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SPECIFIC ELUTION FROM HYDROXYLAPATITE OF THE MITOCHONDRIAL PHOSPHATE CARRIER BY CARDIOLIPIN

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The role of cardiolipin in the purification of the mitochondrial phosphate carrier by hydroxylapatite has been investigated. Without added cardiolipin, the reconstituted phosphate-transport activity in the hydroxylapatite eluate is small and only confined to the first fraction. With cardiolipin added to the extract, the eluted activity is much higher and present until fraction 6. The activity retained by hydroxylapatite in the absence of cardiolipin is eluted after addition of this phospholipid to the column. The requirement of added cardiolipin diminishes on increasing the concentration of solubilized mitochondria. The hydroxylapatite eluate contains five protein bands in the M_r-region of 30000–35000, which are differently distributed in the various fractions. Among these, only the presence and the relative amount of band 3 of M_r 33000 corresponds to the phosphate transport activity. Cardiolipin is the only phospholipid tested which causes elution of band 3 from hydroxylapatite; on the other hand, it prevents the elution of band 2 and retards that of band 5 (the ADP/ATP carrier). Band 1 starts to appear in the second fraction even without cardiolipin. On increasing the concentration of cardiolipin, in the first fraction of the hydroxylapatite eluate band 3 increases and the contamination of band 4 decreases. Under optimal conditions a preparation of band 3 about 90% pure and with high reconstituted phosphate transport activity is obtained. It is concluded that the elution of the phosphate carrier from hydroxylapatite requires cardiolipin and that the phosphate carrier is identical with (or with part of) band 3 of the hydroxylapatite eluate.

Introduction

The *N*-ethylmaleimide-sensitive electroneutral phosphate/proton symporter of the inner mitochondrial membrane is the major transport system for the supply of phosphate for oxidative phosphorylation (see Ref. 1 for review). The mitochondrial phosphate carrier is isolated by solubilization of mitochondria in nonionic detergents and chromatography on hydroxylapatite [2,3]. The phosphate carrier in the pass-through of hydroxylapatite, obtained from different sources (bovine heart, pig heart, flight muscle and rat liver mitochondria) and prepared in different laboratories, apparently consists of only two protein components, namely the ADP/ATP carrier and the phosphate carrier, as shown by standard SDS gel electrophoresis [2–7]. However, when high resolution gradient SDS gel electrophoresis is applied, the pass-through of hydroxylapatite was shown to contain 4–5 protein bands of very similar molecular weight [3,8,9]. To our experience, this holds true for all preparations of purified phosphate carrier reported so far. Several attempts to purify further the phosphate carrier by chromatography

Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

on Celite [3], on Mersalyl-Ultrogel [5] and by using Triton X-114 instead of Triton X-100 [8], were partially successful to remove the ADP/ATP carrier, but they failed to decrease the number of protein bands in the M_r-region of 30000–35000 [8]. With the exception of the protein band with the lowest M_r (30000), which has been identified with the ADP/ATP carrier [10], the function of the other proteins is unknown. It is also not known which of the remaining four bands represents the phosphate carrier. Previously, the unresolved band of a M_r slightly exceeding 30000 was thought to be the phosphate carrier protein on the basis of specific labeling with radioactive *N*-ethylmaleimide after protection with mercurials [11–14]. After resolution of this fraction on SDS gel electrophoresis into several protein components, it has been suggested that the mitochondrial phosphate carrier occurs as a complex of different polypeptides [3] and, more recently, it has been proposed that at least two bands are proteolytic fragments of the native phosphate carrier [8]. In a recent brief communication [9], by using an organomercurial agarose column after hydroxylapatite, we have isolated a fraction which was capable of catalyzing reconstituted phosphate transport and which showed only one main protein after high resolution SDS gel electrophoresis. Although this indicates that a single protein is responsible for phosphate transport, it could not be excluded by these results that the isolated protein is a proteolytic fragment of the native phosphate carrier with higher M_r [9].

