IS THE MITOCHONDRIAL DICYCLOHEXYLCARBODIIMIDE-REACTIVE PROTEIN OF M_r 33 000 IDENTICAL WITH THE PHOSPHATE TRANSPORT PROTEIN?

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1. Introduction

Dicyclohexylcarbodiimide acts at low concentrations as a specific inhibitor of H^+-ATPase due to the covalent binding to a hydrophobic subunit of F_o [1,2]. In mammalian mitochondria, however, an additional protein of different M_r (33 000) is also labelled at H^+-ATPase-inhibitory concentrations of [^{14}C]DCCD [2–4]. In contrast to the DCCD-binding protein of F_o it is neither extracted by chloroform–methanol (2:1, v/v), nor is it detected in the isolated H^+-ATPase complex [3,4]. In different types of mitochondria the 33 000 DCCD-reactive protein is present in a near stoichiometrical quantity with respect to F_o, even though mitochondria differ in the natural content of H^+-ATPase up to 10 times [5].

Here, we describe the separation of the 33 000 DCCD-reactive protein from the DCCD-binding subunit of F_o and its purification. Evidence is presented for its identity with the *N*-ethylmaleimide (NEM)-sensitive phosphate/proton symporter of the mitochondrial membrane.

2. Materials and methods

[^{14}C]DCCD (32 mCi/mmol) was synthesized from [^{14}C]urea as in [6]. *N*-[^{14}C]ethylmaleimide (NEM) (10 mCi/mmol) was obtained from New England Nuclear; Triton X-100 from Merck; celite 535 from Roth; hydroxylapatite (Bio-Gel HTP) from BioRad.

Abbreviations: H^+-ATPase, proton-translocating ATPase; F_o, membrane integral part of H^+-ATPase; DCCD, *N,N'*-dicyclohexylcarbodiimide; NEM, *N*-ethylmaleimide

2.1. *Preparation of mitochondria*

Bovine heart mitochondria were isolated according to procedure 3 in [7] and rat liver mitochondria according to [8]. Prior to further use, bovine heart mitochondria were freeze–thawed 3 times and sedimented (30 000 × *g*, 15 min). They were labelled either with [^{14}C]DCCD (4 nmol/mg protein, 0°C, 16 h) as in [3] or with [^{14}C]NEM (15 nmol/mg protein, 0°C, 2 min) as in [9]. Labelled mitochondria were solubilized for 30 min by 4% Triton X-100, 50 mM KCl, 10 mM KPO_4, 1 mM EDTA, 1 mM dithiothreitol, 10 mM Tris–HCl (pH 7.4) at 15 mg protein/ml. Unsolubilized material was sedimented (100 000 × *g*, 45 min).

2.2. *Adsorbtion chromatography*

Chromatography on hydroxylapatite and celite was performed in Pasteur pipettes. Routinely, 2 mg protein of solubilized mitochondria were applied on the column (0.2 g adsorbent) and elution was performed with the solubilization buffer. Protein-bound radioactivity (90% acetone-precipitate) was determined by liquid scintillation.

2.3. *SDS–polyacrylamide gel electrophoresis*

Electrophoresis of acetone-precipitated samples was performed in the presence of 0.1% SDS on slabs of linear gradient polyacrylamide (12–20%) gel according to [10]. The molecular masses were determined with the help of the following marker proteins (Pharmacia): phosphorylase *b* (94 000); bovine serum albumin (67 000); ovalbumine (43 000); carbonic anhydrase (30 000); soybean trypsin inhibitor (20 100); α-lactalbumin (14 400). Radioactivity of the stained gels (Coomassie brilliant blue R 250) was detected by

fluorography according to [11] and fluorograms were quantified as in [3].

Phosphate transport was measured by passive swelling as in [12]. Protein was determined according to [13] except that 0.5% SDS was present.

