reconstitution of the ATPase into bilayers of C_{18}-PC causes no significant change in the ratio E1/E2, so that the parameters K_{H1}, K_{H2}, and K_1 are little changed. Reconstitution into the short-chain lipid C_{14}-PC, however, causes a major change in the E1/E2 ratio (Table II) which can be simulated reasonably well in terms of Scheme II by assuming a change in the K_1 value to 25 (giving E1/E2 = 1.4 at pH 6 and 5.4 at pH 7). As expected, the response to Ca^{2+} (and hence the E1/E2 ratio) for a 1:1 mixture of C_{14}-PC and C_{18}-PC is intermediate between the responses in C_{14}-PC and C_{18}-PC alone (Table II).

Addition of low mole ratios of oleic acid, oleyl alcohol, or methyl oleate to the FITC–ATPase reconstituted with C_{18}-PC has relatively little effect on the fluorescence response to Ca^{2+}, with higher concentrations reducing the response to Ca^{2+} at pH 6 with relatively little effect at pH 7 or 8. The origin of this effect is not clear, but it corresponds to the concentration range where enzyme activity is reduced (Figures 1–3). In contrast, addition of oleic acid or methyl oleate to the FITC–ATPase reconstituted with C_{14}-PC causes an increase in the fluorescence response to Ca^{2+} at pH 7 to values close to those observed for the FITC–ATPase reconstituted with C_{18}-PC. These effects can be simulated by assuming that addition of oleic acid or methyl oleate affects the equilibrium constant K_1 of Scheme II, as shown in Figure 9. By comparison with Figures 1 and 3, it is clear that K_1 is approaching the value typical for the native ATPase over the concentration range over which activity is markedly simulated.

Variation in the equilibrium constant K_1 with lipid implies differences in the rates of the transitions between the E1 and E2 conformations. However, the observed dependence of ATPase activity on the concentration of ATP (Figures 6–8) cannot be reproduced in terms of Scheme I, assuming that the major effect of lipid substitution follows from an effect on the rate of the E2–E1 transition (not shown). However, an effect of lipid on the equilibrium constant E1/E2 implies an equal and opposite effect on the equilibrium constant E1′PCa_2/E2′PCa_2, since overall the reaction cycle of Scheme I corresponds to the hydrolysis of ATP and there is no suggestion that this is in any way dependent on the lipid present in the system. Variation in the rate of the E1′PCa_2–E2′PCa_2 transition has very marked effects on ATPase activity. The data on ATPase activity for the ATPase reconstituted into a series of phosphatidylcholines of different chain lengths can be simulated by assuming that the rate of the E1′PCa_2–E2′PCa_2 transition is greatest for a C_{20} chain length and decreases with increasing or decreasing chain length from this optimum value (Table III). It is postulated that binding of MgATP to

Table III: Kinetic Parameters Obtained by Simulation for the Reconstituted ATPase at 25 °C, pH 7.2[a]

ATPase	lipid	oleyl alcohol:lipid/molar ratio	equilibrium constant MgATP to E1′PCa_2, K_7	rate constants (s^{-1}) E1′PCa_2–E2′PCa_2,[b] K_{24}	rate constants (s^{-1}) E1′PMgATPCa_2–E2′PMgATPCa_2, K_9	ATP stimulation	E2P[c] dephosphorylation
low activity	native		2.2×10^3	11.5	41.4	3.6	26.4
	C_{14}-PC		2.2×10^3	2.3	8.3	3.6	52.8
	C_{14}-PC	1:1	5.0×10^3	46.0	322.0	7.0	52.8
	C_{18}-PC		2.2×10^3	13.8	50.0	3.6	31.7
	C_{18}-PC	1:1	5.0×10^3	5.8	115.0	20	26.4
	C_{22}-PC		2.2×10^3	4.6	16.6	3.6	26.4
	C_{22}-PC	1:1	2.2×10^3	2.3	8.3	3.6	26.4
	C_{24}-PC		2.2×10^3	4.6	16.6	3.6	1.3
	C_{24}-PC	1:1	2.2×10^3	2.3	8.3	3.6	7.9
high activity	native		2.2×10^3	11.5	80.5	7.0	26.4
	C_{20}-PC		2.2×10^3	28.8	201.2	7.0	31.7
	C_{20}-PC	1:1	5.0×10^3	13.8	165.6	12.0	31.7
	C_{22}-PC		2.2×10^3	6.9	48.3	7.0	26.4
	C_{22}-PC	1:1	2.2×10^3	1.7	12.0	7.0	26.4
	C_{22}-PC	1.3:1	5.0×10^3	6.9	69.0	10.0	26.4

[a] Symbols refer to the complete kinetic scheme given in Gould et al. (1986). [b] For the protonated form in the complete scheme presented in Gould et al. (1986). [c] Composite value for [K] = 16 mM, [Mg] = 5 mM, pH 7.2.