Recently, we have shown that the reconstituted transport activity of the isolated phosphate carrier can be increased several-fold by inclusion of cardiolipin in the solubilization buffer [15–16]. In this paper the role of cardiolipin in the purification of reconstitutively active phosphate carrier is investigated. A specific effect of cardiolipin has been found on the elution of the phosphate carrier from the hydroxylapatite column. Evidence has been obtained for the identity of the phosphate carrier with (or with part of) band 3 of the 5 protein bands present in high resolution SDS gels of the hydroxylapatite pass-through. Furthermore, it has been shown that addition of cardiolipin can be successfully used to improve the purification of the phosphate carrier to such an extent that only one main protein band is detectable in gradient SDS gel electrophoresis. A preliminary account of part of this work has been communicated [17].

Materials and Methods

Materials

Hydroxylapatite (Bio-Gel HTP) and Dowex AG 1-X8 (100–200 mesh) were purchased from Bio-Rad, [^{32}Pi]-phosphoric acid from Radiochemical centre (Amersham, U.K.), egg yolk phospholipids (L-α-phosphatidyl choline, type X-E) from Sigma, cardiolipin and phosphatidyl ethanolamine from Serdary, Triton X-114 and *N*-ethylmaleimide from Serva. Mitochondrial phospholipids were isolated by the procedure described in Ref. 18. L-3-phosphatidyl choline dioleyl, DL-α-phosphatidyl choline dipalmitoyl, L-α-phosphatidyl inositol, L-α-phosphatidic acid and L-α-lysophosphatidyl choline were purchased from Sigma.

Isolation of the phosphate transport protein

Pig heart mitochondria, prepared as described in Ref. 19, were washed twice in 20 mM KCl/20 mM KH_2PO_4/1 mM EDTA (pH 6.5). The pellet was suspended in the same medium, containing in addition 2% Triton X-114, at a final concentration of 12 mg protein/ml. After 20 min at 0°C the mixture was centrifuged at 147000 × *g* for 45 min to obtain a clear supernatant referred to as extract.

600 μl of the extract (3–4 mg protein) were applied on cold hydroxylapatite columns (pasteur pipettes containing 600 mg of dry material) and the elution was performed with the solubilization buffer. Deviations from this procedure are indicated in the legends to the figures. One or more fractions of 500 μl were collected from each hydroxylapatite column.

Preparation of liposomes

Liposomes (5 ml) were prepared by sonication of a mixture of 400 mg egg yolk phospholipids and 100 mg mitochondrial phospholipids in a sonication buffer containing 50 mM KCl/20 mM KH_2PO_4/20 mM Hepes/1 mM EDTA (pH 6.5), with a Branson-Sonifier B15 under a stream of N_2 for 30 min at 0°C (20 s sonication, 20 s intermission).

Incorporation of the phosphate transport protein into liposomes

The phosphate transport protein was incorporated into liposomes by the freeze-thaw-sonication procedure [20,21]. 1.0 ml liposomes were mixed with 50 μl of mitochondrial extracts or hydroxylapatite eluates. After 2 min at 0 °C, the mixture was frozen in liquid N_2, thawed in a water bath at 10–15 ° and then pulse-sonicated (0.4 s sonication, 0.6 s intermission) for 6.0 s at 0 °C. The proteoliposomes were diluted with 350 μl of a buffer containing 50 mM KCl/20 mM KH_2PO_4/20 mM Hepes/1 mM EDTA (pH 6.5), and distributed in four Eppendorf cups (320 μl each).

Assay of phosphate transport in proteoliposomes

The proteoliposomes were tempered at 25 °C for 4 min. The phosphate-phosphate exchange was initiated by the addition of 20 μl 20 mM [^{32}P]-phosphate (150 000–200 000 cpm) and terminated with 10 μl *N*-ethylmaleimide (final concentration , 2 mM), followed by immediate cooling at 0 °C. In two control samples *N*-ethylmaleimide was added 30 s before the labeled substrate. 300 μl of each sample were applied to a Dowex AG1-X8 column, chloride-form (0.5 × 4 cm equilibrated with 170 mM sucrose). The liposomes, eluted with 1.2 ml 170 mM sucrose, were collected in a scintillation vial, vortexed with 4 ml of scintillation mixture (Maxifluor, Backer, The Netherlands) and counted. The phosphate-phosphate exchange activity was calculated by subtracting the control values from the experimental samples.

