Biochimica et Biophysica Acta 860 (1986) 354–360
Elsevier

BBA 72932

Effects of lipid fatty acyl chain structure on the activity of the $(Ca^{2+} + Mg^{2+})$-ATPase

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(Received March 26th, 1986)

Key words: $(Ca^{2+} + Mg^{2+})$-ATPase; Lipid-protein interaction; Fluorescence quenching; Phytanoyl phospholipid; Acyl chain structure; (Rabbit muscle)

The $(Ca^{2+} + Mg^{2+})$-ATPase purified from rabbit muscle sarcoplasmic reticulum has been reconstituted into a series of phosphatidylcholines in the liquid crystalline phase. For phosphatidylcholines containing mono-unsaturated fatty acyl chains, optimal activity is observed for a chain length of C18, with longer or shorter chains supporting lower activities. Phospholipids with methyl-branched chain saturated fatty acids support somewhat lower activities than the corresponding phospholipids with mono-unsaturated fatty acids. Mixed chain phospholipids support ATPase activities comparable to those shown by an unmixed chain phospholipid with the same average chain length. However, the response of the ATPase reconstituted with mixed chain phospholipids to the addition of oleyl alcohol is dominated by the longest fatty acyl chain. Based on their ability to displace brominated phospholipids, relative binding constants to the ATPase of a series of phosphatidylcholines have been determined. Binding to the ATPase is virtually unaffected by fatty acyl chain length or the presence of methyl branches.

Introduction

In the fluid mosaic model of the biological membrane, membrane proteins are pictured as embedded in a lipid bilayer [1]. It seems likely, therefore, that the activities of membrane proteins will depend on the chemical structures of the phospholipids in the membrane. The complexity of the lipid composition of natural membranes makes this dependency difficult to study, and thus the most detailed information about lipid protein interactions has come from studies of simplified, reconstituted membrane systems, consisting of single species of phospholipid and protein. Using such a system it has been shown that the ATPase activity of the $(Ca^{2+} + Mg^{2+})$-ATPase purified from muscle sarcoplasmic reticulum is independent of the fluidity of the surrounding phospholipid, as long as the phospholipid remains in the liquid-crystalline phase [2]. The activity of the ATPase is, however, markedly dependent on the chemical structure of the surrounding phospholipid, with dioleoylphosphatidylcholine supporting the highest activity [3–5]. Surprisingly, however,

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Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; BRPC, 1,2-di(9,10-dibromostearoyl)phosphatidylcholine; C_{14}-PC, 1,2-dimyristoleoylphosphatidylcholine; C_{16}-PC, 1,2-dipalmitoleoylphosphatidylcholine; C_{18}-PC, 1,2-dioleoylphosphatidylcholine; C_{20}-PC, 1,2-dieicosenoylphosphatidylcholine; C_{22}-PC, 1,2-dierucoylphosphatidylcholine; C_{24}-PC, 1,2-dinervonylphosphatidylcholine; $C_{14}C_{18}$-PC, 1-myristoyl-2-oleoylphosphatidylcholine; $C_{18}C_{14}$-PC, 1-oleoyl-2-myristoylphosphatidylcholine; $3MeC_{16}$-PC, 1,2-di(3-methylhexadecanoyl)phosphatidylcholine; Me_4C_{16}-PC, diphytanoylphosphatidylcholine; $4MeC_{16}$PC, 1,2-di(4-methylhexacanoyl)phosphatidylcholine.

there is little selectivity in binding of phospholipids to the ATPase [4–6]. Here we study the effect of phospholipid fatty acyl chain length in more detail, and also study the effect of branched fatty acyl chains.

