ISOLATION AND RECONSTITUTION OF THE PHOSPHATE-TRANSPORT SYSTEM FROM PIG HEART MITOCHONDRIA

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1. Introduction

Phosphate is transported through the inner mitochondrial membrane mainly by an electroneutral proton symporter [1–6]. This phosphate carrier has been identified either by specific labelling with *N*-[^{3}H]ethylmaleimide (NEM) after protection with mercurials [7–11], or by direct labelling with radioactive mersalyl [10,12]. The carrier protein(s) can be partially purified by adsorption chromatography on hydroxylapatite from Triton X-100-solubilized mitochondria [13–15]. This method has also been successfully used for the purification of the ATP,ADP-carrier [16,17]. Purification by hydroxylapatite leads to a fraction still containing both, the phosphate carrier and the ATP,ADP-carrier as well [13–15]. After reconstitution in liposomes, this fraction catalyzed phosphate–phosphate exchange [13].

We describe the separation of the phosphate carrier protein(s) from the ATP,ADP-carrier and demonstrate its reconstitution in liposomes. The phosphate-transport protein designated in [13], identified as a single, [^{3}H]NEM-labelled band on standard sodium dodecylsulfate (SDS) gel electrophoresis [13–15], is shown to be separated into 4 different protein components by gradient SDS gel electrophoresis.

2. Materials and methods

[^{3}H]NEM (>500 mCi/mmol) was obtained from New England Nuclear; [^{32}P]phosphate (carrier-free) from Amersham; acrylamide, Li-dodecylsulfate and NEM from Serva; *N,N'*-methylene-bisacrylamide, Dowex AG 1-X8 and hydroxylapatite (Bio-Gel HTP) from Bio-Rad; Triton X-100 from Merck; Celite 535 from Roth; L-α-phosphatidylcholine (soy bean Typ II-S) from Sigma; and scintillation liquid (Unisolve) from Zinsser. SDS was used as a mixture of 1 part from Pierce and 2 parts from Fluka.

2.1. *Preparation of protein fractions*

Pig heart mitochondria were prepared according to [18] and stored frozen. They were labelled with [^{3}H]NEM (20 nmol/mg protein) as in [19]. Labelled (gel electrophoresis) or unlabelled mitochondria (reconstitution) were solubilized in buffer A (8% Triton X-100, 0.1 mM EDTA, 0.5 mM dithiothreitol, 20 mM LiCl, 20 mM H_3PO_4, adjusted to pH 7.0 with LiOH) to 12 mg protein/ml fined conc., diluted with water (1:1) and centrifuged for 1 h at 100 000 × *g*. 70% of protein and radioactivity were solubilized. Separations on hydroxylapatite and celite (washed twice with hot water) were performed in Pasteur pipettes filled with ~600 mg dry material. About 1 ml mitochondrial extract was applied to the columns and eluted with an equal volume of diluted (1:1) buffer A.

2.2. *SDS–polyacrylamide gel electrophoresis*

SDS gel electrophoresis was performed according to [20] with the following modifications: Ratio of acrylamide (12.5%) to *N,N'*-methylene-bisacrylamide was 37/1. Gradient gels (12–18% acrylamide) contained in addition 15% sucrose. Column eluates (10 μl) were mixed with 10 μl of the following sample buffer: 8% Li-dodecylsulfate, 400 mM Tris–HCl (pH 6.8), 30% glycerol (w/v) and 5% mercaptoethanol (w/v). Radioactivity was visualized by fluorography as in [21]. Gel slices were digested according to [22].

2.3. *Reconstitution of phosphate transport*

Reconstitution was based on the method in [23].

Liposomes were prepared by sonication of 100 mg L-α-phosphatidylcholine (partially purified by extraction with acetone) in 1 ml buffer B (50 mM KCl, 1 mM EDTA, 20 mM Hepes, 10 mM KH_2PO_4, pH 7.0) for 1–1.5 h at 0–4°C under N_2 (20 s pulse, 20 s intermission) with a Branson sonifier, type B-15 P, until the suspension became clear. 50–100 μl of mitochondria, dissolved in buffer A, or of the column eluates were mixed with 1 ml liposomes, frozen in dry ice/acetone, thawed in water, and pulse-sonicated (0.55 s pulse, 0.45 s intermission) for 22 s in Eppendorf cups. After preincubation of 250 μl proteoliposomes with 63 μl buffer B for 5 min at 25°C, 10 μl 66 mM NEM was added to the control and 10 μl $^{32}P_i$ to the sample and control. After the indicated times the exchange was stopped with 10 μl 66 mM NEM. Free $^{32}P_i$ was removed at 0°C by passing the samples through Dowex AG 1-X8 columns (0.5 × 4 cm, acetate form), equilibrated and eluted (1.2 ml) with 160 mM sucrose. The eluates were mixed with 10 ml 'Unisolve' and counted in a scintillation counter (Betaszint BF 5000). Protein was determined by a modified Lowry method [24].

