IDENTIFICATION OF A MERSALYL- AND *N*-ETHYLMALEIMIDE-SENSITIVE PROTEIN FROM PIG HEART MITOCHONDRIAL SUBPARTICLES

Relationship to the ADP *N*-ethylmaleimide-sensitive protein

Sylvie TOURAILLE, Yves BRIAND and Roger DURAND+
Laboratoire de Biochimie, Université de Clermont-Ferrand II, BP 45, 63170 Aubière, France

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1. Introduction

SH-reagents are potent inhibitors of the mitochondrial phosphate transport system. It was firstly postulated that mercurials inhibited phosphate transport in rat liver mitochondria [1,2]. Furthermore, it was demonstrated that phosphate can enter mainly by a mersalyl- (and *N*-ethylmaleimide)-sensitive transport system [3–9] catalysing a phosphate–hydroxide exchange. Similar results were found with pig heart mitochondria [10–12].

In attempts to isolate proteins involved in the mitochondrial phosphate transport system, different methods were developed.

(1) Kadenbach et al. [13] isolated some chloroform soluble proteins containing free SH-groups which bind phosphate. This approach has recently also been used by Guérin et al. [14] for the yeast mitochondrial phosphate transport system.

(2) As described by Fonyo [15,16], mercurials can protect against the ethylmaleimide reaction. Consequently, specific proteins of phosphate transport can be labeled by first protecting them with a mercurial and irreversibly blocking unassociated SH groups with unlabeled NEM. Then, after mercurial removal, the specific SH groups can be labeled with radioactive NEM. Thus, Coty and Pedersen [17] using pCMB protection in rat liver mitochondria were able to detect by SDS–polyacrylamide gel electrophoresis a major pCMB- and NEM-sensitive protein (mol. wt 32 000) while Kadenbach et al. [18] by other suitable experiments found a mersalyl- and NEM - sensitive protein (mol. wt 26 500 ± 800). A comparable investigation with mersalyl protection in pig heart mitochondria allowed us [19] to identify a major mersalyl- and NEM-sensitive protein.

(3) More recently Banerjee et al. [20] claimed that they obtained a protein fraction by solubilisation of submitochondrial particles from beef heart with octylglycoside and extraction with ammonium sulfate. After reconstitution into liposomes, this protein fraction catalysed a mersalyl-sensitive phosphate transport.

Our previous work [19] demonstrated that this mersalyl- and NEM-sensitive component was localized in the mitochondrial subparticles. We present here data on the partial purification of this protein. In addition we present some experiments showing that this mersalyl- and NEM-sensitive protein belongs to a pool of proteins of similar molecular weight which includes and ADP–NEM-sensitive protein identified

Abbreviations: NEM, *N*-ethylmaleimide; pCMB, parachloromercuribenzoate; SDS, sodium dodecyl sulfate; MES, 2-(*N*-morpholino) ethane sulfonic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid

+ To whom reprint requests should be addressed

by Klingenberg [21] consecutively to the initial data of Leblanc and Clauser [22].

2. Materials and methods

Pig heart mitochondria were prepared according [23] substituting 10 mM Tris–HCl buffer for the 10 mM phosphate buffer. Proteins were determined by the biuret [24] or Lowry method [25]. Mitochondrial subparticles were obtained by sonicating mitochondria, 6 × 30 s, at the maximum power of an ultrasonic disintegrator MSE. Mitochondria were eliminated by centrifugation at 20 000 × *g* (10 min), and the subparticles recovered by centrifugation at 130 000 × *g* (1 h). The subparticles were rehomogenised in sucrose–Tris buffer, pH 7.2. Proteins were fractionated on an ultrogel column. Subparticles (15–20 mg/ml) were heated to 90°C (3 min) in the presence of 4% SDS, and 1 ml of this solution was directly applied to an ultrogel AcA 54 (LKB) column (2 × 45 cm). Proteins were eluted with buffer containing 10 mM Tris, pH 7.2, 0.1% SDS, 0.02% NaN_3 at 6 ml/h. Fractions were collected every 15 min (collector ISCO). Proteins were detected by monitoring $A_{280\ nm}$ (ISCO) and radioactivity was then measured on an aliquot (400 μl) of each fraction in an Intertechnique liquid scintillation spectrometer, with a scintillation fluid containing 4 g PPO and 100 mg POPOP/litre toluene. Electrophoretic analysis of subparticle proteins and column fractions were done according to [19]. The apparent molecular weights of proteins were determined by using radioactive standard proteins prepared according to [17]. Standard proteins were labeled with [^{14}C]acetic anhydride for use as an internal reference during polyacrylamide gel electrophoresis of [^{3}H]NEM-labeled mitochondrial proteins.

