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Specific and reversible activation and inactivation of the mitochondrial phosphate carrier by cardiolipin and nonionic detergents, respectively

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The phosphate carrier of pig heart mitochondria was solubilized with Triton X-100 and purified by chromatography on hydroxylapatite. Incubation of the phosphate carrier fraction with cardiolipin stimulated the reconstituted [^{32}P]phosphate exchange activity in liposomes, whereas increased Triton X-100 concentrations inhibited it. The effects of cardiolipin and Triton X-100 are reversible. The activation by cardiolipin is highly specific and could not be obtained with any other applied phospholipid.

1. INTRODUCTION

We have isolated and characterized the phosphate-transport system from heart mitochondria [1,2], and described the optimal conditions for reconstitution of the transport activity in liposomes [3]. During these studies it was found that the phosphate carrier has an essential requirement for cardiolipin [3,4]. This conclusion was based on the improved reconstituted activity, which was obtained, if cardiolipin was included in the extraction buffer. From these data it could not be excluded, however, that the increase of activity was due to an improved extraction of the phosphate carrier in the presence of cardiolipin. Furthermore it was concluded that the phosphate carrier is irreversibly inactivated by high concentrations of non-ionic detergents.

Here, we show that the inactivation of the phosphate carrier by Triton X-100 can be reversed by cardiolipin in a competitive manner. The reactivation of the activity is highly specific and cannot be obtained by any other of the investigated phospholipids.

2. MATERIALS AND METHODS

Cardiolipin (from bovine heart), L-α-phosphatidylcholine (from egg yolk), L-α-phosphatidylethanolamine (from egg yolk), L-α-phosphatidylserine (from bovine brain) and L-α-D,L-dipalmitoyl-phosphatidylglycerol (ammonium salt, synthetic) were obtained from Sigma. The sources of the other chemicals were as in [3,4]. Mitochondrial phospholipids were isolated by chloroform/methanol extraction of bovine heart mitochondria as in [5]. Mitochondria from pig heart or bovine heart were prepared by the usual procedures.

The phosphate carrier was isolated from pig heart mitochondria as described in previous publications by extraction with a buffer containing 3.5% Triton X-100, 1 mM EDTA, 30 mM K-phosphate, pH 6.5 [3,4], except that elution of the protein from the hydroxyapatite column was accelerated by application of a slight pressure of nitrogen, resulting in an increased elution speed of about 1 ml/min instead of 2 ml/h. By this method more protein could be eluted, which showed the same SDS-gel electrophoretic protein pattern as the fraction obtained by the previous method.

Incubation of the isolated phosphate carrier fraction with cardiolipin (or with other phospholipids) was performed in the following manner: the phospholipid solution in ethanol or chloroform was evaporated to dryness in small plastic reaction tubes (2 ml total volume);

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300–1000 μl of the hydroxyapatite eluate, containing 0.22–0.36 mg protein/ml, were pipetted into the cups and the phospholipid was immediately solubilized by shaking on a vortex mixer for a few seconds.

After incubation for the indicated times, 40–100 μl aliquots were used for reconstitution of phosphate transport activity in liposomes prepared from egg yolk phospholipids and cardiolipin as indicated or mitochondrial phospholipids (20%) as described in [3]. Measurement of [^{32}P]phosphate exchange activity was performed as in [3]. Protein was determined by a modified Lowry procedure [6].

3. RESULTS AND DISCUSSION

The reversible activation of the solubilized mitochondrial phosphate carrier by cardiolipin and its inactivation by Triton X-100 is demonstrated in fig.1. All data of the figure were obtained from one hydroxyapatite eluate. At the indicated times aliquots were taken for reconstitution of the carrier in liposomes, and the [^{32}P]phosphate exchange activity was measured for 4 min. It was established [3] that the [^{32}P]-phosphate exchange is not saturated after 4 min. Without addition of cardiolipin the carrier exhibits an activity which decreases slightly during incubation of the hydroxyapatite eluate for 2 h at 25°C. If cardiolipin was added to the extract immediately after elution from the column (d), an approximate 2-fold activity is found which decreases during further incubation almost in parallel to that of the control (a). Addition of cardiolipin after 60 min of incubation increases the activity, which, after further incubation for 60 min, approaches that of the eluate which obtained cardiolipin at 0 min (c). Addition of Triton X-100 at 30 min leads to a time-dependent decrease of activity which can be reversed by subsequent addition of cardiolipin (b). The reactivation by cardiolipin seems to be time-dependent, and the final activity is higher than that of the control, which did not obtain additional Triton X-100.