Gradient SDS gel electrophoresis

Polyacrylamide slab gel electrophoresis of acetone-precipitated samples was performed in the presence of 0.1% sodium dodecyl sulfate according to Laemmli [22] with the following modifications: the separation gel contained a linear gradient of acrylamide from 14 to 20% and 15% sucrose (acrylamide/*N*,*N*-methylene bisacrylamide, 49 : 1) [8]. Alternatively, the separation gel contained 17.5% acrylamide and an acrylamide/bisacrylamide ratio of 150. Both systems give a high resolution of polypeptides of M_r close to 30 000. The gels were stained with silver nitrate [23] except that of fig. 4 which was stained with Coomassie blue R 250. Absorbance traces of the stained gels were performed with the LKB 2202 ultroscan laser densitometer. The molecular weights were determined with the help of the following marker proteins: bovine serum albumin (67 000), ovalbumin (43 000), glycerol-3-phosphate dehydrogenase (36 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100) and cytochrome *c* (12 500).

Other methods

Protein was determined in the presence of 1% sodium dodecyl sulfate to eliminate the interference of Triton X-114 in the Lowry method [24].

Results

In our previous studies on the reconstitution of the phosphate carrier in liposomes [15,16], mitochondria were solubilized in the presence or in the absence of cardiolipin, the extracts were applied to hydroxylapatite columns and the activity of the reconstituted phosphate carrier was measured in the hydroxylapatite eluates. In preliminary experiments of this study, it was found that the inclusion of cardiolipin in the solubilization buffer has no significant effect on the activity and the stability of the phosphate carrier when reconstituted from 2% Triton X-114 extracts directly [17]. Therefore, the importance of cardiolipin in the purification of the phosphate carrier by hydroxylapatite was tested.

Fig. 1a shows that, without addition of cardiolipin, the small amount of reconstituted phosphate exchange present in the hydroxylapatite eluate is nearly only confined to the first fraction. The SDS gel electrophoresis analysis (Fig. 1b) reveals that the whole eluate contains five bands of apparent M_r 35 500, 35 000, 33 000, 31 500 and 30 000, and they are differently distributed in the various fractions. Fraction 1 contains bands 2–5. Band 5 is presumably identical with the ADP/ATP carrier by its staining intensity and its apparent M_r of 30 000 both in the extract and in the various fractions of the eluate. Band 1 appears in the second fraction and is then continuously present; band 2 increases in fraction 2 and then progressively decreases; band 3 is present only in fraction 1 and to some extent in fraction 2 and disappears in fractions 3–6 simultaneously with the disap-

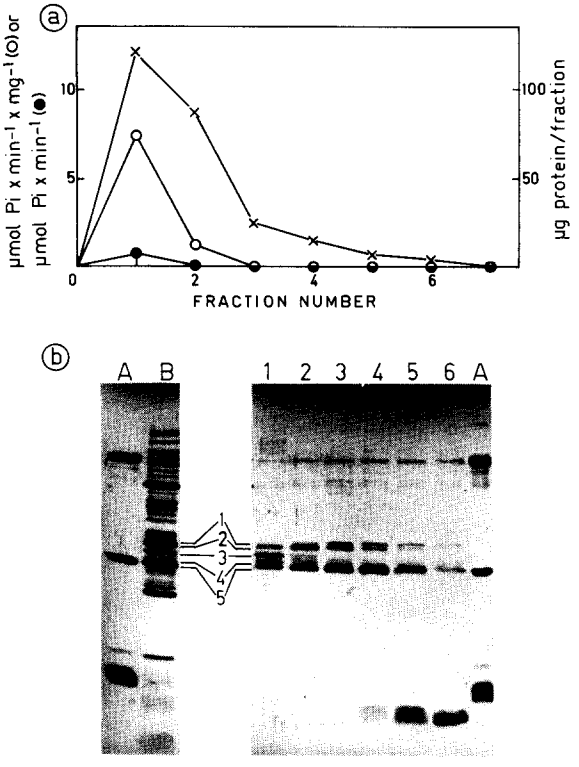

Fig. 1. (a) Elution of reconstituted phosphate exchange activity from hydroxylapatite in the absence of cardiolipin. Mitochondria were solubilized as described in Materials and Methods, and the extract was applied to hydroxylapatite. Elution was performed with the solubilization buffer. (●), total activity of reconstituted inorganic phosphate (Pi) transport; (○), specific activity; (×), protein content/fraction. (b) SDS gel electrophoresis of fractions 1–6 of Fig. 1a. A, marker proteins (bovine serum albumin, carbonic anhydrase, cytochrome *c*), B, mitochondrial extract; 1–6, fractions 1–6 of Fig. 1a.

