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STUDIES ON THE ACTIVATION OF PURIFIED MITOCHONDRIAL ATPase BY PHOSPHOLIPIDS

PATRIK SWANLJUNG*, LUCIANO FRIGERI**, KRISTINA OHLSON and LARS ERNSTER

Department of Biochemistry, University of Stockholm, Box 6409, S-113 82 Stockholm (Sweden)

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SUMMARY

1. An ATPase which is activated by phospholipids and inhibited by oligomycin, has been purified from beef heart submitochondrial particles using affinity chromatography. Phospholipid and detergent are removed by washing the enzyme with a solution of serum albumin while it is attached to the biospecific adsorbent.

2. The ATPase is activated up to 18-fold by lysolecithin and to a smaller extent by cardiolipin, phosphatidylinositol and phosphatidylethanolamine. The amount required of each of these phospholipids to give half-maximal activation is apparently inversely related to the number of fatty acid chains in the lipid. Lecithin, which is a poor activator of the ATPase, competitively inhibits the activation by cardiolipin.

3. The activation of the ATPase consists of an increase in both the maximal velocity of the reaction and the affinity for substrate ATP. The pH optimum of the reaction is not influenced by the charge of the lipid.

4. Arrhenius plots of ATPase activated with lysolecithin show a transition to a higher activation energy at temperatures below 19 °C. The sensitivity of the lysolecithin-activated enzyme to oligomycin is markedly reduced below the same temperature. With cardiolipin the transition is observed at 13 °C.

5. ADP,Mg^{2+} and to a smaller extent ATP,Mg^{2+} enhance the activation of ATPase by suboptimal amounts of phospholipid.

INTRODUCTION

Phospholipids are required for the catalytic activity of the mitochondrial ATPase when the enzyme is bound to the inner membrane or to membrane factors giving a complex with the functional characteristics of the membrane-bound activity retained[1–6]. This requirement is common to many other enzymes from various biological membranes[7].

Abbreviations: TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid; F_1, soluble ATPase or coupling factor 1 (ref. 8).

* Present address: Imperial Chemical Industries Ltd, Corporate Laboratory, P.O. Box No. 11, The Heath, Runcorn WA7 4QE, England.

** Present address: Istituto di Patologia Generale, Università di Padova, 16 via Loredan, Padova, Italy.

The reason for the phospholipid requirement is not clearly understood. In the case of the ATPase, no hydrophobic substrates or products appear to be involved and the reaction is even more efficiently catalyzed by a solubilized enzyme (F_1) that neither contains nor requires lipid[8,9]. Thus, lipid cannot be needed to furnish a favourable environment for the reaction in itself, but possibly for a hydrophobic enzyme protein. An alternative explanation could be that lipid is needed for a different function of the enzyme complex, such as the coupling of the ATPase to other energy-yielding or energy-requiring processes.

The activating effect of various phospholipids on lipid-depleted mitochondrial ATPase has been studied in a number of laboratories[2,4,5,10,11]. No single phospholipid appears to be responsible for the activating effect. The relative efficiency of different phospholipids seems to vary from one study to another. All the lipid-activated ATPase preparations studied can be inhibited by oligomycin, a property characteristic for the mitochondrial membrane ATPase, but absent in the soluble enzyme. The sensitivity of the enzyme to oligomycin is dependent on the lipid used for activation[5,10,11].

These studies permit us to form a vague idea about the role of phospholipids in the mitochondrial ATPase. Evidently, the activating effect is due to physical rather than purely chemical properties of the lipids. It seemed to us that more information on this point might be gained by studying how the activation of the ATPase by phospholipids responds to alterations in lipid type and in the assay conditions.

MATERIALS AND METHODS

Tris of Trizma grade, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid (TES), Triton X-100, bovine serum albumin, sodium phosphoenolpyruvate, nucleotides and protein standards for molecular weight determinations were purchased from Sigma Chemical Company, St. Louis, Missouri, U.S.A. Sepharose 6B and CNBr-activated Sepharose 4B were from Pharmacia Fine Chemicals, Uppsala, Sweden. Pyruvate kinase and lactate dehydrogenase (both from rabbit muscle) were from Boehringer, Mannheim, Germany. All general chemicals were of *pro analysi* or *purissimum* grades.

Nucleotide solutions were neutralized to pH 7 with NaOH. Tris–TES buffers were prepared as described previously[12], while Tris–maleate buffers were prepared by adjusting 0.1 M Tris to the desired pH with maleic acid.

Protein was determined with a biuret method[13] in the case of submitochondrial particles, otherwise by the method of Lowry *et al.*[14].

Triton X-100 bound to the enzyme was determined by extracting the freeze–dried enzyme 3 times with chloroform–methanol (2:1 v/v). The extracts were combined, concentrated and applied to thin-layer plates, using the system described further on for separation of phospholipids. The area containing isolated Triton X-100, identified by parallel runs using standards, was scraped from the plate, the gel was extracted 3 times as above and the Triton X-100 was estimated from the ultraviolet spectrum of the combined extracts.

