Eur. J. Biochem. *169*, 289–293 (1987)

Mitochondrial precursor proteins are imported through a hydrophilic membrane environment

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(Received June 1, 1987) – EJB 870641

We have analyzed how translocation intermediates of imported mitochondrial precursor proteins, which span contact sites, interact with the mitochondrial membranes. F_1-ATPase subunit β ($F_1\beta$) was trapped at contact sites by importing it into *Neurospora* mitochondria in the presence of low levels of nucleoside triphosphates. This $F_1\beta$ translocation intermediate could be extracted from the membranes by treatment with protein denaturants such as alkaline pH or urea. By performing import at low temperatures, the ADP/ATP carrier was accumulated in contact sites of *Neurospora* mitochondria and cytochrome b_2 in contact sites of yeast mitochondria. These translocation intermediates were also extractable from the membranes at alkaline pH. Thus, translocation of precursor proteins across mitochondrial membranes seems to occur through an environment which is accessible to aqueous perturbants. We propose that proteinaceous structures are essential components of a translocation apparatus present in contact sites.

Transport of precursor proteins from the cytosol into mitochondria usually involves the following steps (for review, see [1–3]): synthesis on free cytosolic polysomes; specific binding to the mitochondrial surface; membrane-potential ($\Delta\psi$) dependent import at contact sites between outer and inner membranes; proteolytic removal of amino-terminal presequences; intramitochondrial sorting and functional assembly.

With regard to the question of whether protein components are involved in this processes, the following findings are relevant. There is abundant evidence that proteins on the mitochondrial surface act as receptor sites for import [4–15]. A specific proteolytic enzyme in the mitochondrial matrix (processing peptidase) removes presequences [16–21] (and Hawlitschek, Schmidt and Neupert, unpublished work).

Which components are involved in the translocation of precursor proteins across the mitochondrial membranes? One possibility is that transport occurs directly through the lipid bilayer of the membranes. On the other hand, proteins embedded in the membranes could be essential parts of the translocation apparatus. In order to investigate this question, we accumulated F_1-ATPase subunit β ($F_1\beta$), ADP/ATP carrier and cytochrome b_2 in translocation contact sites of *Neurospora* or yeast mitochondria. These accumulated proteins were in a location where they were spanning both outer and inner membranes.

Treatment of membranes at alkaline pH has been shown to release soluble proteins and peripheral membrane proteins to the supernatant and only to leave proteins in the membrane fraction which are embedded in the lipid phase of the membranes [13, 14, 22–28]. The translocation intermediates described could be released from the mitochondrial membranes at pH 11.5, suggesting that they were located in a hydrophilic environment. A similar result was obtained when urea was used as protein denaturant.

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Enzymes. Proteinase K (EC 3.4.21.14); F_1-ATPase (EC 3.6.1.34).

MATERIALS AND METHODS

Transport of proteins into Neurospora *mitochondria*

Published procedures were used for the following steps: growth of *Neurospora crassa* (wild-type strain 74A) [29]; isolation of mitochondria [13, 30, 31]; protein determination [32]; synthesis of precursor proteins in rabbit reticulocyte lysates [29, 30, 33]; pretreatment of mitochondria and reticulocyte lysate with apyrase and addition of nucleoside phosphates [31]; incubation of mitochondria with reticulocyte lysate to import precursors into mitochondria, addition of potassium ascorbate (pH 7), *N,N,N′,N′*-tetramethylphenylenediamine, antimycin A, and oligomycin, treatment of samples with proteinase K, reisolation of mitochondria, immunoprecipitation of $F_1\beta$ and ADP/ATP carrier [14, 30, 34]; SDS/polyacrylamide gel electrophoresis [29, 35]; fluorography [36]; quantitation of results [30, 34].