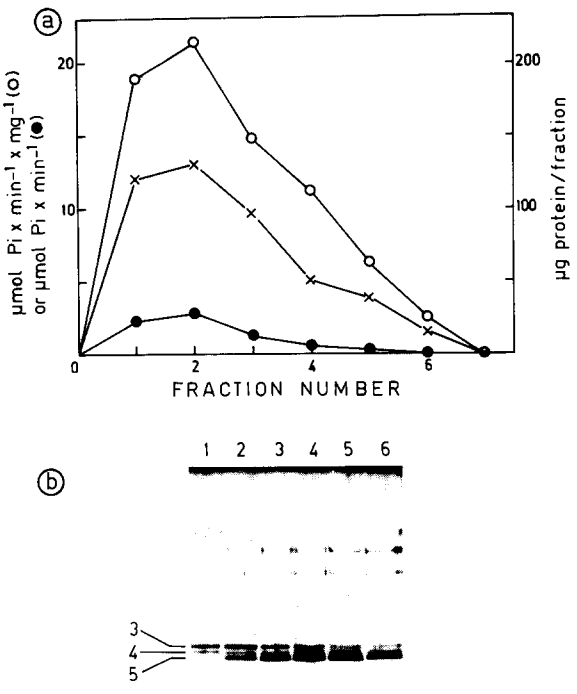

Fig. 2. (a) Elution of reconstituted phosphate exchange activity from hydroxylapatite in the presence of cardiolipin. Conditions as in Fig. 1a except that cardiolipin (3 mg/ml) was added to the extract and to the elution buffer. (●), total activity of reconstituted inorganic phosphate (Pi) transport; (○), specific activity; (×), protein content/fraction. (b) SDS gel electrophoresis of fractions 1–6 of Fig. 2a. 1–6: fractions 1–6 of Fig. 2a.

pearance of the phosphate transport activity; band 4 decreases progressively from fraction 1 to fraction 3, whereas band 5 is always present.

The total and the specific activity of the reconstituted phosphate exchange in the hydroxylapatite eluate are much higher when the mitochondrial extract is supplemented with cardiolipin, i.e., when the elution buffer contains cardiolipin (cf. Fig. 2a and Fig. 1a). Furthermore, with added cardiolipin the activity shows a maximum in the second fraction of the pass-through and is also present in subsequent fractions. The polypeptide pattern of the hydroxylapatite eluate obtained in the presence of 3 mg/ml cardiolipin is also very different from that obtained in the absence of this phospholipid (Fig. 2b). Fraction 1 contains only bands 3 and 4, band 3 being the more intensive one. In the subsequent fractions, the ADP/ATP carrier (band 5) is also present. Whereas the amount of band 5 seems to increase, band 3 decreases from fraction 2 to fraction 6; simultaneously, the reconstituted phosphate transport activity decreases. Bands 1 and 2 are virtually absent in all the fractions, in striking contrast to their presence without added cardiolipin. Besides in fraction 1, band 4 is also present in fraction 2; in the subsequent fractions it is however difficult to say to what extent it is covered by the more

abundant ADP/ATP carrier.

The higher phosphate transport activity in Fig. 2a with respect to Fig. 1a can be interpreted either by assuming that in the absence of cardiolipin the phosphate carrier is eluted largely inactivated, or by retention of the carrier in the column. In order to verify which of these two interpretations is correct, the experiment illustrated in Fig. 3 was carried out. Mitochondria, solubilized with 2% Triton X-114 in the absence of cardiolipin, were applied to an hydroxylapatite column and the fractions were analyzed for total activity, specific activity and protein content. It can be seen that these fractions (peak on the left of Fig. 3a) contain a small amount of carrier. The active phosphate carrier is effectively eluted after addition of cardiolipin to the elution buffer, as shown by the increase in both the total and the specific activity (Fig. 3a). These results clearly demonstrate that the addition of cardiolipin to hydroxylapatite causes elution of the active phosphate carrier retained in the column in its absence. The analysis of the various fractions of the hydroxylapatite eluate by gel electrophoresis can be correlated with these findings (Fig. 3b). The fractions collected before the addition of cardiolipin to the column are similar to the first three fractions of Fig. 1b. The important point is that band 3 of M_r 33 000 is absent in fractions 2 and 3, which are devoid of transport activity. After the addition of cardiolipin band 3 appears in fraction 4, increases in fractions 5 and 6, and disappears again in the following fractions. In contrast to the behaviour of band 3, band 2 is present before and disappears after the addition of cardiolipin to the column. Because of the abundance of the ADP/ATP carrier, it is not clear from the results of Fig. 3b whether band 4 is present in fractions 4–6. In other experiments, however, carried out in slightly different conditions (extraction with 1.2% Triton X-114 to decrease the amount of band 5, i.e. the ADP/ATP carrier), band 4 is clearly absent, after the addition of cardiolipin to the hydroxylapatite column, in the active fractions. Since only the presence of band 3 corresponds to the phosphate transport activity, it may be suggested that band 3 is (or comprises) the phosphate carrier.