Polyacrylamide gel electrophoresis was performed essentially as described by Lenard[15]. The gel compositions are given in total acrylamide concentration T and cross-linking C, as defined by Hjertén[16]. Molecular weights of subcomponents were

calculated by comparison with monomers and dimers of bovine serum albumin (M_r 67 000 and 134 000) and horse heart cytochrome *c* (M_r 12 400 and 24 800), with bovine pancreas chymotrypsinogen A (M_r 23 650) and with myoglobin (M_r 16 900), using a plot of migration *vs* the logarithm of the molecular weight[17].

Preparation of ATPase

ATPase was purified from a Triton X-100 extract using affinity chromatography on a column of ATPase inhibitor protein covalently coupled to Sepharose 4B[12]. The procedure was modified as follows:

The inhibitor–Sepharose gel was prepared using 3.6 mg inhibitor and 1.5 g CNBr-activated Sepharose 4-B. The column, of 3.8 cm length and 1.4 cm diameter, was equilibrated with 0.2 M sucrose, 15 mM Tris–TES (pH 6.6), 3 mM KCl, 0.5 mM $MgSO_4$, 0.5 mM ATP, 5 mM phosphoenolpyruvate, 60 μg/ml pyruvate kinase and 10 mg/ml Triton X-100. 120 mg of EDTA-submitochondrial particles[18] were sedimented at 104 000 $\times g$, 30 min, 0 °C, and were resuspended in 6 ml 0.2 M sucrose, 15 mM Tris–TES buffer (pH 6.6) and 10 mg/ml Triton X-100. The suspension was centrifuged as above and 5 ml of yellowish supernatant was collected. To this extract KCl, $MgSO_4$, phosphoenolpyruvate, pyruvate kinase and ATP were added, in that order, to reach the final concentrations of the solution used for equilibrating the column. The extract was applied to the column, followed by 5 ml equilibrating solution, at a flow rate of 6.3 ml/h. The flow rate was then raised to 12.7 ml/h. 10 ml 0.2 M sucrose, 15 mM Tris–TES (pH 6.6), 10 mg/ml bovine serum albumin was passed through the column at this rate, followed by 10 ml 0.2 M sucrose, 15 mM Tris–TES (pH 6.6). Finally, the flow rate was decreased to 2.3 ml/h and the purified ATPase was eluted from the column with 0.2 M sucrose, 45mM Tris–TES (pH 8.75), 0.2 M KCl.

The elution pattern was similar to that previously described[12], but the slower flow rate during the final elution step resulted in the sharpening of the second peak, which contains the purified ATPase. A protocol of a typical preparation is shown in Table I. The ATPase used in some parallel experiments was prepared by detergent gradient chromatography[19].

Phospholipids

Lecithin (phosphatidylcholine), phosphatidylethanolamine and cardiolipin (diphosphatidylglycerol) were purified from beef heart mitochondria. Heavy-layer mitochondria, prepared by the method of Löw and Vallin[20], were extracted with chloroform–methanol and subjected to Folch partition as described by Rouser and Fleischer[21]. A concentrated extract was applied as a streak onto silica gel G thin-layer plates of 0.25 mm thickness (E. Merck, Darmstadt, Germany). The plates were developed with chloroform–methanol–water (65:30:5, by vol.). After drying, areas containing lipids were visualized by spraying the plates with water. The gel from each area was scraped into a separate test tube and extracted 3 times with 2 ml chloroform–methanol (2:1, v/v). Extracts containing phosphatidylethanolamine, cardiolipin and lecithin were identified against standards by repeating the thin-layer chromatography. The lecithin prepared this way contained a small amount of phosphatidylinositol. Work was carried out as much as possible under a N_2 atmosphere. No appreciable peroxidation was detected in ultraviolet spectra of the extracts.

Lysolecithin prepared enzymically from egg lecithin was purchased from

Koch-Light Laboratories, Colnbrook, England. Phosphatidylserine (bovine) and phosphatidylinositol (plant) were from Supelco, Inc., Bellafonte, Pennsylvania, U.S.A.

Organic solvents were removed by exposing lipid extracts to a stream of dry N_2 gas at room temperature. When all solvent had evaporated, 0.25 ml 0.2 M sucrose, 0.1 M Tris–TES (pH 7.5), 0.1 mM EDTA was immediately added. The lipid was dispersed by sonicating for 15 s at Position 4 of a Branson sonifier. Lipid dispersions were kept at 0 °C and used within 2 days.

Phosphorus in phospholipid dispersions was estimated by a semi-micro Bartlett procedure[22]. The amount of lipid was calculated assuming a content of 2 P atoms in cardiolipin and 1 in all other phospholipids.

Preincubation of ATPase with phospholipid

50 μl enzyme in 0.2 M sucrose, 45 mM Tris–TES (pH 8.75), 0.2 M KCl, was incubated for 10 min at room temperature (about 23 °C) with 50 μl lipid in 0.2 M sucrose, 0.1 M Tris–TES (pH 7.5), 0.1 mM EDTA. The pH of the combined solutions was 7.9.