3. Results and discussion

3.1. *Labeling by [^{14}C]mersalyl or [^{3}H]NEM of a major subparticular protein component*

Figure 1A shows that [^{14}C]mersalyl was essentially incorporated in peak 1 (3.3 nmol/mg subparticular protein). Figure 1B shows that when the cold mersalyl

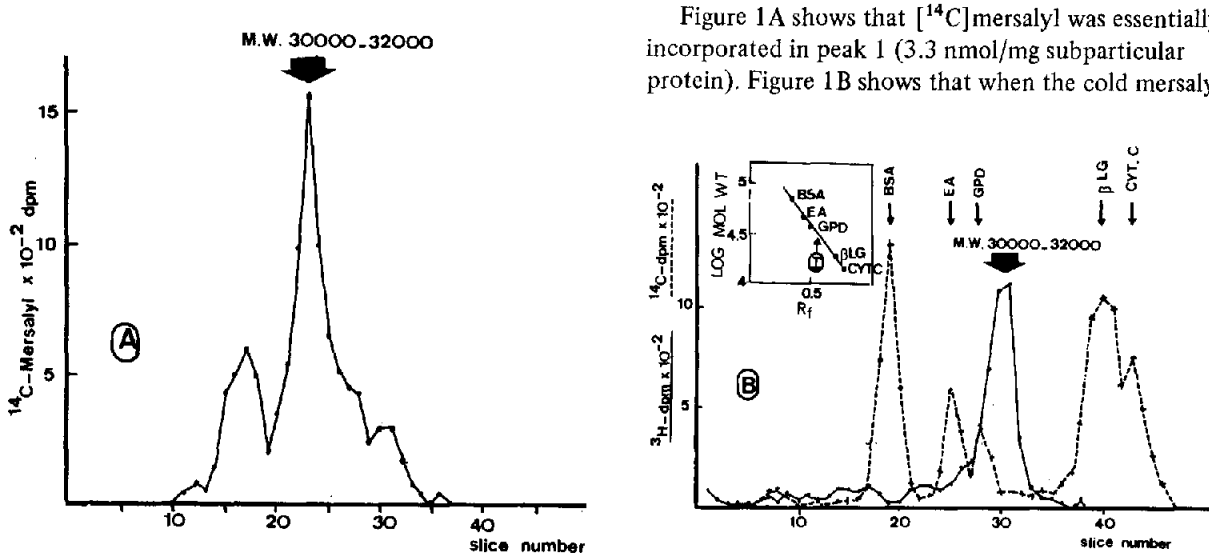

Fig.1. Electrophoresis analysis of submitochondrial particles. Mitochondria (80 mg) in 10 ml Tris–HCl buffer, 0.25 M sucrose, pH 7.2, 30°C, were treated with [^{14}C]mersalyl (15 nmol/mg protein) (fig.1A) in conditions such that phosphate transport was inhibited to about 70–80% [19]. For [^{3}H]NEM labeling (fig.1B) whole mitochondria were first treated with cold mersalyl (15 nmol/mg protein) and 30 s later with cold NEM (12.5 nmol/mg protein, 1.5 min). Then the cold mersalyl was removed by cystein and the mitochondria were washed in sucrose–Tris buffer and treated with [^{3}H]NEM (12.5 nmol/mg protein, 1.5 min). Following labeling, subparticles were obtained (see Materials and methods) and examined by gel electrophoresis. Gels were cut into 2 mm slices and assayed for radioactivity. The inset shows a log mol. wt vs R_F plot of [^{14}C]acetyl-labeled proteins: bovine serum albumin 68 000 (BSA), egg albumin 45 000 (EA), glyceraldehyde 3-phosphate dehydrogenase 36 000 (GPD), β-lactoglobulin 18 400 (βLG), cytochrome c 13 000 (cyt. C).