The experiment of Fig. 4 was performed in an attempt to explain why the phosphate carrier is

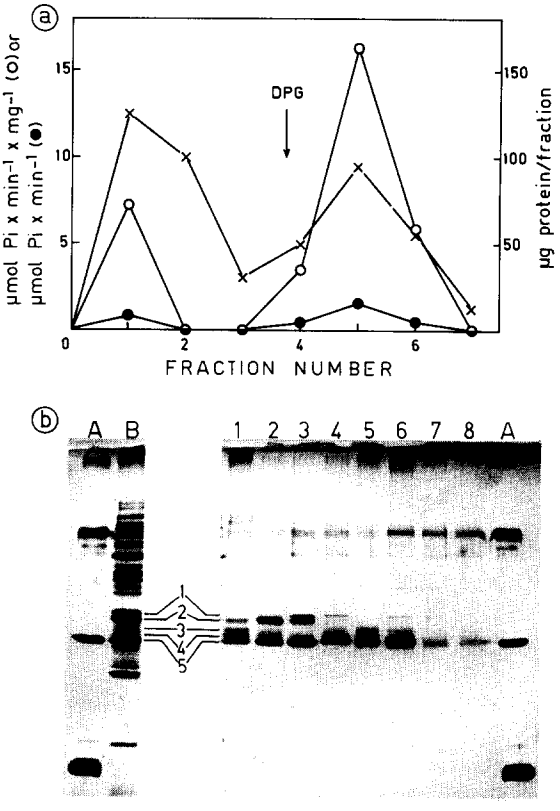

Fig. 3. (a) Elution of phosphate transport activity from hydroxylapatite before and after the addition of cardiolipin. Mitochondria were solubilized as described in Materials and Methods, the extract was applied to hydroxylapatite and eluted with the solubilization buffer. Where indicated, 2 mg/ml cardiolipin were added to the elution buffer. (●), total activity of reconstituted inorganic phosphate (Pi) transport; (○), specific activity; (×) protein content/fraction. DPG, cardiolipin. (b) SDS gel electrophoresis of fractions 1–8 of Fig. 3a. A, markers; B, mitochondrial extract; 1–8, fractions 1–8 of Fig. 3a.

eluted, to a limited extent, without added cardiolipin (Fig. 1). The extracts derived from the solubilization of mitochondria at different concentrations, either with or without the addition of cardiolipin, were applied to hydroxylapatite columns and the first 500 µl (fraction 1) of the eluates were analyzed for reconstituted phosphate transport (Fig. 4a). When 15 mg of mitochondrial protein/ml of solubilization buffer were used, the activity of reconstituted phosphate transport without added cardiolipin is nearly as high as with cardiolipin. In contrast, when less than 10 mg

