DISCRIMINATION BETWEEN THE *N*-ETHYLMALEIMIDE MERSALYL-SENSITIVE PROTEIN(S) AND THE NUCLEOTIDE TRANSLOCATOR IN PIG HEART MITOCHONDRIA

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

Received 26 September 1980

1. Introduction

The protection of mercurials against *N*-ethylmaleimide has allowed the identification of a group of M_r 3.0–3.2 × 10^4 proteins present in the internal membrane of mitochondria [1–6]. These proteins have also been located using radioactively labelled mercurials [4,7]. Pig heart mitochondria were shown [4] to contain these proteins, including nucleotide translocase, by the fixation of NEM in the presence of ADP. Electrophoretic separation of a protein sensitive to mersalyl and *N*-ethylmaleimide from nucleotide translocase was demonstrated in [8]. The results presented here:

(i) Show that the phenomenon of protection preserves all the essential properties of phosphate transport, and notably its activation or its inhibition by ionophorous antibiotics.
(ii) Confirm that it is possible to make a distinction between the mersalyl *N*-ethylmaleimide-sensitive protein and nucleotide translocase.
(iii) Show that the mixture of these two proteins can be purified on hydroxyapatite.

2. Materials and methods

2.1. *Isolation of mitochondria*

Pig heart mitochondria were prepared according to [9] substituting 10 mM Tris–HCl buffer for the 10 mM phosphate buffer. Proteins were determined by the biuret method [10].

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

* To whom reprint requests should be addressed

2.2. *[^{32}P]Phosphate uptake*

Mitochondrial suspension (1 mg in 100 μl Tris–sucrose buffer) was mixed with 100 μl of a medium containing 0.25 M sucrose, 10 mM Hepes, 12 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA. Phosphate transport was then initiated by adding [^{32}P]phosphate (0.5 mM) and stopped 10 s later with mersalyl (20 nmol/mg protein). Mitochondria were precipitated in an Eppendorf 3200 centrifuge. The pellet was solubilised in 200 μl formic acid and the radioactivity measured in a liquid scintillation counter (Intertechnique, France). Controls were made by adding mersalyl before phosphate. In each experiment, extra-mitochondrial space was measured with [^{14}C]sucrose.

2.3. *Mitochondrial fractionation*

Mitochondria were treated by [^{3}H]NEM with or without effector and the reaction was stopped with cysteine according to [4]. Mitochondria were fractionated as in [11].

2.4. *Electrophoresis of proteins*

SDS gel electrophoresis was done as in [12]. Gels were cut in 2 mm slices and solubilised in 200 μl H_2O_2 for 10 h at 60°C for radioactivity. Standard proteins were labelled with [^{14}C]anhydride acetic as internal standard (M_r: bovine serum albumin, 68 000; egg albumin, 45 000; glyceraldehyde 3-P-dehydrogenase, 36 000; chymotrypsinogen, 25 700; trypsin inhibitor, 20 000; hemoglobin, 16 000).

2.5. *Purification on hydroxyapatite*

Submitochondrial particles were treated with 4% Triton X-100, 0.5 MNaCl and after centrifugation (130 000 × *g*, 45 min) 2 ml supernatant was treated batchwise with hydroxyapatite at 4°C for 15 min and centrifuged. The resulting supernatant was analysed by electrophoresis.

Elsevier/North-Holland Biomedical Press

3. Results

3.1. *Protective effect of mersalyl against the inhibition of phosphate transport by NEM in mitochondria*

The protective effect of mersalyl was measured as follows: mitochondria were incubated in the presence of mersalyl, a reversible inhibitor of -SH groups, then in the presence of the inhibitor *N*-ethylmaleimide which gives rise to the formation of covalent bonds with the -SH groups which did not react with mersalyl. The mersalyl was then eliminated by the addition of cysteine, and the mitochondrial phosphate transport activity was measured.

