THE MITOCHONDRIAL PHOSPHATE CARRIER HAS AN ESSENTIAL REQUIREMENT FOR CARDIOLIPIN

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1. Introduction

The phosphate carrier of mitochondria has been purified by chromatography of a Triton X-100 lysate on hydroxylapatite, and the [^{32}P]phosphate exchange activity reconstituted in liposomes by the freeze–thaw–sonication procedure [1–4]. The reconstituted exchange activity, however, was rather low (~1 μmol . min^{-1}. mg^{-1} at 25°C).

Here, the effect of cardiolipin on the reconstituted activity of the isolated phosphate carrier is described. We show that cardiolipin is required for full activity and also for preventing an irreversible inactivation of the carrier occurring during its extraction from mitochondria with Triton X-100 or Triton X-114. In the presence of cardiolipin the maximal rate of the [^{32}P]-phosphate exchange can be enhanced to ~30 μmol . min^{-1}. mg protein^{-1} at 25°C [5].

2. Materials and methods

Triton X-100 was purchased from Merck, Triton X-114 from Sigma, Dowex AG 1 X-8 and hydroxylapatite (Bio-Gel HTP) from Bio-Rad. [^{32}P]Phosphate (carrier-free) was obtained from Amersham Buchler, *N*-ethylmaleimide, L-α-dipalmitoyl phosphatidylcholine, L-α-dioleyl phosphatidylcholine, L-α-dipalmitoyl phosphatidylethanolamine and L-α-dipalmitoyl phosphatidylserine from Serva and egg yolk phospholipids (L-α-phosphatidylcholine from chicken eggs) and cardiolipin (from bovine heart) from Sigma.

Mitochondrial lipids were extracted from bovine or pig heart mitochondria as in [6]. Pig heart mitochondria were isolated as in [7] and stored at −80°C. Mitochondria were (partly) solubilized either in a medium containing 2% Triton X-100, 50 mM KCl, 20 mM Hepes (pH 7.0), 10 mM KP_i, 1 mM EDTA and 1 mM dithioerythrol (final conc.) (table 1, fig.1), or in 1.8% Triton X-114, 1 mM EDTA, 20 mM KCl, 20 mM KP_i (pH 6.5) (tables 2,3, fig.2) as in [5]. Chromatography on dry hydroxylapatite columns of clear supernatants (30 min, 100 000 × *g*) was performed as in [2,5]. Reconstitution of the phosphate transport system was performed with liposomes prepared from egg yolk lipids (table 1, fig.1), or 80% (w/w) egg yolk lipids and 20% (w/w) mitochondrial phospholipids (tables 2,3, fig.2) as in [2,5]. [^{32}P]Phosphate exchange activity was measured at 25°C as in [2].

3. Results

The stimulatory effect of mitochondrial lipids or cardiolipin on the reconstituted [^{32}P]phosphate exchange activity is presented in table 1. With liposomes containing 25% mitochondrial lipids or 5% cardiolipin ~50% higher activity is obtained than with control liposomes.

To improve the extraction of the phosphate carrier from mitochondria, the effect of various concentrations of Triton X-100 in the solubilization buffer on the reconstituted activity of [^{32}P]phosphate exchange was studied. An optimal activity was obtained with 2.5% Triton X-100 (fig.1). Increasing Triton X-100 to >4% resulted in almost complete inhibition of activity, although even more protein was solubilized. Since a possible inactivation by peroxides, known to be present in commercial detergents [8], was excluded [5], an inactivating effect of the detergent in itself was suggested. We assumed that Triton X-100 (or Triton X-114) might specifically extract cardiolipin

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Table 1
Influence of the lipid composition of liposomes on the reconstituted [^{32}P]phosphate exchange activity

Lipid composition	[^{32}P]Phosphate exchange (nmol . min^{-1} . mg protein^{-1})
EYPL	1870
EYPL + MPL (75:25, w/w)	3100
EYPL + DPG (95:5, w/w)	2800

Mitochondria were solubilized in a buffer containing 2.25% Triton X-100. After centrifugation and chromatography on hydroxylapatite, reconstitution was performed with liposomes prepared with the indicated phospholipids

Abbreviations: EYPL, egg yolk phospholipids; MPL, mitochondrial phospholipids; DPG, cardiolipin

from the phosphate carrier, as demonstrated for cytochrome *c* oxidase [9,10].