The ATPase is reconstituted into bilayers of defined phospholipid composition by mixing the purified ATPase with excess phospholipid in cholate, followed by a 1200-fold dilution into buffer, so that the concentration of cholate drops much below its critical micelle concentration, forming fragments of membrane containing the ATPase inserted into bilayers with a phospholipid composition predominantly that of the added phospholipid [5]. Binding constants of phospholipids to the ATPase are determined using a fluorescence quenching method [5]. When the ATPase is reconstituted into bilayers containing the brominated lipid 1,2-di(9,10-dibromostearoyl)phosphatidylcholine (BRPC), 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 phospholipid to the system which can bind strongly to the lipid-protein interface will result in displacement of BRPC from the protein surface and thus to an increase in fluorescence intensity. Addition of a phospholipid to the system which can bind only weakly to the lipid-protein interface will result in displacement of less BRPC and thus to a smaller increase in fluorescence intensity. These measurements can be quantitated readily to give relative lipid binding constants for the ATPase [5].

Materials and Methods

Dimyristoleoyl (C_{14}-PC), dipalmitoleoyl (C_{16}-PC), dieicosenoyl (C_{20}-PC), dierucoyl (C_{22}-PC), dinervonoyl (C_{24}-PC), 1-myristoyl-2-oleoyl ($C_{14}C_{18}$-PC) and 1-oleoyl-2-myristoyl-phosphatidylcholines ($C_{18}C_{14}$-PC) were obtained from Avanti Polar Lipids and dioleoylphosphatidylcholine (C_{18}-PC) was obtained from Lipid Products. Lipids were used as obtained. Di(9,10-dibromostearoyl)phosphatidylcholine (BRPC) was synthesised from C_{18}-PC as described in East and Lee [5].

Phytanic acid (> 99% purity) was prepared from phytol by the Dumas-Stass reaction as described [7]. 3-Methylhexadecanoic acid (> 99% purity) was made by a multistep reaction sequence in which methylmalonation of tridecylmesylate to give 2-methylpentadecanoic acid [8] was followed by chain elongation by one carbon [9]. 4-Methylhexadecanoic acid (> 99% purity) was synthesised via the intermediate γ-dodecyl-γ-methylbutyrolactone by the method of Cason et al. [10] with modification [11].

Diphytanoyl (Me_4C_{16}-PC), di(3-methylhexadecanoyl)- ($3MeC_{16}$-PC) and di(4-methylhexadecanoyl)-phosphatidylcholine ($4\text{-}MeC_{16}$-PC) were prepared by the method described by Hermetter and Paltauf [12].

(Ca^{2+} + Mg^{2+})-ATPase was prepared from female rabbit (New Zealand White) hind leg muscle as described in East and Lee [5]. Lipid substitutions were carried out according to the methods described in East and Lee [5]. Lipid (1 μmol) was sonicated to clarity with cholate (0.5–0.6 mg) in 40 μl of buffer (250 mM sucrose, 1 M KCl, 5 mM MgATP and 50 mM potassium phosphate (pH 8.0)). ATPase (0.125 mg) in a volume of 3–10 μl was then added and the mixture incubated for 1 h at 5°C (for lipid chain lengths C_{14}–C_{20}) or incubated for 20 min at room temperature followed by 40 min at 5°C (for lipid chain lengths C_{22}–C_{24}). After incubation, samples were diluted with 200 μl of buffer (250 mM sucrose, 1 M KCl, and 50 mM potassium phosphate (pH 8.0)) and stored on ice until assayed.

For ATPase assay, 12 μl of the ATPase sample was added to 2.45 ml of buffer (40 mM Hepes (pH 7.2)) containing $MgSO_4$ (5.1 mM), ATP (2.1 mM), phospho*enol*pyruvate (0.53 mM), EGTA (1.02 mM), NADH (0.152 mM), pyruvate kinase (7.5 IU) and lactate dehydrogenase (18 IU). In the final assay medium, the ATPase concentration was 0.02 μM, the molar ratio of phospholipid to ATPase was 950 : 1 and the cholate concentration 0.01 mg/ml: cholate at this concentration has no effect on ATPase activity. The same conditions were used for all lipid substitutions. After incubation at 37°C for 10 min, the reaction was started by addition of $CaCl_2$ to a total concentration of 0.92 mM and assayed by the decrease in absorbance at 340 nm. For experiments with oleic acid, oleic acid in methanol was added directly to the ATPase after dilution into the assay mixture.