3. Results and discussion

As demonstrated by SDS–polyacrylamide gel electrophoresis followed by fluorography, the DCCD-binding protein of F_o (radioactive bands of M_r 9000 and 16 000 – see [3]) and an unidentified protein of M_r 30 000 which also reacts with [^{14}C]DCCD [2–4] could be solubilized from bovine heart mitochondria by the non-ionic detergent Triton X-100 (fig.1D). In comparison with original mitochondria, their relative proportions were not influenced. Of the 3 labelled proteins only that of M_r 33 000 stained well with Coomassie blue (fig.1C). The adsorbtion chromatography on hydroxylapatite of a detergent-solubilized mitochondria has been repeatedly used for purification of several carrier proteins, all with $M_r \sim 30\,000$ [14–18]. The solubilized [^{14}C]DCCD-labelled mitochondria were therefore chromatographed on hydroxylapatite (2 mg protein/0.2 g adsorbent). No matter whether the preequilibrated or dry-bed hydroxylapatite was used, the majority (~90%) of the membrane proteins was removed, but the 33 000 M_r DCCD-reactive protein was nearly quantitatively recovered in the pooled eluate (fig.1B,E, table 1). Interestingly, also the other two radioactive bands were mostly present in the eluate (fig.1E). Regarding their poor staining, it

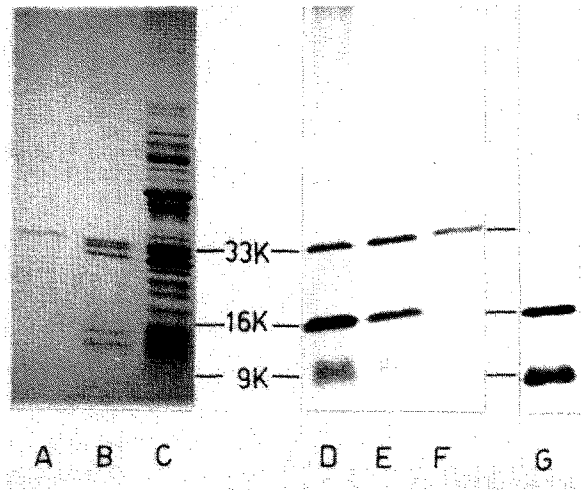

Fig.1. Electrophoretic pattern of the pass-through of solubilized bovine heart mitochondria after adsorbtion chromatography – correlation of [^{14}C]DCCD radioactivity with Coomassie blue staining. SDS-gel electrophoresis was done as in section 2: (C,D) solubilized mitochondria; (B,E) hydroxylapatite eluate; (A,F) hydroxylapatite/celite eluate; (G) material extracted from hydroxylapatite/celite column by 2.5% SDS (2 h, 50°C); (A–C) protein staining; (D–G) fluorography.

is therefore quite probable that the DCCD-binding protein of F_o might contaminate various preparations obtained by the hydroxylapatite-based isolation procedures. The 3 radioactive bands were found in a similar proportions throughout the radioactivity containing fractions of the eluate. The attempts to separate them by means of a gel filtration (Sephadex G-50) or electrophoresis (non-dissociating conditions)

Table 1
Recovery of protein and of the 33 000 M_r protein-bound radioactivity in the eluate of hydroxylapatite and hydroxylapatite/celite columns

	Protein (%)	Radioactivity present in the 33 000 M_r protein (%)	Purification (-fold)
[^{14}C]DCCD			
Solubilized mitochondria	100	100	1
Hydroxylapatite eluate	8.2	80	9.8
Hydroxylapatite/celite eluate	4.5	70	15.6
[^{14}C]NEM			
Solubilized mitochondria	100	100	1
Hydroxylapatite eluate	9.0	67	7.4
Hydroxylapatite/celite eluate	5.0	61	12.2

were not successful. When the ratio of proteins applied/hydroxylapatite was decreased a selective adsorbtion of the 9000 and 16 000 radioactive bands was observed. The complete separation could not be, however, achieved.

