THE ROLE OF PHOSPHOLIPID ACYL CHAINS IN THE ACTIVATION OF MITOCHONDRIAL ATPase COMPLEX

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SUMMARY

1. The role of length and unsaturation of phospholipid acyl chains in the activation of ATPase complex was studied with synthetic phosphatidylcholines and a phospholipid-dependent preparation obtained after cholate-extraction of submitochondrial particles (Kagawa, Y. and Racker, E. (1966) J. Biol. Chem. 241, 2467–2474).

2. Micelle-forming, short-chain phosphatidylcholines produced activation only at critical micellar concentration. The reactivated complex was cold-stable but the oligomycin sensitivity was low.

3. Bilayer-forming saturated phosphatidylcholines produced activation which was maximal at 9 carbon atoms in each chain but decreased sharply as the chain-length was increased and essentially disappeared at 14 carbon atoms. By contrast the oligomycin-sensitivity increased with the increase in chain length.

4. Activation of ATPase complex reappeared when bilayers were formed with long-chain unsaturated phosphatidylcholines. The activity was oligomycin sensitive. Significant inhibition of activity was observed also after incorporation of cholesterol into the bilayers.

5. By contrast the activation induced by negatively charged liposomes of diacylphosphatidylglycerol was independent on acyl-chain composition and occurred at very low amounts of phospholipid.

6. The discontinuity in the Arrhenius plot of activity of the ATPase complex reactivated with saturated phospholipids was found at temperatures close to the gel-to-liquid crystalline transition of the lipid showing that the activity of ATPase complex was sensitive to the physical state of membrane phospholipids.

7. It is concluded that (a) reactivation of ATPase complex by isoelectric phospholipids is an interfacial activation, the minimum requirement for the lipid effect being micelle formation. (b) In order to gain the properties of the native complex a stable lamellar phase is needed. Both activity and oligomycin sensitivity are regulated by the chain length and degree of unsaturation of phospholipid acyl chains.

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INTRODUCTION

The mechanism of phospholipid-induced activation of membrane-bound mitochondrial ATPase [1–4] is relevant to the specific problem of organization of membrane-fragments catalyzing the synthesis of ATP and more in general the function of phospholipids in a biological membrane. The contribution of the phospholipid polar-head-group to the activation process has been documented [3–5]. Negatively charged liposomes can induce a variety of effects, ranging from activation to detachment of soluble ATPase [6, 7]. Influence on the association between the soluble enzyme and the endogenous ATPase inhibitor is believed to be important in these effects [5, 7, 8]. Although clearly indicated since the early experiments of Kagawa and Racker [1] the role of hydrocarbon-chain composition has not been systematically investigated.

The developments in the studies of membrane lipids have established that the properties of bilayer organization which forms an essential part of cellular membranes are largely dependent on phospholipid acyl chain composition [9–12]. Chain-length and degree of unsaturation regulate to a large extent the fluidity and molecular ordering of membranes which have a marked influence on the membrane architecture, permeability properties and function of associated enzymes [13–15].

In this study it will be shown that the length and unsaturation of phospholipid acyl chains influence both the activity of mitochondrial membrane-bound ATPase (ATPase complex [16]) and the effect of oligomycin, a well-known inhibitor of oxidative phosphorylation.

MATERIALS AND METHODS

The synthetic 1,2-diacyl-*sn*-glycero-3-phosphorylcholines used in this study were obtained in this laboratory according to published procedures [17]. Dimyristoyl and dioleoylphosphatidylglycerol were prepared from dimyristoyl- and dioleoyl-phosphatidylcholine by phospholipase D catalyzed choline-glycerol exchange and purification on a silicic acid column [18]. Egg-phosphatidylcholine and lysophosphatidylcholine were extracted and purified as described [19]. The purity of phospholipids was tested by thin-layer chromatography with Silica gel G and a solvent of chloroform/methanol/H_2O (65 : 35 : 4, by vol.). Oxidation of unsaturated phospholipids was checked by determination of the oxidation index (ratio of the absorbance at 233 nm to the absorbance at 215 nm) according to Klein [20]. Sodium dicetylphosphate (Sigma) was recrystallized from ethoxyethanol. ATP(Na)$_2$, pyruvate kinase (glycerol solution) and phosphoenolpyruvate were from Boehringer Mannheim. Oligomycin, 15 % A and 85 % B, from Sigma.