Oleyl alcohol, however, because of its insolubility in water, was mixed with lipid in cholate before the reconstitution procedure.

Fluorescence measurements were made using a Spex Fluorolog fluorimeter, exciting fluorescence at 285 nm and measuring the fluorescence intensity at 340 nm. For these experiments, 36 μl of the ATPase sample was added to 3 ml of buffer (40 mM Hepes, 100 mM NaCl, 1 mM EGTA (pH 7.2)) at 37°C, followed by a 10 min incubation before measurement.

Protein was estimated using the extinction coefficient given by Hardwicke and Green [13].

Results

In the lipid titration procedure [14] the purified ATPase is incubated with a large excess of test lipid, followed by dilution 1200-fold into buffer to ensure that most of the cholate dissociates away from the lipid-protein complex. In the dilution step, the membrane reforms into unsealed membrane fragments, so that the ATPase activity is fully uncoupled from the accumulation of Ca^{2+}.

In Table I are listed ATPase activities for the ATPase reconstituted into various phospholipid bilayers at 37°C, pH 7.2 with MgATP = 2.1 mM. In previous publications it has been shown that addition of oleic acid or oleyl alcohol to these reconstituted systems can have marked effects on activity [15,16]. Figs. 1–3 illustrate the effects of these molecules on the activity of the ATPase reconstituted into bilayers of C_{14}-PC and C_{18}-PC, of the mixed lipids $C_{14}C_{18}$-PC and $C_{18}C_{14}$-PC and of a 50/50 mixture of C_{14}PC and C_{18}-PC.

TABLE I

ATPase ACTIVITIES AT 2.1 mm MgATP AND 37°C AND RELATIVE LIPID BINDING CONSTANTS

Lipid system	ATPase activity (I.U./mg)	Relative annular binding constant [a]	Bilayer thickness (nm) [b]
Native	18.4	–	–
C_{14}-PC	3.7	0.8	3.8
C_{16}-PC	19.1	1.1	4.3
C_{18}-PC	24.1	1.0 [c]	4.3
C_{20}-PC	18.2	–	4.6
C_{22}-PC	11.5	1.3	5.0
C_{24}-PC	3.3	1.0	5.3
3Me C_{16}-PC	17.4	1.0	–
4Me C_{16}-PC	16.1	1.0	–
Me_4 C_{16}-PC	12.2	0.8	–
$C_{14}C_{18}$-PC	21.0	–	–
$C_{18}C_{14}$-PC	20.4	–	–
C_{14}-PC/C_{18}-PC (1:1)	17.9	–	–

[a] Measured relative to the binding constant of C_{18}-PC.
[b] Taken from Ref. 4.
[c] By definition.

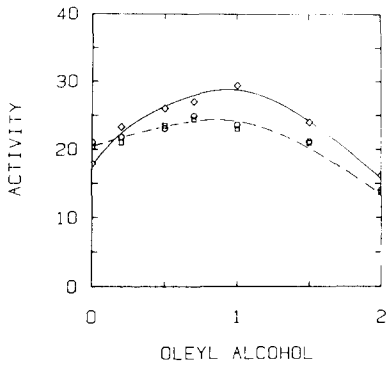

Fig. 1. ATPase activities (I.U./mg) for the reconstituted ATPase as a function of the concentration of oleyl alcohol, at the given molar ratio of oleyl alcohol to lipid, measured at 37°C and MgATP = 2.1 mM. ATPase reconstituted with ○, $C_{14}C_{18}$-PC; □, $C_{18}C_{14}$-PC; ◇, an equimolar mixture of C_{14}-PC and C_{18}-PC.