Import of cytochrome b_2 *into yeast mitochondria*

Yeast cells of wild-type *Saccharomyces cerevisiae* (D273-10B) were grown on 2% lactate and mitochondria were isolated essentially as described [37]. Mitochondria were suspended in sucrose/Mops/EDTA (250 mM sucrose, 1 mM EDTA, 10 mM Mops/KOH, pH 7.2) at a protein concentration of 5 mg/ml. The precursor of cytochrome b_2 was synthesized by coupled transcription/translation [38]. The genomic clone described previously [39] was used. Import of cytochrome b_2 into isolated mitochondria was performed under

the same conditions used for import into mitochondria of *N. crassa* [13], except that 2 mM ATP was included.

Treatment of membranes with aqueous perturbants

For treatment with sodium carbonate, mitochondria were reisolated from the import reaction by centrifugation, resuspended in 100 mM Na_2CO_3 (pH 11.5), at a mitochondrial protein concentration of 50–125 μg/ml and incubated for 30 min at 0°C. Separation of pellets and supernatants and immunoprecipitation from these fractions was performed as described [13, 14, 40].

For treatment with urea, the reisolated mitochondria were incubated in 2–6 M urea for 20 min at 0°C. Separation into supernatants and pellets and immunoprecipitation was performed as described above.

RESULTS

$F_1\beta$ translocational intermediate, spanning contact sites of Neurospora *mitochondria, is accessible to aqueous perturbants*

When the *in vitro* import system, i.e. reticulocyte lysate containing mitochondrial precursor proteins and isolated mitochondria, was depleted of nucleoside triphosphates (NTPs) by pre-incubation with apyrase (an adenosine 5′-triphosphatase and an adenosine 5′-diphosphatase), the import of $F_1\beta$ into mitochondria was inhibited [31]. After addition of low concentrations of ATP or ADP (which led to formation of ATP via the adenylate kinase) to the apyrase-treated system, $F_1\beta$ was partially imported into mitochondria [34]: it was processed by the matrix-located processing peptidase, but a part of the $F_1\beta$ polypeptide was still accessible to externally added proteases which did not cross the outer membrane barrier [13, 14, 40, 41]. Thus, this $F_1\beta$ translocational intermediate was spanning contact sites between outer and inner membranes.

Interaction of this intermediate with the membranes was studied in the experiment described in Fig. 1. Reticulocyte lysate containing radiolabeled *Neurospora* precursor proteins and isolated *Neurospora* mitochondria were pretreated with apyrase (0.6 U/ml; reactions **2** and **3**) or received an apyrase preparation which had been inactivated by heating to 95°C prior to use (reaction **1**). Reticulocyte lysate and mitochondria were mixed and incubated at 25°C in the absence (reaction **1**) or presence of 4 mM ADP (reaction **2**) or 8 mM ADP (reaction **3**). The reactions were then divided into three portions each (**a, b, c**). From portions **a**, mitochondria were reisolated, $F_1\beta$ was immunoprecipitated and samples were analyzed by SDS gel electrophoresis; mature-sized $F_1\beta$ was quantified (Fig. 1, curve **a**). Portions **b** were treated with proteinase K, the mitochondria were reisolated and further treated as described for portions **a**. Compared to the control reaction **1**, only a small fraction of the processed $F_1\beta$ was protected against proteinase K in reactions **2** and **3**, i.e. was completely imported into mitochondria. Most of the processed $F_1\beta$ of reactions **2** and **3**, however, was accessible to proteinase K and was therefore spanning translocation contact sites. From portions **c**, mitochondria were reisolated and incubated in 100 mM sodium carbonate, pH 11.5, for 30 min at 30°C. The membranes were pelleted by centrifugation at 166000 × *g*. Most of the $F_1\beta$ spanning translocation contact sites (reactions **2** and **3**) was extracted from the membranes at pH 11.5, as was the case with the completely imported $F_1\beta$ (reaction **1**).