Preparation of phospholipid dispersions

Known amounts of phospholipids dissolved in organic solvent were taken to dryness under a stream of pure nitrogen. Residual traces of solvent were removed under vacuum. Liposomes were prepared in 0.2 M sucrose, 25 mM Tris · HCl, 1 mM EDTA (pH 7.4) by 3 min sonication (Branson Sonifier Mod. B$_{12}$, 60 W output) at phospholipid concentrations of 1–3 μmol/ml and at temperatures above the gel-to-liquid crystalline phase transition. Sonication of unsaturated phospholipids was

performed in a nitrogen atmosphere. The choice of a brief sonication (3 min) was suggested by the absolute need to avoid hydrolysis of phospholipids or oxidation of unsaturated bonds, events which greatly would have altered the results. Appropriate control experiments, however, showed that longer time of sonication would not have improved the activation.

Micelles of lysophosphatidylcholine, dihexanoyl-, diheptanoyl- and dioctanoyl-phosphatidylcholine were obtained by addition of the sucrose, Tris · HCl, EDTA solution to the dry phospholipids and mixing on a Vortex mixer at room temperature. In all cases the phospholipid dispersions were used within few hours after their preparation.

Reactivation of ATPase complex

Bovine heart mitochondria [21] and submitochondrial particles prepared in the presence of pyrophosphate [22], were obtained according to published procedures. The phospholipid dependent ATPase complex was prepared as described earlier [5] after extraction of submitochondrial particles with cholate [1]. The residual content of phospholipid phosphorus was 0.1 μmol/mg protein (0.7 μmol/mg protein in the original particles). The residual activity was 0.15–0.2 μmol/mg protein per min at 37 °C (intact submitochondrial particles, 3.6 μmol/mg per min).

Reconstitution of the depleted preparation with phospholipids was performed by addition of 1 μl (25 μg) of the clear preparation of ATPase complex to 0.3 ml of 0.13 M sucrose, 16 mM Tris · HCl, 0.6 mM EDTA containing the phospholipids and prewarmed at 37 °C. The dilution of the cholate-containing preparation resulted in a precipitation in the presence of phospholipids. After 10 min at 37 °C the ATPase reaction was started by addition of 0.7 ml of a stock solution containing ATP, Mg^{2+} and the other reagents. The final composition in a volume of 1.0 ml was 40 mM sucrose, 0.2 mM EDTA, 55 mM Tris · HCl (pH 7.4), 2.5 mM $MgCl_2$, 3 mM $ATP(Na)_2$, 2 mM phosphoenolpyruvate, 20 μg pyruvate kinase. After 20 min incubation at 37 °C the reaction was stopped by addition of 0.25 ml cold 50 % trichloroacetic acid. Variations in this standard procedure are indicated in the legends to tables and figures.

Analytical procedures

Inorganic phosphate was determined according to Fiske and SubbaRow [23], phospholipid phosphorus after the wet-ashing procedure described by Bartlett [24], protein according to Lowry et al. [25], with crystalline bovine serum albumin as standard. Interference by dihexanoyl- and diheptanoylphosphatidylcholine with the inorganic phosphate determination was avoided [26] by addition of 1.0 ml 3 % bovine serum albumin to the incubation mixture 30 sec before the trichloroacetic acid solution which was increased to 0.5 ml. Differential scanning calorimetric determinations were performed as described previously [27].