Reconstitution of the ATPase into bilayers of the brominated phospholipid BRPC results in quenching of the fluorescence of tryptophan residues in the ATPase, and it has been shown that the fluorescence intensity F' for the ATPase re-

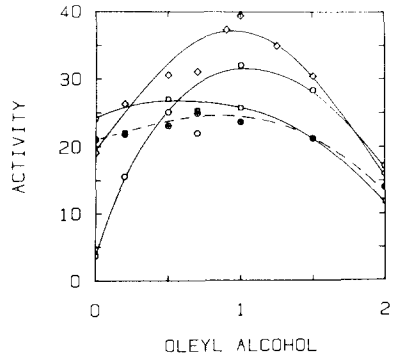

Fig. 2. ATPase activities (I.U./mg) for the reconstituted ATPase as a function of the concentration of oleyl alcohol at the given molar ratio of oleyl alcohol to lipid, measured at 37°C and MgATP = 2.1 mM. ATPase reconstituted with: ○, C_{14}-PC; ◇, C_{16}-PC; □, C_{18}-PC; ⊕, $C_{14}C_{18}$-PC.

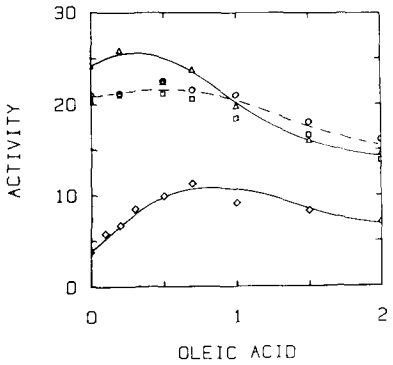

Fig. 3. ATPase activities (I.U./mg) for the reconstituted ATPase as a function of the concentration of oleic acid at the given molar ratio of oleic acid to lipid, measured at 37°C and MgATP = 2.1 mM. ATPase reconstituted with: ◇, C_{14}-PC; △, C_{18}-PC; ○, $C_{14}C_{18}$-PC; □, $C_{18}C_{14}$-PC.

constituted into a mixture of BRPC and a non-quenching lipid can be fitted to the equation

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

where F_0 is the fluorescence intensity in the absence of BRPC and F is the fluorescence intensity when the fraction of sites at the lipid-protein interface (annular sites) occupied by BRPC is f_a [5,15].

Binding of non-quenching lipid L and BRPC at the annular sites can be described by a series of displacement reactions of the type:

$$\text{ATPase}\cdot\text{BRPC} + \text{L} \rightleftharpoons \text{BRPC} + \text{ATPase}\cdot\text{L} \quad (2)$$

The relative binding constant of L with respect to BRPC, K_a, is given by

$$K_a = \frac{[\text{ATPase}\cdot\text{L}]}{[\text{ATPase}\cdot\text{BRPC}]} \cdot \frac{[\text{BRPC}]}{[\text{L}]} \quad (3)$$

and thus

$$f_a = 1/(1 + K_a x_L) \quad (4)$$

where x_L is the mole ratio of L to BRPC in the membrane. Since it is assumed that BRPC and C_{18}-PC bind equally to the ATPase [5], K_a is also equal to the relative binding constant of L with respect to C_{18}-PC.

Fig. 4 illustrates the observed fluorescence quenching for the ATPase reconstituted with mixtures of phospholipids and BRPC, expressed for convenience as $(F - F_{min})/(F_0 - F_{min})$ where F_{min} and F_0 are the fluorescence intensities observed for the ATPase reconstituted with BRPC and non-quenching lipid L, respectively, and F is the fluorescence intensity for the ATPase reconstituted with mixtures of L and BRPC. The figure also shows the best fits to Eqns. 1 and 4 with the relative binding constants given in Table I. Fig. 5 illustrates the activity of the ATPase reconstituted into lipid mixtures.

