PURIFICATION OF A PHOSPHATE CARRIER IN PIG HEART MITOCHONDRIA BY AFFINITY CHROMATOGRAPHY ON MERSALYL–ULTROGEL

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1. Introduction

Mersalyl is a powerful inhibitor of phosphate transport in mitochondria. In addition, by its reversible reaction with thiol groups, it can protect the latter from irreversible reaction with *N*-ethylmaleimide [1,2]. Use of this property enabled a group of proteins of M_r 3.0–3.2 × 10^4 to be identified in the internal mitochondrial membrane [3–8]. This group of proteins could be directly labeled using radioactive mersalyl [6,9]. It was subsequently shown that these proteins consisted of a mersalyl and *N*-ethylmaleimide-sensitive protein (M_r 3.2 × 10^4) and the nucleotide translocase (M_r 3.0 × 10^4) [10,11]. These two proteins can be purified on hydroxyapatite [11,12]. The mixture of the two proteins when integrated into liposomes catalyzes a P_i–P_i exchange which is sensitive to thiol-group reagents [12].

This work describes the complete separation of the mersalyl and *N*-ethylmaleimide-sensitive protein from the nucleotide translocase. This purification was carried out by combining fractionation on a mersalyl–ultrogel column with purification on hydroxylapatite.

2. Materials and methods

Pig heart mitochondria were isolated according to [13] substituting 10 mM Tris–HCl buffer for the 10 mM phosphate buffer. Proteins were assayed by the biuret method [14]. Mitochondria were fractionated as in [15]. SDS gel electrophoresis was done as in [16].

Abbreviations: EGTA, ethyleneglycol tetraacetic acid; HEPES, *N*-2-hydroxy-ethylpiperazine-*N'*-2, ethane sulfonic acid; NEM, *N*-ethylmaleimide; P_i, inorganic phosphate; Tris, Tris (hydroxymethyl) amino methane

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3. Results

3.1. *Preparation of mersalyl–ultrogel [17]*

The matrix derived from AM ultrogel A-4-R (reactifs IBF, Pharmindustrie), a 4% crosslinked agarose, by successive reactions with epichlorhydrine and ammonia: agarose–*O*–CH_2–CH(OH)–CH_2–NH_2.

Mersalyl (sodium *o*-((3-(hydroxymercuri)-2-methoxypropyl)-carbonyl)-phenoxyacetate) was coupled to the matrix by Boschetti's method [18], using EEDQ (*N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline) as a coupling agent. Gel (25 ml) was equilibrated in a water–ethanol (50:50) mixture. Mersalyl (0.06 M) in water–ethanol (50:50) was obtained by dissolving mersalyl acid (Sigma) in diluted NaOH, adjusting the pH to 7.5 and adding an equal volume of ethanol. This solution (max. 10 ml) was quickly added to EEDQ dissolved in the minimal volume of ethanol, under vigorous stirring. The mixture was then added to the gel. After gentle agitation for 16 h, mersalyl–ultrogel was successively washed with ethanol–water (50:50), 1 M NaCl and distilled water. The affinity gel used in this work contained 24 nmol mersalyl/ml.

3.2. *Purification of the mersalyl and* N*-ethylmaleimide-sensitive protein*

If the mixture of proteins from the internal mitochondrial membrane is run through a mersalyl–ultrogel column, practically all the proteins with –SH groups would be expected to be retained. Retention

of the nucleotide translocase may be avoided by the following preliminary treatment on whole mitochondria: the mitochondrial phosphate carrier is first protected with mersalyl, and then the –SH groups of the nucleotide translocase are irreversibly blocked with *N*-ethylmaleimide in the presence of ADP [19–21]. Under these conditions the latter protein would not be expected to be retained on the mersalyl–ultrogel column.

The subparticulate proteins are then prepared from mitochondria and dissolved in the presence of Triton. The solution is fractionated on a mersalyl–ultrogel column, and the retained proteins are eluted with dithiothreitol. Further purification of the eluted fraction on hydroxylapatite then yields the completely pure phosphate carrier.

3.2.1. Protection of phosphate carrier and fixation of *N*-ethylmaleimide on translocase

Mitochondria (80 mg protein) were treated with 20 nmol mersalyl/mg protein. The suspension of mitochondria was then treated with 1 mM NEM for 2 min in the presence of 80 μM ADP. The reaction was stopped and the mersalyl removed by addition of cysteine. The mitochondria were found to still be able to catalyze phosphate transport as measured by swelling.

3.2.2. Preparation of subparticulate proteins and fractionation on the mersalyl–ultrogel column

Subparticulate proteins were prepared as in [6,11]. The proteins were dissolved in 6% Triton in 0.1 M NaCl (10 min, 4°C). Proteins (4–6 mg) were run through a 0.5 × 5 mersalyl–ultrogel column pretreated with a 0.15 M NaCl, 1% Triton, 10 mM phosphate buffer (pH 7.2). Flow rate was very low (1 drop/min). After washing out unretained proteins with the same buffer, retained proteins were eluted with 30 mM dithiothreitol. Mersalyl–ultrogel may be regenerated by washing the column with 10 mM $HgCl_2$ and 20 mM EDTA in 50 mM sodium acetate at pH 4.8. Excess $HgCl_2$ is removed with 0.15 M NaCl in 10 mM sodium phosphate (pH 7.2).