was removed and [^{3}H]NEM added the radioactivity was incorporated in the same peak I (3.5 nmol/mg protein). The relative protein concentration of this band has been estimated at about 4–5% total mitochondrial protein component [19].

Previously we determined mol. wt 39 000 for this major mersalyl- and NEM-sensitive protein from polyacrylamide disc gel electrophoresis, using one disc for standard proteins and another for subparticular proteins. Later experiments showed some variation in the positions of subparticular proteins. Consequently, following [17], we used as internal reference [^{14}C]acetic anhydride labeled standard proteins. These experiments gave a probably more accurate mol. wt 30 000–32 000 (fig.1B).

3.2. *Purification of the mersalyl–NEM-sensitive protein on an ultrogel column*

Better fractionation was obtained when mitochondria were treated as follows: after addition of [^{14}C]-mersalyl (15 nmol/mg proteins), cold NEM was added in order to limit unspecific labeling of proteins by [^{14}C]mersalyl during fractionation.

15–20 mg of the [^{14}C]mersalyl subparticular protein fraction were applied to an ultrogel column. The elution profile is shown in fig.2. SDS polyacrylamide gel electrophoresis revealed that the mersalyl sensitive protein appeared with elution vol. 82–98 ml, but the most highly purified fraction was obtained between 87–93 ml elution volume (fig.3). Traces of some other labeled proteins with smaller molecular weights can also be seen.

During fractionation a considerable peak of radioactivity was found at 130–140 ml elution volume (on the right of fig.2). We assume that it corresponds to some free [^{14}C]mersalyl released either during SDS heat treatment of [^{14}C]mersalyl subparticular proteins, or during fractionation on the ultrogel column. This peak was not present when the same protein was labeled with [^{3}H]NEM according to the experimental conditions of fig.1B (result not shown).

3.3. *Identification of an ADP–NEM-sensitive protein in the subparticular fraction*

It has been demonstrated that ADP translocation can be inhibited by NEM when mitochondria were