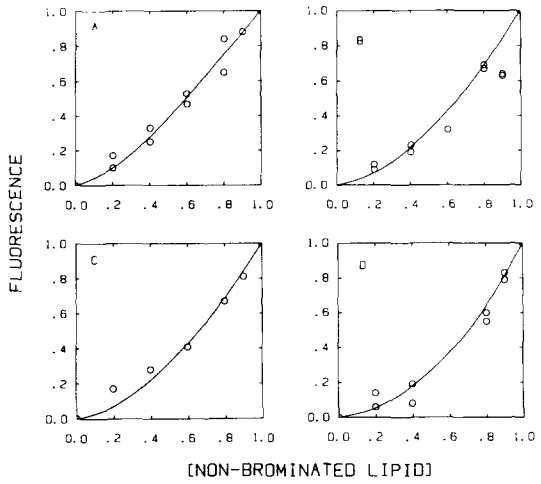

Fig. 4. Fluorescence quenching plots for the ATPase reconstituted with mixtures containing BRPC at the given mole fraction of the non-bromine containing lipid: points, experimental; solid lines, theoretical calculations (see text). (A) C_{22}-PC; (B) $3MeC_{16}$-PC; (C) $4MeC_{16}$-PC; (D) Me_4C_{16}-PC. Fluorescence expressed as $(F - F_{min})/(F_0 - F_{min})$ (see text).

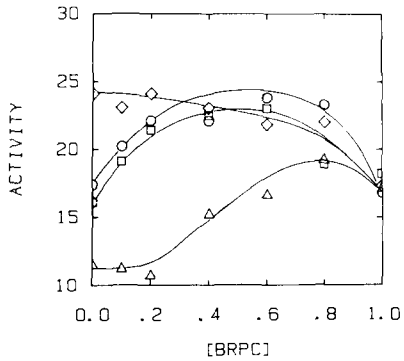

Fig. 5. ATPase activities (I.U./mg) for the ATPase reconstituted into phospholipid mixtures containing BRPC at the given mole fractions, measured at 37°C and MgATP = 2.1 mM: ○, $3MeC_{16}$-PC; □, $4MeC_{16}$-PC; ◇, C_{18}-PC; ◇, C_{22}-PC.

The gel to liquid-crystalline phase transition temperatures for $3MeC_{16}$-PC and $4MeC_{16}$-PC were determined by measuring the temperature dependence of fluorescence polarisation of the hydrophobic probe diphenylhexatriene (DPH) incorporated into liposomes of the lipids. A sharp phase transition was found for $3MeC_{16}$-PC, centred on 18.5°C. The phase transition for 4 MeC_{16}-PC was less distinct, occurring at approximately 10°C between a less ordered gel state and a more ordered liquid-crystalline state (Earl, C.R.A., unpublished data), see also Ref. 16. Thus these lipids will be in the liquid-crystalline state at the temperature of our experiments, 37°C. The reported phase transition temperatures of the other lipids used are also such that they will be in the liquid-crystalline phase at 37°C [17].

Discussion

The results presented in Table I show that the binding constants of phosphatidylcholines to the lipid-protein interface of the ATPase are independent of fatty acyl chain length and are unaffected by the presence of methyl branches in the chains. The lack of dependence on chain length has been demonstrated previously by Caffrey and Feigenson [4] using a similar fluorescence quenching method but employing spin-labelled phospholipids. The lack of effect of methyl branches is also interesting. It has been shown that diphytanoylphosphatidylcholine produces a more than usually expanded bilayer, and that there is a loss of the gel to liquid-crystalline phase transition, both presumably as a consequence of the presence of the four methyl groups along the chain [18]. Although the methyl groups, therefore, seem to affect packing in the lipid bilayer they evidently do not affect packing of lipid and protein.