Fig.1 gives the inhibition-protection kinetics in terms of the mersalyl concentration. At 20 nmol mersalyl/mg mitochondrial protein 100% inhibition the entry of $^{32}P_i$ into the mitochondria was observed. The protection was ~70% for the same mersalyl concentration. This graph is analogous to that obtained in phosphate penetration studies by swelling measurement [13]. That protection by mersalyl is limited to 70% could be caused by the mersalyl which is not accessible to cysteine. This observation is consistant in all experiments.

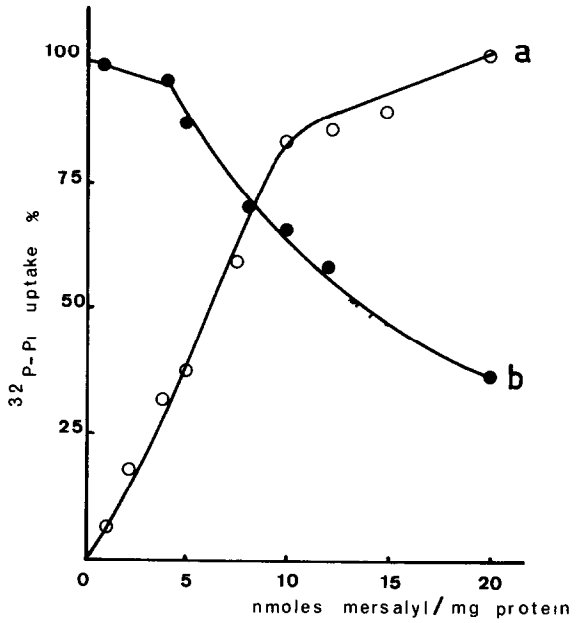

Fig.1. Effect of mersalyl on [^{32}P]phosphate uptake. (a) Inhibition by mersalyl (○): 1 mg mitochondria were incubated as in section 2. Mersalyl was added at different concentrations. Phosphate uptake was initiated by potassium [^{32}P]phosphate (0.5 mM) and stopped by 20 nmol mersalyl 10 s later. (b) Protection by mersalyl against NEM inhibition (●). Mitochondria were preincubated with 12.5 nmol NEM/mg protein in presence of different concentrations of mersalyl for 1.5 min. Mersalyl was eliminated and NEM reaction stopped by cysteine. Mitochondria were washed and [^{32}P]phosphate uptake measured.

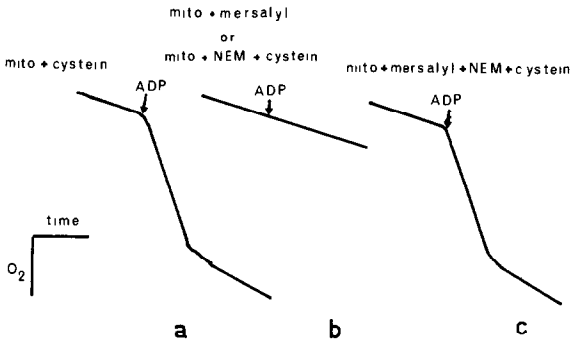

Fig.2. Effect of mersalyl and NEM on respiratory control. Respiratory control of mitochondria was measured in an Hansatech O_2 electrode using 3 mM glutamate as substrate, 5 mM P_i and 0.2 mM ADP. (a) Control; (b) Inhibition in presence of mersalyl (20 nmol/mg protein) or NEM (12.5 nmol/mg protein, 1.5 min); (c) protection by mersalyl (20 nmol/mg protein) against NEM (12.5 nmol/mg protein). The reaction was stopped by cysteine.

Fig.2 shows that mersalyl protects respiratory control. Mersalyl therefore protects the entry of phosphate, which is essential for ATP synthesis. The fixation of mersalyl to block the entry of phosphate, the subsequent addition of *N*-ethylmaleimide and then the elimination of mersalyl did not affect the coupling properties of the mitochondria.