RESULTS

Phosphatidylcholines forming micelles

Diacylphosphatidylcholines with short, saturated paraffin chains aggregate in micelles in aqueous solutions [28]. The effect of these phospholipids in comparison to
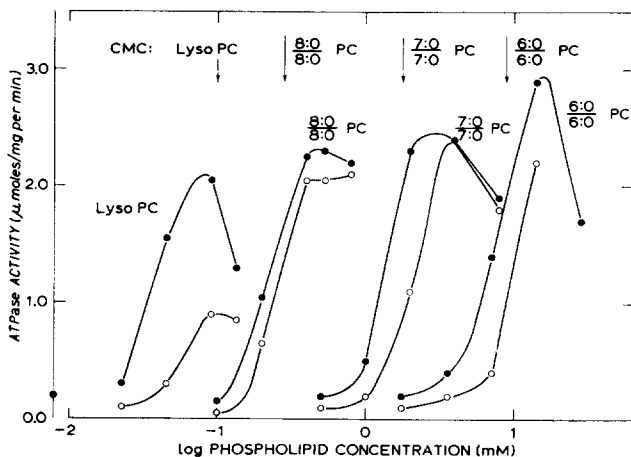
Fig. 1. Activation of ATPase complex by phospholipids with critical micellar concentration. The dry phospholipids were suspended in 0.20 m sucrose, 25 mM Tris · HCl, 1 mM EDTA (pH 7.4) at concentrations above the critical micellar concentration. The phospholipid-dependent ATPase complex was preincubated 10 min at 37 °C in 0.3 ml of 0.13 M sucrose, 16 mM Tris · HCl, 0.6 mM EDTA (pH 7.4) containing the phospholipids at the concentrations indicated. After preincubation, the measurement of ATPase activity was performed as described in Materials and Methods. Interference by the large amounts of dihexanoyl- and diheptanoylphosphatidylcholines with inorganic phosphate determination was avoided by addition to the tubes of 30 mg bovine serum albumin 30 sec before stopping the reaction with trichloroacetic acid [26]. The arrows indicate the values of critical micellar concentrations (CMC) as reported by Lewis and Gottlieb [29] for lysophosphatidylcholine and Tausk et al. [28] for short-chain diacylphosphatidylcholines. ($\bullet - \bullet$), without oligomycin. ($\bigcirc - \bigcirc$), with 1 μg/tube oligomycin (40 μg/mg protein).

lysophosphatidylcholine, a long-chain monoacylphospholipid which also aggregates in micelles, is shown in Fig. 1. A close correlation was observed between activation and the critical micellar concentrations. With dihexanoylphosphatidylcholine, monomers were present up to 20–30 μmol/mg protein, yet no activation ensued till the aggregation value was reached. The oligomycin sensitivity, generally low, was more evident with lysophosphatidylcholine, perhaps reflecting the importance of chain length in the effect of inhibitor. Separate experiments showed that the activation induced by lysophosphatidylcholine, dihexanoyl- and diheptanoylphosphatidylcholine was cold-stable even at concentrations where the oligomycin-sensitivity was low or absent. This constituted evidence that the activation was not concomitant with release of the soluble, cold-labile F_1-ATPase [30] but reflected the activity of a membrane-associated complex. Testing the cold stability with dioctanoylphosphatidylcholine inconsistent results were obtained, likely due to the particular behavior of this phospholipid in aqueous solution at various temperatures [28, 31].

Phosphatidylcholines forming bilayers

Phosphatidylcholines with increased number of carbon atoms in the paraffin chains aggregate in a lamellar phase [32]. At 10 carbon atoms in each chain the bilayer structure is still unstable and very permeable but the stability increases with the chain-length [33]. In Fig. 2 it can be seen that at 9 carbon atoms the activation was very high but decreased as the length of saturated acyl chains was increased. The
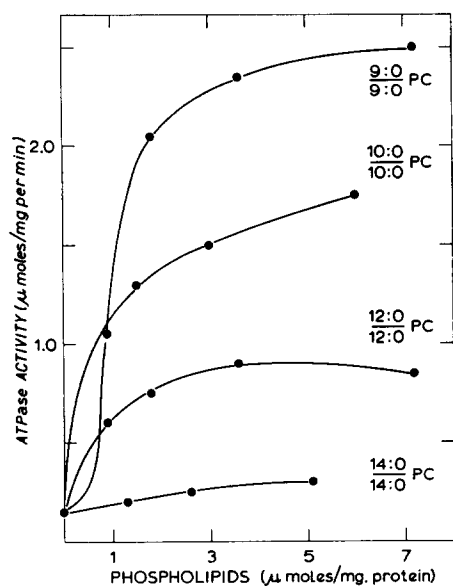
Fig. 2. Activation of ATPase complex by bilayer-forming phosphatidylcholines. The dry phospholipids were suspended at concentrations of 1–3 μmol/ml in 0.2 M sucrose, 25 mM Tris · HCl, 1 mM EDTA (pH 7.4) and sonicated 3 min above the transition temperature. Reconstitution of ATPase complex and measurement of ATPase activity as described in Materials and Methods.
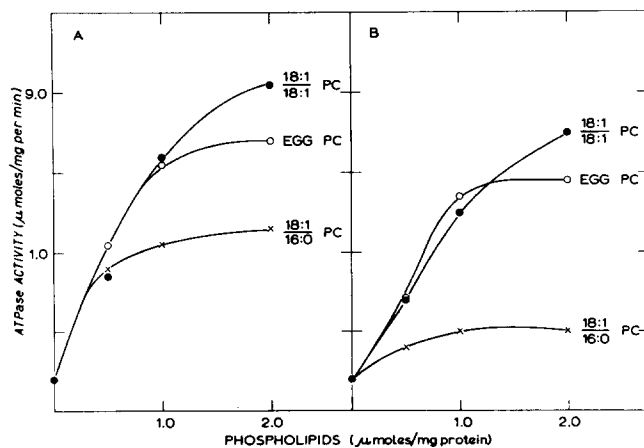

