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NEGATIVELY CHARGED PHOSPHOLIPID REQUIREMENT OF THE OLIGOMYCIN-SENSITIVE MITOCHONDRIAL ATPase

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The highly-purified, oligomycin-sensitive mitochondrial adenosine triphosphatase has been reconstituted with phosphatidylserine. Treatment of the phosphatidylserine-reconstituted ATPase with phosphatidylserine decarboxylase produced a 3-fold decrease in the specific activity of the resulting phosphatidylethanolamine-enriched ATPase complex. Subsequent control experiments indicated that the resulting phosphatidylethanolamine was responsible for the lowered ATPase specific activity. These observations indicate that acidic phospholips do more than facilitate an interaction between the highly-purified, lipid-depleted ATPase and phospholipid. The negatively charged phospholipid appears to be essential for maintaining high levels of oligomycin-sensitive activity even after the initial interaction between phospholipid and the ATPase complex has occurred.

The oligomycin-sensitive ATPase from beef heart mitochondria has been prepared in a phospholipid-deficient form by several laboratories [1–3]. The ATPase and ATP-P_i exchange activities of these preparations are quite low, but can be stimulated several-fold by reconstitution with added phospholipids [1–4]. Earlier studies carried out with ATPase preparations of relatively low purity indicated that acidic phospholipids reactivated the lipid-depleted ATPase to higher levels of specific activity than either neutral or basic phospholipids [5–8].

The superior stimulatory activity of acidic phospholipids may indicate that a negatively charged ionic environment facilitates the interaction between the ATPase and phospholipid more readily than the environment provided by neutrally charged phospholipids. For example, reactivation may be disaggregation, as appears to be the case when cytochrome b_5 is reconstituted into phospholipid vesicles [9]. Under this circumstance the enzyme, once combined with phospholipid, should require the lipid only as a matrix from which it expresses its enzymatic activity. Alternatively, acidic phospholipids may elicit higher activities from the ATPase by influencing the catalytic capacity of the enzyme complex. The following experiments were carried out to differentiate between the two possibilities mentioned above.

A highly-purified mitochondrial ATPase preparation [2] was reactivated with the acidic phospholipid, phosphatidylserine (PS). The PS in the enzyme-phospholipid complex was enzymatically converted to phosphatidylethanolamine (PE), a neutral phospholipid, using phosphatidylserine decarboxylase. The activity of the ATPase was mea-

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Abbreviations: PS, phosphatidylserine; PE, phosphatidylethanolamine.

sured before and after phosphatidylserine decarboxylase treatment to determine if its activity could be maintained once the phospholipid in the complex was converted from an acidic to a neutral species. This approach has been utilized previously to study the phospholipid requirements of the $(Na^+ + K^+)$-ATPase [10].

The highly-purified mitochondrial ATPase (38–45 p) was isolated by the method of Serrano et al. [2]. The specific activity of the preparation used in this study was 10.5 μmol/min per mg protein with asolectin added as an activator; specific activities as high as 12.5 were obtained in other Serrano preparations. The oligomycin-sensitive ATPase activity of this preparation is directly dependent upon the addition of exogenous phospholipid [2]. The phosphatidylserine decarboxylase was kindly provided by Drs. Larry Rizzolo and E.P. Kennedy of the Department of Biological Chemistry, Harvard Medical School; the specific activity of the preparation was 120 μmol $^{14}CO_2$/min per mg protein.

As had been noted by other investigators [10,11], the phosphatidylserine decarboxylase activity was highly dependent on the presence of Triton X-100. However, incubation of the mitochondrial ATPase in 1.6 mM (0.1%) Triton X-100 resulted in an almost complete loss of ATPase activity (Table I). Therefore, a survey of alternative detergents was performed. Of the detergents tested (Tween 20, 40, 60, and 80 (J.T. Baker), Brij 30, 56, 76, and 99 (Sigma), recrystallized sodium cholate and deoxycholate (Matheson, Coleman, and Bell), and octyl glucoside (Calbiochem)), the octyl glucoside stimulated the best phosphatidylserine decarboxylase activity (about 80% of the activity stimulated with 1.6 mM Triton X-100). Maximal activity with PS liposomes was obtained at 17.1 mM (0.5%) octyl glucose (data not shown).