Pig heart mitochondria were extracted with Triton X-114 under optimal conditions (see [5]) in the absence or presence of increasing concentrations of cardiolipin (fig.2). The use of Triton X-114 instead of Triton X-100 results in a purer hydroxylapatite eluate, which is virtually free of the ATP/ADP-carrier [11].

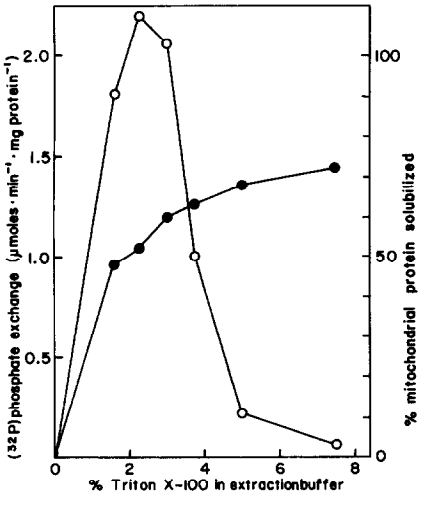

Fig.1. Influence of Triton X-100 concentration of solubilization buffer on the reconstituted [^{32}P]phosphate exchange activity. Mitochondria were solubilized in a buffer (section 2) containing the indicated amounts of Triton X-100. After centrifugation and chromatography on hydroxylapatite, the eluates were used for reconstitution of the carrier with liposomes (80% egg yolk phospholipids, 20% mitochondrial lipids). (o——o) [^{32}P]phosphate exchange activity; (•——•) solubilized protein.

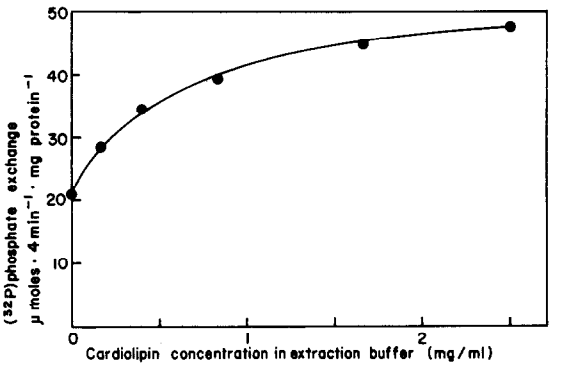

Fig.2. Effect of cardiolipin concentration in the solubilization buffer on the reconstituted [^{32}P]phosphate exchange activity. Mitochondria were solubilized in a buffer (section 2) containing 2.5% Triton X-114 and the indicated amounts of cardiolipin. After centrifugation and chromatography on hydroxylapatite, the eluates were used to reconstitute the carrier with liposomes (80% egg yolk phospholipids and 20% mitochondrial phospholipids). [^{32}P]phosphate exchange activity was measured for 4 min as in [2,5].

Inclusion of increasing concentrations of cardiolipin in the solubilization buffer leads to increased exchange activity after reconstitution. All samples were reconstituted with liposomes containing sufficient cardiolipin to maintain full exchange activity (20% mitochondrial lipids). Therefore, we suggested that cardiolipin prevents an irreversible inactivation of the phosphate carrier during solubilization. This was further demonstrated by the experiment shown in table 2:

Table 2
Effect of cardiolipin in the solubilization buffer on the activity of repeatedly extracted phosphate carrier

Conditions	Protein in hydroxylapatite eluate (mg . ml^{-1})	Total act. (nmol . 4 min^{-1} . 0.1 ml^{-1})	Spec. act. (μmol . 4 min^{-1} . mg protein^{-1})
1. Extract – DPG	0.24	395	16.5
2. Extract + DPG	0.14	571	40.8
2. Extract – DPG	0.15	21	1.4
2. Extract + DPG	0.05	300	60.0