Fig. 3. Activation of ATPase complex by phosphatidylcholines with unsaturated chains. In (A) the experimental conditions were those indicated in Fig. 2. In (B) the liposomes were prepared in 0.2 M sucrose, 25 mM Tris/HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 1 mM EDTA (pH 7.4). The reconstitution of ATPase complex was performed in 0.3 ml of 0.13 M sucrose, 16 mM Tris/HEPES, 0.6 EDTA (pH 7.4) and the ATPase activity measured in 1.0 ml of 40 mM sucrose, 0.2 mM EDTA, 5 mM Tris/HEPES, 2.5 mM MgSO$_4$, 3 mM ATP, 2 mM phosphoenolpyruvate, 20 μg pyruvate kinase (pH 7.4).

low effectiveness of dimyristoylphosphatidylcholine was not improved after increasing the proton conductance or ionic fluxes through the bilayer by addition of uncoupler or uncoupler plus valinomycin in the presence of potassium chloride.

In Fig. 3 it is shown that activation by long-chain phosphatidylcholines reappeared to a large extent provided *cis*-unsaturated bonds were present in the hydrocarbon chains. The role of unsaturation was clearly appreciated using a phosphatidylcholine with a saturated chain at the 2 position and an unsaturated chain at the 1 position. The activity was substantially decreased especially at low levels of chloride anions (Fig. 3B) since a higher concentration of Cl⁻ stimulated the reactivation (Fig. 3A) in accordance with earlier results [5]. From an inspection of Fig. 3 it is also apparent that the activity of egg phosphatidylcholine used in this study was higher than that reported before [3, 5]. Considering the importance of chain length and the dependence on the unsaturation, a great variability can be expected in the activation by natural phosphatidylcholines depending on the length of the acyl chains and the extent and preservation of unsaturated bonds. The great difference in the degree of saturation among natural phosphatidylcholines has been emphasized recently [34]. Experiments have been made to test the effect of cholesterol on the activation induced by unsaturated phosphatidylcholines. Cholesterol is known to induce a condensing effect on monolayers of unsaturated phospholipid species and to reduce the mobility of hydrocarbon chains in bilayers [9, 12]. Inhibition by cholesterol of phospholipid-induced activation of sodium and potassium-stimulated ATPase has been demonstrated [35]. In agreement with these results it was found that inclusion of cholesterol into bilayers of dioleoylphosphatidylcholine at a mole ratio of 1 : 1 produced 32–35 % inhibition of stimulated ATPase activity while the oligomycin-sensitivity was not changed.
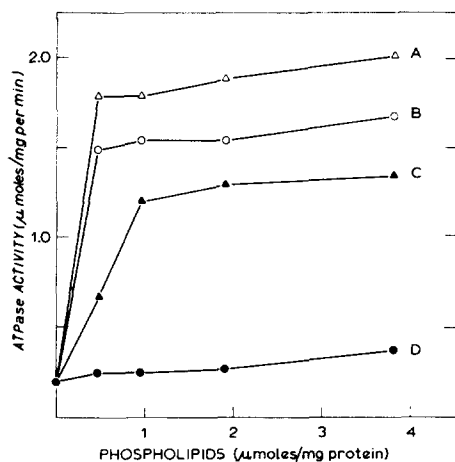