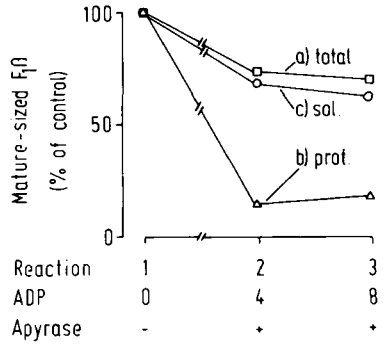

Fig. 1. *Contact site intermediate of $F_1\beta$, generated at decreased levels of nucleoside triphosphates, is solubilized at pH 11.5.* Reticulocyte lysate (containing radiolabeled precursor proteins) and isolated *Neurospora* mitochondria were pretreated with apyrase (0.6 U/ml) (reactions **2** and **3**) or received an apyrase preparation which had been heated to 95°C for 10 min prior to use (reaction **1**). Reticulocyte lysate and mitochondria were mixed and incubated in the presence of antimycin A, oligomycin, potassium ascorbate, tetramethylphenylenediamine, and a buffer containing 3% (w/v) bovine serum albumin as published previously [31]. Reaction **2** contained 4 mM ADP, and reaction **3** contained 8 mM ADP. After 20 min at 25°C, the reactions wer divided into three portions each. Mitochondria from portions **a** were reisolated and $F_1\beta$ was immunoprecipitated (total). Portions **b** were treated with proteinase K (15 μg/ml final concentration); then mitochondria were reisolated and $F_1\beta$ was immunoprecipitated (prot. = protease-protected). Mitochondria of portions **c** were reisolated and incubated in 100 mM Na_2CO_3, pH 11.5 (see Materials and Methods); $F_1\beta$ was immunoprecipitated from pellets and supernatants (sol. = $F_1\beta$ which was solubilized at pH 11.5). Samples were resolved by SDS/polyacrylamide gel electrophoresis. Mature-sized $F_1\beta$ was quantified by densitometry

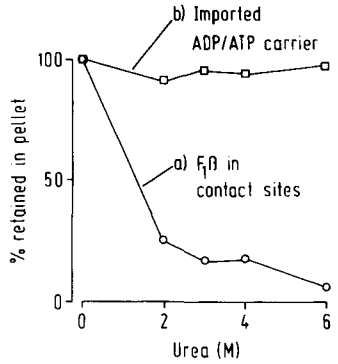

Fig. 2. *Contact site intermediate of $F_1\beta$ is extracted with urea.* Reaction **a** was performed as described for reaction **2a** of Fig. 1 except that 6 mM ADP was present. The reaction was divided into five portions and mitochondria were reisolated. The mitochondria were resuspended either in sucrose/Mops/EDTA or in the indicated concentrations of urea, and further treated as described in Materials and Methods. Mature-sized $F_1\beta$ which was retained in the membrane pellet is shown. Reaction **b** was performed as described for **a** except that apyrase, antimycin A, oligomycin, and ADP were omitted, and that the reaction was treated with proteinase K following import [14]; ADP/ATP carrier remaining in the membrane pellet is shown

In order to have an independent control, we also used urea as protein denaturant (Fig. 2). $F_1\beta$ was accumulated in translocation contact sites as described above. The mitochondria were reisolated and incubated in 2–6 M urea. The membranes were then separated from the supernatant by high-speed centrifugation. Reactions *a* of Fig. 2 show that 2 M urea were sufficient to extract this $F_1\beta$ from the mem-

