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PHOSPHOLIPID REQUIREMENT OF THE MEMBRANE-BOUND Mg^{2+}-DEPENDENT ADENOSINETRIPHOSPHATASE IN *ACHOLEPLASMA LAIDLAWII*

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Summary

1. Treatment of membranes of *Acholeplasma laidlawii* B with phospholipase A_2 from pig pancreas and phospholipase C from *Bacillus cereus* results in complete hydrolysis of phosphatidylglycerol.

2. Phosphatidylglycerol is not required for the activity of two membrane-bound enzymes: NADH oxidase and *p*-nitrophenylphosphatase. A slight increase in activity of those enzymes is observed upon complete hydrolysis of phosphatidylglycerol.

3. 90% of the phosphatidylglycerol can be hydrolysed with phospholipases A_2 and C without loss in activity of Mg^{2+}-dependent adenosinetriphosphatase (Mg^{2+}-ATPase). Hydrolysis of the residual 10% phosphatidylglycerol strongly reduces the Mg^{2+}-ATPase activity.

4. Modification of phosphatidylglycerol to phosphatidic acid by phospholipase D from cabbage does not effect the Mg^{2+}-ATPase activity.

5. The inactivated Mg^{2+}-ATPase in phosphatidylglycerol-depleted membranes can be reactivated by adding phosphatidylglycerol, phosphatidic acid or phosphatidylserine but not with phosphatidylcholine, phosphatidylethanolamine nor any of the *A. laidlawii* lipids except phosphatidylglycerol.

6. The ability to restore full Mg^{2+}-ATPase activity in membranes which contain less than 2% of their original amount of phosphatidylglycerol is lost gradually upon prolonged incubation times. This irreversible loss of Mg^{2+}-ATPase activity is not accompanied by a measurable hydrolysis of the residual phosphatidylglycerol.

7. Reconstitution experiments show that the fatty acid composition of both the (residual) phosphatidylglycerol present in the membrane as well as the added phosphatidylglycerol, determine the activation energy of the Mg^{2+}-ATPase and the temperature at which a break in the Arrhenius plot occurs.

Introduction

Phospholipases have been used successfully in studies concerning the role of phospholipids in membrane structure and function. We have demonstrated earlier that complete hydrolysis of phosphatidylglycerol in *Acholeplasma laidlawii* cells hardly affects the integrity of the cell membrane of this organism [1]. The present report describes the effects of phosphatidylglycerol hydrolysis on membrane-bound enzymatic activities. *A. laidlawii* is a suitable organism for this type of experimental approach. The membrane contains only one substrate for phospholipases (phosphatidylglycerol) which accounts for 30% of the total lipid content. This implies that alterations in membrane-bound enzymatic activities following hydrolysis must be ascribed to the modification or removal of this specific phospholipid or to the accumulation of hydrolysis products. The latter possibility can be investigated by using various phospholipases.

Three enzymes, belonging to the group of intrinsic membrane proteins [2], have been studied: NADH oxidase, *p*-nitrophenylphosphatase and Mg^{2+}-dependnet ATPase. As could be expected from the results of de Kruyff et al. [17] we can conclude that the first two enzymatic activities are not effected by the removal of phosphatidylglycerol. The reported lipid dependence of the Mg^{2+}-ATPase can be described more specifically as a requirement for phosphatidylglycerol. This is shown both by depletion and reconstitution studies.

Materials and Methods

Organism. A. laidlawii strain B was cultured on a lipid poor tryptose medium supplemented with various fatty acids at a final concentration of 0.12 mM [3]. In a number of experiments [1-^{14}C]elaidic acid was added to the growth medium.

Preparation of cell membranes. Membranes were isolated according to the method described by van Golde et al. [4] and finally suspended in a 1 : 20 diluted β-buffer containing 0.15 M NaCl, 0.05 M Tris and 0.01 M β-mercaptoethanol adjusted at 24°C to pH 7.4 with HCl. The protein concentration in these preparations was 5 mg per ml and was determined with the method of Lowry et al. [5]. Membrane preparations were stored at −80°C.