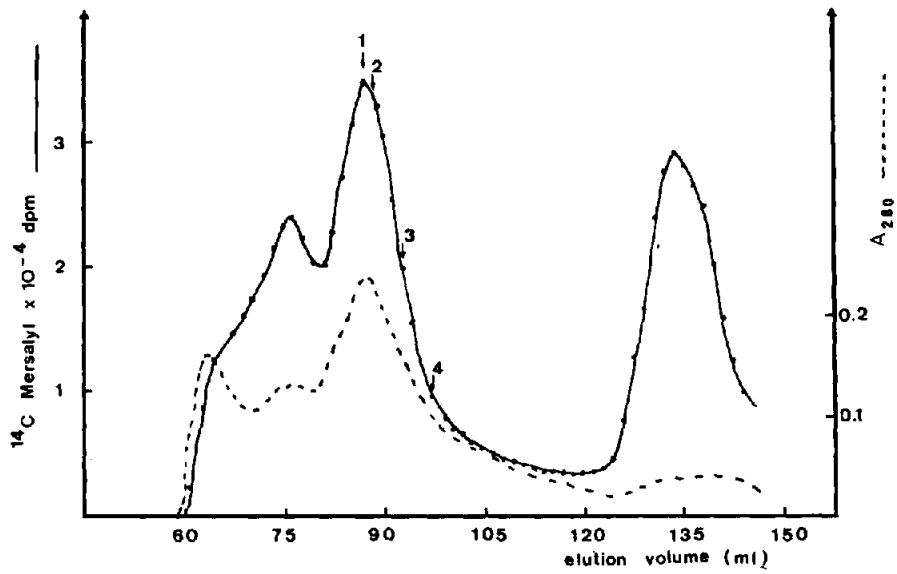

Fig.2. Fractionation of subparticle proteins on ultrogel column. Mitochondria (80 mg) in 10 ml Tris–HCl buffer pH 7.2, 0.25 M sucrose were treated with 1.2 μmol [^{14}C]mersalyl for 30 s, then with 1 μmol NEM for 1.5 min. NEM reaction was stopped by centrifugation at 39 000 × *g* 5 min. Subparticles were obtained (see Materials and methods). The [^{14}C]mersalyl subparticles (15–20 mg/ml) in sucrose–Tris buffer, 4% SDS, were heated to 90°C for 3 min and 1 ml of this solution was applied to an ultrogel column (2 × 45 cm) equilibrated with 10 mM Tris–HCl, pH 7.2, 0.1%, SDS, 0.2% NaN_3. Radioactivity was measured on a 400 μl sample of each fraction and fractions were analysed by SDS–polyacrylamide gel electrophoresis.

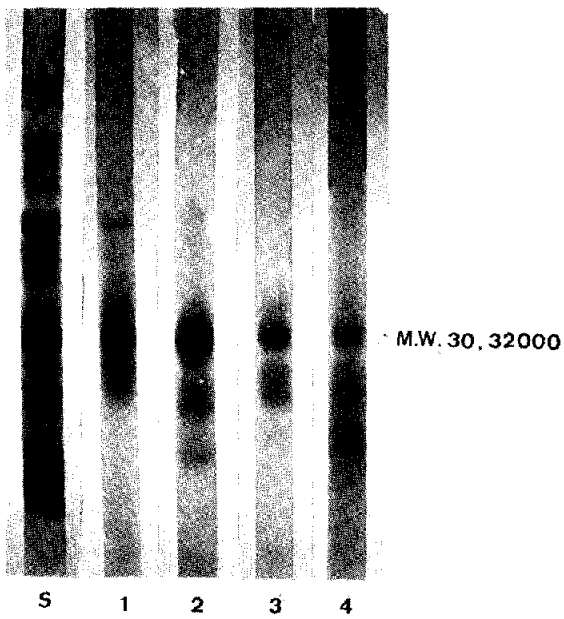

Fig.3. SDS–polyacrylamide gel electrophoresis of subparticles and ultrogel fractions. SDS–polyacrylamide gel electrophoresis of subparticles (S) and fractions indicated by arrows on fig.2 and corresponding to elution vol. 86 ml (1), 88.5 ml (2), 93 ml (3) and 97.5 ml (4), are made as indicated in Materials and methods.

incubated in presence of ADP [22,26] and an activation of NEM incorporation was observed in presence of ADP. In pig heart mitochondria, the effect of ADP on NEM incorporation is shown in fig.4A. Incorporation of NEM was increased by 1 nmol/mg protein in the subparticular fraction. This ADP effect was prevented by addition of carboxyatractylate to the medium (result not shown).

SDS gel electrophoresis analysis (fig.4B) showed that the ADP–NEM-sensitive protein is found in the same band as the mersalyl–NEM-sensitive protein (mol. wt 30 000–32 000).

