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A 28 kDa mitochondrial protein is radiolabelled by crosslinking with a ^{125}I-labelled presequence

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A 13-residue peptide containing the first 12 amino acids of the N-terminal part of the signal sequence of yeast cytochrome *c* oxidase subunit IV is shown by chemical crosslinking to interact with a mitochondrial protein. This result is obtained with mitochondria from four different origins. Submitochondrial localization experiments suggest that the 28 kDa labelled component is present on the outer face of the inner membrane. Since such addressing peptides are imported into mitochondria through the same machinery as protein precursors, the 28 kDa protein might be a component of the translocation apparatus.

Addressing sequence; Mitochondria; Crosslinking

1. INTRODUCTION

Most of the nuclear-encoded mitochondrial proteins are synthesized as precursors containing N-terminal extensions that direct them to the correct mitochondrial compartment [1,2]. These signal sequences lack acidic amino acids, are rich in positively charged residues and show a tendency to fold in an amphiphilic α-helix. When such sequences are attached to cytosolic proteins, the chimeric proteins are imported into mitochondria. More recently, it has been shown that peptides possessing such sequences are imported into mitochondria and that they block the import of homologous or heterologous precursors [3–6].

We have recently shown that a peptide of 13 amino acids containing the first twelve residues of the N-terminal part of the signal sequence of yeast cytochrome *c* oxidase subunit IV (peptide M) blocks an ionic channel of mitochondrial outer membrane [7]. This channel is different from the classical VDAC since it is cationic, and since it is present in porin-deficient yeast mutants [8]. Electrophysiological data are consistent with the translocation of the peptide M through the cationic channel. This observation may be interesting since it has recently been suggested that precursor proteins may be imported into mitochondria through a hydrophilic pore [9]. In the present communication, the same 13-residue peptide is used to identify by a crosslinking approach the molecular components interacting with this peptide and to determine their possible relationship with the cationic channel.

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Abbreviations: peptide M, MLSLRQSIRFFKY; EGS, ethyleneglycol bissuccinimidyl succinate; ClCCP, carbonylcyanide *m*-chlorophenylhydrazone

2. MATERIALS AND METHODS

2.1. *Materials*

Peptide M, synthesized by Appligène (Strasbourg, France) was labelled by solid phase oxidation using Iodogen (Pierce) and Na^{125}I [10]. Mitochondria from rabbit heart, rat liver and bovine adrenal cortex were isolated in 0.25 M sucrose/1 mM EGTA/10 mM Hepes, pH 7.4 [7,11]; mitochondria from *Schizosaccharomyces pombe* were kindly supplied by Dr J.M. Jault (Villeurbanne). The microsomal pellet from rat liver was obtained by centrifugation of a post-mitochondrial supernatant at 100 000 × *g* for 1 h.

2.2. *Cross-linking experiments*

To mitochondria (200 μg of protein in 0.3 ml or 0.25 M sucrose/10 mM Hepes, pH 7.4) preincubated for 1 min at room temperature with 10 μM ^{125}I-labelled peptide M, EGS (0.5 mM) was added and the mixture was incubated at the same temperature for 1 min. The reaction was stopped by addition of 10 mM glycine (final concentration). To remove free peptide, the mixture was centrifuged for 5 min at 15 000 × *g*, the pellet was resuspended in 0.85 ml of the same buffer and centrifuged again under the same conditions. The washed pellet was analysed by SDS-PAGE (10% polyacrylamide). After electrophoresis, gels were stained with Coomassie blue, dried and autoradiographied for 10–20 days at −70°C with an intensifying screen.

2.3. *Other methods*

Digitonin treatment was performed as in [11]. For trypsin treatment, mitochondria (200 μg of protein) were incubated with 50 μg of trypsin (Sigma, type IX) for 30 min at 0°C, in 0.3 ml of 10 mM Hepes

buffer, pH 7.4, with or without 0.25 M sucrose. The reaction was stopped by addition of 150 μg soybean trypsin inhibitor (Sigma), centrifugation and resuspension of the pellet in 0.3 ml of sucrose buffer containing 0.5 mM PMSF. Adenylate kinase, hexokinase, malate deshydrogenase and protein were assayed as indicated in reference [11].