Phospholipase treatment. Incubations with purified phospholipase A_2 (EC 3.1.1.4) from pig pancreas (a gift from Dr. G.H. de Haas) were carried out at 37°C in the presence of 20 mM $CaCl_2$. In experiments with very short incubation periods (in the order of minutes), EDTA (ethylenediaminetetraacetic acid) was used to stop phospholipase action. Other phospholipase A_2 treatments were stopped by diluting the incubation mixture with a large volume of ice-cold 1 : 20 diluted β-buffer. It was necessary to avoid EDTA as much as possible because it strongly reduced the activity of Mg^{2+}-ATPase in phospholipase-treated as well as untreated membranes. This reduction in enzymatic activity was due presumably to a disruption of membrane structure by complexation of bivalent cations.

Treatment of membranes with purified phospholipase C (EC 3.1.4.3) from *Bacillus cereus* (kindly provided by Dr. R.F.A. Zwaal) was carried out as described for phospholipase A_2. In addition 1 mM $ZnCl_2$ was present in these

incubations. Phospholipase D (EC 3.1.4.4) from cabbage was obtained from Boehringer (Mannheim). Incubations were carried out in the presence of 100 mM $CaCl_2$. At the end of each phospholipase incubation, membranes (also untreated controls) were washed three to four times with diluted β-buffer. No phospholipase activity could be detected in the final membrane suspensions. Determinations of the extent of hydrolysis of phosphatidylglycerol and fatty acid analysis were carried out as described before [1].

Enzyme assays. Mg^{2+}-ATPase activity (EC 3.6.1.3) was measured according to Pollack et al. [6]. 650 μl membrane preparation containing 50—300 μg protein was mixed with 250 μl of a buffer containing 1.25 μmol $MgCl_2$, 1.10 μmol NaCl and 50.0 μmol Tris · HCl adjusted to pH 8.0 at 24°C. The pH of this buffer appeared to vary between pH 7.7 and 8.2 at 37 and 5°C, respectively. No alterations in the specific activity of ATPase could be measured due to this limited shift in pH. After 2 min preincubation at the desired temperature, incubations were started by adding 100 μl ATP solution (1.25 μmol). The reaction was stopped with 1 ml ice-cold 10% trichloroacetic acid after 15 or 30 min. The protein precipitate was removed by centrifugation and a phosphate determination according to Chen et al. [7] was carried out on the supernatant. In order to correct for a non-enzymatic hydrolysis of ATP all measurements were standardized with respect to time and compared with appropriate blanks.

For the construction of Arrhenius plots of the Mg^{2+}-ATPase activity, activities were determined for at least eight different temperatures and three different incubation periods at each temperature. From the initial velocities of the ATP hydrolysis, specific activities were calculated and used for the Arrhenius plots. NADH dehydrogenase (oxidase) activity (EC 1.6.99.3) was measured continuously at 37°C as described by Pollack et al. [6] in a double beam spectrophotometer. *p*-Nitrophenylphosphatase activity was assayed according to Ne'eman et al. [8]. The incubation mixture consisted of 0.4 ml buffer containing 100 μmol Tris · HCl (pH 7.6) and 5 μmol $MgCl_2$ to which 0.5 ml membrane suspension was added containing 200—400 μg membrane protein. The reaction was started by addition of 0.1 ml *p*-nitrophenylphosphate (10 μmol). The adsorbance at 420 nm was measured continuously in a double beam spectrophotometer against the same mixture without substrate.

Preparation of lipid dispersions. A. laidlawii lipids were isolated from a 10 l culture by extraction of isolated membranes according to Bligh and Dyer [9] followed by preparative thin-layer chromatography on 0.5 mm silica gel H plates with chloroform/methanol/water/acetic acid (65 : 25 : 4 : 1, v/v) as developing system. Lipids were stained with iodine vapour and isolated by scraping off the silica gel and elution with chloroform/methanol mixtures. Phosphatidylcholine and phosphatidylethanolamine were obtained from egg yolk [10,11]. Phosphatidic acid was prepared from egg yolk phosphatidylcholine by digestion with phospholipase D from cabbage [11]. Phosphatidylserine was isolated from pig brain according to the procedure of Sanders [12].

Handshaken liposomes were prepared by drying 1 or 2 mg lipid as a thin film on the wall of a round bottom flask and suspended with a glass bead in 1 ml 1 : 20 diluted β-buffer.