The effect of the octyl glucoside on the mitochondrial ATPase activity was also examined (Table I). It should be noted that the detergent incubation concentrations listed in Table I (1.6 mM Triton X-100 and 17.1 mM octyl glucoside) were diluted 1:6 during the ATPase assay procedure. From Table I, it is evident that the octyl glucoside was only mildly inhibitory towards the ATPase compared to Triton X-100 when both were added at concentrations which elicit maximal activity from phosphatidylserine decarboxylase. Also, more oligomycin sensitivity was retained with octyl glucoside than with Triton X-100. It is apparent, however, the octyl glucoside lowers the oligomycin sensitivity of the ATPase reconstituted with phospholipids. Based on the above observations octyl glucoside (17.1 mM) was utilized to enhance the enzymatic conversion of PS to PE in the reconstituted mitochondrial ATPase during all subsequent experiments.

TABLE I

THE EFFECT OF EXTENDED INCUBATION WITH NONIONIC DETERGENTS ON THE PHOSPHOLIPID-STIMULATED ATPase

The phospholipid (360 nmol) was suspended in a solution containing ATPase buffer (10 mM Tris sulfate (pH 7.5), 1 mM $MgSO_4$, 0.5 mM EDTA, 0.5 mM dithiothreitol) 10% sucrose, 5 mM 2-mercaptoethanol, and 5% glycerol along with the amount of detergent indicated below. After bath sonicating for 5 min under nitrogen with a Heat Systems Ultrasonic Sonifier (Model 13) at an output of 240 watts (25°C), 38–45 p ATPase (24 μg) was added. The resulting suspension (final volume of 0.3 ml) was incubated at 30°C for the times designated. Aliquots containing 60 nmol phospholipid and 4.0 μg protein was assayed for ATPase activity. ATPase assays were performed as described by Serrano et al. [2] with the following modifications. No asolectin was added. The ascorbic acid was replaced with 50 μl of a saturated solution of 1-amino-2-naphtholsulfonic acid in 13.2% $Na_2S_2O_5$ and 0.5% Na_2SO_3. Color development was achieved by incubation for 10 min at 37°C.

Incubation conditions	ATPase activity (μmol/min/mg protein)	%ATPase activity oligomycin sensitive
No detergent (12.75 h) [a]		
PS	7.7	74
PE	4.1	84
1.6 mM Triton X-100 (6 h) [b]		
PS	0.4	8
PE	0.9	5
17.1 mM octyl glucoside (6 h) [b]		
PS	5.7±0.2 [c]	33±4
PE	1.7±0.2	36±7

[a] Time of incubation in absence of detergent does not influence ATPase activity.

[b] Detergents in the incubation medium were diluted 1:6 during the ATPase assay procedure.

[c] The values are the average ±S.E.M. from four separate experiments.

The effect of phosphatidylserine decarboxylase on the activity of the mitochondrial ATPase previously reconstituted with PS is shown in Table II. When 26% of the PS was converted to PE, the ATPase maintained the level of specific activity characteristic of PS stimulation. However, after 87% of the PS had been converted to PE, the ATPase specific activity dropped to levels characteristic of PE stimulation. The variability of oligomycin sensitivity observed in Tables II and III appears to be a function of the combination of octyl glucoside and phospholipid in the reconstituted ATPase. The oligomycin sensitivity measurements are included to demonstrate the susceptibility of the reconstituted system to this classical inhibitor of the ATP synthetase complex.

The fact that the PE was responsible for the decreased ATPase specific activity was suggested from two lines of evidence. First, control experiments (Table III, part B) indicated that PE, at concentrations obtained after 87% hydrolysis of PS by phosphatidylserine decarboxylase, was able to activate the ATPase to low levels of activity when the lipid-depleted ATPase was presented simultaneously with PE and octyl glucoside. This indicated that the PE was able to associate with the ATPase in the presence of octyl glucose. Therefore, the decrease in ATPase activity observed (Table II) upon conversion of PS to PE was due to PE interaction with the ATPase rather than the physical displacement of phospholipid by octyl glucoside during enzymatic hydrolysis. Second, reconstitution of the lipid-depleted ATPase (Table III) with the same amounts of PS and PE remaining after incubation with phosphatidylserine decarboxylase (Table II) indicated that the PE was responsible for the decreased ATPase activity observed. When reconstituted with a 3:1 molar ratio of PS:PE (Table III, part A), the ATPase activity was elevated to levels characteristic of stimulation by PS. However, when the molar ratio of PS to PE was shifted to 1:7 (Table III, part B), the ATPase activity dropped to levels characteristic of stimulation by PE. Moreover, the decrease in ATPase

TABLE II

TREATMENT OF THE PHOSPHATIDYLSERINE-RECONSTITUTED ATPase WITH PHOSPHATIDYLSERINE DECARBOXYLASE AND THE EFFECTS ON ATPase SPECIFIC ACTIVITY