In fig.2 the concentration-dependent reactivation of the isolated phosphate carrier by cardiolipin is demonstrated. As expected, a saturation curve is obtained. It should be mentioned that the maximal reconstituted activity of the carrier is only

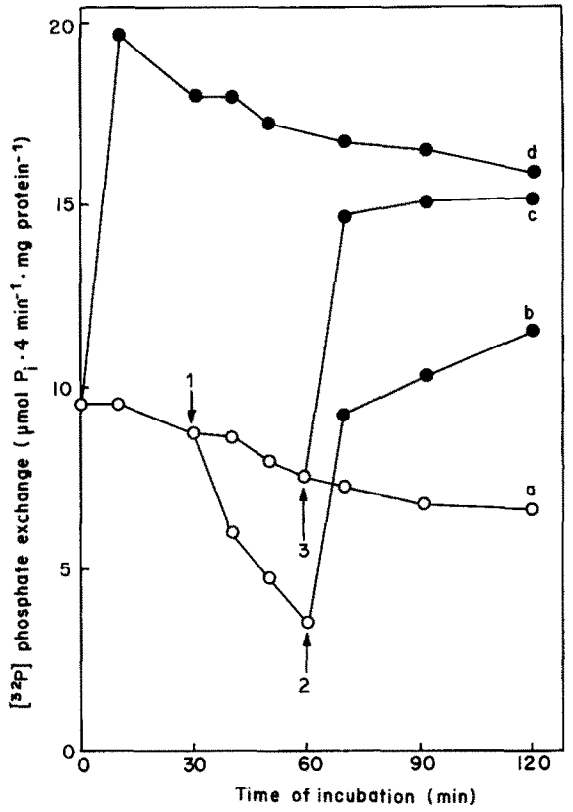

Fig.1. Reversible activation by cardiolipin and inactivation by Triton X-100 of the isolated phosphate carrier. A hydroxyapatite eluate (containing 2.3% Triton X-100) was incubated for 10 min at 0°C in the presence (d) or absence (a) of 5 mg/ml cardiolipin. Further incubations were performed at 25°C. To aliquots of the hydroxyapatite eluate 23 mg/ml Triton X-100 (1), 9.2 mg/ml (2) or 5 mg/ml (3) cardiolipin were added and at the indicated times aliquots were taken for reconstitution with liposomes containing 20% mitochondrial phospholipids. [^{32}P]Phosphate exchange was measured for 4 min.

obtained at a large molar excess of cardiolipin in respect to the carrier protein (1 mg/ml added cardiolipin corresponds to 280 mol cardiolipin/mol phosphate carrier, assuming that half of the protein in the hydroxyapatite eluate represents carrier protein with an M_r of 34000 [1]).

The concentration-dependent inactivation of the phosphate carrier by Triton X-100 is demonstrated in fig.3. Incubation of the hydroxyapatite eluate for 50 min at 25°C with 50 mg/ml Triton X-100 results in almost complete inactivation of the

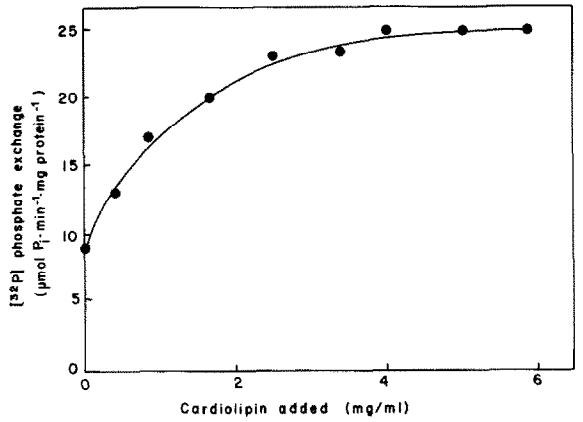

Fig.2. Concentration-dependent reactivation of the isolated phosphate carrier by cardiolipin. A hydroxyapatite eluate was incubated at 25°C for 20 min with the indicated amounts of cardiolipin. [^{32}P]Phosphate exchange was measured for 1 min after reconstitution with liposomes containing 20% mitochondrial phospholipids.

reconstituted phosphate exchange activity. It should be mentioned that the highest amount of applied Triton X-100 (50 mg/ml) is far below that amount of detergent which impairs the measurement of [^{32}P]phosphate exchange in proteoliposomes. The molar lipid/Triton X-100 ratio after reconstitution is 33:1 (at 50 mg/ml Triton X-100), whereas impairment of liposome structure

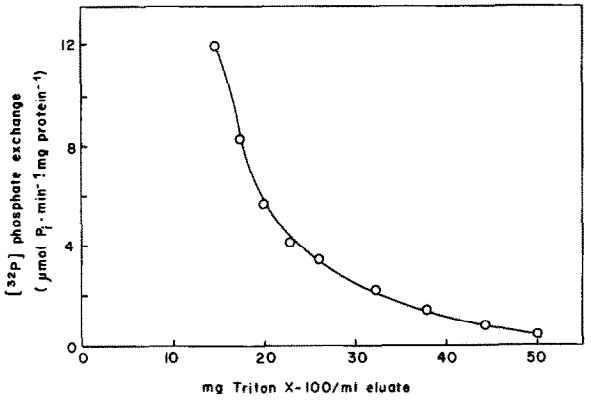

Fig.3. Concentration-dependent inactivation of the isolated phosphate carrier by Triton X-100. A hydroxyapatite eluate was incubated at 25°C for 50 min in the presence of the indicated final concentration of Triton X-100. Reconstitution and activity measurements were done as described in the legend to fig.2.

was found to occur at a ratio less than 10:1 [7].