3.2.3. Purification of the mersalyl- and *N*-ethylmaleimide-sensitive protein

The protein fraction eluted with dithiothreitol was treated with hydroxylapatite (0.5 g/ml fraction), then centrifuged (10 min at 27 000 × *g*). The pure protein was left in the supernatant liquid.

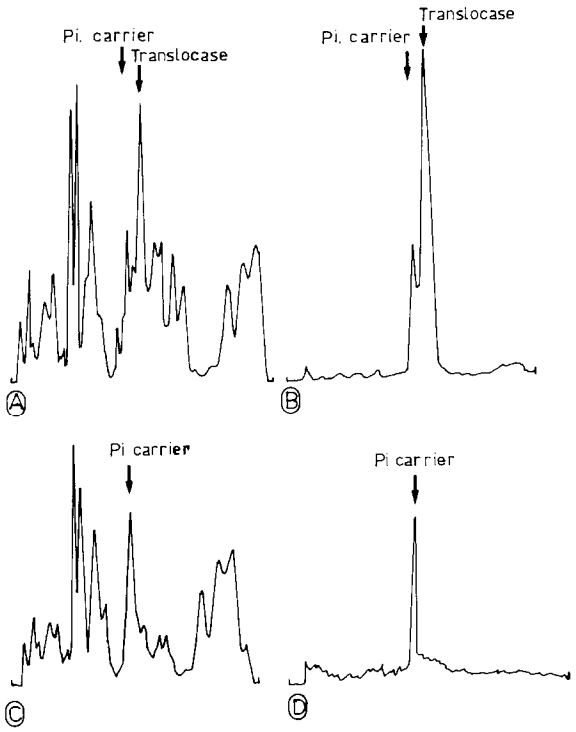

Fig.1. Densitometric peaks of electrophoresis of the different protein fractions: (A) subparticular proteins; (B) proteins obtained after fractionation on hydroxyapatite column; (C) proteins retained by the mersalyl–ultrogel column; (D) protein of M_r 3.2 × 10^4 obtained after fractionation on the mersalyl–ultrogel column and the fractionation on hydroxyapatite.

3.2.4. Electrophoresis of the different protein fractions

Densitometric peaks of electrophoresis of protein fractions at the different stages of purification are shown in fig.1; (A) shows the protein bands of the subparticulate fraction. It is possible to clearly distinguish between the phosphate carrier and the nucleotide translocase [11]. The 2 proteins can be purified on a hydroxylapatite column (B). Photographs of both types of electrophoresis have been published [11]. These results enable the relative proportions of phosphate carrier and nucleotide translocase to be estimated (20–25% and 75–80%, respectively). (C) shows the profile of the proteins retained on the mersalyl–ultrogel column. About 18 protein bands may be distinguished. The protein of M_r 3.2 × 10^4 can be seen to present in almost the same proportion as in the initial subparticulate fraction (A). At M_r 3.0 × 10^4, a protein band is present which could

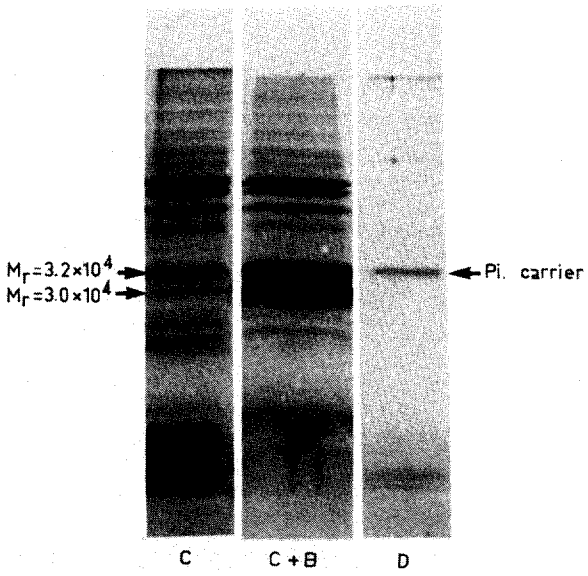

Fig.2. Electrophoresis of: (C) proteins retained by the mersalyl–ultrogel column; (C + B) proteins resulting from the mixture of (C) plus purified proteins on hydroxyapatite column (B); (D) protein of M_r 3.2 × 10^4 obtained after fractionation on the mersalyl–ultrogel column and the fractionation on hydroxyapatite.

correspond either to residual unblocked nucleotide translocase, or to some other protein with the same molecular mass. The second explanation is apparently the correct one since subsequent purification on hydroxylapatite yielded only the phosphate carrier (D).

An experiment using an internal standard was carried out to further check that the protein eluted from the mersalyl–ultrogel column was indeed the phosphate carrier. The fraction eluted with dithiothreitol was combined with the mixture of the 2 proteins purified on hydroxyapatite as internal standard. Electrophoresis (fig.2 (B + C)) shows a strong increase in the intensity of both the nucleotide translocase band (M_r 3.0 × 10^4) and the phosphate carrier band (M_r 3.2 × 10^4).

4. Conclusions

Protection by mersalyl against NEM completely protects active phosphate transport [11]. This protection enabled a M_r 3.2 × 10^4 protein to be located, which could be prepared on hydroxylapatite along with a nucleotide translocase [11]. Phosphate transport could be reconstituted by integrating the mixture of the 2 proteins into liposomes [12].

This paper describes a complete purification of the phosphate carrier by successive fractionation of subparticulate proteins on an affinity column prepared with mersalyl–ultrogel and on hydroxyapatite. Reconstitution of the mitochondrial phosphate transport system with the pure protein is being investigated.

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