Recent isolation of the mitochondrial phosphate transporter [17] introduced the separation of the carrier protein from the contaminants that remained present in the hydroxylapatite eluate by means of an adsorbtion chromatography on infusorial earth (celite). This approach proved to be also effective in further purification of the 33 000 M_r DCCD-reactive protein. The chromatography of the hydroxylapatite eluate on celite, as well as the one-step procedure chromatography of the solubilized mitochondria on a mixture of hydroxylapatite and celite (ratio of 1/1 (w/w) was sufficient) gave essentially the same result. As shown in fig.1F, the isolation of the 33 000 M_r radioactive band and a complete removal of the 9000 and 16 000 M_r radioactive bands was achieved. As shown in fig.1G, in the course of chromatography on celite, the 9000 and 16 000 M_r bands did not aggregate to form the 33 000 M_r band but were adsorbed. In this step, also other contaminants that remained after hydroxylapatite were mostly removed, except for traces of two proteins with M_r 31 000 and 32 000 (fig.1A,2). Of the radioactivity, originally present in 33 000 M_r band, 70% was recovered after hydroxylapatite/celite chromatography (table 1). This means a 16-fold purification of the 33 000 M_r DCCD-reactive protein. An equal separation of this protein was also achieved using mitochondria of rat liver and hamster brown fat (not shown).

To determine whether the 33 000 M_r DCCD-reactive protein is compatible with the NEM-reactive protein, isolated in [15,17,18], the parallel experiments with [^{14}C]NEM-labelled mitochondria were performed. As shown in fig.2, [^{14}C]DCCD label in the 30 000 M_r region corresponded exactly to the 33 000 M_r [^{14}C]-NEM-labelled protein, which was purified and recovered in a comparable way by the isolation procedure (table 1). An additional NEM-reactive protein of M_r 31 000, which accounted in solubilized mitochondria for 25% of radioactivity (50% were present in the 33 000 M_r protein), was mostly adsorbed by celite (fig.2D–F). A coelectrophoresis of the isolated [^{14}C]-DCCD-labelled protein and of the isolated [^{14}C]NEM-labelled protein resulted in a single radioactive band (fig.2H). The same result was obtained using an alternative [19] electrophoresis system.

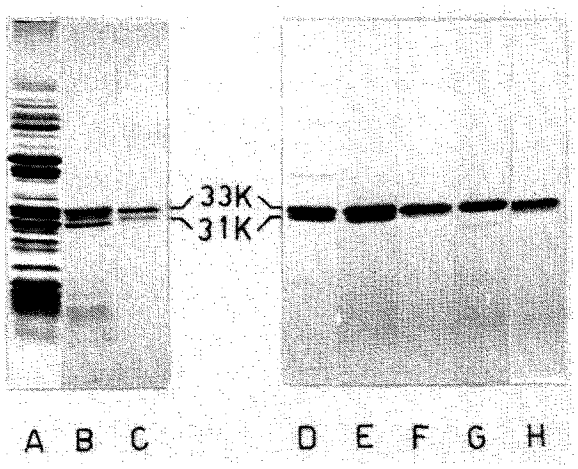

Fig.2. Electrophoretic pattern of the pass-through of solubilized bovine heart mitochondria after adsorbtion chromatography – correlation of [^{14}C]NEM and [^{14}C]DCCD radioactivity. SDS-gel electrophoresis was performed as in section 2: (A,D) solubilized mitochondria ([^{14}C]NEM); (B,E) hydroxylapatite eluate ([^{14}C]NEM); (C,F) hydroxylapatite/celite eluate ([^{14}C]NEM); (G) hydroxylapatite/celite eluate ([^{14}C]-DCCD); (H) coelectrophoresis of the sample (F) and (G) ratio 1/1; (A–C) Coomassie blue staining; (D–H) fluorography.

In a preliminary experiments, the effect of DCCD and NEM on phosphate-induced swelling of isolated rat liver mitochondria was compared. Preincubation of mitochondria with DCCD (20 nmol/mg protein, 30 mg protein/ml, 1 h, 0°C) decreased the rate of swelling by 30–35%. The inhibition by NEM (20 nmol/mg protein, 2 min, 0°C) was 92%. Based on:

(i) The striking similarity in behaviour of both the DCCD-reactive and the NEM-reactive protein in the course of isolation, which is essentially equal to that yielding so far the purest and active phosphate carrier [17];
(ii) The same electrophoretic mobility of the DCCD- and NEM-reactive protein under all conditions tested;

it is concluded that the DCCD-reactive protein of M_r 33 000 does not represent an aggregate of the DCCD-binding subunit of F_o [4,20] but rather is identical with the NEM-sensitive phosphate/H^+ transporter. The modification of the carrier by DCCD seems to have only a moderate effect on its transport activity, nevertheless it might help to specify further the physical–chemical properties of the carrier.

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