E1′PCa_2 increases the rate of transition to the E2 conformation (Gould et al., 1986). The stimulation caused by binding of MgATP has been found to vary between preparations, with a stimulation of 3.6 for a "low-activity" preparation and 7.0 for a "high-activity" preparation (Table III). Although the reason for this variation has not yet been established, one possibility is that the stimulation caused by MgATP depends on thiol groups in the enzyme: transient activation of the ATPase has been observed during thiol modification with 5,5′-dithiobis(2-nitrobenzoate) (Swoboda & Haselbach, 1983), and inhibition of the E1′PCa_2–E2′PCa_2 transition has been observed after modification with *N*-ethylmaleimide (Nakamura & Tonomura, 1982). The degree of stimulation caused by binding of MgATP appears to be largely independent of phospholipid (Table III). Effects of changing the phospholipid fatty acyl chain length on ATPase activity are different at high and low concentrations of MgATP (Figures 6–8), and this pattern cannot be reproduced, assuming that only one step in the reaction sequence is modified by changing phospholipid. Previous studies have shown that the rate of dephosphorylation of the phosphorylated ATPase is slower than normal in delipidated preparations of the ATPase or for the ATPase reconstituted into lipid bilayers in the gel phase (Martonosi et al., 1974; Hidalgo et al., 1976; Nakamura et al., 1976). Assuming that dephosphorylation is also affected by changing phospholipid structure, it is possible to simulate the ATP dependence of ATPase activity for all the reconstituted systems (Figures 6–8), with the rate of dephosphorylation decreasing with increasing chain length (Table III).

The effects of oleyl alcohol on the activity of the ATPase reconstituted into bilayers of phosphatidylcholines of different chain lengths can then be simulated in terms of changes in the rates of these same steps (Table III). Thus, addition of oleyl alcohol to the ATPase reconstituted with C_{14}-PC causes a large increase in the rate of the transition E1′PCa_2–E2′PCa_2, to above that for the native ATPase: results with FITC–ATPase reconstituted into C_{14}-PC show a large increase in the E1/E2 ratio (Table II). For the ATPase reconstituted into phosphatidylcholines with chain lengths of C_{18} or longer, oleyl alcohol at a molar ratio of 1:1 with phospholipid causes a decrease in the rate of the E1′PCa_2–E2′PCa_2 transition, the net result being that the rate of the E1′PCa_2–E2′PCa_2 transition in the presence of oleyl alcohol increases with decreasing fatty acyl chain length. Binding of oleyl alcohol also affects the stimulation caused by binding of MgATP so that the rate of the E1′PMgATPCa_2–E2′PMgATPCa_2 transition also increases with decreasing fatty acyl chain length. For the ATPase reconstituted with C_{24}-PC, where the rate of dephosphorylation is very slow, addition of oleyl alcohol causes some increase in the rate of dephosphorylation, but for the other reconstituted systems, where the rates of dephosphorylation are little changed from that in the native ATPase, oleyl alcohol appears to have no significant effect on the rate of dephosphorylation. Finally, oleyl alcohol affects the affinity of E1′PCa_2 for MgATP, the affinity doubling for the ATPase reconstituted into short-chain lipids at a 1:1 molar ratio of oleyl alcohol to lipid, and for the ATPase reconstituted into longer chain lipids at a 1.3:1 molar ratio of oleyl alcohol to lipid. The effect of the increase in affinity for MgATP is to cause a large increase in ATPase activity at high concentrations of MgATP. If the effect on MgATP affinity were to come from an "all or none" conformation change on the ATPase, it would explain the sharp and discontinuous increase in activity with increasing concentrations of oleyl alcohol observed in Figure 2: the different concentration range over which the effect occurs in Figures 2 and 7 is a consequence of the different temperatures for the two experiments. For the ATPase reconstituted with C_{24}-PC, effects on the rate of the E1′PCa_2–E2′PCa_2 transition will have much less effect on the net ATPase activity, because activity is much reduced by the much reduced rate of dephosphorylation (Table III).

The ATP dependence of activity for the ATPase reconstituted with C_{14}-PC in the presence of oleyl alcohol is reminiscent of that for the ATPase dissolved in the detergent $C_{12}E_8$ (Kosk-Kosicka et al., 1983). Since the ATPase is monomeric in $C_{12}E_8$ (Kosk-Kosicka et al., 1983), both annular and nonannular sites will be occupied, as postulated in the C_{14}-PC/oleyl alcohol system. In contrast, it has been found that those detergents which dissolve the ATPase in aggregated form support only very low activities (Dean & Suarez, 1984). From the published data, it can be estimated that the rate of dephosphorylation for the ATPase dissolved in $C_{12}E_8$ (Kosk-Kosicka et al., 1983) or in Triton X-100, which also supports high activity (McIntosh & Davidson, 1984), is about twice that for the native ATPase; the same increase in rate was suggested for the ATPase reconstituted into C_{14}-PC (Table III).

Clearly, a full understanding of the effects of binding at the postulated annular and nonannular sites on the ATPase will require much further study. Nevertheless, we have shown here

that the complex effects observed for at least one molecule, oleyl alcohol, can be understood in terms of a coherent kinetic model for the ATPase. The model indicates that binding at nonannular sites has marked effects on the rate of the transition $E1'PCa_2$–$E2'PCa_2$ which is the transport step. Since it has been suggested that these nonannular sites lie at protein–protein interfaces in dimers of ATPase molecules (Froud et al., 1986), it would seem that protein–protein interactions are involved in maintaining the native conformation of the ATPase found in the sarcoplasmic reticulum and that changes in protein–protein interaction could occur on substitution of the native lipids around the ATPase.

Registry No. C_{14}-PC, 77285-90-6; C_{16}-PC, 65206-87-3; C_{18}-PC, 10015-85-7; C_{20}-PC, 104757-69-9; C_{22}-PC, 56649-39-9; C_{24}-PC, 86288-10-0; ATPase, 9000-83-3; Ca, 7440-70-2; oleyl alcohol, 143-28-2; methyl oleate, 112-62-9; myristoleic acid, 544-64-9; palmitoleic acid, 373-49-9; oleic acid, 112-80-1; eicosenoic acid, 28933-89-3; oleylamine, 112-90-3; vanadate, 14333-18-7; decane, 124-18-5.

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