Fig. 4. Effect of negative charges on the stimulation by saturated and unsaturated phosphatidylcholines. A solution of dicetylphosphate in chloroform was mixed with the phospholipid solution to give a mole ratio of 1 : 9 (dicetylphosphate to phospholipid). The samples were taken to dryness, resuspended in 0.2 M sucrose, 25 mM Tris · HCl, 1 mM EDTA (pH 7.4) and sonicated. Reconstitution of ATPase complex and measurement of ATPase activity as described in "Materials and Methods". (A), dipalmitoleoylphosphatidylcholine, dicetylphosphate. (B), dipalmitoleoylphosphatidylcholine alone. (C), dimyristoylphosphatidylcholine, dicetylphosphate. (D), dimyristoylphosphatidylcholine alone.
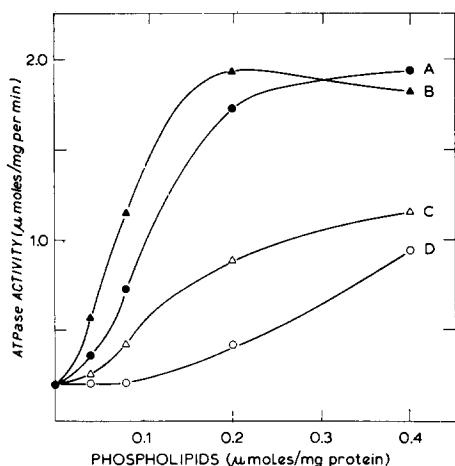
Fig. 5. Stimulation of ATPase complex by dimyristoylphosphatidylglycerol (A, D) and dioleoylphosphatidylglycerol (B, C). Experimental conditions as described in Fig. 2. Closed symbols, without oligomycin. Open symbols, incubation with 40 μg oligomycin/mg protein.

In agreement with previous results [5], it can be seen in Fig. 4 that introduction of negative charges into the bilayer gave rise to a stimulation of oligomycin-sensitive activity, with both saturated and unsaturated phospholipids.

The role of acyl chain length and unsaturation in bilayers with strong negative charge was also examined (Fig. 5). In this case the activity was largely independent on the chain length and unsaturation. Maximal reactivation of ATPase complex occurred at amounts of phospholipids lower than the amount present in undepleted submitochondrial particles. These observations may support the idea that the activation induced by isoelectric or strongly charged liposomes originated from a different interaction with components of ATPase complex.

The effect of temperature in the presence of activating phospholipids is shown in Fig. 6 as Arrhenius plots. With the original submitochondrial particles used in this study a change in slope was evident at an average value of 14.2 °C, only slightly different from that previously reported with frozen bovine-heart mitochondria (Fig. 2 in ref. 36). The ATPase complex reactivated by dimyristoylphosphatidylcholine plus dicetylphosphate (2.0 μmol/mg protein) or dimyristoylphosphatidylglycerol (0.2 μmol/mg protein) showed discontinuities shifted to higher temperatures, 23.9 °C and 19.7 °C, respectively. In the first case this corresponded to the temperature of gel-to-fluid state transition found for this mixture in calorimetric determinations. In the second, the change in slope occurred slightly below the midpoint of the endothermic peak (23 °C), perhaps reflecting the influence of residual lipids in the ATPase preparation (note the low amount of lipid added for reactivation). By contrast, a discontinuity at 16 °C was retained when the stimulation was induced by unsaturated dioleoyl-phosphatidylglycerol (0.2 μmol/mg protein). It is of interest to note that a break at 13 °C was observed with a preparation of phospholipid-dependent ATPase complex made from a Triton X-100 extract and reactivated by highly unsaturated diphosphatidylglycerol [4]. When stimulated by lysophosphatidylcholine the transition was shifted to 19 °C [4].
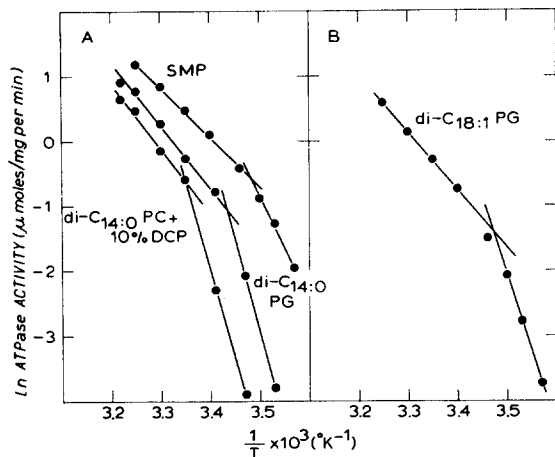
Fig. 6. Effect of temperature on the activity of submitochondrial particles and ATPase complex reconstituted with various phospholipids. The ATPase complex was reconstituted at 37 °C with phospholipids as described in Materials and Methods. 0.3 ml of the sucrose, Tris · HCl solution containing the reconstituted complex or the submitochondrial particles were added to 0.6 of a mixture equilibrated at different temperatures and containing 50 μmol Tris · HCl, 2 μmol phosphoenolpyruvate and 20 μg pyruvate kinase. After 2–3 min thermoequilibration the reaction was started with 0.1 ml of 30 mM ATP and 25 mM MgCl$_2$. When desired for appropriate control, 1 μg oligomycin was included in this last addition. The final composition of incubation medium was that described in Materials and Methods. Incubation time, 20 min (10 min with submitochondrial particles). (A) 27 μg submitochondrial particles (SMP) prepared in presence of pyrophosphate, 25 μg ATPase complex reconstituted with 0.2 μmol/mg protein dimyristoylphosphatidylglycerol, 25 μg ATPase complex reconstituted with 2 μmol/mg protein dimyristoylphosphatidylcholine plus dicetylphosphate (9 : 1 mol/mol). (B), 25 μg ATPase complex reconstituted with 0.2 μmol/mg protein dioleoylphosphatidylglycerol.