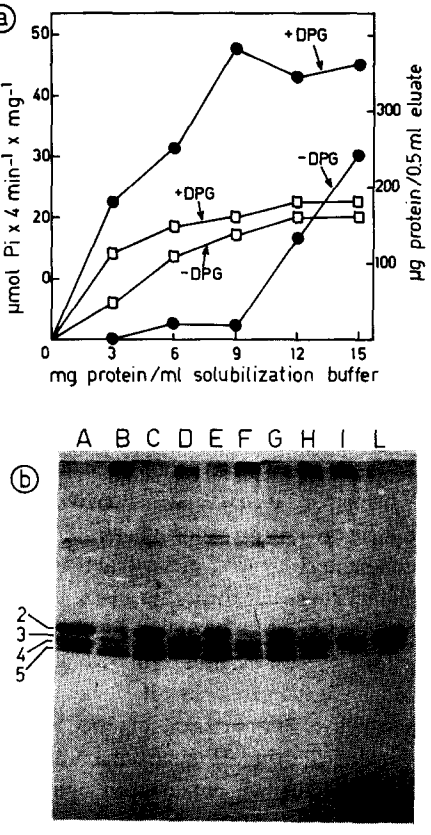

Fig. 4. (a) Influence of the mitochondrial protein concentration in the solubilization buffer on the reconstituted phosphate exchange activity of hydroxylapatite eluates. The indicated concentrations of mitochondria were solubilized as described in Materials and Methods. The extracts, with or without the addition of cardiolipin (2 mg/ml), were applied to hydroxylapatite columns. The first 500 µl of the pass-throughs were collected and assayed for inorganic phosphate (Pi) exchange activity. (●), specific activity of reconstituted inorganic phosphate transport; (□), protein content/0.5 ml eluate. DPG, cardiolipin. (b) SDS gel electrophoresis of the first 500 µl (fraction 1) of the hydroxylapatite eluates of Fig. 4a. (A) and (B) 3 mg protein/ml of solubilization buffer; (C) and (D) 6 mg/ml; (E) and (F) 9 mg/ml; (G) and (H) 12 mg/ml; (I) and (L) 15 mg/ml. (A), (C), (E), (G), (I) without cardiolipin; (B), (D), (F), (H), (L) with cardiolipin added to the extracts applied to the columns.

protein/ml were used, the phosphate carrier activity is almost negligible if cardiolipin is not added. It is likely that at high protein concentrations the endogenous cardiolipin is sufficient to elute the carrier from hydroxylapatite, whereas at low protein concentrations the elution of the phosphate carrier, and therefore the activity in the eluates, is completely dependent on the addition of cardiolipin. This conclusion is supported by the analysis of the SDS gel electrophoresis of this experiment (Fig. 4b). Without added cardiolipin, band 3 is absent at 3 mg protein/ml, is present in very small amounts at 6 mg/ml and also, in relation to the other proteins, at 9 mg/ml. In contrast, with added cardiolipin, band 3 is already present at 3 mg/ml. At 15 mg/ml the amount of band 3 is approx. the same with and without added cardiolipin. In this experiment again (cf. Fig. 4a and b) the presence and the relative amount of band 3 corresponds well to the activity of the phosphate carrier. In contrast to the behaviour of band 3, band 2 is always absent if cardiolipin is added to the column, whereas without added cardiolipin it diminishes on increasing the protein concentration because of the presence of endogenous cardiolipin. These results, together with those shown in Figs. 1–3, indicate that cardiolipin prevents the elution of band 2, whereas it is required for the elution of band 3. In agreement with these observations, only band 2 of M_r 35 000 is labeled by low concentrations of dicyclohexylcarbodiimide and the radioactive label bound to this polypeptide only appears in the hydroxylapatite pass-through in the absence of cardiolipin [25].

The specificity of the elution of the phosphate carrier from hydroxylapatite by cardiolipin has been investigated by testing the effect of other phospholipids. In these experiments (not shown), the mitochondrial extract was supplemented with different phospholipids (2 mg/ml) and applied to hydroxylapatite columns. The first fractions of the pass-throughs were assayed for phosphate transport activity. The results, showing the specific effect of cardiolipin on the elution of the phosphate transport activity from hydroxylapatite, are comparable to those obtained by addition of phospholipids during solubilization [15], except that, in the present conditions, phosphatidylethanolamine decreases the specific and the total activity by 22% and 64%, respectively. Phosphatidylinositol and phosphatidic acid (not tested in Ref. 15) decrease the total activity without influencing the specific activity. Lysophosphatidylcholine (also not tested before) increases somewhat both the specific and the total activity, but considerably less than cardiolipin. In agreement with these data, the SDS

