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Mitochondrial preproteins en route from the outer membrane to the inner membrane are exposed to the intermembrane space

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Mitochondrial precursor proteins are known to be imported at sites of close contact between mitochondrial outer and inner membranes. We have identified translocation intermediates exposed to the intermembrane space, including the precursor of the ADP/ATP carrier accumulated at the general insertion site GIP, and the precursor of F_1-ATPase subunit β accumulated on its import pathway at low levels of ATP. These results suggest that mitochondrial contact sites are not sealed structures, but that polypeptides pass (at least partly) through the intermembrane space on their route from the outer membrane to the inner membrane.

Mitochondria; Protein translocation; Intermembrane space; Contact site

1. INTRODUCTION

The typical biogenesis pathway of nuclear-encoded proteins of the mitochondrial inner membrane or matrix includes the following steps [1–3]: (i) synthesis on cytosolic polysomes; (ii) recognition by receptor proteins on the outer membrane surface; (iii) insertion into the outer membrane at the general insertion site GIP; (iv) membrane potential-dependent insertion into the inner membrane; (v) interaction with the heat shock protein hsp70 in the matrix (mt-hsp70); (vi) proteins carrying amino-terminal presequences are proteolytically cleaved by the processing peptidase in the matrix. Translocation intermediates of preproteins could be accumulated in a 2-membrane-spanning fashion, indicating that at the import sites the 2 mitochondrial membranes were in such close contact that they could be spanned by a single polypeptide chain [4–11]. These results suggested that mitochondrial contact sites were the major site for the import of preproteins.

Unexpectedly, import receptors and components of GIP were not exclusively located in the morphological contact sites, but distributed over the entire outer membrane [12–16]. Moreover, some precursor proteins could be directly translocated across the inner membrane without a requirement for receptors or GIP [17,18] (B. Ségui-Real, J.R., W. Neupert and N.P., in preparation). These findings raised the possibility that the protein import sites in the outer and inner membranes could also be located outside translocation contact sites and may be able to function independently of each other. We thus asked if, in intact mitochondria, polypeptides get access to the aqueous intermembrane space while being transported from the outer membrane to the inner membrane. We analysed the import pathways of 2 abundant mitochondrial proteins, the inner membrane protein ADP/ATP carrier and the subunit β of the F_1- ATPase ($F_1\beta$), a protein located on the matrix side of the inner membrane. We indeed found that precursor polypeptides passed at least partly through the intermembrane space, leading to a dynamic model of the formation and function of the mitochondrial protein import machinery.

Abbreviations: $\Delta\Psi$, membrane potential; GIP, general insertion site ('general insertion protein'); $F_1\beta$, F_1-ATPase subunit β; mt-hsp70, heat shock protein of 70 kDa in the mitochondrial matrix.

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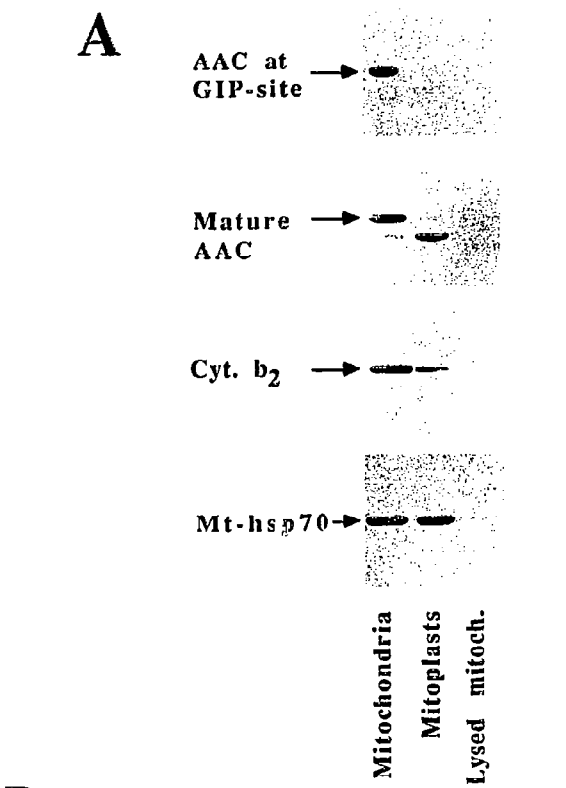
2. MATERIALS AND METHODS