3. Results and discussion

The labelling of pig heart mitochondria with an amount of [^{3}H]NEM, sufficient to inhibit phosphate transport, results in a rather specific labelling of a protein with app. M_r 34 000, as shown in fig.1. The figure compares Coomassie blue staining with fluorography of total pig heart mitochondrial proteins, separated by SDS gel electrophoresis. A less intensively labelled band (M_r 33 000) contains ~20% of the amount of radioactivity present in the upper band (M_r 34 000), as measured by scintillation counting. The lower protein band (M_r 33 000) is different from the ATP,ADP-carrier, which is identified by its staining intensity and apparent molecular weight (M_r 31 000). This contradicts the conclusion in [13] correlating the second labelled band to the ATP,ADP-carrier. Labelling of both proteins is protected by preincubation with mersalyl [14,15].

To isolate the phosphate carrier protein, various adsorbents were investigated (fig.2). It was found advantageous to apply the mitochondrial extract to columns, filled with dry adsorbents, since no dilution of the fractions occurs. The use of hydroxylapatite results in elution of 3 proteins, as visualized by stand-

Fig.1. Correlation of [^{3}H]NEM fluorography with Coomassie blue staining of total pig heart mitochondrial proteins on a slab gel. SDS gel electrophoresis was performed as in section 2. The indicated molecular weights were determined with the following marker proteins (Boehringer): carbonic anhydrase (31 000); glycerol-3-phosphate dehydrogenase (36 000); glyoxylate reductase (43 000); aspartate aminotransferase (46 500); fumarase (49 000); citrate synthase (50 000). (A) Coomassie blue staining; (B) fluorography.

ard SDS gel electrophoresis, the ATP,ADP-carrier, the 'phosphate-transport protein' (see [13]) and the 33 000 M_r protein (lane B). Passing the hydroxylapatite eluate through celite leads to complete adsorption of the ATP,ADP-carrier (lane C). A quantitative analysis of protein and radioactivity recovered in the eluates is presented in table 1. To differentiate between protein-bound radioactivity and [^{3}H]NEM bound to low molecular weight compounds, the eluates were acetone-precipitated (1:9). After hydroxylapatite/

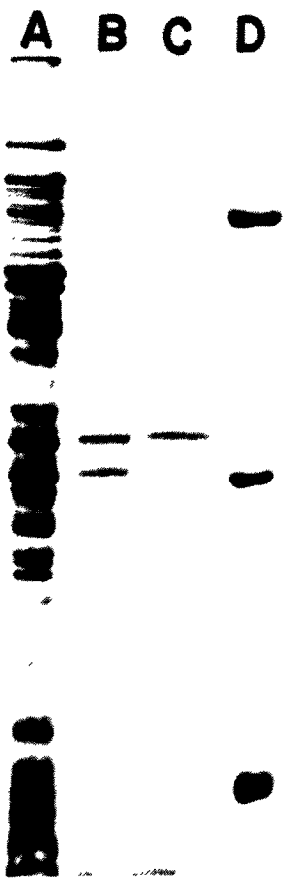

Fig.2. Standard SDS gel electrophoretic patterns of the flow-through of solubilized pig heart mitochondria after column chromatography: (A) solubilized pig heart mitochondria; (B) eluate from hydroxylapatite; (C) eluate from hydroxylapatite/celite; (D) marker proteins: cytochrome *c* (12 000, Sigma); carbonic anhydrase (31 000, Boehringer); bovine serum albumin (68 000, Roth).