Results

Treatment with phospholipase A_2 and C

Phospholipase A_2 treatment of isolated membranes of *A. laidlawii* results in hydrolysis of phosphatidylglycerol only. Table I shows that the phosphatidylglycerol content of treated membranes is reduced from 30 to 1% of the total lipids. No lysophosphatidylglycerol can be observed after incubation with phospholipase A_2 which can be explained by the presence of membrane-bound lysophospholipase [4]. Furthermore Table I shows that none of the other *A. laidlawii* lipids is affected by phospholipase A_2. Also for phospholipase C from *B. cereus* there appears to be only one suitable substrate present in the membrane of *A. laidlawii*. As can be seen from Table I treatment of these membranes with phospholipase C results in an almost complete conversion of phosphatidylglycerol into diglycerides, the other lipids remaining unaltered. And finally, phospholipase D from cabbage converted phosphatidylglycerol into phosphatidic acid.

The activity of three different membrane-bound enzymes was tested on membranes treated with phospholipase A_2 and C. Table II shows that the activity of both NADH oxidase and *p*-nitrophenylphosphate is increased when phosphatidylglycerol is hydrolysed.

In contrast, removal of phosphatidylglycerol strongly reduces the activity of the membrane-bound Mg^{2+}-ATPase in *A. laidlawii*. As can be seen from Table II only 35—45% of the original activity is left. To exclude the possibility that the decrease in Mg^{2+}-ATPase activity is due to an inhibitory effect of the free fatty acids, liberated during phospholipase A_2 action, membranes were washed with lipid poor bovine serum albumine. The results show that the presence of free fatty acids does not interfere with the ATPase activity (Table II). Removal of diglycerides produced during phospholipase C treatment by anhydrous ether extraction as described by Roelofsen and van Deenen [13] was not possible.

TABLE I

LIPID COMPOSITION OF *A. LAIDLAWII* MEMBRANES AFTER TREATMENT WITH VARIOUS PHOSPHOLIPASES

Data are expressed as percentages of the total lipid content of the membrane. Membranes were isolated from cells grown on [1-^{14}C]elaidate and incubated with excess phospholipase for 60 min at 37°C.

Lipid	Control	Treated with phospholipase A_2 from pig pancreas	Treated with phospholipase C from *B. cereus*	Treated with phospholipase D from cabbage
Diglyceride	1	1	31	
Fatty acid	2	33	3	70 *
Monoglucosyldiglyceride	38	38	38	
Diglucosyldiglyceride	18	17	16	18
Phosphatidylglycerol	30	1	1	1
Diglucosyldiglyceride-glycero 3-phosphate	4	3	4	4
Unidentified	6	6	6	6

* Including the released phosphatidic acid.

TABLE II

EFFECT OF PHOSPHOLIPASE TREATMENT ON THE ACTIVITY OF MEMBRANE-BOUND ENZYMES IN *A. LAIDLAWII*

Membranes were incubated with excess phospholipase for 2 h at 37°C. In this case 98% hydrolysis of phosphatidylglycerol could be measured.

Membrane treatment	Percentage of specific activity of untreated controls		
	NADH oxidase	*p*-Nitrophenylphosphatase	Mg^{2+}-ATPase
Phospholipase A_2	126	106	35
Phospholipase A_2, washed with bovine serum albumine	130	124	39
Phospholipase C	118	145	45

Restoration of Mg^{2+}-ATPase activity

Phospholipase A_2-treated membranes were used for reactivation experiments with various lipids. Removal of free fatty acids from phospholipase A_2-treated membranes, prior to reactivation with lipids did not influence the extent of reactivation of the Mg^{2+}-ATPase. A number of preliminary experiments were carried out to find the optimal conditions for reactivation. Different lipid dispersions were tested: handshaken liposomes prepared as described in Materials and Methods; vesicles obtained by sonication of handshaken liposomes; liposomes prepared according to Batzri and Korn [14] and finally handshaken liposomes prepared with a membrane suspension in stead of a 1 : 20 diluted β-buffer, with additional sonication. Optimal results were obtained with handshaken liposomes in a 1 : 20 diluted β-buffer, sonicated for 5 min. The presence of Mg^{2+} (10 mM) does not influence the efficacy of reactivation of Mg^{2+}-ATPase. Different preincubation times (up to 3 h) prior to the ATPase assay, did not improve the reactivation. A preincubation time of 15 min was allowed before each ATPase assay was started.