Assay of ATPase

The enzyme was assayed in the presence of an ATP-regenerating system, either by determination of P_i liberated or by following the formation of ADP enzymically[8]. In the former method 1.9 ml 0.1 M Tris–TES (pH 7.5), 3 mM $MgSO_4$, 3mM KCl, 0.75 mM phosphoenolpyruvate, 10 μg/ml pyruvate kinase, were added to 100 μl enzyme preincubated with lipid. After 10 min at 37 °C the reaction was stopped by adding 0.2 ml 50% trichloroacetic acid. P_i was determined after centrifugation, $1200 \times g$, 10 min, by the Fiske–SubbaRow method as described by Lindberg and Ernster[23].

For enzymic determination, up to 40 μl ATPase were directly added to 1.0 ml of the above medium supplemented with 0.21 mM NADH and 1.5 μg/ml lactate dehydrogenase. The decrease in absorbance at 340 nm was followed in a semi-micro cuvette.

RESULTS

Some properties of purified lipid-depleted ATPase

The ATPase eluted from the inhibitor–Sepharose column is a clear colourless solution, but as shown in Table I the protein content of this solution is quite low. The ATPase activity, tested with or without added activating lipid, is labile and is lost with a half-time of about 4 h at room temperature or about 18 h at 0 °C. The inactivation is parallelled by an increase in absorbance of the enzyme solution, which is probably due to an aggregation of the protein. The same phenomenon can be observed with the detergent gradient preparation[10].

Spectral analysis of the present ATPase preparation reveals a very low content of cytochromes, and about 0.13 nmole/mg protein flavin. This flavin content corresponds to one fifth of that in submitochondrial particles.

The presence of Triton X-100, used in the preparation of ATPase by affinity chromatography, is unsuitable for studies on activation of the ATPase by lipids or inhibition by oligomycin since, as can be seen in Table II, Triton X-100 is a potent

TABLE I

PURIFICATION OF MITOCHONDRIAL ATPase BY AFFINITY CHROMATOGRAPHY

ATPase was measured by enzymic determination of ADP formed as described in Materials and Methods.

Fraction	*Vol.* (*ml*)	*Protein* (*mg*)	*Total ATPase* (*μmoles/min*)	*Specific activity* (*μmoles/min per mg protein*)	*Yield* (%)
Submitochondrial particles	6	120	252	2.1	100
Triton X-100 extract	5	7.5	22.5	3.0	8.9
Purified enzyme*	6	1.1	9.4	8.5	4.2

* 15 mg Triton X-100/mg enzyme added.

TABLE II

ACTIVATION OF MITOCHONDRIAL ATPase BY LIPIDS

16 μg enzyme was preincubated with lipid and assayed by determination of liberated P_i as described in Materials and Methods. The preincubation medium contained 0.5% (v/v) ethanol and, in one set of experiments, 1 μg oligomycin. N.T., not tested.

Added lipid	*Amount* (*μmoles/mg protein*)	*ATPase* (*μmoles/min per mg protein*)	
		− Oligomycin	*+ Oligomycin*
None		0.49	0.25
Lecithin from ox heart mitochondria	2.41	0.62	N.T.
Phosphatidylethanolamine from ox heart mitochondria	2.91	1.26	0.56
Cardiolipin from ox heart mitochondria	1.10	4.1	3.0
Lysolecithin from egg	3.20	8.5	3.5
Phosphatidylserine from bovine brain	2.75	0.72	N.T.
Phosphatidylinositol from plant	2.10	2.2	0.91
Triton X-100	Approx. 5*	7.1	6.7

* 5 mg/mg protein.

inducer of oligomycin-insensitive activity. In order to overcome this problem the inhibitor–Sepharose column was washed with bovine serum albumin while the enzyme was attached. This removed nearly all Triton X-100 together with most of the phospholipids, as evidenced by analyzing chloroform–methanol extracts of the enzyme for phosphorus and Triton X-100 content. The values obtained were 66 nmoles and 15 μg per mg protein, respectively.

The elution profile of purified lipid-depleted ATPase applied to a Sepharose 6-B column and eluted in the presence or absence of Triton X-100, is shown in Fig. 1. It can be seen that in the presence of detergent the enzyme elutes as a single but fairly broad peak, ahead of the largest protein marker used. A rough estimate of the molecular weight of the ATPase, obtained by extrapolation of a plot[24] of elu-