Fig.3 shows the effects of valinomycin and nigericin on the penetration of $^{32}P_i$ into the mitochondria. Test (A) showed that valinomycin stimulated the entry of the phosphate, and nigericin inhibited it. When mitochondria were incubated in the presence of *N*-ethylmaleimide (B), the entry of the phosphate was observed to be inhibited and the ionophoric effects were suppressed. In (C) mitochondria had been preincubated in the presence of mersalyl, then NEM, and the mersalyl then eliminated by cysteine. In these conditions the entry of phosphate was inhibited by ~50%, on the other hand the stimulating effects of valinomycin or the inhibiting effects of nigericin were the same as in the control tests. These ionophores are known to stimulate (valinomycin) or to collapse (nigericin) the transmembrane ΔpH. Our results suggest that mersalyl can protect the control of active phosphate transport by the transmembrane ΔpH.

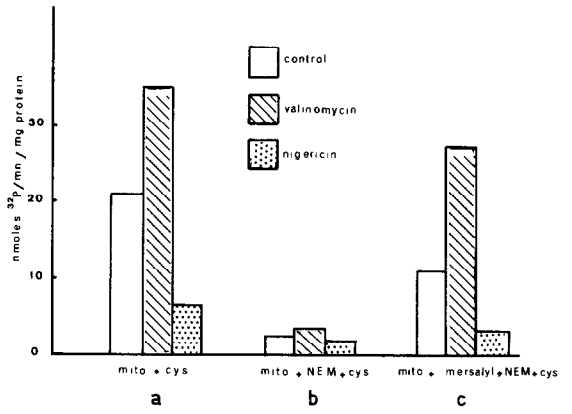

Fig.3. Valinomycin and nigericin effect on [^{32}P]phosphate uptake. (a) [^{32}P]phosphate uptake was measured in presence or absence of valinomycin (12.5 ng/mg protein) or nigericin (12.5 ng/mg protein). Essays were made in presence of cysteine. (b) [^{32}P]phosphate uptake was measured after mitochondria had been treated with NEM (12.5 nmol/mg protein). (c) Mitochondria were incubated in presence of mersalyl (20 nmol/mg protein) and NEM (12.5 nmol/mg protein). NEM reaction was stopped and mersalyl removed by added cysteine. Then [^{32}P]phosphate uptake was measured in presence or absence of antibiotic ionophores.

3.2. *Electrophoretic differentiation of mersalyl* N-*ethylmaleimide-sensitive proteins and nucleotide translocase*

Two types of experiment were carried out using mitochondria:

(A) Nucleotide translocase was identified by the activation of *N*-[^{3}H]ethylmaleimide fixation in the presence of ADP [4,13–15]. These results are given in fig.4A. A peak corresponding to a protein band of M_r 30 000 can be shown. This protein band is located in the internal membrane of mitochondria as shown in fig.5A (band I).

(B) The proteins protected by mersalyl were identified as follows: mitochondria were incubated with mersalyl and non-radioactive *N*-ethylameimide. The mersalyl was then eliminated by the addition of cysteine and after washing the mitochondria, *N*-[^{3}H]ethylmaleimide was added, a parallel test was done without the initial addition of mersalyl. The different protein fractions were analysed by electrophoresis, and the radioactivity was measured in each protein band. The results are given in fig.4B. Mersalyl protects the fixation of [^{3}H]-NEM at the level of the M_r 31 000 and 32 000 proteins (bands II, III).

3.3. *Purification of a mersalyl* N-*ethylmaleimide-sensitive protein and nucleotide translocase*

Mitochondria were fractionated and the submitochondrial particles fraction were solubilised in Triton X-100, proteins I and III could be purified on hydroxyapatite (fig.5B). It must be noted that protein II (M_r 31 000) disappeared at the time of purification