Various lipids including the *A. laidlawii* lipids were tested for their ability to restore the Mg^{2+}-ATPase activity. It is shown in Table III that from the *A. laidlawii* lipids only phosphatidylglycerol is able to induce a partial restoration of the Mg^{2+}-ATPase activity. The negatively charged phospholipids, phosphatidic acid and phosphatidylserine, exhibit the same activity whereas the neutral phospholipids appear to be unable to reactivate the enzyme.

Supporting evidence for the ability of phosphatidic acid to restore the ATPase activity comes from an experiment with phospholipase D from cabbage. Modification of the total phosphatidylglycerol content of the membrane into phosphatidic acid (see Table I) has no effect on the Mg^{2+}-ATPase activity. From these results it can be concluded that phosphatidylglycerol is involved in the Mg^{2+}-ATPase activity in *A. laidlawii* membranes.

Relation between Mg^{2+}-ATPase activity and phosphatidylglycerol content of the membrane

A number of experiments were carried out to establish the approximate amount of phosphatidylglycerol which is required for enzymatic activity. Samples were taken from membrane preparations which were incubated with phos-

TABLE III

ABILITY OF VARIOUS LIPIDS TO RESTORE THE Mg^{2+}-ATPase ACTIVITY OF PHOSPHOLIPASE A_2-TREATED MEMBRANES FROM *A. LAIDLAWII*

3.75 μmol lipid was added per mg membrane protein. In all experiments control incubations with untreated membranes were carried out. Addition of lipids to these membranes resulted in a variable reduction of ATPase activity between 5 and 15%. To correct for this the data are expressed as percentages of the specific activity of Mg^{2+}-ATPase of untreated controls to which the same amount of lipid was added.

Lipid added to the membrane	Specific activity of Mg^{2+}-ATPase
No lipid added	37
Phosphatidylglycerol	74
Monoglucosyldiglyceride	36
Diglucosyldiglyceride	38
Diglucosyldiglycerideglycero 3-phosphate plus unidentified lipid	43
Phosphatidic acid	74
Phosphatidylserine	72
Phosphatidylcholine	28
Phosphatidylethanolamine	38

pholipase A_2 or C at different intervals and assayed for residual phosphatidylglycerol and Mg^{2+}-ATPase activity. About 90% of the phosphatidylglycerol can be hydrolysed without a substantial decrease in enzyme activity (Fig. 1). By contrast even a 25% increase in activity has been measured which might be due to the exposure of more enzymatic sites of the Mg^{2+}-ATPase or a facilitation of the penetration of ATP to the active centre of the enzyme. It is obvious from these data that the majority of phosphatidylglycerol is not required for Mg^{2+}-ATPase activity. Hydrolysis of the residual 10% phosphatidylglycerol results in a sharp decrease in Mg^{2+}-ATPase activity. Not only the extent of phosphatidylglycerol hydrolysis, but also the incubation time which was required to obtain an extensive hydrolysis, effected the residual Mg^{2+}-ATPase activity. Fig. 2A shows that a prolonged incubation in the presence of phospholipase A_2 results in a gradual decrease of the enzymatic activity without a measurable hydrolysis of the small amount of phosphatidylglycerol left. Reconstitution experiments demonstrate that the initial loss of Mg^{2+}-ATPase activity is reversible (Fig. 2B). Maximal reactivation can be obtained with phosphatidylglycerol at a lipid to protein ratio of 4 to 1. At longer incubation periods, however, the enzymatic activity is irreversibly lost. After 5 h incubation only 20% of the Mg^{2+}-ATPase activity (as compared with a control incubation without phospholipase) remains and reconstitution of the Mg^{2+}-ATPase activity is hardly possible. These data strongly suggest that phosphatidylglycerol is required not only for Mg^{2+}-ATPase activity but also for maintaining the stability of the enzyme.