3. RESULTS

3.1. *Identification of mitochondrial proteins interacting with the signal peptide*

Incubation of bovine adrenal cortex mitochondria with ^{125}I-labelled peptide M, followed by stabilization of the interacting components by crosslinking with EGS labels a band with an apparent molecular mass of 30 kDa (arrow in Fig. 1, lane a). In addition, some high molecular mass material is usually found at the top of the stacking and separating gels which may indicate highly crosslinked proteins. Assuming that one mol of peptide is bound to one mol of protein in the presence of the crosslinking agent, the apparent molecular mass of the protein is estimated to be 28 kDa (molecular mass of the iodinated peptide plus crosslinking reagent: 2 kDa).

The 30 kDa labelled protein is not observed in the absence of EGS (Fig. 1A, lane c). On the other hand, the labelling is not affected by the addition of an excess of NaI to the incubation mixture (Fig. 1A, lane b). Such control experiments are important since when the experiment is conducted with a high specific activity ^{125}I-labelled peptide M, obtained by iodination at nanomolar peptide concentration range, with high [^{125}I]specific activity, two additional components of 44 and 54 kDa are labelled; however, these two labelled bands are visible even in the absence of EGS. With the experimental procedure used, excess free iodine is not separated from the peptide and these bands may result from an iodination of proteins, catalysed by mitochondrial peroxidase. At variance with the 28 kDa component (see below), the labelling of these components does not decrease in the presence of a 4000 molar excess of unlabelled peptide.

The specificity of the interaction of the peptide is tested in two different ways. First, no labelling is observed when the experiment is repeated with a microsomal fraction (Fig. 1A, lane d). Secondly, incubation in the presence of a 20-fold excess of cold peptide decreases markedly the labelled material (Fig. 1B, lane b) compared to control (Fig. 1B, lane a), thus indicating that the 28 kDa protein is a saturable site. The interaction revealed by chemical crosslinking is independent of the mitochondrial membrane potential, since the labelling is not affected by the addition of ClCCP (Fig. 1C, lane a).

The same type of interaction is also detected in other mitochondria from different sources. A 30 kDa component is heavily labelled under the same experimental conditions in rabbit heart mitochondria (Fig. 2, lane a); the labelling is EGS-dependent (Fig. 2, lane b). A band