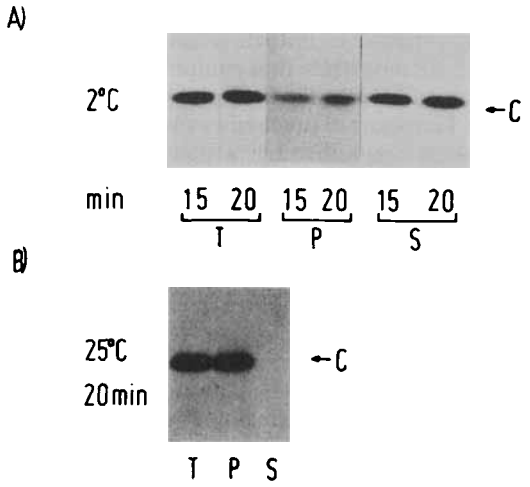

Fig. 3. *ADP/ATP carrier accumulated in contact sites at low temperature is solubilized at pH 11.5.* Reticulocyte lysate, *Neurospora* mitochondria, buffer containing bovine serum albumin, ascorbate, and tetramethylphenylenediamine were incubated for 15 min or 20 min at 2°C (A) or for 20 min at 25°C (B). Reaction B was treated with proteinase K [14]. The reactions were divided in half, and mitochondria were reisolated. From one half, ADP/ATP carrier was immunoprecipitated (T, total). The other half was treated with sodium carbonate as described in Materials and Methods; ADP/ATP carrier was immunoprecipitated from pellets (P) and supernatants (S). Samples were resolved by SDS/polyacrylamide gel electrophoresis. A fluorograph of the dried gel is shown. C, ADP/ATP carrier

branes to a large extent. As a control, imported ADP/ATP carrier (see below) was used which was resistant to extraction at all concentrations of urea tested (up to 6 M) (Fig. 2, reactions *b*).

This suggests that the $F_1\beta$ spanning translocation contact sites is in a location accessible to aqueous perturbants.

ADP/ATP carrier accumulated in contact sites of Neurospora *mitochondria can be extracted with sodium carbonate*

When reticulocyte lysate containing radiolabeled *Neurospora* precursors was incubated with isolated mitochondria having an intact membrane potential at 2°C, 75% of the precursor associated with mitochondria was spanning translocation contact sites: the precursor was still accessible to externally added proteases, but it could be chased to the mature form in the inner membrane by warming to 25°C in the absence of a membrane potential [14]. Thus, the $\Delta\psi$-dependent insertion into the inner membrane had already taken place at 2°C.

We incubated reticulocyte lysate with mitochondria at 2°C for 15 or 20 min in the presence of ascorbate plus tetramethylphenylenediamine, which supplies electrons to complex IV of the respiratory chain thereby generating a membrane potential [42, 43]. Mitochondria were reisolated and samples were halved. One half was left on ice while the other half was treated with 100 mM Na_2CO_3, pH 11.5, and separated into pellet and supernatant. ADP/ATP carrier was then immunoprecipitated. The majority of ADP/ATP carrier was extracted from the membranes at pH 11.5 (Fig. 3A). The minor fraction which remained in the membranes correlated well with the 25% of ADP/ATP carrier which was either already completely imported into the inner membrane or unspecifically inserted into the lipid phase of the outer membrane (see below) [14]. Thus, practically all of the contact-site intermediates of the ADP/ATP carrier were extracted from the membranes at pH 11.5.

In a control experiment, the ADP/ATP carrier was imported at 25°C; the mitochondria were then treated with high concentrations of proteinase K to remove all precursor which was not completely imported. All of the fully imported ADP/ATP carrier was resistant to extraction at pH 11.5 as was expected for an integral membrane protein (Fig. 3B) [14]. Precursor to the ADP/ATP carrier which was unspecifically inserted into the lipid phase of the outer membrane was also resistant to extraction at pH 11.5 [14].

Cytochrome b_2 *can be accumulated in contact sites of yeast mitochondria*

Yeast cytochrome b_2 is synthesized as a precursor with a long amino-terminal presequence (80 amino acid residues) [39]. The first part of the presequence is cleaved off by the processing peptidase of the mitochondrial matrix, whereas the second part is assumed to be removed by a processing activity on the outer face of the inner membrane [44]. Mature cytochrome b_2 is a soluble protein in the intermembrane space [37].