Separate experiments have shown that with dioleoyl- or dipalmitoleoyl phosphatidylcholine (2.0 μmol/mg protein) no sharp discontinuities were detectable. In these cases however deviations from linear Arrhenius kinetics were constantly observed. The formation of quasi-crystalline clusters in bilayers of dioleoylphosphatidylcholine at temperatures below 30 °C [37], might provide the basis for their explanation. A curved Arrhenius plot was reported measuring the Mg^{2+}-stimulated ATPase of sonicated rat-liver mitochondria [38] whereas in a more recent report [39], no discontinuous changes in slope were recorded, with a similar preparation.

Sensitivity to oligomycin

The low detergent activity of phosphatidylcholines forming bilayers allowed to obtain a reconstituted ATPase complex after incubation with phospholipids, centrifugation and washing. This procedure was preferred to test the oligomycin sensitivity since it avoided possible ambiguity due to occasional detachment of oligomycin-insensitive F_1-ATPase from the membrane. In Table I it is shown that reconstituting the complex with saturated phosphatidylcholines the inhibition by oligomycin followed the reverse order with respect to the activation and was more manifest at longer chain length. A marked inhibition by oligomycin was retained also with long-chain, unsaturated phosphatidylcholines in spite of higher activation. It

TABLE I

OLIGOMYCIN SENSITIVITY OF ATPase COMPLEX ACTIVATED BY DIFFERENT PHOS-PHATIDYLCHOLINES

0.75 mg phospholipid-dependent ATPase complex was added to 6.5 ml of a medium containing 25 mM Tris · HCl, 1 mM EDTA, 50 mM sucrose and the phospholipids (pH 7.4). After 10 min at 37 °C the samples were centrifuged at 4 °C 30 min at 40 000 rev./min and the supernatant discarded. The sediments were homogenized in 1.0 ml of 25 mM Tris · HCl, 1 mM EDTA (pH 7.4) and centrifuged 15 min at the same speed. Final precipitates in 0.2 ml of the Tris · HCl, EDTA solution. The ATPase activity was measured as described in Materials and Methods but the preincubation period was omitted. Oligomycin 1 μg/tube (about 30 μg/mg protein).