gels of the eluted fractions (Fig. 5a) demonstrate that cardiolipin strongly increases the elution of band 3 (i.e., the phosphate carrier), but it decreases that of band 5 and prevents elution of band 2. In the presence of dipalmitoyl phosphatidylcholine, phosphatidylinositol and (not shown) dioleyl phosphatidylcholine all four bands are eluted without significant change in their relative amount with respect to the control. Lysophosphatidylcholine, which to some extent increases the phosphate transport activity in the hydroxylapatite eluate, increases the amount of band 3 but it does not change the overall number of the eluted proteins. Interestingly, the elution pattern in the presence of phosphatidylethanolamine is also characteristic; thus, the hydroxylapatite eluate consists mainly of band 4. In control experiments the various phospholipids were added to the hydroxylapatite eluate instead of to the extract. Under these conditions the increase of the reconstituted transport activity of the hydroxylapatite eluate by cardiolipin is much smaller, virtually only confirmed to the first fraction (i.e., where band 3 is present), and, therefore, cannot account for the large effect induced by the addition of cardiolipin to the extract. The other phospholipids tested have no effect at all.

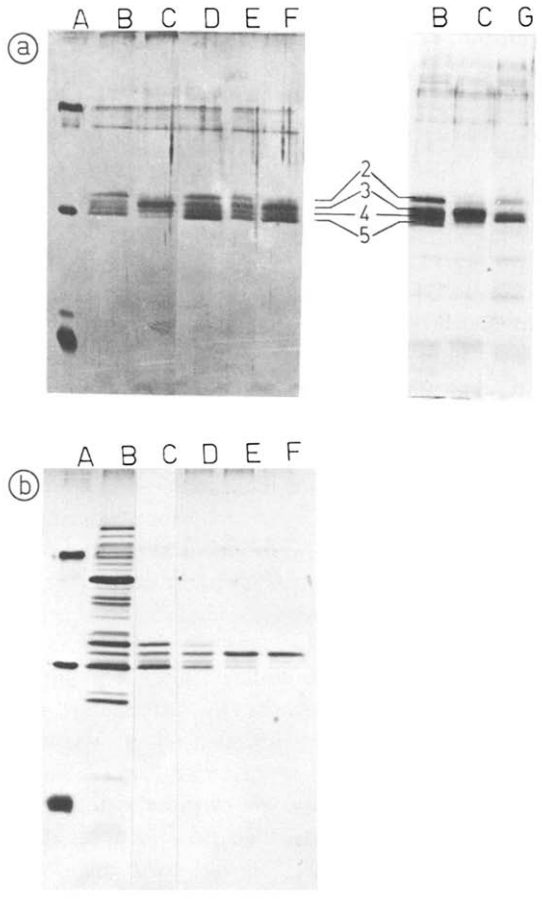

Fig. 5. (a) SDS gel electrophoresis of the first 500 μl (fraction 1) of the hydroxylapatite eluates obtained in the presence of various phospholipids. Mitochondria were solubilized as described in Materials and Methods. The extracts, with or without the addition of the indicated phospholipids (2 mg/ml), were applied to hydroxylapatite columns. A, markers; B, without addition to the mitochondrial extract, C, with cardiolipin; D, with dipalmitoyl phosphatidylcholine; E, with phosphatidylinositol; F, with lysophosphatidylcholine; G, with phosphatidylethanolamine. (b) SDS gel electrophoresis of the first 500 μl of the hydroxylapatite eluates obtained in the presence of increasing concentrations of cardiolipin. Mitochondria were solubilized as described in Materials and Methods. To the extract applied to hydroxylapatite, no (C), 1 mg/ml (D), 2 mg/ml (E) and 4 mg/ml (F) cardiolipin were added. Protein content/500 μl eluate was 120 μg (C), 130 μg (D), 145 μg (E) and 135 μg (F). A, markers; B, mitochondrial extract.

Fig. 5b shows the dependence of the protein pattern of the first fractions of the hydroxylapatite eluates on the amount of cardiolipin which is added to the mitochondrial extract applied to the columns. On increasing the concentration of cardiolipin from 1 to 4 mg/ml, band 3 increases, bands 2 and 5 disappear and the contamination of band 4 decreases. With 4 mg/ml cardiolipin a substantially pure preparation of band 3 (i.e., the phosphate carrier) is obtained. Under these conditions, from densitometric traces of stained gels a purity of about 90% for band 3 is calculated. According to the degree of purification of the respective preparations, the reconstituted phosphate-transport activity increases progressively by increasing the concentration of cardiolipin added to the extract from 7.3 (without added cardiolipin) to 26.0 μmol phosphate per min per mg protein (with 4 mg/ml cardiolipin).