Mitochondria were extracted in a medium containing 2.5% Triton X-114 (section 2) and 3 mg cardiolipin/14 mg protein if indicated. After centrifugation the pellet was again extracted with the same media and centrifuged as above. The 4 supernatants were chromatographed on hydroxylapatite and the eluates were used for reconstitution of the carrier with liposomes (80% egg yolk lipids, 20% mitochondrial lipids)

Table 3
Effect of various phospholipids in the solubilization buffer on the activity of repeatedly extracted phosphate carrier

Addition	Protein in hydroxylapatite eluate (mg . ml^{-1})	Total activity (nmol . min^{-1}. 0.1 ml^{-1})	Spec. act. (μmol . min^{-1}. mg $protein^{-1}$)
1. Extract	0.14	112	8.0
+ DPG	0.16	437	27.3
+ DPPC	0.16	161	10.1
+ DOPC	0.15	204	13.6
+ PE	0.14	182	13.0
+ PS	0.14	185	13.2
2. Extract	0.16	3.8	0.2
+ DPG	0.09	160	17.8
+ DPPC	0.12	0	0
+ DOPC	0.09	0	0
+ PE	0.12	0	0
+ PS	0.10	0	0

Mitochondria were solubilized twice as in table 2 with buffers containing 2 mg indicated phospholipid/13 mg protein. Further treatment was as in table 2

Abbreviations: DPG, cardiolipin; DPPC, dipalmitoyl phosphatidylcholine; DOPC, dioleyl phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine

Mitochondria were extracted in the presence or absence of cardiolipin with 1.8% Triton X-114, and after centrifugation the pellet was again extracted with the same buffers. After reconstitution in liposomes, containing again 20% mitochondrial lipids, the hydroxylapatite eluate of the first extract showed ~50% more total exchange activity if cardiolipin was included in the extraction buffer although less protein was eluted. The hydroxylapatite eluate of the second mitochondrial extract showed almost no exchange activity, if cardiolipin was omitted in the solubilization buffer. In the presence of cardiolipin, however, 50% of the exchange activity of the first extract was obtained. Again, if cardiolipin was included in the extraction buffer, less protein was eluted from hydroxylapatite resulting in a very high specific exchange activity, even higher than that obtained after the first extraction in the presence of cardiolipin. Since sufficient cardiolipin was present in the liposomes during reconstitution, this result indicates an irreversible inactivation of the phosphate carrier during solubilization by Triton X-114 in the absence of cardiolipin.

To demonstrate that the protecting effect is specific for cardiolipin, the effect of other phospholipids was studied. As demonstrated in table 3, addition of any phospholipid to the solubilization buffer resulted in an increased absolute as well as specific exchange activity after the first extraction. But with cardiolipin the exchange activity was >2-fold of that obtained with any other phospholipid. After the second extraction, however, only in the presence of cardiolipin an exchange activity was obtained. None of the other phospholipids could prevent the inactivation of the phosphate carrier during the second solubilization step.

4. Discussion

The phosphate carrier of mitochondrial represents an integral membrane protein. This follows from its function to transport the hydrophilic phosphate anion across the inner mitochondrial membrane, and from its very hydrophobic protein nature [12]. For some integral membrane proteins of mitochondria (NADH-dehydrogenase complex [13], cytochrome bc_1 complex [13] and cytochrome *c* oxidase [9,10]) a specific requirement for cardiolipin was demonstrated. Here, a specific dependence on cardiolipin is also shown for the mitochondrial phosphate carrier.

Beside the stimulating effect of cardiolipin on the reconstituted activity, an additional effect was found.

Extraction of the phosphate carrier with Triton X-100 or Triton X-114 leads to a complete and irreversible inactivation of the carrier (table 3), which could be prevented by the presence of cardiolipin. No other phospholipid, including acidic (e.g., phosphatidylserine) and unsaturated ones, could prevent the irreversible inactivation of the phosphate carrier.