Influence of the fatty acid composition of phosphatidylglycerol on the specific activity of Mg^{2+}-ATPase

Membrane lipids of *A. laidlawii* membranes can be varied in their fatty acid composition by the addition of fatty acids to the growth medium of this organism [15]. Two types of membranes have been used in the following experiments. The phosphatidylglycerol of linoleate-enriched cells contained

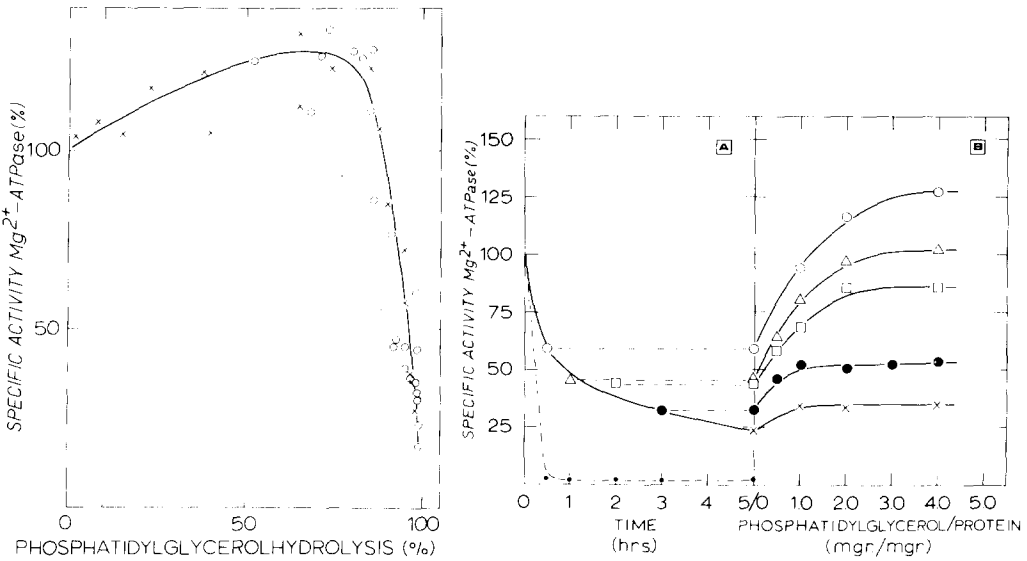

Fig. 1. Relation between phosphatidylglycerol and Mg^{2+}-ATPase activity of *A. laidlawii* membranes. Membranes were isolated from cells grown on [1-^{14}C]elaidate and incubated with phospholipase A_2 (○) and C (×). Samples were assayed for phosphatidylglycerol content and residual Mg^{2+}-ATPase activity. The data are expressed as percentage of the initial phosphatidylglycerol content and as percentage of the specific ATPase activity of appropriate control incubation.

Fig. 2. Relation between phosphatidylglycerol content and Mg^{2+}-ATPase activity of *A. laidlawii* membranes and reactivation by phosphatidylglycerol. Membranes were isolated from cells grown on [1-^{14}C]-elaidic acid and incubated with phospholipase A_2. The extent of phosphatidylglycerol hydrolysis is given in Fig. 3A (○- - - -○) and expressed as percentage of the initial amount of phosphatidylglycerol present. Samples taken from this incubation at different time intervals were washed three times to remove phospholipase (no EDTA was added to stop phospholipase action). The final membrane suspension was used to determine phosphatidylglycerol content and Mg^{2+}-ATPase activity (A) and the Mg^{2+}-ATPase activity after mixing the membranes with various amounts of phosphatidylglycerol (B). Membranes were tested after 30 min (○), 1h (△), 2 h (□), 3 h (●) and 5 h (×) incubation with phospholipase A_2.

15% of linoleate, 10% of oleate and 50% of palmitate as major fatty acids. Enrichment with palmitate resulted in phosphatidylglycerol containing nearly exclusively palmitate (72%) and myristate (10%) and only a small amount of oleic acid (4%). An Arrhenius plot of the Mg^{2+}-ATPase activity of linoleate-enriched membranes is linear over a temperature range from 0 to 40°C as can be seen from Fig. 3. The activation energy of the Mg^{2+}-ATPase in these membranes appeared to be 13.6 ± 0.1 kcal/mol. Treatment of these membranes with phospholipase A_2 results in a lowering of the specific activity at all temperatures but no significant change in activation energy can be observed (Fig. 3, Table IV). Reactivation of this residual Mg^{2+}-ATPase with phosphatidylglycerol isolated from cells grown on palmitic acid, results in a different Arrhenius plot in which a discontinuity at 19°C is observed with activation energies of 11.3 and 19.1 kcal/mol above and below this temperature, respectively. Moreover incubation of untreated membranes isolated from linoleic acid-grown cells, with phosphatidylglycerol enriched in palmitic acid gives rise to a similar change in the Arrhenius plot (Table IV). The discontinuity temperature for these membranes is somewhat lower while the activation energy below this