Import of proteins into mitochondria via translocation contact sites has been shown in *Neurospora* mitochondria [13, 14, 40, 41]. In order to examine whether protein import into yeast mitochondria can occur at translocation contact sites, we incubated reticulocyte lysate containing radiolabeled cytochrome b_2 precursor and yeast mitochondria at different temperatures. The reactions were subsequently divided into three portions, each. The portions **a** were analyzed to determine the total amounts of mature-sized cytochrome b_2 (Fig. 4A) and of intermediate-sized cytochrome b_2 (Fig. 4B) which were generated at varying temperatures. The second portions (**b**) were treated with proteinase K to assay for mature (Fig. 4A) and intermediate-sized cytochrome b_2 (Fig. 4B) that was not exposed to the mitochondrial surface. Portions **c** were subjected to treatment with sodium carbonate and the amounts of mature (Fig. 4A) and intermediate-sized (Fig. 4B) cytochrome B_2 which could be solubilized at pH 11.5 were determined. Mature cytochrome b_2 generated at all temperatures was protected against protease. Furthermore, it was soluble at pH 11.5 as expected of a soluble intermembrane space protein (Fig. 4A). After disruption of the outer membrane, mature cytochrome b_2 was sensitive to externally added proteases (not shown). Practically all of the intermediate-sized cytochrome b_2 was extracted at pH 11.5 (Fig. 4B, portions **c**). The intermediate-sized cytochrome b_2 generated at lower temperature, however, was digested completely (7°C) or partly (10° and 12°C) by added proteases (Fig. 4B, portions **b**). This intermediate cytochrome b_2 was therefore spanning translocation contact sites: the amino-terminus was accessible to the processing peptidase in the matrix, but a major part was still outside the outer membrane. The contact-site intermediate of cytochrome b_2 can be completely imported and processed to mature cytochrome b_2 in the absence of a membrane potential (Hartl and Neupert, unpublished).

Thus, the contact site intermediate of cytochrome b_2 in yeast mitochondria is also accessible to aqueous perturbant.

DISCUSSION

The observation that import intermediates in translocation contact sites are accessible to aqueous perturbants may

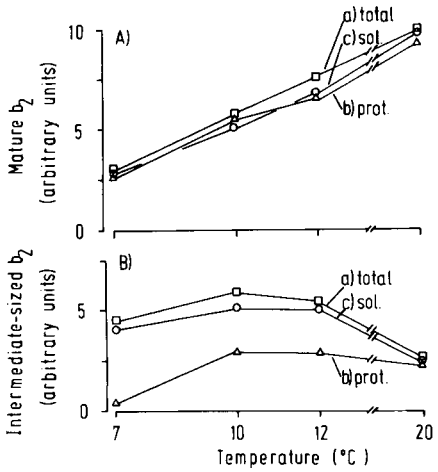

Fig. 4. *Cytochrome* b_2 *accumulated in contact sites at low temperature is solubilized at pH 11.5.* Isolated yeast mitochondria (100 µg per reaction) were incubated with reticulocyte lysate containing the labeled precursor to cytochrome b_2 in a total volume of 150 µl. Import was performed for 30 min at the indicated temperatures. Afterwards, each reaction was cooled to 0 °C, divided into three portions (**a, b, c**), and mitochondria were reisolated by centrifugation. Mitochondria of portions **a** and **b** were resuspended in sucrose/Mops/EDTA. Portions **b** were treated with proteinase K. Mitochondria of portions **c** were resuspended in 100 mM sodium carbonate, pH 11.5, and solubilized cytochrome b_2 was determined (see Materials and Methods). After SDS electrophoresis and fluorography, intermediate-sized and mature cytochrome b_2 were quantified by densitometry. Amounts are expressed in arbitrary units. (A) Mature cytochrome b_2; (B) intermediate-sized cytochrome b_2. (a) Total mitochondria-associated mature or intermediate-sized b_2 (total); (b) protease-protected b_2 (prot.); (c) b_2 solubilized at pH 11.5 (sol.)

seem surprising since these intermediates traverse two membranes. In order to exclude the possibility that we had analyzed an exceptional situation, we examined the import pathways of three proteins which end up in different mitochondrial compartments ($F_1\beta$, ADP/ATP carrier and cytochrome b_2); we used two distinct methods for the reversible accumulation of translocation intermediates in contact sites (import at decreased levels of nucleoside triphosphates or at lower temperatures) and investigated two different organisms (*Neurospora crassa* and yeast).