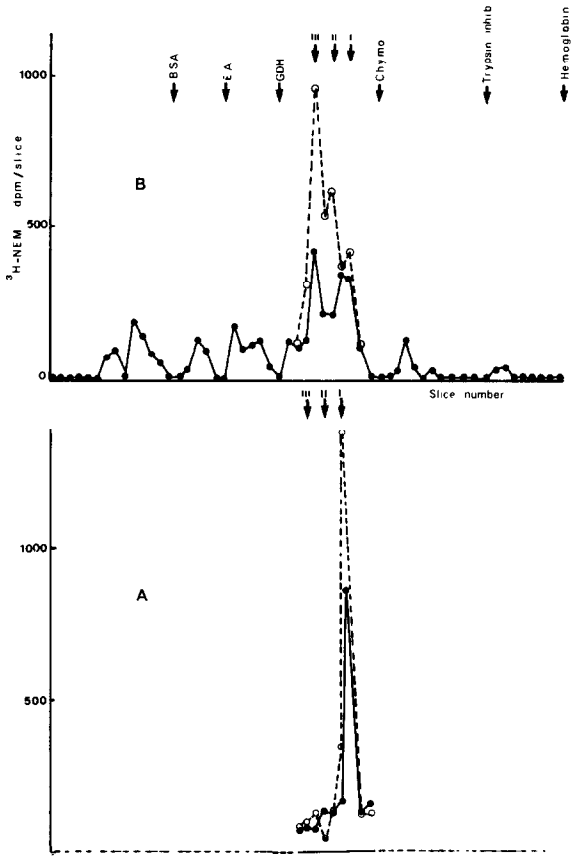

Fig.4. Radioactive analysis of gel electrophoresis of submitochondrial particles. (A) Mitochondria were treated by cold NEM (25 nmol/mg protein, 3 min) then by [^{3}H]NEM (12.5 nmol/mg protein, 3 min) in the presence (0) or absence (•) of ADP (50 μM). Submitochondrial particles were prepared and analysed by electrophoresis. Radioactivity in gel slices was measured. (B) Mitochondria were treated in presence of NEM 12.5 nmol/mg protein without (•) or with (○) mersalyl (20 nmol/mg protein). Then mersalyl was removed by cysteine and mitochondria were incubated in presence of [^{3}H]NEM (12.5 nmol/mg protein). Submitochondrial particles were prepared and analysed as in (A). M_r: I, 30 000; II, 31 000; III, 32 000; bovine serum albumin (BSA), 68 000; egg albumin (EA), 45 000; glyceraldehyde 3-P-dehydrogenase, (GDH) 25 700; chymotrypsinogen (Chymo), 25 700; trypsin inhibitor, 20 000; hemoglobin, 16 000.

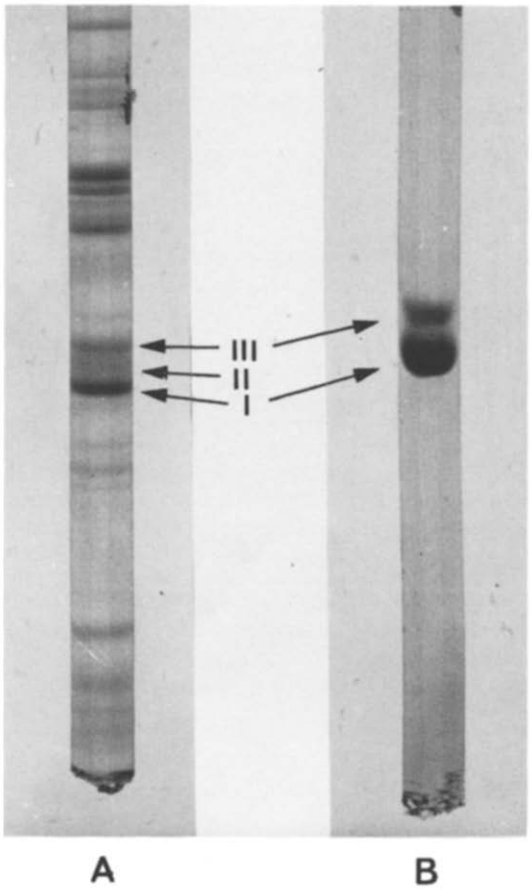

Fig.5. Electrophoretic pattern of subparticles (A) and proteins after hydroxyapatite treatment (B). Mitochondria were treated as in fig.4. Electrophoresis of submitochondrial particles was as in section 2. Submitochondrial particles were solubilized by Triton X-100 and proteins fractionated on hydroxyapatite. (A) Electrophoresis of subparticulate proteins. (B) Electrophoresis of proteins after separation on hydroxyapatite. M_r: I, 30 000; II, 31 000; III, 32 000.

on hydroxyapatite. Moreover, the relative concentration of protein III compared with protein I (nucleotide translocase) can vary between one preparation and another.