| Phospholipids (2 μmoles/mg protein)	ATPase activity (μmol/mg per min)		
	Without oligomycin	With oligomycin	% inhibn
None	0.16	0.15	–
Dinanoylphosphatidylcholine	2.26	1.60	29
Didecanoylphosphatidylcholine	1.60	0.79	50
Dilauroylphosphatidylcholine	0.90	0.34	62
Dimyristoylphosphatidylcholine	0.65	0.21	68
Dipalmitoleoylphosphatidylcholine	1.65	0.34	79
Dioleoylphosphatidylcholine	1.65	0.42	74
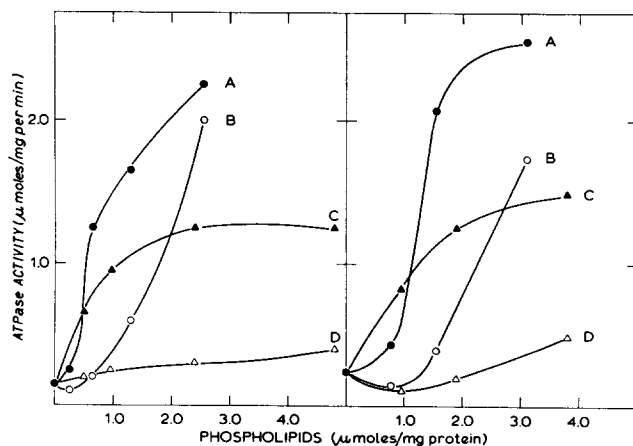
Fig. 7. Activation and oligomycin sensitivity of ATPase complex reconstituted with mixed phospholipids. Mixtures of egg-lysophosphatidylcholine with egg-phosphatidylcholine and dilauroylphosphatidylcholine with dinanoylphosphatidylcholine were prepared in chloroform, taken to dryness, suspended in 0.2 M sucrose, 25 mM Tris · HCl, 1 mM EDTA (pH 7.4) and sonicated in the cold. Reconstitution of ATPase complex and measurement of ATPase activity as described in Materials and Methods. Oligomycin 40 μg/mg protein. Left, (A) lysophosphatidylcholine with phosphatidylcholine (1 : 1 mol/mol). (B) Same plus oligomycin. (C) phosphatidylcholine alone. (D) Same plus oligomycin. Right, (A) dinanoylphosphatidylcholine alone. (B) Same plus oligomycin. (C) dinanoylphosphatidylcholine with dilauroylphosphatidylcholine (65 : 35 mol/mol) (D) Same plus oligomycin.

TABLE II

EFFECT OF ACYL CHAIN LENGTH ON THE OLIGOMYCIN SENSITIVITY

0.5 mg phospholipid-dependent ATPase complex was reconstituted with 4 μmol phospholipids/mg protein as described in Table I. Phospholipid phosphorus and ATPase activity measured as described in Materials and Methods. Separate samples showed that liposomes without enzyme complex gave no sediment under these conditions. Oligomycin 1 μg/tube (about 30 μg/mg protein).

Phospholipids added	Phospholipids bound (μmol/mg protein)	ATPase activity (μmol/mg per min)		
		Without oligomycin	With oligomycin	% inhibn
Dinanoyl-phosphatidylcholine	2.5	1.9	1.7	10
plus dilauroyl-phosphatidylcholine (75 : 25 mol/mol)	1.6	1.5	0.9	40
plus dilauroyl-phosphatidylcholine (50 : 50 mol/mol)	1.25	0.7	0.25	64

should be pointed out however that a high inhibition by the antibiotic was always found when sub-optimal levels of phospholipids were used. This can be appreciated in the results shown in Fig. 7 in which the role of structure assumed by lipid in water was studied. Converting the lamellar phase of egg-phosphatidylcholine in a population of mixed micelles and lamellae of phosphatidylcholine plus lysophosphatidylcholine [40, 41], the pattern of activation changed and a strong sigmoidal curve appeared. The oligomycin-sensitivity was rapidly lost as the level of phospholipid was increased. In addition, the sigmoidal, micelle-type of stimulation induced by dinanoylphosphatidylcholine was converted into a bilayer-type by addition of 35 % dilauroylphosphatidylcholine with improvement of oligomycin sensitivity. It should be pointed out that a critical micellar concentration is still detectable in aqueous dispersions of dinanoyl-phosphatidylcholine [28].

Liposomes formed by mixtures of dinanoyl and dilauroylphosphatidylcholine gave water clear dispersions which could not be sedimented at high-speed centrifugation. This allowed precise determination of phospholipid bound to the complex at different oligomycin sensitivity. In Table II it is seen that more phospholipids were bound when the effect of inhibitor was low. This result is reminiscent of that obtained by Kagawa and Racker [1] with an F_1-depleted, phospholipid-dependent, preparation (CFo), although aspecific reincorporation of F_1 into the complex in the presence of an excess of phospholipids was the most likely explanation for the decreased oligomycin sensitivity in that case.

The correlation between phospholipid bound to the complex and oligomycin sensitivity raised the possibility that the low effect of inhibitor could be due to protection by the excess of lipid against oligomycin penetration. However, control experiments showed that pretreatment of ATPase complex with oligomycin before addition of phospholipids did not change the values of inhibition.