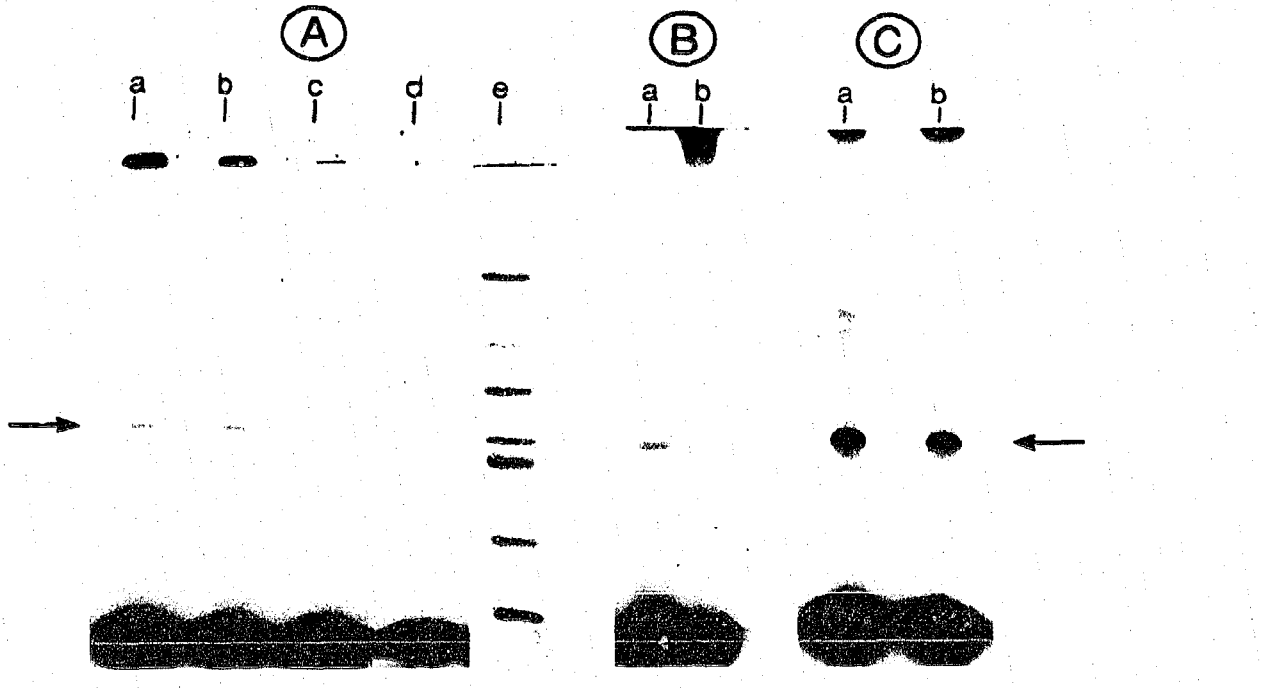


Fig. 1. Crosslinking of ^{125}I-labelled peptide M to adrenal cortex mitochondria. (A) Incubations have been performed with (lanes a, b, d) or without (lane c) 0.5 mM EGS, on mitochondria (lanes a–c) or microsomes (lane d). In lane b, 2 mM NaI was added before crosslinking. Lane e, molecular mass markers (66, 45, 36, 29, 24, 20.1 and 14.2 kDa). (B) Effect of 200 μM unlabelled peptide M (lane b); lane a, control at 10 μM ^{125}I-labelled peptide M. (C) Crosslinking in the presence (lane a) or in the absence (lane b) of 3.3 μM ClCCP.

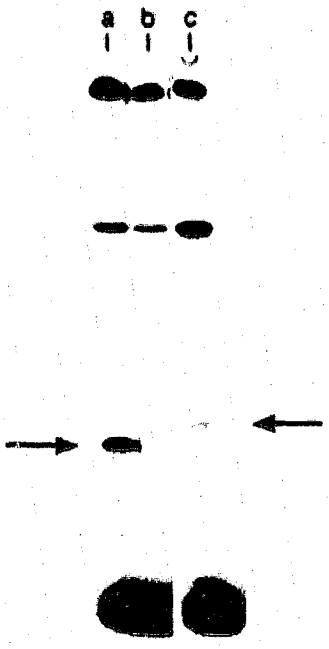
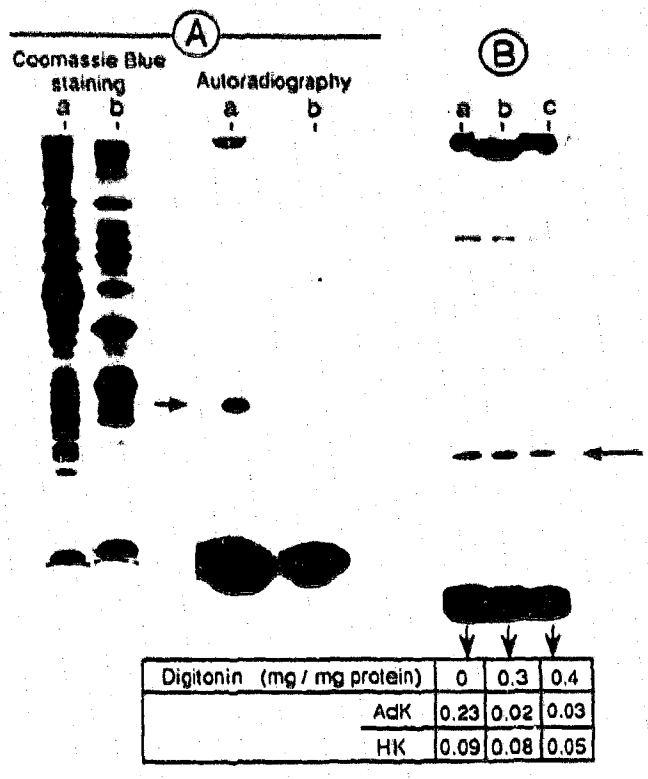


Fig. 2. Labelling of mitochondria from different origins. Rabbit heart mitochondria in the presence (lane a) or in the absence (lane b) of EGS; *Schizosaccharomyces pombae* mitochondria (lane c).