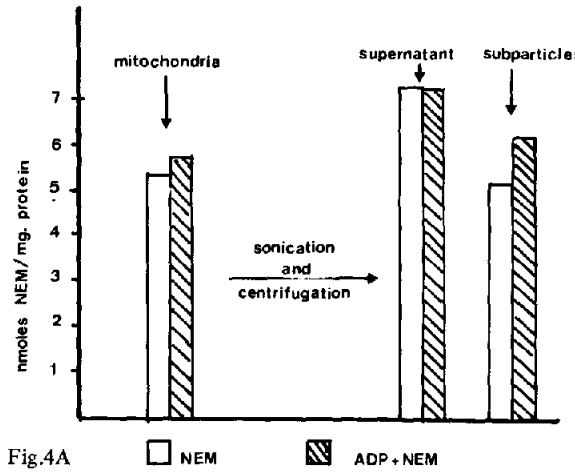

Fig.4A

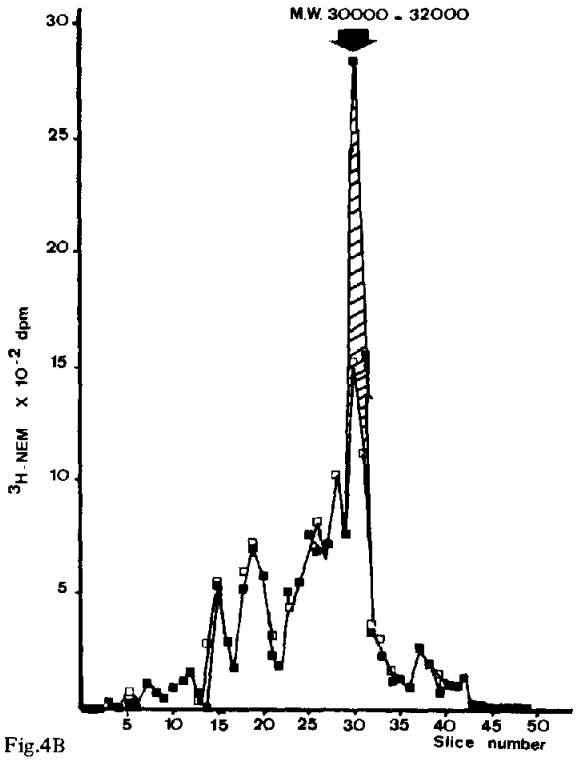

Fig.4B

Fig.4A. ADP effect on NEM incorporation in pig heart mitochondrial fractions. Mitochondria (200 mg) in 10 ml, 10 mM Tris–HCl buffer, 0.25 M sucrose pH 7.2, 30°C, were treated for 3 min with 0.1 mM cold NEM and centrifuged. The pellet was suspended in 20 ml Tris–HCl buffer. Then 10 ml of the suspension were added to 10 ml ETK [26] (120 mM KCl, 10 mM Tris, 10 mM Mes, 0.1 mM EDTA, 4 mM succinate, 3 μg/ml oligomycin, 2 μg/ml rotenone) with 0.1 mM [^{3}H]-NEM or 0.1 mM [^{3}H]NEM and 40 μM ADP. NEM reaction was stopped by adding cystein 2.75 min after. Subparticles were obtained (see Materials and methods) and radioactivity was measured as described [11]. Fig.4B. Electrophoretic analysis of subparticles. Subparticles obtained as explained in fig.4A were analysed by SDS–gel electrophoresis. Gels were cut into 2 mm slices and assayed for radioactivity.