Table 1
Recovery of protein and [^{3}H]NEM radioactivity in the flowthrough of hydroxylapatite and hydroxylapatite/celite columns

	Protein (%)	Radioactivity (%)		
		Total	Acetone insoluble	Acetone soluble
Mitochondrial extract	100	100	49.4	39.2
Hydroxylapatite eluate	5.9	67.2	27.6	39.4
Hydroxylapatite/ celite eluate	3.5	58.7	21.0	35.9

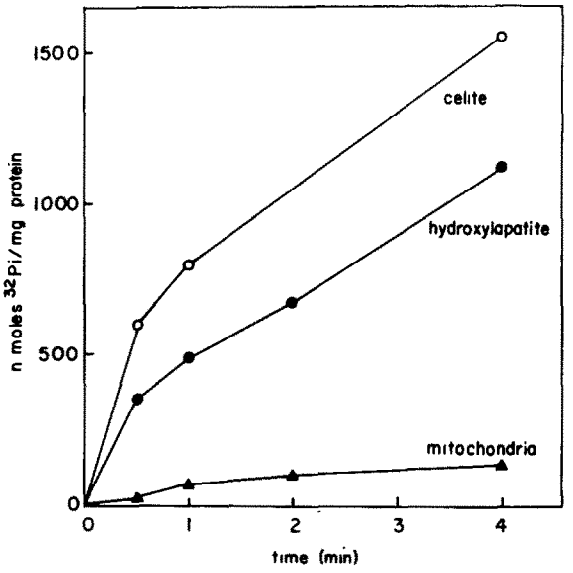

Fig.3. Kinetics of reconstituted NEM-sensitive phosphate–phosphate exchange of different protein fractions. Each point represents the difference between 2 exchange and 2 control samples. The controls showed values 30–60% of the exchange samples. Each sample contained 150 μg (mitochondrial extract), 25 μg (hydroxylapatite eluate) or 10 μg (hydroxylapatite/celite eluate) protein and 1.5 μCi [^{32}P]phosphate: (○) eluate from hydroxylapatite/celite; (●) eluate from hydroxylapatite; (▲) Triton X-100-dissolved mitochondria.

celite only 3.5% of total applied mitochondrial protein, but 42.5% of protein-bound radioactivity are recovered.

Reconstitution of phosphate transport activity in liposomes was performed applying a high lipid/protein ratio as in [23]. Fig.3 compares the kinetics of NEM-sensitive phosphate–phosphate exchange of 3 different protein fractions. The highest specific exchange rate is obtained with the eluate from hydroxylapatite/celite.

The hydroxylapatite eluate gives a lower exchange rate, but also with the total mitochondrial Triton X-100 extract phosphate transport could be reconstituted. The initial exchange rate of 700 nmol $^{32}P_i$. min^{-1} . mg $protein^{-1}$, obtained with the eluate from the hydroxylapatite column, is a little lower, that obtained with the eluate after hydroxylapatite/celite (1200) higher, than the value of 990 found in [13]. It may be noted, however, that our reconstitution system was not optimized.

In order to investigate whether the eluate from hydroxylapatite/celite, showing only one band by

Fig.4. Gradient SDS gel electrophoretic patterns of the flow-through of solubilized pig heart mitochondria after column chromatography: (A) solubilized pig heart mitochondria; (B) eluate from hydroxylapatite; (C) eluate from hydroxylapatite/celite. The apparent molecular weights of the 4 proteins (35 000 (1), 34 000 (2), 33 000 (3) and 31 500 (4)) were determined using the marker proteins listed in fig.1.

standard SDS gel electrophoresis, consists of a single polypeptide chain, a gradient SDS gel electrophoresis was performed (fig.4). Four different protein bands can be detected with app. M_r 35 000 (1), 34 000 (2), 33 000 (3) and 31 500 (4) (lane C). The [^{3}H]NEM radioactivity in the 4 bands as determined by scintillation counting from 3 gels resulted in the following distribution: (1) 50%; (2) 28%; (3) 22%; (4) 0%. The appearance of labelled band 3 after hydroxylapatite/celite contradicts the statement in [15]. As to the identity of the phosphate carrier protein(s), different possibilities have to be envisaged:

(i) The phosphate carrier of mitochondria occurs as a complex of up to 4 different polypeptide chains;
(ii) Some of the 4 proteins represent proteolytically or otherwise modified forms of the same protein;
(iii) The phosphate carrier is one protein, the other proteins represent different functional proteins, not related to the phosphate transport.

At present we cannot decide between the 3 possibilities.

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