Digitonin (mg / mg protein)	0	0.3	0.4
AdK	0.23	0.02	0.03
HK	0.09	0.08	0.05

Fig. 3. Submitochondrial localization of the labelled protein. (A) Coomasie blue staining and autoradiography of the proteins from adrenal cortex whole mitochondria (lanes a) or outer mitochondrial membranes (lanes b) crosslinked with ^{125}I-labelled signal peptide. (B) Effect of digitonin treatment on the presence of the labelled 30 kDa band in the mitochondrial pellet, and comparison with its effect on the specific activities of adenylate kinase (AdK) and hexokinase (HK). Mitochondria from rabbit heart are treated with the indicated amount of digitonin as described in Materials and Methods. After centrifugation, enzymatic activities are estimated in an aliquot of the resuspended pellet and crosslinking experiments are performed with another aliquot as described in Materials and Methods. Specific activities are expressed in U/mg protein.

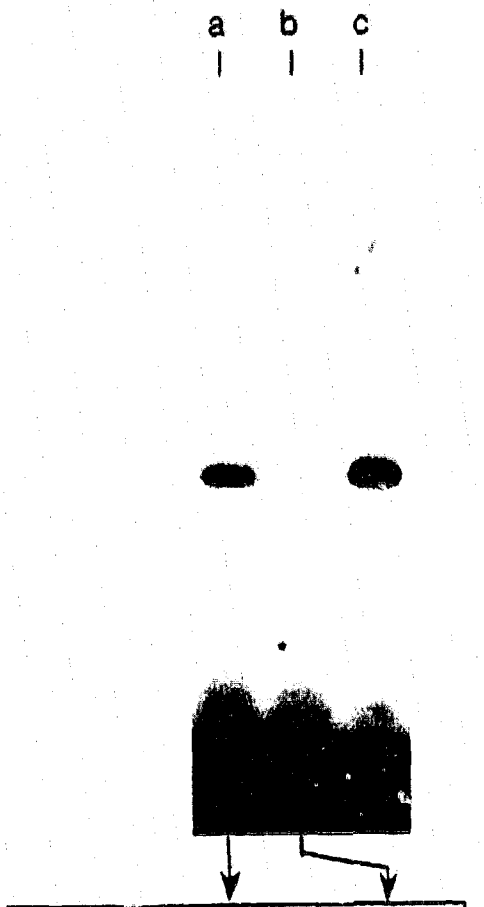
of similar molecular mass, but of weaker intensity is also found in rat liver mitochondria (data not shown). In mitochondria from *Schizosaccharomyces pombæ*, the same procedure labels a component with a molecular mass of 33 kDa (Fig. 2, lane c). The experiments described thereafter are performed with mitochondria prepared either from adrenal cortex or from heart.

3.2. *Submitochondrial localization of the labelled protein*

In a first series of experiments, the labelling procedure is applied to submitochondrial fractions. Outer membranes from adrenal cortex mitochondria are separated by an osmotic shock and purified by centrifugation on discontinuous gradients [12]. No 30 kDa ^{125}I-labelled component can be detected when this material is incubated with [^{125}I]peptide M and EGS (Fig 3A, lane b). In contrast, mitoplasts derived from the same material are still labelled (data not shown). The failure encountered with the outer membrane preparation does not originate in the absence of a transmembrane potential in these vesicles sice mitochondria labelling is not affected by ClCCP addition (Fig. 1C, lane a).

In a second type of experiment, digitonin is used to remove the outer membrane (Fig. 3B). Increasing the detergent concentration releases the intermembrane marker, adenylate kinase, and then solubilizes 45% of the outer membrane hexokinase, without any decrease of the 30 kDa component labelling.

In a third type of experiment, advantage is taken of the fact that the 30 kDa labelled component is sensitive to trypsin. Crosslinking experiments are performed on mitochondria trypsinized in media which are either isotonic or hypotonic (Fig. 4). The isotonic treatment does not affect the outer membrane integrity, since only 7.5% of the adenylate kinase is sensitive to trypsin under these conditions; the 30 kDa component is clearly labelled after this treatment (Fig. 4, lane a). On the other hand, when mitochondria are incubated with trypsin under hypotonic conditions, 74% of the adenylate kinase activity is proteolysed without any parallel inhibition of malate dehydrogenase (a matrix enzyme marker which is 83% inhibited in hypotonic medium only in the presence of 0.008% Lubrol), thus indicating that the outer but not the inner membrane is ruptured, and that the intermembrane space is accessible to trypsin; under these conditions, the 30 kDa component almost totally disappears or gives rise to lower molecular mass fragments (Fig. 4, lane b).