Since the Gibbs free energy of exposure of hydrophobic residues to water is high [19], it is unlikely that any mismatch between the thickness of the hydrophobic portions of the ATPase and of the lipid bilayer will result in significant exposure of these regions to water. Rather, changes in conformation of either the protein or the lipid can be expected to minimise any mismatch in thickness. The ATPase contains a number of well-defined hydrophobic α-helical sequences flanked by charged residues [20] so that it seems likely that the 'thickness' of the hydrophobic, membrane-penetrant part of the ATPase will be similarly well defined. In general, a structural change in the ATPase causing a change in its hydrophobic thickness by several Å will then be unlikely. However, the ATPase is thought to exist in two different conformations E_1 and E_2. In the E_1 conformation, calcium binding sites are of high affinity and exposed to the outside surface of the sarcoplasmic reticulum whereas in the E_2 conformation, the calcium binding sites are of low affinity and exposed to the inside surface: transition between the E_1 and E_2 conformations constitutes the transport event [21]. It has been suggested that the equilibrium constant E_1/E_2 can be determined from measurements of the fluorescence of the ATPase modified with fluorescein isothiocyanate (Ref. 21; Froud, R.J., East, J.M., Jones, O.T. and Lee, A.G., unpublished observations). The equilibrium constant E_1/E_2 has been found to change from 0.4 for the ATPase reconstituted with C_{18}-PC to 5.2 for the ATPase reconstituted with C_{14}-PC, at pH 7, implying a difference in relative affinity of the E_1 and E_2 conformations for C_{18}-PC and C_{14}-PC (Froud, R.J., East, J.M., Jones, O.T. and Lee, A.G., unpublished observations). The equilibrium in this system can be defined by the following scheme:

$$\begin{array}{ccc} (C_{18}\text{-PC})E_1 & \xrightleftharpoons{K_{E1}} & (C_{14}\text{-PC})E_1 \\ \Big\| K_1 & & \Big\| K_2 \\ (C_{18}\text{-PC})E_2 & \xrightleftharpoons{K_{E2}} & (C_{14}\text{-PC})E_2 \end{array}$$

where K_{E1} and K_{E2} are binding constants of C_{14}-PC relative to C_{18}-PC for the E_1 and E_2 conformations, respectively, and K_1 and K_2 are the equilibrium constants E_1/E_2 for the ATPase reconstituted with C_{18}-PC and C_{14}-PC, respectively. The experimentally measured binding constant for C_{14}-PC relative to C_{18}-PC, K', is given by:

$$K' = (K_{E2} + K_1 K_{E1})/(1 + K_1)$$

With $K_1 = 0.4$ and $K_2 = 5.2$, $K_{E1} = 13\ K_{E2}$ and $K' = 4.4\ K_{E2}$. K' has been determined to be 0.83

[5] so that $K_{E1} = 2.5$ and $K_{E2} = 0.2$. These values indicate a stronger binding of C_{14}-PC relative to C_{18}-PC to the E_1 conformation but a weaker binding of C_{14}-PC compared to C_{18}-PC to the E_2 conformation, suggesting that the hydrophobic thickness of the ATPase is less in the E_1 conformation than in the E_2 conformation. It is, therefore, interesting that time-resolved X-ray diffraction studies have been interpreted as showing that the volume of the hydrophobic portion of the ATPase is greater in the phosphorylated E_2 conformation than in the E_1 conformation [22].

The relative binding constants of C_{14}-PC and C_{18}-PC for the E_1 and E_2 conformations of the ATPase derived above correspond to very small differences in the free energies of binding of C_{14}-PC and C_{18}-PC to the ATPase. The relative binding constants listed in Table I suggest that free energies of binding to the ATPase are generally largely independent of fatty acyl chain structure. Since free energies of distortion of proteins can be expected to be large, the above results suggest that it must be the bilayer that distorts to match the hydrophobic thickness of the ATPase. Lewis and Engleman [23] have suggested that only small free energy changes are involved in thickening or thinning a bilayer.

Because the changes in free energy for the transitions between the E_1 and E_2 conformations and between the corresponding phosphorylated forms is small, and because changes in the rates of these transitions cause significant changes in ATPase activity, small differences in the binding energies of phospholipids to these two conformations can cause large changes in ATPase activity [21]. The data in Table I indeed shows that the activity of the ATPase is markedly dependent on the chemical structure of the lipid fatty acyl chains. We find that the highest activity is supported by C_{18}-PC, with either longer or shorter fatty acyl chains supporting lower activity. This is in general agreement with earlier studies [3,4] except that Caffrey and Feigenson [4] report that the activities in C_{18}-PC and C_{22}-PC are virtually identical and Johannsson et al. [3] report that the highest activity is supported by C_{20}-PC. The data in Table I shows that the methyl-branched derivatives $3MeC_{16}$-PC and $4MeC_{16}$-PC support slightly lower activities than C_{16}-PC and that the activity of diphytanoylphosphatidylcholine (Me_4C_{16}-PC) is considerably lower than expected. This presumably reflects a relatively poor interaction of the phytyl chain with the protein surface. The presence of the large bromine atoms in BRPC may similarly explain the lower activity for the ATPase reconstituted with BRPC than with C_{18}-PC (Fig. 5).

