Purification of the active mitochondrial phosphate carrier by affinity chromatography with an organomercurial agarose column

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1. INTRODUCTION

The general procedure for the isolation of the phosphate carrier from mitochondria involves solubilization with non-ionic detergents and chromatography on hydroxylapatite [1–3]. With high resolution SDS-gel electrophoresis this preparation can be separated into 4–5 protein bands [2]. Further purification, in particular removal of the ADP/ATP-carrier, was obtained by chromatography on Celite [2], on Mersalyl–Ultrogel [3,4] and by using Triton X-114 instead of Triton X-100 [5]. However, after all these procedures the purified phosphate carrier fraction still contained 4–5 protein bands in the M_r-region of 30 000–35 000 [5].

Here, affinity chromatography with an organomercurial agarose gel (Affi-Gel 501) was used to purify the phosphate carrier from the hydroxylapatite eluate. The same principle was used in [3,4], where total mitochondrial extract was applied to a Mersalyl-Ultrogel column. The purified phosphate carrier fraction, however, had a low specific activity [4] and showed 4 bands after high resolution SDS-gel electrophoresis [5]. With our procedure, using a mercaptoethanol gradient for the elution of retained proteins, a phosphate carrier fraction with high specific activity is obtained which shows one main protein band after high-resolution SDS gel electrophoresis.

* *Abbreviations*: SDS, sodium dodecylsulfate; NEM, *N*-ethylmaleimide

2. MATERIALS AND METHODS

Affi-Gel 501 (an organomercurial agarose gel), Dowex AG 1-X8 and hydroxylapatite (Bio-Gel HTP) were obtained from Bio-Rad; [^{32}P]phosphate (carrier free) from Amersham; [^{3}H]NEM from New England Nuclear; acrylamide, *N,N'*-methylenebisacrylamide, SDS, Triton X-100 and NEM from Serva; scintillation liquid (Maxifluor) from J. Baker; L-α-phosphatidylcholine (from egg yolk, Type X-E) from Sigma and cardiolipin from Serdary.

Pig heart mitochondria prepared as in [6] and washed twice with buffer A (20 mM LiCl, 10 mM H_3PO_4 neutralized with LiOH (pH 7.0) and 0.1 mM EDTA) were extracted with buffer A containing in addition 3% Triton X-100 and 3 mg cardiolipin/ml (120 mg protein in 8 ml) for 20 min at 0°C. After centrifugation 600 μl supernatant was applied to small hydroxylapatite columns followed by 1 ml buffer A containing 3% Triton X-100 and 1 mg cardiolipin/ml. The first 0.4–0.5 ml eluted from several columns were pooled and 5–6 ml (1–1.5 mg protein) were applied to an Affi-Gel column (6 × 0.7 cm) equilibrated with buffer A containing 3% Triton X-100 and 1 mg cardiolipin/ml. The column was washed with 10–12 ml of the same buffer and the retained proteins were eluted by the addition of mercaptoethanol as indicated in the legends to the figures. Fractions of 600 μl were collected.

Liposomes were prepared by sonication of egg

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yolk phospholipids together with 20% mitochondrial phospholipids as in [7]. Reconstitution was performed as in [2,7] using 70–100 μl of column eluates except that the amount of NEM used to stop the [^{32}P]phosphate/phosphate exchange was increased when mercaptoethanol was present.

Radioactive labelling of intact mitochondria was performed by incubating 5 mg mitochondria/ml, suspended in 0.25 M sucrose, 10 mM Tris–HCl (pH 7.4) and 1 mM EDTA, with [^{3}H]NEM for 2 min at 0°C, followed by addition of 5 mM cysteine and washing of mitochondria.

SDS gel electrophoresis was performed as in [8] using 17.5% acrylamide and an acrylamide/bisacrylamide ratio of 150.

Protein was determined by the Lowry procedure modified for samples containing sulphydryl compounds [9] and adapted to the presence of Triton [10].

3. RESULTS AND DISCUSSION

The protein components of the phosphate carrier fraction, obtained after chromatography on hydroxylapatite, were shown to contain cysteine [5]. The reactivity of membrane SH-proteins to organomercurials is different and depends also on the solubilization stage during isolation [11]. Therefore a further purification of the phosphate carrier fraction was attempted by chromatography on an organomercurial column. About half of the proteins pass through the column unretarded (fig.1). These fractions have very little reconstituted phosphate-exchange activity. After elution of the column with a mercaptoethanol step gradient, most of the reconstituted activity appears together with a small amount of protein, followed by a large protein peak, devoid of reconstituted activity. It should be mentioned that the total recovered reconstituted activity corresponded to 16.5% of the total activity applied to the column.