Medium	0.25 M sucrose	10 mM Hepes
AdK	7.5	74
MDH	3.6	0

Fig. 4. Effects of trypsin on the labelled protein. Mitochondria (200 μg) are incubated with trypsin, either in 0.25 M sucrose/10 mM Hepes, pH 7.4 (lane a), or 10 mM Hepes, pH 7.4 (lanes b and c). The reaction is stopped by addition of trypsin inhibitor; enzymatic activities, adenylate kinase (AdK) and malate dehydrogenase (MDH) are measured in aliquots whereas the mitochondrial pellet from another aliquot is used for crosslinking experiments. The table indicates the percentage of inhibition of the enzymatic markers. Lane c, control experiment without trypsin.

4. DISCUSSION

Crosslinking agents are interesting tools to reveal protein/protein interaction because they induce covalent intermolecular links between interacting molecules. However, experimental conditions have to be carefully controlled to prevent unspecific crosslinking by random collisions. As reported by Middaugh et al. [13], collisional crosslinking increases at high protein concentration and for long periods of incubation. Therefore, in the present experiments we have used low protein concentrations (≤0.7 mg/ml) and a short time of incubation with the crosslinking agent (1 min); significant label is still detected for a shorter incubation time (15 s, data not shown).

Under these experimental conditions, and with mitochondria from four different origins, we observe the specific interaction of only one protein which the labelled signal peptide corresponding to the first 12 residues of the N-terminal part of the precursor of the cytochrome oxidase subunit IV. Similar results have been reported by Gillespie [14] using heart mitochondria and a different synthetic peptide (amino acids 1-27 of pre-ornithine carbamyl transferase, an enzyme which is localized in the matrix space). In adrenal cortex and heart mitochondria, we label a protein with an apparent molecular mass of 28 kDa, similar to the 30 kDa described by Gillespie. The peptide is bound to saturable sites and this interaction requires a well-defined conformation of the binding protein since when mitochondrial proteins are isolated by electrophoresis under denatured conditions and transferred to nitrocellulose, no specific interaction can be detected by a ligand blotting method (data not shown). The labelled 28 kDa protein is likely to be a mitochondrial protein since: (i) it is not present in microsomes (Fig. 1A, lane d), and (ii) it is sensitive to trypsin only under hypotonic conditions (Fig. 4, lane b) and thus it is unlikely to be a contaminant adsorbed on mitochondrial surface.

The labelled protein appears to be membrane bound and not to be located on the outer membrane since: (i) the band is not revealed on isolated outer mitochondrial membrane (Fig. 3A, lane b); (ii) the labelling is not significantly reduced in digitonin treated mitochondria (Fig. 3B, lanes b and c); (iii) the labelled band is hydrolysed by trypsin only in hypotonic medium (Fig. 4, lane b). From our data, a localization on the outer face of the inner membrane seems to be the most likely hypothesis.

This localization disagrees with the results of Gillespie [14] which suggested a localization of the 30 kDa component on the outer membrane. This discrepancy might originate in a loose control of the integrity of the outer membrane in her experiments. It is also possible that two different proteins are labelled. Since the two bifunctional reagents used have different hydrophobicity and thus might have access to different proteins, the agent used in our experiments might be accessible to more internal proteins than that previously used, which is membrane impermeant.

In previous experiments on adrenal cortex mitochondria, we showed the existence in this organelle of a new cationic channel. The electrical activity of this channel is blocked by peptide M [7] and by ^{125}I-labelled peptide (data not shown). However, the localization of the 28 kDa protein rules out any structural relationship with the cationic channel which has recently been located on the outer membrane [15]. However, the present data in-

dicate that peptide M crosses the outer membrane. A possible role of ionic channels, such as the cationic channel, will have to be evaluated in this respect. It may be noted that the import of several peptides into the mitochondrial matrix has recently been demonstrated, including the presequence of ornithine aminotransferase (34 residues) [4], cytochrome *c* oxidase subunit IV (22 residues) [5], aldehyde dehydrogenase (22 residues) and ornithine transcarbamylase (27 residues) [6]. The import is specific since other unrelated sequences are not imported and, for the three last peptides, the translocation is not potential-dependent.

Finally, is the 28 kDa protein part of the import machinery? The present data show only the interaction of a signal peptide with a protein of the inner membrane. However, similar observations have been made, describing the interactions of addressing peptides [16] with the inner membrane [6,15]. This binding inhibits the translocation of precursor proteins at a step common to the import pathway of many different precursors. It is thus tempting to speculate that this 28 kDa protein is a component of the import machinery.

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