Published procedures were used for: (i) isolation of mitochondria from *Saccharomyces cerevisae* and *Neurospora crassa* [19–22]; (ii) synthesis of precursor proteins in rabbit reticulocyte lysate in the presence of [^{35}S]methionine and import into isolated mitochondria at 25°C [11,23,24]; (iii) generation of the GIP-intermediate of the ADP/ATP carrier in the absence of a membrane potential [11,23]; (iv) pretreatment of mitochondria and reticulocyte lysate with apyrase at 0°C and 25°C, respectively [11,20]; (v) swelling of *S. cerevisiae* mitochondria in 150 mM sorbitol [19,22] and fractionation of *N. crassa* mitochondria with digitonin [21]; (vi) treatment with trypsin and proteinase K [11,23]; (vi) analysis by SDS-polyacrylamide gel electrophoresis, fluorography and laser densitometry [11].

3. RESULTS AND DISCUSSION

The import pathway of the ADP/ATP carrier can be experimentally divided into several sequential steps:



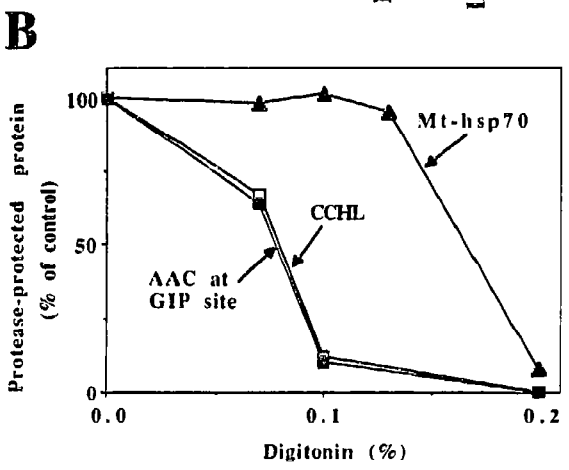


Fig. 1. The GIP-intermediate of the ADP/ATP carrier is exposed to the intermembrane space. (A) Reticulocyte lysate containing ^{35}S-labeled ADP/ATP carrier (AAC) was incubated with isolated *S. cerevisiae* mitochondria in the absence of a membrane potential to form the GIP-intermediate [11,23]. The mitochondria were re-isolated and divided into 3 aliquots: control mitochondria (no treatment); mitoplasts (generated by incubation in 150 mM sorbitol); and lysed mitochondria (1% Triton X-100). All samples were treated with trypsin (15 μg/ml) and analysed as described in Materials and Methods. The endogenous ADP/ATP carrier, cytochrome b_2 (Cyt.b_2) and mt-hsp70 were analysed by immunodecoration. (B) ^{35}S-labeled ADP/ATP carrier was accumulated at the GIP-site of *N. crassa* mitochondria [11]. The re-isolated mitochondria were incubated in digitonin [21,22], treated with proteinase K (10 μg/ml) and analysed as described in Materials and Methods. Endogenous cytochrome *c* heme lyase (CCHL) and mt-hsp70 were analysed by immunodecoration. The amount of protease-protected protein in intact mitochondria was set to 100%. The GIP-intermediate was fully membrane-associated also after opening of the intermembrane space, both with *S. cerevisiae* and *N. crassa*.

binding to surface receptors, insertion into the outer membrane at the GIP-site, and $\Delta\Psi$-dependent insertion into the inner membrane [14–16,23,25,26]. The GIP-intermediate was formed by incubating the precursor, synthesized in rabbit reticulocyte lysate in the presence of [^{35}S]methionine, with isolated mitochondria in the presence of ATP, but in the absence of a membrane potential. By re-establishing $\Delta\Psi$, the precursor arrested at this intermediate stage was completely imported into the inner membrane and correctly assembled [6,23,25].