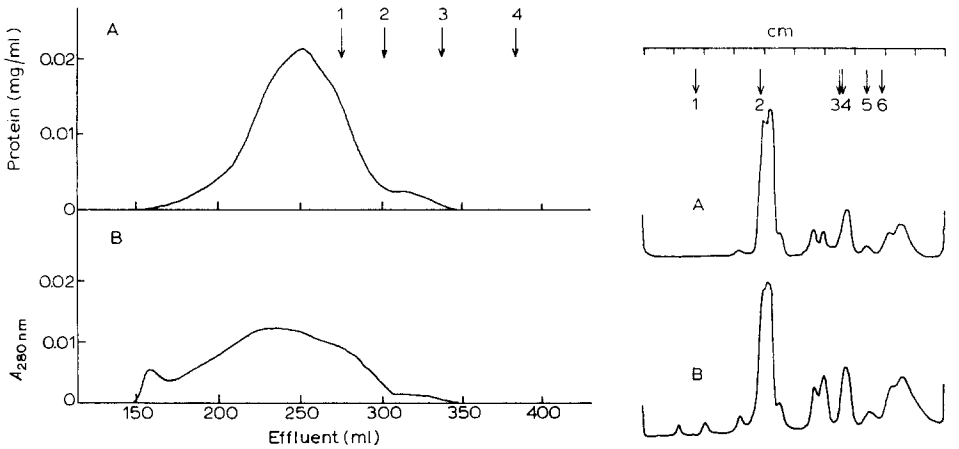

Fig. 1. Sepharose 6B gel chromatography of lipid-depleted ATPase purified by affinity chromatography. The column (92 cm × 2.5 cm) was equilibrated with 0.2 M sucrose, 45 mM Tris–TES (pH 8.75), 0.2 M KCl and, in A but not in B, 10 mg/ml Triton X-100. (A) 10 ml sample containing 3.1 mg enzyme, purified as described in ref. 12 but increasing the Triton X-100 concentration to 10 mg/ml throughout the procedure, was applied. (B) 10 ml sample containing 2.5 mg enzyme purified as described in Materials and Methods, was applied. In both experiments the column was then eluted with the buffer used for equilibration at a flow rate of 22 ml/h. The arrows mark the position in parallel runs of: 1, jack bean urease (M_r 480000); 2, beef liver catalase (M_r 250000); 3, bovine serum albumin dimer (M_r 134000) and 4, bovine serum albumin (M_r 67000).

Fig. 2. Densitometric tracings of stained polyacrylamide gels of purified lipid-depleted ATPase. (A) 1 ml enzyme containing 0.26 mg protein, prepared by affinity chromatography as described in Materials and Methods, was dialyzed against distilled water at 4 °C overnight. The dialyzed solution was freeze-dried. To the freeze-dried material 100 μl 5 mM EDTA, 5 mM 2-mercaptoethanol, 2 mg sucrose and 3 mg sodium dodecyl sulfate was added and the solution was heated 3 min at 100 °C. 50 μl of solution was then applied to a gel (10 cm × 0.5 cm) of composition $T = 6\%$ (w/v), $C = 5\%$ (w/w). The gel was run 6 h at 8 mA. (B) 0.5 ml enzyme containing 0.31 mg protein, prepared by detergent gradient chromatography as described in ref. 19, was treated, applied and run on a gel as in A. The gels were stained with Coomassie Brilliant Blue and scanned at 610 nm using a Gilford spectrophotometer with a gel scanning attachment. The arrows mark the positions in parallel runs of: 1, serum albumin dimer; 2, serum albumin; 3, cytochrome *c* dimer; 4, chymotrypsinogen; 5, myoglobin; 6, cytochrome *c*.

tion volume *vs* the logarithm of molecular weight, would give a value in the range 500 000–800 000. The lower figure is comparable to that obtained for a preparation of oligomycin-sensitive ATPase from yeast by Tzagoloff and Meagher[25].

The broadness of the peak may possibly be due to a formation of dimers even though detergent is present. In the absence of Triton X-100 the distribution of the enzyme is still wider and some material is eluted with the void volume of the column.

ATPase purified both by affinity chromatography as described in this paper and by the detergent gradient chromatography technique[19] was examined by polyacrylamide gel electrophoresis in a depolymerising system. Densitometric tracings of the gels are shown in Fig. 2. The two preparations are similar and display 10–12 clearly visible bands with apparent molecular weights of 178 000, 126 000, 84 000, 58 000, 55 000, 43 000, 30 000, 27 000, 20 000, 15 000, 12 000, and 10 000. The first two high molecular weight bands may represent firmly aggregated material and

are not present in the ATPase prepared by the milder affinity chromatography procedure.

The 58 000 and 55 000 mol. wt components probably correspond to the 2 main subunits of F_1, which have molecular weights in this range[26,27]. This impression is strengthened by ultraviolet scanning of an unstained gel.

Unfortunately determination of molecular weights by gel electrophoresis appears too variable and subject to interference from other components, such as fatty acids[28], to allow us to identify the bands of lower molecular weight in our preparations with those described for other subcomponents of F_1 and for the ATPase inhibitor protein, oligomycin sensitivity conferring protein and *N,N*-dicyclohexylcarbodiimide-binding protein[26,27,29–32].

Activation of the ATPase with different lipids

The ATPase activity of the protein purified by affinity chromatography is quite low but can be enhanced some 20-fold by the addition of a suitable lipid, as demonstrated in Table II. In agreement with previous findings in different laboratories[2,4,5,10,11], there is no pronounced specificity for any given lipid. Lysolecithin and cardiolipin are the best activators among the phospholipids we have tested. The same result was obtained with the detergent gradient preparation. Inhibition of the enzyme with oligomycin varies with the phospholipid, but there is no correlation with the degree of activation like that suggested by our earlier data[10].