The gel electrophoretic analysis of the various fractions from the organomercurial column (fig.1) is presented in fig.2. Beside the polypeptide pattern obtained after Coomassie blue staining, the figure also shows the pattern after subsequent silver staining of the gel. The hydroxylapatite eluate, applied to the column, shows 4 protein bands, as found in [2,5]. Band 4 has been shown to contain the ADP/ATP-carrier and at least one other protein [5]. Whereas in the pass-through fractions all 4 bands are visible, in the fraction to which most of the reconstituted activity is associated (lane K) only band 2 is observed after Coomassie blue staining. The silver stain of the gel, which detects trace amounts of protein, shows also some weak bands in the upper part of the gel, but no staining in the region of bands 3 and 4 (lane K). In the lower part of the gel no staining at all was detected. This result clearly shows that band 4 is not involved in the phosphate transport activity and that a single protein, band 2 (lane K), is responsible for the reconstituted phosphate exchange activity. It should be pointed out that the highest amount of band 2 is found in the inactive fractions (lanes E–H) eluted after the active one (lanes D,J–L). In the inactive fractions band 2 is mainly associated with band 4, but also with some proteins of lower and higher app.-M_r, visible by the silver stain.

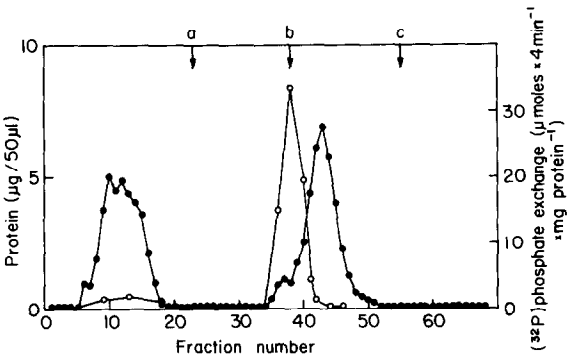

Fig.1. Purification by affinity chromatography of the active phosphate carrier from a hydroxylapatite eluate of Triton X-100 solubilized mitochondria. Hydroxylapatite eluate (5.2 ml) corresponding to a total activity of 14 μmol/4 min, were applied to the column. At the indicated arrows increasing concentrations of mercaptoethanol were added to the elution buffer: 1.5 mM (a), 3 mM (b) and 6 mM (c). Aliquots of 50 μl were used for protein determination (•—•) and 75 μl for measurements of reconstituted [^{32}P]phosphate exchange activity (○—○). Further conditions as in section 2.

It is well known that the phosphate carrier is inhibited by NEM. Therefore it was of interest to demonstrate that incubation of mitochondria with NEM results in inhibition of reconstituted phosphate-transport activity after isolation of the carrier. Fig.3 shows a titration curve of reconstituted

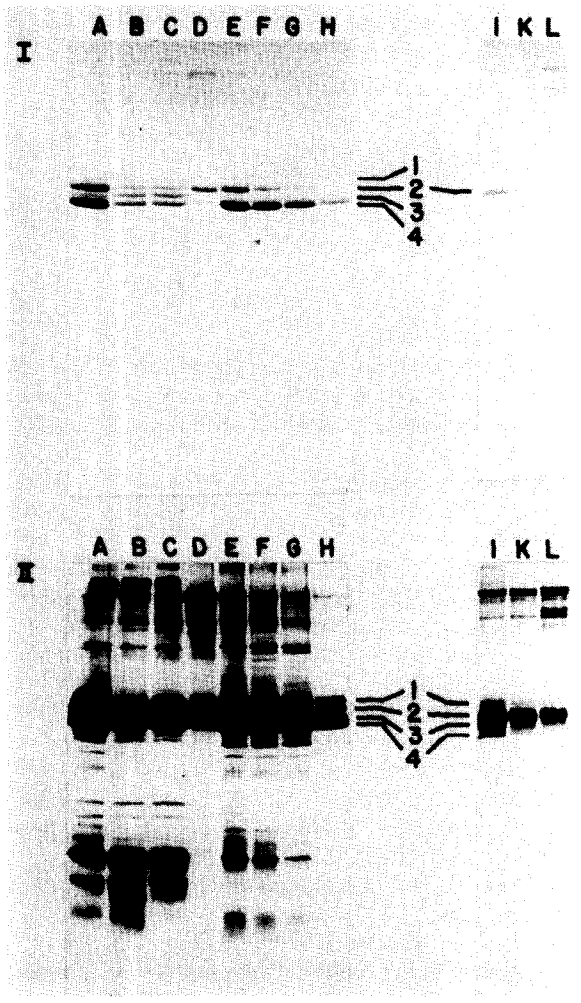

Fig.2. SDS gel electrophoresis of fractions obtained from the affinity column of fig.1: (A) hydroxylapatite eluate; (B) fractions 7–8; (C) fractions 9–12; (D) fractions 35–40; (E) fractions 41–42; (F) fractions 43–44; (G) fractions 45–46; (H) fractions 47–48. (I,K,L) Taken from a separate gel: (I) fractions 39–40; (K) fractions 37–38; (L) fractions 35–36. I, Coomassie blue stain; II, silver stain, performed after Coomassie blue staining.