Phospholipids containing methyl-branched fatty acids occur in the tissue lipids of animals given diets including phospholipids containing methyl-branched fatty acids [24]. Phytanic acid is found in quantity in tissue lipids of humans having the rare neurological disorder, Refsum's Disease [25]. Phospholipids with methyl-branched fatty acyl chains exhibit decreased gel to liquid crystalline phase transition temperatures compared to the corresponding phospholipids containing unbranched saturated fatty acids. The phase transition temperature for dipalmitoylphosphatidylcholine is at 42°C (see Ref. 17), and for $3MeC_{16}$-PC, and $4MeC_{16}$-PC at 18.5°C and approx. 10°C, respectively. It has been suggested that methyl-branched saturated fatty acids can act like unsaturated fatty acids in prokaryotic microorganisms to maintain the membrane in the liquid crystalline phase [26,27]. We show here (Table I) that these methyl-branched saturated fatty acids are able to support reasonable activities for the ATPase, at a temperature where dipalmitoylphosphatidylcholine would support a very low activity [14].

The activities supported by the mixed chain phospholipids 1-myristoyl-2-oleoylphosphatidylcholine and 1-oleoyl-2-myristoylphosphatidylcholine are close to that expected for C_{16}-PC (which has the same average chain length), which in turn is close to that supported by an equimolar mixture of C_{14}-PC and C_{18}-PC: the activities in these systems are not close to the average value observed for the C_{14}-PC and C_{18}-PC systems (13.9 I.U./mg). In this sense, therefore, the importance of the lipid fatty acyl chains could be said to be to determine the thickness of the lipid bilayer. However, other experiments suggest that it is not useful to characterise the membrane by an 'effective' average thickness. In previous studies it has been shown that addition of oleic acid or oleyl alcohol to the reconstituted ATPase can have marked

effects on ATPase activity, depending on the phospholipids present in the membrane (Ref. 15; Froud, R.J., East, J.M., Jones, O.T. and Lee, A.G., unpublished observations). Fig. 3 shows that the effect of oleic acid on the activity of the ATPase reconstituted with the mixed chain lipids $C_{14}C_{18}$-PC and $C_{18}C_{14}$-PC are closer to effects on the ATPase reconstituted with C_{18}-PC than with C_{14}-PC. This is also apparent in Figs. 1 and 2 which show the effect of addition of oleyl alcohol. It is clear that the response to oleyl alcohol of the ATPase reconstituted in an equimolar mixture of C_{14}-PC and C_{18}-PC is similar to that seen with C_{16}-PC, although the activities are consistently lower (Fig. 2). The response of the ATPase reconstituted with $C_{14}C_{18}$-PC and $C_{18}C_{14}$-PC, however, are unlike those seen for the ATPase reconstituted with C_{16}-PC and again are like those observed for the ATPase reconstituted with C_{18}-PC. For the mixed chain lipid it is clear that the oleyl chain is having the dominant effect on activity. In the case of the mixed chain lipids, therefore, a description of the membrane in terms of an average chain length or thickness would not be helpful. If interaction of phospholipid fatty acyl chains with membrane proteins does involve some distortion of the phospholipid to match the protein surface then phospholipid with one C_{14} and one C_{18} chain is not equivalent to a phospholipid with two C_{16} chains.

Despite the non-equivalence of the fatty acyl chains at the 1- and 2-positions of the phospholipid [28], the activity of the ATPase is independent of the positions of the C_{14} and C_{18} chains in the mixed lipid (Table I).

Acknowledgements

We thank the ARFC, SERC and Wellcome Trust for financial support. J.M.E. is a Wellcome Trust Lecturer.

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