Some detergents and free fatty acids activate the enzyme but the resulting ATPase is not, or to a very small extent, inhibited by oligomycin.

The amount of phospholipid required for activation was determined using three different types of phospholipid: cardiolipin with 2 negative charges and 4 hydrocarbon chains, phosphatidylethanolamine and phosphatidylinositol, both zwitterions with 2 hydrocarbon chains, and lysolecithin, a zwitterion with only 1 hydrocarbon chain. The result of the titrations is plotted in Fig. 3, using a double-reciprocal plot. The intercept on the abcissa is interpreted as the inverse amount of phospholipid giving rise to half-maximal activity. Thus, for half-maximal activity with each phospholipid 0.18 μmole cardiolipin, 0.25 μmole phosphatidylethanolamine, 0.26 μmole phosphatidylinositol or 1.1 μmoles lysolecithin would be required per mg protein.

When lipids giving a high degree of activation are mixed with a lipid giving no or a poor activation, the activating effect of the former is subdued. The double-reciprocal plot in Fig. 4 shows the effect of adding lecithin to a titration of the enzyme with cardiolipin. It can be seen that lecithin, a poor activator of the ATPase, competitively inhibits the activation with cardiolipin.

Comparison of lysolecithin-activated ATPase with cardiolipin-activated ATPase

As already mentioned, both lysolecithin and cardiolipin are good activators of the ATPase. It is of considerable interest to compare the activation by these two phospholipids, since they differ from one another in several respects. The difference in charge and number of hydrocarbon chains has already been mentioned. In addition some 80% of the fatty acids in beef heart cardiolipin is diunsaturated linoleic acid[33], whereas most of the fatty acid in our lysolecithin derived from egg yolk was found to be saturated palmitic acid.

Cardiolipin- and lysolecithin-activated ATPase activities were compared

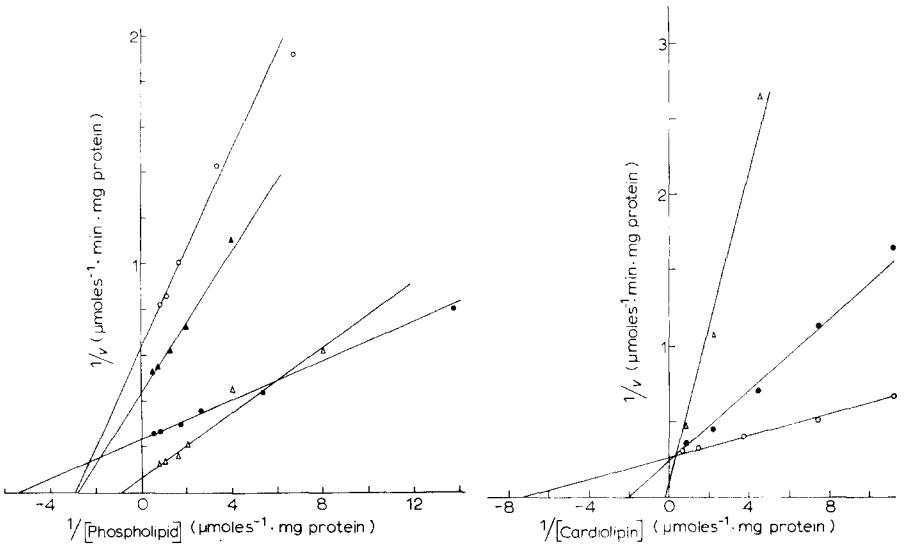

Fig. 3. Double-reciprocal plot of titrations of ATPase with different phospholipids. 16 μg enzyme was preincubated with lipid and assayed by determination of P_i liberated as described in Materials and Methods. ●—●, cardiolipin; △—△, lysolecithin; ▲—▲, phosphatidylinositol and ○—○, phosphatidylethanolamine.

Fig. 4. Double-reciprocal plot of the effect of lecithin on titrations of ATPase with cardiolipin. Experimental conditions as in Fig. 3. ○—○, control; ●—●, 0.89 μmole lecithin/mg protein; and △—△, 2.22 μmoles lecithin/mg protein.