The specificity of cardiolipin for the reactivation of phosphate carrier activity is shown in table 1. Addition of 1 μmol cardiolipin to the solubilized phosphate carrier fraction increased the [^{32}P]phosphate exchange activity after reconstitution in liposomes 3-fold. The same molar amount as well as the double amount of phosphatidylserine did not stimulate the activity. The same holds for phosphatidylethanolamine and phosphatidylcholine. Phosphatidylglycerol diminished the [^{32}P]phosphate exchange activity which could be partly due to the use of the ammonium salt, since NH_4Cl alone showed also some inhibitory effect. The stimulatory effect of cardiolipin cannot be due to its negative charge since the negatively charged phosphatidylserine could not increase the activity. Also the nature of the fatty acids in the phospholipids seems not to influence the reactivation. The phospholipids used in table 1 were from natural sources. In [4] dipalmitoyl- as well as dioleylphosphatidylcholine were without effect on the activity of the phosphate carrier. From these

Table 1

Specific activation of the isolated phosphate carrier by cardiolipin

Addition	mg	μmol	^{32}P-exchange (μmol P_i.4 min^{-1}. mg $protein^{-1}$)
None	–	–	7.4
Cardiolipin	1.43	1	22.3
Phosphatidylserine	0.76	1	7.7
Phosphatidylserine	1.52	2	7.5
Phosphatidylethanolamine	0.71	1	7.2
Phosphatidylcholine	0.75	1	6.7
Dipalmitoyl-phosphatidylglycerol	0.76	1	5.4
Dipalmitoyl-phosphatidylglycerol	1.52	2	4.1
NH_4Cl		1	6.3
NH_4Cl		2	5.0

300 μl hydroxyapatite eluate, containing 90 μg protein, were incubated for 40 min at 25°C with the indicated phospholipids. The phosphate transport was reconstituted as described in the legend to fig.1

data it is concluded that cardiolipin is specifically required for the phosphate transport activity.

In [4] we concluded that the phosphate carrier is irreversibly inactivated by high concentrations of nonionic detergents, because the activity could not be reconstituted with liposomes containing an excess of cardiolipin. In fig.4, a full reactivation of the completely inhibited transport activity is obtained if cardiolipin is added to the carrier before reconstitution. In contrast, no reactivation is obtained if increasing amounts of cardiolipin are incorporated into liposomes before addition of the inhibited carrier. The amount of cardiolipin added to the solubilized carrier corresponds to only 0.23% cardiolipin in liposomes (fig.4) and

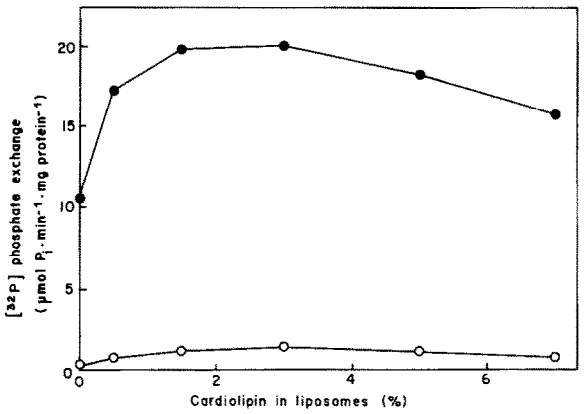

Fig.4. Reactivation of the Triton X-100-inhibited phosphate carrier by cardiolipin is only possible in the solubilized state. A hydroxyapatite eluate was incubated for 60 min at 25°C with 85 mg/ml Triton X-100 (final concentration). To one part of the eluate 7 mg cardiolipin/ml was added and both parts were furthermore incubated for 30 min at 25°C. Reconstitution was performed with liposomes prepared from egg yolk phospholipids and the indicated amounts of cardiolipin. [^{32}P]Phosphate exchange was measured for 1 min. Similar results were obtained if the phosphate exchange activity was measured for 1 min: (○---○) incubation of the eluate without cardiolipin; (●---●) with cardiolipin.

represents the saturation concentration of 7 mg/ml as found in fig.2.

Increasing amounts of cardiolipin in the liposomes lead to a further increase of the reactivated activity with a maximum at about 3% cardiolipin. The stimulating effect of liposomal cardiolipin seems to be unspecific and due to its negative charge. A similar effect of cardiolipin and other negatively charged phospholipids on the activity of the mitochondrial ADP/ATP carrier was described in [8]. The reactivating effect of cardiolipin, added to the solubilized carrier, on the other hand, is suggested to represent a specific effect. It is observed at an approximate 10-fold lower total amount of cardiolipin in the reconstituted system and is not obtained with any other phospholipid (table 1). The inability of liposomal cardiolipin to reactivate the Triton X-100-inhibited phosphate carrier is not understood, and will be the subject of future work.

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