DISCUSSION

Reactivation of ATPase complex

An organized lipid-water interface was found to be the minimum requirement for the stimulation of ATPase complex by neutral diacylphospholipids. Formation of mixed micelles of phospholipid plus the ATPase complex might provide the hydrophobic environment around the membrane component needed to activation. Alternatively, the orientation of polar group on micelle surface makes possible a polar interaction of the ATPase complex with the lipid interface. Other examples of interfacial activation are found with phospholipase A_2 from porcine pancreas [32, 42] and *Crotalus adamanteus* [31] and indicate a phenomenon of general interest in lipid-protein interaction.

The pattern of activation by neutral diacylphospholipids was greatly changed when these molecules in aqueous solution arranged in more complex structures. Judging from the possibility to reconstitute a particulate fragment with oligomycin-sensitive, cold-stable ATPase activity, it is apparent that the lamellar phase, formed as the chain length was increased, made the reactivated complex similar to the native enzyme. The loss of activity as the chain length of saturated phosphatidylcholines is increased and the higher effectiveness of unsaturated members rise the question as to whether the activation of the ATPase complex is linked to the possibility to have a permeable bilayer [13, 14]. However two observations argue against this interpretation (a) activation is already lost with dimyristoylphosphatidylcholine. This phospholipid forms very permeable bilayers and vesicles obtained after sonication are reported to be unable to trap solutes [43]. (b) Increasing the proton conductivity of the bilayer with uncouplers or inducing an increased ionic-flux with uncouplers plus valinomycin, failed to activate the low ATPase activity. From these results it appears that the structure of bilayer rather than the permeability properties is of prevalent importance.

cis-Unsaturation and reduction in length have the effect of reducing the interaction among the fatty acid chains. As a consequence, short-chain and unsaturated phosphatidylcholines form at a given temperature more expanded monolayers [9]. It is therefore conceivable that the condensed structure of the lamellar phase formed by long chain saturated phosphatidylcholines is not optimal for the correct lipid-protein interaction. Expanded bilayers would allow the membrane component of ATPase complex to penetrate deeply into the hydrophobic region of the lamellae and come in contact with fluid and mobile paraffin chains, a condition needed to induce activation in the absence of surface charges.

Temperature-induced changes

The changes in slope in Arrhenius plots seen with saturated phospholipids (thermotropic phase transition above the temperature of discontinuity in the original submitochondrial particles) indicated that the mitochondrial ATPase complex, as other ATPases associated with membranes [35], requires the fluid state of membrane lipids. Furthermore they constituted evidence that the activation was due to the added exogenous phospholipids and excluded other possible mechanism of activation such as removal of bound cholate or reactivation of residual undepleted membrane fragments. The discontinuity in the Arrhenius plot seen with original submitochondrial particles containing very unsaturated endogenous lipids [44, 45] and in the dioleoyl-

phosphatidylglycerol-stimulated ATPase complex, are of difficult interpretation. It is possible that immobilization of phospholipid by proteins through polar and apolar interaction results in a change of the transition temperature [46] but the association of protein with lipids heterogeneously distributed within the membrane [47, 36], the influence of upper and lower limits of transition [48], the cluster formation [37] or a change in the structure of water [49] near the ATPase are other possibilities which deserve consideration.

Oligomycin sensitivity

The possibility that oligomycin interferes with the phospholipid-induced activation of ATPase complex was originally considered by Kagawa and Racker [1] who showed that phospholipids can remove the inhibitor from the membrane fragment and restore the ATPase activity. Further studies [50–55] have confirmed the role of phospholipids in the oligomycin effect and support the suggestion [52] that the inhibitor can find in the lipid part of the membrane the suitable environment to reach the inhibitory site. This possibility is also indicated by more recent findings of Bertina et al. [56] who, however, were unable to detect reactivation by phospholipids of ATPase activity in oligomycin-pretreated submitochondrial particles.

In this paper it is shown that the inhibition by oligomycin was sensitive to the composition of phospholipids used for reactivation. The most evident change was in the transition between micelle-forming and bilayer-forming phospholipids. Further studies are needed to establish whether the sensitivity to oligomycin, induced by phospholipids forming a lamellar structure, is linked to an appropriate organization of ATPase complex or that the low oligomycin-sensitivity with short-chain phosphatidylcholines reflects a greater affinity of the inhibitor for a micellar phase with dilution in a larger lipid area.

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