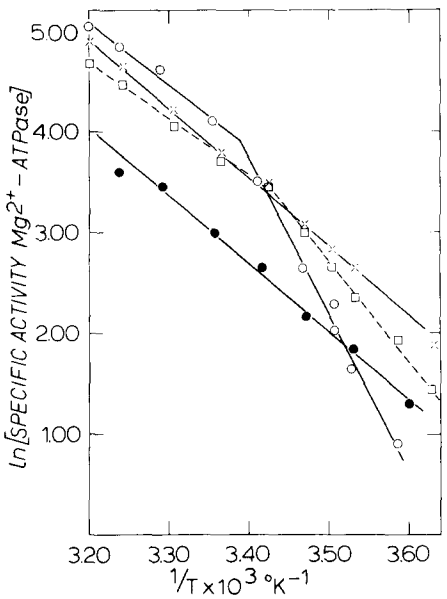

Fig. 3. Arrhenius plots of the Mg^{2+}-ATPase activity of various membrane preparations. Membranes, isolated from cells grown on linoleic acid were treated with phospholipase A_2, such that a fair amount of ATPase activity was lost, but upon adding phosphatidylglycerol a good reactivation is still possible (see also Fig. 2). Shown are the Arrhenius plots of Mg^{2+}-ATPase activity of linoleic acid-enriched membranes (X), linoleic acid-enriched membranes treated with phospholipase A_2 (•), phospholipase A_2-treated, linoleic acid-enriched membranes, reactivated with palmitic acid-enriched phosphatidylglycerol (□) and palmitic acid-enriched membranes (○).

temperature is increased to 25.5 kcal/mol. Fig. 3 and Table IV show furthermore the transition temperature of palmitic acid-enriched membranes from which the phosphatidylglycerol used for the reactivation of Mg^{2+}-ATPase, was isolated. The break was found at 21.4°C and the activation energy below and above the transition are 32.0 and 12.7 kcal/mol, respectively. The results demonstrate that the physical state of the phosphatidylglycerol which is added to the *A. laidlawii* membranes, has a pronounced effect on the activation energy of the Mg^{2+}-ATPase in these membranes.

TABLE IV

INFLUENCE OF FATTY ACID COMPOSITION OF PHOSPHATIDYLGLYCEROL ON THE ACTIVATION ENERGY OF Mg^{2+}-ATPase IN *A. LAIDLAWII* MEMBRANES

T_k is the temperature at which a break in the Arrhenius plot occurs. E_a is the activation energy of Mg^{2+}-ATPase. Data are presented ± S.E. of the mean. Fatty acid compositions of the phosphatidylglycerol present in the above membrane preparations are given in the text.

	T_K (°C)	$E_a > T_k$ (kcal/mol)	$E_a < T_k$ (kcal/mol)
Linoleate membranes	—	13.6 ± 0.1	13.6 ± 0.1
Phosphatidylglycerol-depleted linoleate membranes	—	12.9 ± 0.5	12.9 ± 0.5
Palmitate membranes	21.4 ± 0.6	12.7 ± 0.5	32.0 ± 1.0
Phosphatidylglycerol-depleted linoleate membranes + palmitate phosphatidylglycerol	19.0 ± 0.5	11.3 ± 0.2	19.1 ± 0.6
Linoleate membranes + palmitate phosphatidylglycerol	16.0 ± 0.3	11.6 ± 0.6	25.5 ± 1.9

Discussion

From the results described in this paper it is obvious that phosphatidylglycerol is not required for the function of two membrane-bound enzymes, NADH oxidase and *p*-nitrophenylphosphatase (Table II). The increase in activity which is observed upon hydrolysis of the phosphatidylglycerol might have a similar explanation as given for the initial increase in Mg^{2+}-ATPase activity (see Results). There has been some doubt about the nature of the *p*-nitrophenylphosphatase activity and the Mg^{2+}-ATPase activity [16]. The different behaviours of these two activities towards phospholipases indicate that they are not to be described to a single enzyme. Other observation which are in agreement with this conclusion are the different behaviours of these activities towards detergents as described by Ne'eman et al. [8] and the difference in Arrhenius plots of both activities as shown by de Kruyff et al. [17]. It is not surprising that neither NADH oxidase nor *p*-nitrophenylphosphatase require phosphatidylglycerol for activity. Indications that both enzymes although membrane bound, are not lipid dependent were already obtained by de Kruyff et al. [17] who showed that both enzymes have a constant activation energy over a temperature range in which the membrane lipids undergo a phase transition. Moreover, Ne'eman et al. [8] showed that it is possible to solubilize these two enzymes without great loss in activity, using selected detergents.