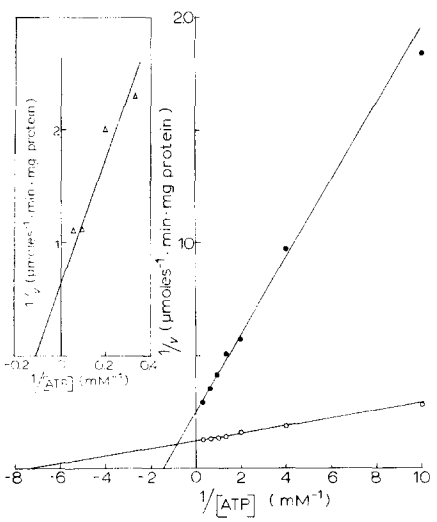

Fig. 5. Double-reciprocal plot of the effect of ATP on ATPase activated with lysolecithin or cardiolipin. 12 μg enzyme was preincubated with lipid and assayed by determination of P_i liberated as described in Materials and Methods, except that the concentration of ATP in the assay medium was varied. The concentration of Mg^{2+} was kept constant at 3 mM, except in experiments with no added lipid where it was equal to the concentration of ATP. ●—●, 1.1 μmoles cardiolipin/mg protein; ○—○, 3.2 μmoles lysolecithin/mg protein; and △—△, no lipid added.

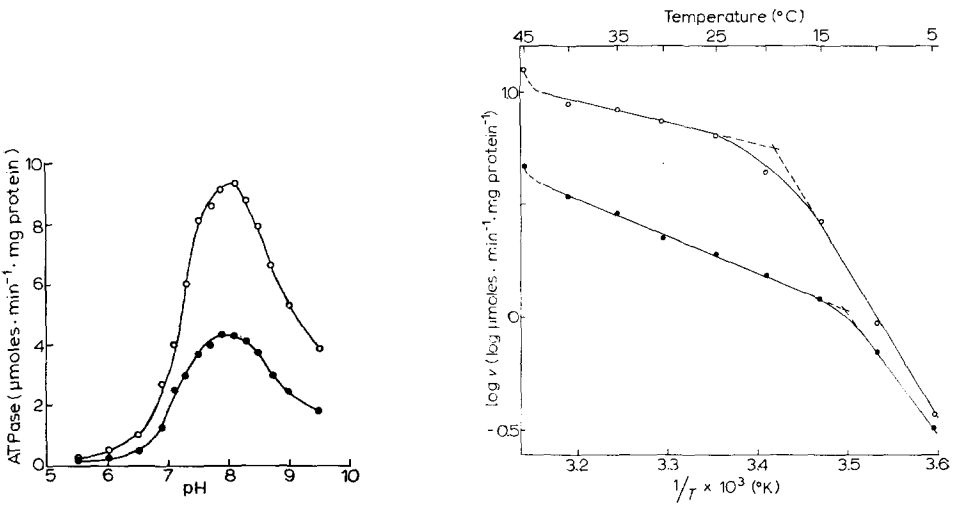

Fig. 6. The effect of pH on ATPase activated with lysolecithin or cardiolipin. 12 μg enzyme was preincubated with lipid and assayed by determining P_i liberated as described in Materials and Methods, except that the Tris–TES buffer in the assay medium was replaced with 0.1 M Tris–maleate of varying pH. ●—●, 1.1. μmoles cardiolipin/mg protein; and ○—○, 3.2 μmoles lysolecithin/mg protein.

Fig. 7. Arrhenius plot of the temperature dependence of ATPase activated with lysolecithin or cardiolipin. 12 μg enzyme was preincubated with lipid and assayed by determining P_i liberated as described in Materials and Methods, except that the temperature was varied. ●—●, 1.1 μmoles cardiolipin/mg protein; and ○—○, 3.2 μmoles lysolecithin/mg protein.

in three respects: ATP, pH and temperature dependence. Fig.5 shows a Lineweaver–Burk plot of the ATP dependence of the reaction. It can be seen that activation with cardiolipin gives rise to a much lower affinity for the substrate than with lysolecithin. The K_m for ATP is 0.71 mM and 0.14 mM, respectively. We also found that the ATPase activity of the enzyme can be increased in the absence of phospholipid by using very high concentrations of substrate, with a K_m for ATP around 9 mM.

When the ATPase is activated with cardiolipin a large number of negative charges are introduced in the immediate vicinity of the enzyme. This may be expected to cause a shift in the pH optimum of the reaction when compared with the zwitterionic lysolecithin. However, pH profiles of the ATPase reaction, in Fig. 6, are roughly the same with the two phospholipids. Maximal activity is obtained around pH 8.

Thermodynamic studies of reactions catalyzed by many membrane-bound enzymes have shown that the apparent activation energy of the reaction may vary in different temperature ranges. This effect has been attributed to phase changes in the lipid part of the enzymes[34,35]. The behaviour of the mitochondrial ATPase studied here is also complex in this respect. Fig. 7 shows Arrhenius plots of cardiolipin- and lysolecithin-activated ATPase reactions assayed at different temperatures. With both phospholipids a break in the slopes can be seen. The lysolecithin-activated ATPase reaction has an apparent activation energy of 130 kJ/mole (31 kcal/mole) at low temperatures. When the temperature is increased the apparent

activation energy falls to 20 kJ/mole (4.8 kcal/mole). The transition occurs gradually around 19 °C. With cardiolipin the corresponding values are 109 kJ/mole (26 kcal/mole) and 33 kJ/mole (7.9 kcal/mole) and the transition occurs around 13 °C.