4. Discussion and conclusion

In the internal membrane of pig heart mitochondria there are 3 proteins of similar M_r. One protein of M_r 30 000, (considering activation of the fixation of *N*-ethylmaleimide by ADP) corresponds to nucleotide translocase: its purification and isolation in other types of mitochondria had been carried out and the transport system was reconstituted [16,17]. The proteins of M_r 31 000 and 32 000 (considering the protection of specific -SH groups by mersalyl against *N*-ethylmaleimide) were identified by the electrophoresis of proteins in the internal membrane of mitochondria. We cannot exclude that protein II (M_r 31 000) results from partial proteolysis of protein III. The M_r 32 000 protein can be purified on hydroxyapatite at the same time as the nucleotide translocase. The relationship of these proteins with phosphate transport is suggested by two types of results:

(1) Mersalyl is shown to protect the entry of phosphate and the control of this transport system by the transmembrane ΔpH against *N*-ethylmaleimide.

(2) In [18] phosphate transport was reconstituted with a mixture of two proteins (the proteins protected by mersalyl and nucleotide translocase) obtained on a hydroxyapatite column, as in this case.

The separation and isolation of the two proteins is in progress.

Acknowledgement

This work was supported in part by DGRST (convention 79.7.1032).

References

[1] Coty, W. A. and Pedersen, P. L. (1975) J. Biol. Chem. 250, 3515–3521.
[2] Briand, Y., Touraille, S., Debise, R. and Durand, R. (1976) FEBS Lett. 65, 1–7.
[3] Hadvary, P. and Kadenbach, B. (1976) Eur. J. Biochem. 67, 573-581.
[4] Touraille, S., Briand, Y. and Durand, R. (1977) FEBS Lett. 84, 119-124.
[5] Wohlrab, H. (1978) Biochem. Biophys. Res. Commun. 83, 1430–1435.
[6] Alziari, S., Touraille, S., Briand, Y. and Durand, R. (1979) Biochimie 61, 891–903.
[7] Hofmann, H. D. and Kadenbach, B. (1979) Eur. J. Biochem. 102, 605–613.
[8] Wohlrab, H. (1979) Biochemistry 18, 2098–2102.
[9] Crane, F. L., Gleen, J. F. and Green, D. E. (1976) Biochim. Biophys. Acta 22, 475–487.
[10] Gornall, A. G., Bardawill, L. H. and David, M. N. (1949) J. Biol. Chem. 177, 751–766.

[11] Fessenden, J. M. and Racker, E. (1967) Methods Enzymol. 10, 194–197.
[12] Laemmli, U. K. (1970) Nature 227, 680–685.
[13] Leblanc, P. and Clauser, H. (1972) FEBS Lett. 23, 107–113.
[14] Vignais, P. M., Chabert, J. and Vignais, P. V. (1974) in: Biomembrane structure and function, FEBS Symp. 35 (Gardos, G. and Szasz, I. eds) Akademiai Kiado, Budapest.
[15] Riccio, P. Aquila, H. and Klingenberg, M. (1975) FEBS Lett. 56, 133–138.
[16] Kramer, R. and Klingenberg, M. (1977) FEBS Lett. 82, 363–367.
[17] Kramer, R. and Klingenberg, M. (1977) Biochemistry 16, 4954–4961.
[18] Wohlrab, H. (1980) 1st Eur Bioenergetics Conf. pp. 301–302, Patron Editore, Bologna.