The GIP-intermediate was protected against protease added to intact mitochondria [6,23,25]. To analyse if the GIP-intermediate was exposed to the intermembrane space, the intermembrane space of *S. cerevisiae* mitochondria was opened by mild swelling, leading to the formation of 'mitoplasts' (Fig. 1A; cytochrome b_2 as marker for the intermembrane space). The GIP-intermediate remained associated with the mitochondrial membranes (Fig. 1, legend), but was now accessible to protease (Fig. 1A). The mitochondrial inner membrane remained fully intact as evidenced by the protection of mt-hsp70. After opening of the inner membrane, mt-hsp70 was digested by protease (Fig. 1A). The mature ADP/ATP carrier in the inner membrane exposes only a few parts to the intermembrane space [27]. After opening of the intermembrane space, only a small fragment of 2–3 kDa was cleaved off from mature ADP/ATP carrier; complete digestion occurred after lysis of the inner membrane (Fig. 1A). Since the GIP-intermediate was degraded from the intermembrane space-side without the formation of fragments that could be resolved on the SDS-polyacrylamide gel (fragments were also not found in the supernatant), major parts of this ADP/ATP carrier-intermediate were apparently accessible from the intermembrane space-side. To substantiate this conclusion with a different organism, we used mitochondria from *N. crassa*. The intermembrane space was opened by the standard procedure established for *N. crassa* mitochondria, i.e. the use of low concentrations of digitonin [21,22]. Fig. 1B shows that the protease-accessibility of the GIP-intermediate correlated with that of cytochrome *c* heme lyase, a marker protein for the intermembrane space [28].

Import of proteins into the mitochondrial matrix requires the ATPase mt-hsp70 [29–31]. We tested if a lowering of the levels of ATP led to an import intermediate exposed to the intermembrane space. The in vitro import system was pretreated with apyrase (an ATPase and ADPase) and oligomycin was included to inhibit the mitochondrial F_0F_1-ATPase [6,20]. The precursor of $F_1\beta$ was then imported in the presence of $\Delta\Psi$ (Fig. 2B). $F_1\beta$, which was accumulated within mitochondria in a protease-protected location, became accessible to protease after opening of the intermembrane space (Fig. 2B, 0.1% digitonin). After addition of ATP, this low-ATP intermediate was fully imported into the mitochondrial matrix and was digested by protease only