At temperatures near 45 °C there appears to be a second deviation from a straight-line plot. Since it is known that mitochondrial ATPase at high temperatures is activated through the formation of F_1-type ATPase[36], it is likely that this deviation reflects such a degradation of the ATPase complex. We have indeed observed that ATPase kept at 45 °C is more cold-labile, a property characteristic for F_1-type ATPase[8], than enzyme kept at 35 °C.

Table III shows the oligomycin-sensitivity of the lysolecithin-activated ATPase at different temperatures. It may be seen that the effect of oligomycin is markedly reduced at temperatures below the break in the Arrhenius plot in Fig.7.

TABLE III

EFFECT OF TEMPERATURE ON THE OLIGOMYCIN SENSITIVITY OF ATPase ACTIVATED WITH LYSOLECITHIN

Assay conditions as in Fig. 7, except that 0.25% (v/v) ethanol and, in one set of experiments, 0.5 μg oligomycin were added to the preincubation medium.

Temperature (°C)	*ATPase* (*μmoles/min per mg protein*)		*Inhibition* (%)
	− Oligomycin	*+ Oligomycin*	
35	8.2	3.2	61
25	6.2	2.3	63
15	2.6	1.6	31
5	0.37	0.36	3

Effect of nucleotides on the lipid-activation of the ATPase

We have earlier observed that the activation of an ATPase by phospholipids is more efficient if ATP is present when lipid is added to the enzyme[10]. In titration curves of the enzyme with phospholipid, the addition of ATP gives a positive cooperative effect. We have further studied the effect of nucleotides on the activation of the present ATPase preparation with a suboptimal amount of phospholipid. As is evident from Table IV, ADP,Mg^{2+} appears to induce an even more effective activation than ATP,Mg^{2+}. No effect can be seen in the absence of Mg^{2+}, nor with AMP, Mg^{2+}.

It should be noted that ADP, Mg^{2+} stimulates the lipid activation of the enzyme only and not the ATPase reaction, which it inhibits. Thus, to observe the effect it is necessary to have an ATP-regenerating system present during the assay, in order to remove the ADP. A concentration of about 2 mM ADP, Mg^{2+} is required for optimal enhancement of lipid activation under the conditions in Table IV.

Several other nucleotides, such as UDP, UTP, GDP, GTP, CDP, CTP and ITP, give some enhancement in the presence of Mg^{2+}, usually in the same order as that observed with ATP or less.

TABLE IV

EFFECT OF Mg^{2+} AND ADENINE NUCLEOTIDES ON ACTIVATION OF THE ATPase BY LYSOLECITHIN

7 μg enzyme was preincubated 10 min with 0.56 nmole lysolecithin in 100 μl 0.2 M sucrose, 0.1 M Tris–TES (pH 7.5) and additions as indicated in the table. The final concentrations of added Mg^{2+} and nucleotides in the preincubation medium were 2 mM. After preincubation ATPase was measured by determination of P_i liberated as described in Materials and Methods.

Additions to preincubation medium	*ATPase (μmoles/min per mg protein)*
None	0.50
Mg^{2+}	0.45
ATP	0.53
ADP	0.51
ATP, Mg^{2+}	0.79
ADP, Mg^{2+}	1.14
AMP, Mg^{2+}	0.55

DISCUSSION

Notes on the purity of the enzyme

The gel electrophoretic profiles of the mitochondrial ATPase preparations, shown in Fig. 2, differ from published profiles of beef heart F_1 (refs 26, 27, 30) in having 5–7 extra bands. Allowing for inexactness in quantitation due to varying affinity for the dye, these bands would seem to be minor components of the complex. The impression that the degree of purification must be considerably above submitochondrial particles though not equal to F_1, is strengthened by the low content of respiratory pigments and an amino acid composition fairly similar to that of F_1. Yet the maximal specific activity obtained is only one tenth of those observed for the soluble ATPase[37,27]. Indeed, when our Triton X-100-extracted preparation is activated with mixed mitochondrial lipid, presumably the natural environment of the enzyme, the ATPase activity is even somewhat lower than that of our submitochondrial particles.

Hammes and Hilborn[38] have shown that the turnover number of F_1-type ATPase is decreased when the enzyme is bound to mitochondrial membranes, but this decrease is not large enough to explain the very low specific activity of apparently reasonably purified preparations. An explanation could be that the enzyme, when removed from its natural milieu—the membrane—rapidly and irreversibly aggregates into inactive polymers. In line with this explanation are the observed instability of the enzyme, the simultaneous increase in turbidity of the solution and the formation of material eluting with the void volume on Sepharose 6B chromatography in the absence of detergent. Electron micrographs of the preparations show the presence of linear arrays of 80 Å spheres and thinner fibers. Possibly only a part of the ATPase protein present is catalytically active, even after activation with lipid.

Nature of the activation of ATPase with phospholipids

Egg lysolecithin and beef heart cardiolipin were in the present work the best activators for both of two ATPases prepared by different methods. This would suggest to us that the reason for the variation found in the literature regarding the ability of certain phospholipids to restore the ATPase[5,10,11], is not due to a difference between the ATPases used but rather in the phospholipid preparations.