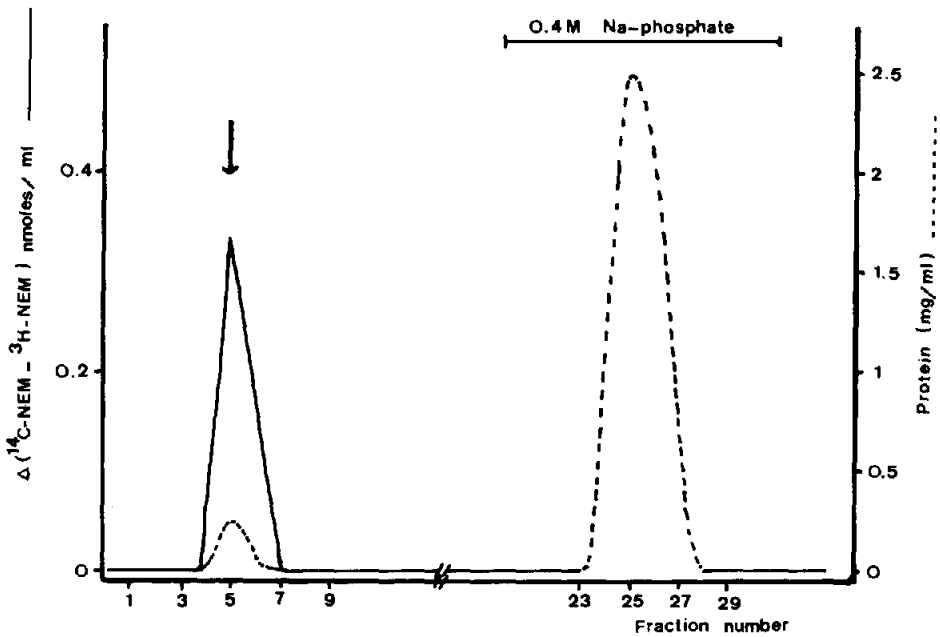

Fig.5. Purification of an ADP–NEM-sensitive protein on hydroxyapatite. Mitochondria (100 mg in 10 ml Tris–sucrose buffer) were treated with non-radioactive NEM (16 nmol/mg protein). After two washings, the mitochondria were resuspended in 9 ml Tris–sucrose buffer and subdivided into two fractions:

(i) 4 ml were mixed with 4 ml ETK buffer containing 0.2 mM [^{14}C]NEM (without ADP).

(ii) 4 ml were mixed with 4 ml ETK buffer containing 80 μM ADP and 0.2 mM [^{3}H]NEM.

After 2.75 min, NEM reaction was stopped by cystein and the two fractions were mixed and centrifuged. After washing, subparticles were obtained (see Materials and methods). Subparticles were then solubilised and fractionated according to [27]. Subparticles were treated by 4% Triton X-100, 0.5 M NaCl, and after centrifugation (130 000 × *g*, 45 min) 2 ml supernatant was applied to a 15 × 1 cm hydroxyapatite column equilibrated in 0.5% Triton X-100, 0.1 M NaCl, 10 mM HEPES, pH 7.2, at 4°C. After elution with this buffer, complete elution was performed by 0.4 M Na-phosphate in the same buffer.

3.4. *Purification of the ADP–NEM-sensitive protein on hydroxyapatite*

The method used for the purification of the ADP–NEM-sensitive protein was that [27] used for the purification of the carboxyatractylate-binding protein.

A double labeling procedure was used. Mitochondria were labeled with [^{14}C]NEM (16 nmol/mg protein) in the absence of ADP and with [^{3}H]NEM (16 nmol/mg protein) in the presence of ADP in conditions described in fig.4A. The two samples were then mixed and subparticles prepared.

The subparticles were solubilised and fractionated on a hydroxyapatite column as described in the legend to fig.5.

Increased radioactivity was observed in the first peak eluted after addition of the proteins (fraction No. 5, fig.5). In contrast there was no increased radioactivity in the fraction resulting from elution by 0.4 M Na-phosphate. On electrophoresis, fraction 5 exhibited one band of mol. wt 30 000–32 000.

4. Discussion

A major NEM- and mersalyl-sensitive protein component has been partially purified from subparticles of pig heart mitochondria in presence of SDS and fractionation on an ultrogel column. It has mol. wt 30 000–32 000.

An ADP–NEM-sensitive subparticular protein has been purified by solubilisation with Triton X-100 and fractionation on a hydroxyapatite column. It also has mol. wt 30 000–32 000.

At least two hypotheses can be suggested to explain our data:

(1) There is a pool of NEM-sensitive subparticular proteins, mol. wt 30 000–32 000 (not separated by our electrophoresis method), including proteins involved in the phosphate transport system and proteins involved in nucleotide translocation.

(2) A major protein (mol. wt 29 000) at the outer face of the inner mitochondrial membrane has been identified [29]. This protein was found to be identical to the carboxyatractylate binding protein [28] and to the ADP–NEM-sensitive protein [21]. It may also be identical to the protein involved in the phosphate transport system.

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