Dioleoylphosphatidyl[U-^{14}C]serine (Amersham) was adjusted to a specific radioactivity of 50 cpm/nmol by mixing with unlabelled brain PS (Serdary). The diluted ^{14}C-labelled PS (360 nmol) was bath sonicated in incubation medium (Table I) which contained 17.1 mM octyl glucoside. After addition of the 38–45 p ATPase (24 μg), the suspension was incubated for 5 min at 30°C. Pure phosphatidylserine decarboxylase (PSD) (20 units, Experiment 1; 60 units, Experiment 2) was added and incubations proceeded for 6 h at 30°C. Controls containing PS and PE were prepared identically, but with PSD omitted. Aliquots containing 60 nmol phospholipid and 4 μg protein were assayed for ATPase activity as described in Table I.

	ATPase activity (μmol/min/mg protein)	% ATPase activity oligomycin sensitive	% Conversion of PS to PE via PSD [a]
Experiment 1			
No phospholipid	1.2	55	–
PS	5.4	32	–
PE	1.5	20	–
PSD digest	5.1	30	26
Experiment 2 [b]			
PS	5.9	43	–
PE	2.1	55	–
PSD digest	2.2	53	87

[a] The phospholipids were extracted [14] and the radioactive PS and PE were separated by one-dimensional thin-layer chromatography (Silica gel H; 0.25 mm thick) using chloroform/methanol/28% ammonia (65:25:5, v/v). The spots (visualized by iodine vapors) were counted for 10 min in scintillation fluid (10 ml) containing 0.4% Omnifluor (New England Nuclear) in toluene/Triton X-100/water (2:1:0.2, v/v).

[b] A 'no phospholipid' sample was not assayed in this experiment. An average value obtained in three separate experiments carried out under identical conditions was 1.1 ± 0.1 μmol P_i/min per mg. Oligomycin sensitivity was 61 ± 7%.

TABLE III

RECONSTITUTION OF THE LIPID-DEPLETED ATPase WITH THE AMOUNTS OF PHOSPHATIDYLSERINE AND PHOSPHATIDYLETHANOLAMINE REMAINING AFTER TREATMENT WITH PHOSPHATIDYLSERINE DECARBOXYLASE (PSD)

The reconstitution of the lipid-depleted ATPase with PS and/or PE was performed as described in Table I in the presence of 17.1 mM octyl glucoside.

	nmol phospholipid per μg 38–45 p ATPase [a]	ATPase activity	% ATPase activity oligomycin sensitive
A. 26% Conversion of PS to PE via PSD			
No phospholipid	–	1.1	56
PS	10.2	5.9	32
PE	3.6	1.3	36
PS and PE	10.2 and 3.6	5.7	37
B. 87% Conversion of PS to PE via PSD			
No phospholipid	–	1.1	56
PS	2.0	5.4	26
PE	13.5	1.7	33
PS and PE	2.0 and 13.5	1.9	34

[a] The values were calculated from the amounts of ATPase and phospholipid utilized in the experiments described in Table II.

activity occurred in spite of the fact that the amount of PS present (2.0 nmol/μg ATPase) was sufficient to stimulate high levels of activity as long as the PE (13.5 nmol/μg ATPase) was not present. Therefore, the ATPase activity in the PS-PE mixture (Table III, part B) was unmistakably due to the PE. These results strongly suggested that the ATPase activity observed upon the enzymatic conversion of PS to PE (Table II) was due to a change in the phospholipid headgroup region surrounding the reconstituted ATPase.

The present study indicates that the superior stimulatory activity of acidic phospholipids is not simply a function of their ability to facilitate an interaction between the ATPase and phospholipid. If this were the case, the high levels of ATPase activity elicited by addition of PS should have been maintained upon conversion of the PS to PE (Table II). The observation that ATPase activity dropped upon phosphatidylserine decarboxylase treatment to levels characteristic of the PE reconstituted ATPase suggests that negatively charged phospholipids are required to maintain high ATPase activity even after the initial reassociation of phospholipid and enzyme has occurred.

Since other investigators [5–8] have demonstrated that it is the net negative charge rather than any one particular phospholipid headgroup structure that is important for ATPase reactivation, the present study implies that it is the net negative charge that is also important for maintaining high levels of ATPase activity. Such an environment might be provided by PS in vivo. However, this acidic phospholipid makes up only about 0.2% of the phospholipid composition in beef heart mitochondria [12]. A more likely candidate might be diphosphatidylglycerol because this phospholipid comprises about 20% of the phospholipid in submitochondrial particles [13].

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