It has been proposed that the role of phospholipids in the activation of the (Na^+,K^+)-ATPase is to provide the enzyme with a hydrophobic or a negatively charged environment[39]. The latter proposal is immediately ruled out for the mitochondrial ATPase, which is activated by lysolecithin having not net charge. It is difficult to imagine how the enzyme can be activated by hydrophobicity *per se* that may be provided by both lysolecithin and cardiolipin, but not by lecithin.

The activating effect of phospholipids can hardly be dependent on their ability to form membranes or vesicular structures. Lecithin, especially together with a negatively charged lipid, readily forms vesicular membranes[40]. However, as is evident from Fig. 3, lecithin together with negatively charged cardiolipin strongly inhibits the activating effect of the latter.

The suggestion put forth by Emmelot and Bos[41] that phospholipids activate by displacing detergent bound to the enzyme during purification, seems less likely since Triton X-100 in itself is a good activator of ATPase prepared with this detergent.

Toson *et al.*[42] have shown that acidic phospholipids may stimulate ATPase in beef heart submitochondrial particles by solubilizing F_1. The phenomenon was only observed with entire submitochondrial particles and not with an oligomycin-sensitive ATPase found in the supernatant after centrifugation of particles which had not been treated with lipid. Though our ATPase preparations, when activated with cardiolipin, are relatively insensitive to oligomycin, we have earlier found that this probably reflects a competition between the lipid and the antibiotic[10]. The enzyme can still be inhibited by using very large amounts of the antibiotic, which is not the case for F_1. Our cardiolipin-activated ATPase is also more stable at 0 °C than at room temperature. Therefore, we do not think that activation of the ATPase with cardiolipin proceeds through the solubilization of F_1 and we can certainly not explain the activation with lysolecithin this way.

Both lysolecithin and cardiolipin form almost clear dispersions after sonication in aqueous buffer and are apparently relatively water-soluble. It could easily be conceived that their effect on the ATPase might be based on this property. An important role of the hydrophilic–hydrophobic balance of the activating lipid has also been suggested by Pitotti *et al.*[11]. The lipids may, for instance, activate the enzyme by solubilizing aggregates and thereby expose buried binding sites. In this case there would be two kinds of aggregation, one irreversible leading to permanent loss of potential activity, and one that can be reversed by phospholipids. An alternative mechanism of lipid activation, based on the assumption that the water solubility of the lipid is an important factor, would be that lipids are required in order to allow hydrophilic reactants to reach or leave a strongly hydrophobic site on the enzyme. Both alternatives agree with our observations.

The hydrocarbon chain is apparently an important part of the phospholipid in the activation of the ATPase. The data in Fig. 3 indicate that the amount of phos-

pholipid required for activation is dependent on the number of fatty acid chains in each lipid, which in turn could mean that the binding to the protein is hydrophobic. When the enzyme is activated with lysolecithin, containing a relatively large proportion of saturated fatty acids, there is a break in the Arrhenius plot at around 19 °C. This break occurs at a lower temperature with the relatively unsaturated cardiolipin. A phase transition in the lipid could influence either its binding to the protein or its solubility.

The marked decrease in oligomycin sensitivity observed with the lysolecithin-activated enzyme at low temperatures would fit with a proposed role of lipid in the mode of action of oligomycin[43–46]. It is interesting to note that the ouabain-sensitivity of (Na^+,K^+)-ATPase similarly is lost below a critical temperature[47].

Nucleotides, especially ADP, facilitate the activation of ATPase by suboptimal amounts of lysolecithin. ADP and ATP are known to bind firmly to F_1 (ref. 48) and coupling factor A contains bound ADP[36]. The ultraviolet spectrum of our preparation shows a maximum at 265 nm that could be due to the presence of bound nucleotide. Possibly bound nucleotides may control the conformation of the enzyme and thereby the accessibility of binding sites to lipid.

As shown in Fig. 7, the activation of the ATPase is achieved by both an increase in the turnover and in affinity for the substrate ATP. It is extremely interesting that our observations are remarkably similar to the phospholipid activation of bacterial pyruvate oxidase as described by Cunningham and Hager[49,50]. Both enzymes are best activated by phospholipids that may be classified as relatively water soluble, in both cases the lipids decrease the K_m for substrate in addition to the increase in V, and for both the activation may be enhanced in the presence of substrates or ligands to the enzymes. These common features of two enzymes having quite different functions may reflect a general behaviour of membrane-bound enzymes.

Recently some purified mitochondrial preparations have been described that catalyze the ATP–$^{32}P_i$ exchange reaction and that are either dependent on or strongly stimulated by phospholipids[51–53]. It might be argued that the involvement of phospholipids in the reversible exchange reaction more closely mimics the conditions of oxidative phosphorylation and that results obtained on this point by studying the ATPase may not have bearing on the physiological situation. Nevertheless, we feel that only by studying and comparing both reactions we can hope to reach complete understanding of the reaction mechanisms involved.

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