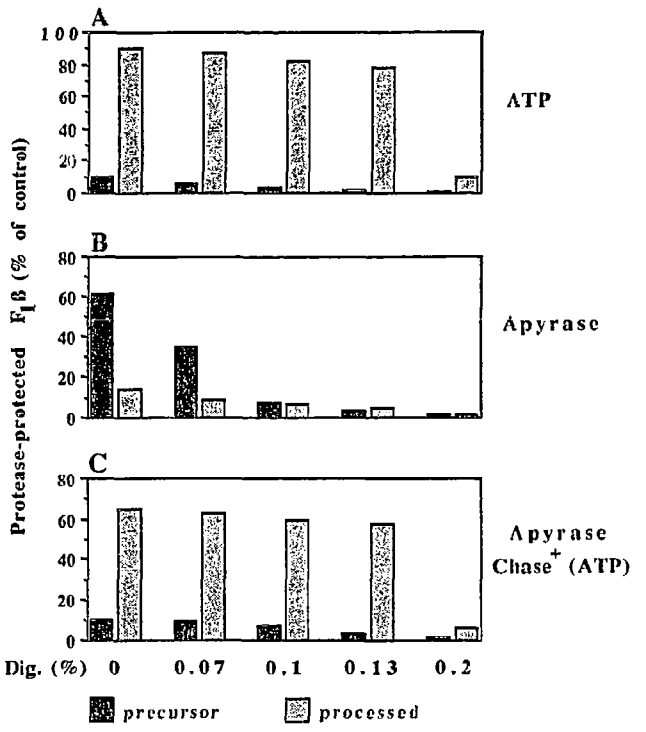


Fig. 2. Accumulation of a $F_1\beta$-intermediate spanning the inner membrane and exposed to the intermembrane space. Reticulocyte lysate containing ^{35}S-labeled $F_1\beta$ was incubated with isolated *N. crassa* mitochondria in the presence of a membrane potential [11]; (A) standard conditions (plus ATP); (B) and (C) low ATP (pretreatment of reticulocyte lysate and mitochondria with 1 U/ml apyrase [11]). All samples were then treated with trypsin (15 μg/ml). The samples of (C) received 3 mM Mg-ATP and were incubated for 10 min at 25°C (chase). The mitochondria of all samples were re-isolated and incubated in digitonin (Dig.) as indicated [21], followed by a treatment with proteinase K (15 μg/ml). Dissipation of the membrane potential [11] did not inhibit the chase. The protease-protection of the marker proteins CCHL and mt-hsp70 was as shown in Fig. 1B (deviations<5%). The total amount of protease-protected $F_1\beta$ (precursor + processed) under control conditions (A, no digitonin) was set to 100%.

after disruption of the inner membrane (Fig. 2C, 0.2% digitonin). This chase confirms that a true translocation intermediate of $F_1\beta$ had been accumulated at low levels of ATP. The chase was also possible when the membrane potential had been dissipated, indicating that the $\Delta\Psi$-dependent interaction of the preprotein with the inner membrane had already taken place at the low ATP levels. By further lowering the levels of ATP (with a higher concentration of apyrase), the completion of transport of $F_1\beta$ across the outer membrane was also inhibited [6,11,20]. This is probably due to the strongly reduced activity of mt-hsp70 and the requirement for cytosolic hsp70s [32–34]. In summary, at least 2 steps in the import of $F_1\beta$ depend on ATP, transport across the outer membrane and transport across the inner membrane. At intermediate levels of ATP, the first step is possible, but the complete transport across the inner membrane is inhibited, leading to an intermediate spanning the inner membrane and exposing parts to the intermembrane space.

We report here on import intermediates of mitochondrial preproteins that are exposed to the intermembrane space: (i) ADP/ATP carrier accumulated in the absence of $\Delta\Psi$, but in the presence of ATP (GIP-intermediate); and (ii) $F_1\beta$ accumulated in the presence of $\Delta\Psi$, but at lowered levels of ATP. Precursors arrested at these intermediate stages could be chased to the fully imported forms, confirming that they were on the correct import pathway. We conclude that mitochondrial preproteins are not exclusively imported through sealed contact sites. Polypeptides in transit get access to the aqueous intermembrane space. This pathway appears to be a major pathway, as most precursors accumulated at the GIP-site are exposed to the intermembrane space, and we have previously shown by competition studies that the ADP/ATP carrier is exclusively imported via the GIP-site [25]. Similarly, most of the $F_1\beta$ that was accumulated at low levels of ATP was exposed to the intermembrane space.

These results lead to a new view of the import sites in intact mitochondria. Several models that are not mutually exclusive are compatible with the findings. (i) The outer and inner mitochondrial membranes are not in physical contact, but there is an aqueous (proteinaceous) space in contact sites. This is supported by electron micrographs that show a space of about 6 nm between both membranes [9]. The import machineries in contact sites should then be of a dynamic nature, as receptors and GIP are also present outside contact sites [12–15]. (ii) Translocation contact sites themselves (at least a fraction of them) are dynamic and can be formed and disassembled. (iii) Precursor proteins can be directly translocated across the outer membrane, pass through the intermembrane space and then insert into the inner membrane. None of the intermediates we obtained were freely soluble in the intermembrane space; they were still membrane-associated (probably with the outer membrane in the case of the GIP-intermediate, and with the inner membrane in the case of the $F_1\beta$ intermediate). This would favour that the passage through the intermembrane space is coupled to the transport across the membranes. It is evident, however, that the mitochondrial import machinery and, in particular, translocation contact sites are more dynamic than previously assumed.

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