The irreversible inactivation of the phosphate carrier by removal of cardiolipin explains the low specific exchange activity of the isolated and reconstituted carrier of ~1 μmol . min^{-1}. mg protein^{-1} found in [1–4]. Addition of cardiolipin to liposomes prepared from egg yolk phospholipids can only partly restore the activity of the isolated carrier (table 1). Addition of cardiolipin to the solubilization buffer, however, stimulates the reconstituted activity under optimal conditions [5] to 30 μmol . min^{-1}. mg protein^{-1} (table 3). This specific activity is higher than that found for the isolated and reconstituted ATP/ADP-carrier of 0.4 μmol . min^{-1}. mg protein^{-1} [14]. A higher turnover number of the reconstituted phosphate carrier should be expected, since for the phosphate carrier from rat liver a molecular activity of 3500 min^{-1} at 0°C was calculated [15], whereas for the ATP/ADP-carrier from bovine heart a molecular activity of only 500 min^{-1} at 18°C was found [16].

The specific requirement of the phosphate carrier for cardiolipin contrasts the lipid dependence of the ATP/ADP-carrier of mitochondria. The activity of the isolated and reconstituted ATP/ADP-carrier is also influenced by the lipid composition of the liposomes, but no specific lipid requirement was found [17]. Thus a stimulating effect of cardiolipin could be replaced by phosphatidylethanolamine or phosphatidylserine.

We conclude that the mitochondrial phosphate carrier is specifically stabilized by cardiolipin. Removal of this phospholipid by Triton X-100 or Triton X-114 leads to a conformational change of the carrier which cannot be reversed by subsequent addition of cardiolipin or any other phospholipid.

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References

[1] Wohlrab, H. (1980) J. Biol. Chem. 255, 8170–8173.
[2] Kolbe, H. V. J., Böttrich, J., Genchi, G., Palmieri, F. and Kadenbach, B. (1981) FEBS Lett. 124, 265–269.
[3] Palmieri, F., Kolbe, H. V. J., Genchi, G., Stipani, I., Mende, P., Kadenbach, B. and Quagliariello, E. (1981) in: Vectorial Reactions in Electron and Ion Transport in Mitochondria and Bacteria (Palmieri, F. et al. eds) pp. 281–290, Elsevier Biomedical, Amsterdam.
[4] Durand, R., Briand, Y. and Touraille, S. (1981) in: Vectorial Reactions in Electron and Ion Transport in Mitochondria and Bacteria (Palmieri, F. et al. eds) pp. 299–302, Elsevier Biomedical, Amsterdam.
[5] Mende, P., Kolbe, H. V. J. and Kadenbach, B. (1982) submitted.
[6] Rouser, G. and Fleischer, S. (1967) Methods Enzymol. 10, 325–339.
[7] Smith, A. L. (1967) Methods Enzymol. 10, 81–86.
[8] Ashani, Y. and Catravas, G. N. (1980) Anal. Biochem. 109, 55–62.
[9] Robinson, N. C., Stry, F. and Talbert, L. (1980) Biochemistry 19, 3656–3661.
[10] Fry, M. and Green, D. E. (1980) Biochem. Biophys. Res. Commun. 93, 1238–1246.
[11] Kolbe, H. V. J., Mende, P. and Kadenbach, B. (1982) submitted.
[12] Hofmann, H.-D. and Kadenbach, B. (1979) Eur. J. Biochem. 102, 605–613.
[13] Fry, M. and Green, D. E. (1981) J. Biol. Chem. 256, 2874–2880.
[14] Krämer, R. and Klingenberg, M. (1980) Biochemistry 19, 556–560.
[15] Coty, W. A. and Pedersen, P. L. (1974) J. Biol. Chem. 250, 3515–3521.
[16] Klingenberg, M. (1976) in: The Enzymes of Biological Membranes. Membrane Transport (Martonosi, A. N. ed) vol. 3, pp. 383–438, Plenum, New York.
[17] Krämer, R. and Klingenberg, M. (1980) FEBS Lett. 119, 257–260.