Controls show the validity of the method (extraction at pH 11.5): mature $F_1\beta$ and mature cytochrome b_2 are extracted at alkaline pH, whereas the mature ADP/ATP carrier, an integral membrane protein, cannot be extracted; precursor proteins which unspecifically interact with the lipid phase of the outer membrane are resistant to extraction at pH 11.5; and precursor proteins in reticulocyte lysate are soluble at pH 11.5 [13, 14]. Similarly, $F_1\beta$ which accumulated in translocation contact sites could be extracted from the membranes with 2 M urea, whereas mature ADP/ATP carrier was resistant to extraction at all concentrations of urea which were tested (up to 6 M).

By several lines of evidence it has been proven that the intermediates in contact sites described here are indeed traversing two membranes at the time when extraction at alkaline pH or with urea is performed [13, 14, 34, 40, 41] (and this study). Parts of the processed precursors are exposed to the outer surface of the outer membrane since they are accessible to externally added proteases. The precursors interact with the inner membrane since they can be completely imported without a requirement for $\Delta\psi$. The intermediates are not released from the membranes in the presence of salt or by subfractionation of mitochondria by sonication.

Our results demonstrate that contact site intermediates are not firmly embedded into the lipid phase of the mitochondrial membranes. Transport of precursors via translocation contact sites appears to occur through a hydrophilic environment. It cannot be excluded that this is only caused by a local rearrangement of the polar lipid headgroups. It is much more likely, however, that distinct proteins in translocation contact sites are the structural and functional components of this environment. Several lines of evidence support this view. Translocation contact sites appear to be stable structures which can be enriched by fractionation of mitochondria into submitochondrial vesicles [41]. The import pathway of the ADP/ATP carrier can be divided into several successive steps: prior to insertion into contact sites, the precursor interacts with proteinaceous sites in the outer membrane which are near contact sites [14]. Notably, for the transport of proteins across the membrane of the endoplasmic reticulum, a proteinaceous pore has been proposed on the basis of experiments employing similar methods [26, 45].

We suggest that specific proteins are essential components of mitochondrial translocation contact sites. These proteins may, on the one hand, form a specific pore-like structure which mediates the translocation of precursor proteins across the mitochondrial membranes. On the other hand, precursors may move by folding of domains through complexes between these proteins and lipids.

Previously, import of precursor proteins via translocation contact sites has been documented for protein transport into *Neurospora* mitochondria [13, 14, 40, 41]. For the study described here, we worked out procedures to accumulate cytochrome b_2 in contact sites of yeast mitochondria. Thus, protein import via translocation contact sites seems to be a general feature in mitochondrial biogenesis.

The results presented here also give new insights into the intramitochondrial sorting of precursors. It has been proposed that the hydrophobic second parts of presequences of precursors of proteins like cytochrome *c* peroxidase, cytochrome c_1 and cytochrome b_2 act as 'stop transfer signals' which anchor the precursors to the lipid phase of the inner membrane [39, 46–49] so that translocation of the mature protein parts into the mitochondrial matrix is prevented. On the basis of the findings presented here we suggest that the precursors interact with specific proteins of the mitochondrial translocation apparatus and that this plays an essential role in the intramitochondrial sorting process.

We thank Dr. Donald W. Nicholson for critically reading the manuscript. We are grateful to Birgit Amely, Christine Forster and Sigrun Meier for expert technical assistance. This study was supported by the *Deutsche Forschungsgemeinschaft (Sonderforschungsbereich* 184, projects B1 and B2) and the *Fonds der Chemischen Industrie.*

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