Complete hydrolysis of phosphatidylglycerol has a profound effect on the Mg^{2+}-ATPase activity.

Furthermore, the reactivation experiments demonstrate that negatively charged phospholipids are able to restore the Mg^{2+}-ATPase activity. None of the *A. laidlawii* lipids can reactivate the enzyme except phosphatidylglycerol, which is the only negatively charged phospholipid present in these membranes. It can be concluded from these results that phosphatidylglycerol is required for Mg^{2+}-ATPase activity. However, the majority of phosphatidylglycerol can be hydrolysed without loss in Mg^{2+}-ATPase activity (Fig. 1). Less than 10% of the phosphatidylglycerol, which means 3% or less of the total membrane lipid is required for Mg^{2+}-ATPase activity. This result resembles the observations by Roelofsen and van Deenen [13] on the $(Na^+ + K^+)$-ATPase in human erythrocytes. They demonstrated that only modification of the residual 13% phosphatidylserine into phosphatidylethanolamine results in a complete loss of activity. Furthermore, Warren et al. [18] showed that the sarcoplasmic reticulum ATPase retains maximal activity until about 30 phospholipid molecules per molecule ATPase remain.

The decrease in Mg^{2+}-ATPase activity during incubation with phospholipase, is a rather slow process and occurs only when the majority of phosphatidylglycerol is hydrolysed (Fig. 2). It is not possible, with the technique we used, to demonstrate further hydrolysis of the residual phosphatidylglycerol during this Mg^{2+}-ATPase inactivation. Therefore, two different explanations might be given for the slow inactivation of Mg^{2+}-ATPase. A small amount of phosphatidylglycerol is necessary to maintain an active conformation of the Mg^{2+}-ATPase and is in turn protected to a certain extent by the protein against phospholipase attack. Only prolonged incubation with phospholipase will hydrolyse also this phosphatidylglycerol with a corresponding loss in enzymatic activity. The

resulting conformational change of the protein can be reversed by adding phosphatidylglycerol to the system. Depending on the amount of phosphatidylglycerol which was withdrawn from the Mg^{2+}-ATPase, the denaturation of the enzyme will be more irreversible. Another explanation might be that phosphatidylglycerol is required for the stability of the Mg^{2+}-ATPase but is not protected by the protein. Therefore all phosphatidylglycerol can be hydrolysed but because this lipid is not directly involved in the ATP hydrolysis, the enzyme will initially retain full activity. Lack of phosphatidylglycerol will induce a time-dependent denaturation of the protein which is partially reversible upon adding phosphatidylglycerol.

de Kruyff et al. [17] and Hsung et al. [19] demonstrated that the Mg^{2+}-ATPase activity of *A. laidlawii* membranes exhibit discontinuities in the slope of the Arrhenius plot when the lipids undergo a change from the lipid-crystalline to the gel state. Fig. 3 and Table IV show that the activation energy of this enzyme is constant over a temperature range from 0 to 40°C for membranes enriched with linoleic acid. This is in agreement with observations of de Kruyff et al. [17] that lipids in linoleic acid-enriched membranes exist in the liquid-crystalline state in this temperature region. Hydrolysis of phosphatidylglycerol, accompanied by a reduction in Mg^{2+}-ATPase activity does not result in a change in activation energy. Restoration of the activity with palmitic acid-enriched phosphatidylglycerol gives rise to a change in the Arrhenius plot. A discontinuity is now observed, meaning that the physical state of the newly added phosphatidylglycerol influences the activation energy of the Mg^{2+}-ATPase. Moreover a similar change in Arrhenius plot was observed when untreated linoleic acid-enriched control membranes are mixed with palmitic acid-enriched phosphatidylglycerol. From these results it can be concluded that the original Mg^{2+}-ATPase-surrounding phosphatidylglycerol is mixed with the newly added phosphatidylglycerol. This is in agreement with observations of Thilo and Overath [20] demonstrating the mixing of newly synthesized lipids with pre-existing lipids surrounding the β-galactoside transport system in *Escherichia coli*. These results were surprising when compared with findings of de Kruyff et al. [17]. They suggest that the Mg^{2+}-ATPase in *A. laidlawii* membranes is associated with the most liquid lipid species in the membrane. This implies that it should be impossible to find a discontinuity in the Arrhenius plot of untreated linoleic acid-enriched membranes, supplemented with palmitic acid-enriched phosphatidylglycerol, which is in contrast with the present results. It is obvious that this contradiction will remain unexplained as long as we do not know if and how the added phosphatidylglycerol is interacting with the membrane in general and the Mg^{2+}-ATPase in particular.