Discussion

In contrast to the bulk of mitochondrial protein, the phosphate carrier from mitochondria is not adsorbed to hydroxylapatite when special elution conditions are applied [2–6]. This is very important for the purification of the carrier pro-

tein. The data presented in this paper show that cardiolipin is required for the elution of the active phosphate carrier from hydroxylapatite. With added cardiolipin not only a higher amount of phosphate carrier appears in the pass-through of hydroxylapatite than in its absence, but also the retained phosphate carrier can be eluted by the addition of cardiolipin. Furthermore, the small amount of phosphate carrier which appears in the hydroxylapatite eluate without addition of cardiolipin to the extract can be easily explained by the presence of endogenous cardiolipin. We have previously suggested a specific requirement of the phosphate carrier for cardiolipin. The inhibition of the phosphate carrier by high concentrations of Triton was explained by assuming a removal of mitochondrial cardiolipin from the protein [15,16]. The apparent need of cardiolipin for the elution of the phosphate carrier from hydroxylapatite strongly supports the idea of a specific interaction of the carrier protein with cardiolipin. One can assume that the specific binding of cardiolipin to the carrier neutralizes positive charges, making the protein more hydrophobic and hence unable to bind to hydroxylapatite.

The effect of cardiolipin on the elution of the phosphate carrier from hydroxylapatite is specific, since none of the other proteins adsorbed to hydroxylapatite in the absence of this phospholipid are eluted by cardiolipin. A specific interaction between the phosphate carrier and cardiolipin, possibly important to maintain the protein in its native state, is also suggested by the finding that no other phospholipid tested is able to cause appreciable elution of the phosphate carrier from hydroxylapatite nor to increase the degree of purification.

The pass-through of hydroxylapatite contains not only the ADP/ATP carrier and the phosphate carrier [2–7], but altogether at least 5 protein bands, as revealed by application of high resolution gel electrophoresis. Our data demonstrate that the presence and the relative amount of these bands vary in the individual fractions of the pass-through and greatly depend on the presence of cardiolipin. Furthermore, they give evidence that band 3 of the hydroxylapatite eluate (the second band of the first fraction) is (or comprises) the phosphate carrier. A strong argument in favour of this conclusion is the close relationship between the reconstituted phosphate exchange activity and the presence or absence of band 3. The other protein bands present in the hydroxylapatite eluate are not related to the active phosphate carrier, since there are fractions devoid of transport activity in which one or more of them are present and, vice versa, there are fractions with high activity in which they are absent. The apparent identification of the phosphate carrier protein with band 3 (the second band of the first fraction) is in contrast to the hypothesis [8] that the phosphate carrier corresponds to three protein bands present in the hydroxylapatite eluate, two of which are proteolytic fragments of the native carrier assumed to be identical with the highest M_r band. It agrees, on the other hand, with our previous evidence [9] that a single band of the hydroxylapatite eluate is responsible for the reconstituted phosphate transport activity. It should be emphasized that, due to the appearance of band 1 in the second and subsequent fractions of the hydroxylapatite eluate, band 3 (i.e., the second band of the first fraction) of the present paper is identical with band 2 of the previous report [9] in which only the first fraction was considered.

The data reported also show that cardiolipin can be successfully used to improve the purification of the phosphate carrier by hydroxylapatite if the very first part of the pass-through is collected. This result is based and justified by the following experimental observations: band 1 is absent in the first fraction, band 2 is retained and band 5 retarded in the presence of cardiolipin, the phosphate carrier is eluted only in the presence of cardiolipin, and the contamination of band 4 is diminished by increasing the amount of cardiolipin added to the column. Under the optimal conditions described here (Fig. 5b, lane F) it is possible to obtain a preparation of the active phosphate carrier, suggested to be identical with band 3 of the hydroxylapatite eluate (the second band of the first fraction), which is about 90% pure upon gradient SDS gel electrophoresis. This preparation represents a considerable progress with respect to all the purifications of the phosphate carrier available in the literature [2–9]. It is also superior to the only preparation consisting of a main protein band after high resolution SDS gel electrophoresis

[9], since the present procedure is simpler, faster and leads to a higher yield of the carrier protein.

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