phosphate-exchange activity of the hydroxylapatite eluate, obtained from mitochondria treated with increasing concentrations of NEM. Half maximal inhibition is obtained with ~20 nmol NEM/mg protein, which corresponds well with the inhibition of phosphate transport in intact pig heart mitochondria [5]. In fig.4, mitochondria were labeled with 12 nmol [^{3}H]NEM/mg protein, and the radioactivity in the various fractions, obtained

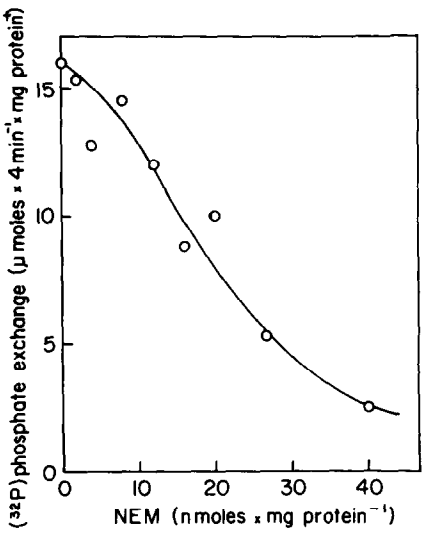

Fig.3. Inhibition of the reconstituted phosphate exchange activity of hydroxylapatite eluate by increasing concentrations of NEM added to mitochondria. Fresh pig heart mitochondria were incubated with the indicated amounts of NEM, extracted with Triton X-100, passed through hydroxylapatite, and the activity was reconstituted as in section 2.

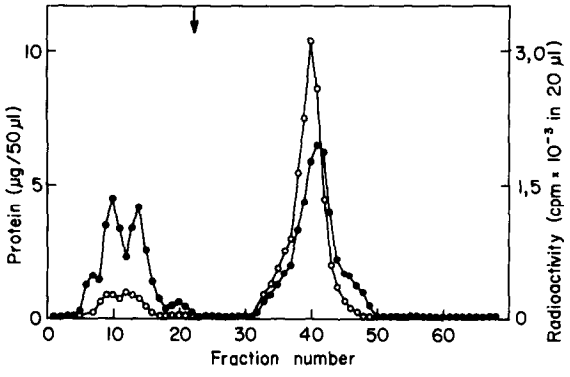

Fig.4. Affinity chromatography of a hydroxylapatite eluate obtained from [^{3}H]NEM-labeled mitochondria. Fresh pig heart mitochondria were labeled with 12 nmol [^{3}H]NEM/mg protein. Hydroxylapatite eluate (5 ml) was applied to the column; (→) linear mercaptoethanol gradient from 0–25 mM (30 ml) was included in the elution buffer. Aliquots (50 μl) were used for protein determinations (•—•), and 20 μl for radioactivity measurements (○—○).

after chromatography of the hydroxylapatite eluate on an organomercurial column, was measured. The protein fractions of the first peak contain some labeled NEM. However, most of the radioactivity is present in the fractions which are eluted with mercaptoethanol. The first part of these fractions (fraction 33–37), which corresponds to the active phosphate carrier (see fig.1), exhibited some activity after reconstitution (not shown). Most of the radioactivity, however, is present in the inactive fractions. The gel electrophoretic protein pattern was similar to that shown in fig.2 (not shown). It therefore appears that the radioactivity is associated with the protein of band 2 which occurs in all fractions.

It might be difficult to understand why the phosphate carrier was bound to the organomercurial column after blocking its SH-groups with NEM. However, it has been found that the P_i carrier contains 'two equivalent SH-groups, each of them is able in itself to maintain carrier function' [12]. Therefore, it may be assumed that at suboptimal inhibitory concentrations of NEM, used in the experiment of fig.4, the free SH-group of the carrier is involved in the binding to the column.

Furthermore it was found that after SDS-gel electrophoresis of a [^{3}H]NEM-labeled phosphate carrier fraction, band 2 does not contain radioactivity, and it was postulated that band 2 represents a proteolytic fragment of the native carrier (band 1) from which a small peptide, containing the NEM-sensitive SH-group, was split off [5]. Unless the protein is unfolded by SDS, it was suggested, that the SH-peptide remains attached to the protein, without loss of activity [5]. This proposal is compatible with the presence of NEM in the active fractions of fig.4 (corresponding to lanes D,J–L of fig.2) where mainly band 2 is present.

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