The function of Mg^{2+}-ATPase in *A. laidlawii* is not yet understood (for a discussion, see ref. 21). The present data, however, indicate a close resemblance to the $(Na^+ + K^+)$-ATPase of eukaryotic cells with respect to their dependence on acidic phospholipids [13,22—25] and their disposition in the membrane [2]. This in contrast to the Mg^{2+}-ATPases in other microbial systems, like *E. coli*, which are stimulated generally by neutral lipids and belong to the group of peripheral membrane proteins.

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References

1 Bevers, E.M., Singal, S.A., Op den Kamp, J.A.F. and van Deenen, L.L.M., Biochemistry, in press
2 Ne'eman, Z. and Razin, S. (1975) Biochim. Biophys. Acta 375, 54—68
3 de Kruyff, B., Demel, R.A. and van Deenen, L.L.M. (1972) Biochim. Biophys. Acta 255, 331—347
4 van Golde, L.M.G., McElhaney, R.N. and van Deenen, L.L.M. (1971) Biochim. Biophys. Acta 231, 245—249
5 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
6 Pollack, J.D., Razin, S. and Cleverdon, R.C. (1965) J. Bacteriol. 90, 617—622
7 Chen, P.S., Toribara, T.Y. and Warner, N. (1956) Anal. Chem. 28, 1756—1760
8 Ne'eman, Z., Kahane, I., Kovartovsky, J. and Razin, S. (1972) Biochim. Biophys. Acta 266, 255—268
9 Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911—917
10 Singleton, W.S., Gray, M.S., Brown, M.L. and White, J.L. (1965) J. Am. Oil Chem. Soc. 42, 53—58
11 Ansell. G.B. and Hawthorne, J.N. (1964) in Phospholipids, pp. 91—101, Elsevier Publ. Co., Amsterdam
12 Sanders, H. (1967) Biochim. Biophys. Acta 144, 485—487
13 Roelofsen, B. and van Deenen, L.L.M. (1973) Eur. J. Biochem. 40, 245—257
14 Batzri, S. and Korn, E.D. (1973) Biochim. Biophys. Acta 298, 1015—1019
15 McElhaney, R.N. (1974) J. Mol. Biol. 84, 145—157
16 Ne'eman, Z., Kahane, I. and Razin, S. (1971) Biochim. Biophys. Acta 249, 169—176
17 de Kruyff, B., van Dijck, P.W.M., Goldbach, R.W., Demel, R.A. and van Deenen, L.L.M. (1973) Biochim. Biophys. Acta 330, 269—282
18 Warren, G.B., Bennett, J.P., Hesketh, T.R., Houslay, M.D., Smith, G.A. and Metcalfe, J.C. (1975) in Fed. Proc. Eur. Bioch. Soc., 10th Meeting, Paris, Vol. 41, p. 3, Elsevier, Amsterdam
19 Hsung, J., Huang, L., Hoy, D.J. and Haug, A. (1974) Can. J. Biochem. 52, 974—980
20 Thilo, L. and Overath, P. (1976) Biochemistry 15, 328—334
21 Rottem, S. and Razin, S. (1966) J. Bacteriol. 92, 714—722
22 Hokin, L.E. and Hexum, I.D. (1972) Arch. Biochem. Biophys. 151, 453—463
23 Goldman, S.S. and Albers, R.W. (1973) J. Biol. Chem. 248, 867—874
24 Kimelberg, H.K. and Papahadjopoulos, D. (1974) J. Biol. Chem. 249, 1071—1080
25 Walker, A. and Wheeler, K.P. (1975) Biochim. Biophys. Acta 394, 135—144