Sorting pathways of mitochondrial inner membrane proteins

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Two distinct pathways of sorting and assembly of nuclear-encoded mitochondrial inner membrane proteins are described. In the first pathway, precursor proteins that carry amino-terminal targeting signals are initially translocated via contact sites between both mitochondrial membranes into the mitochondrial matrix. They become proteolytically processed, interact with the 60-kDa heat-shock protein hsp60 in the matrix and are retranslocated to the inner membrane. The sorting of subunit 9 of *Neurospora crassa* F_0-ATPase has been studied as an example. F_0 subunit 9 belongs to that class of nuclear-encoded mitochondrial proteins which are evolutionarily derived from a prokaryotic ancestor according to the endosymbiont hypothesis. We suggest that after import into mitochondria, these proteins follow the ancestral sorting and assembly pathways established in prokaryotes (conservative sorting). On the other hand, ADP/ATP carrier was found not to require interaction with hsp60 for import and assembly. This agrees with previous findings that the ADP/ATP carrier possesses non-amino-terminal targeting signals and uses a different import receptor to other mitochondrial precursor proteins. It is proposed that the ADP/ATP carrier represents a class of mitochondrial inner membrane proteins which do not have a prokaryotic equivalent and thus appear to follow a non-conservative sorting pathway.

Most proteins are encoded by nuclear genes, synthesized on cytosolic polysomes and are then transported to the various intracellular and extracellular compartments [1, 2]. A basic problem in biogenesis of nuclear-encoded mitochondrial proteins is the sorting of precursor proteins into the four mitochondrial subcompartments (outer membrane, intermembrane space, inner membrane and matrix) [3–7]. Outer membrane proteins are directly inserted and assembled into the outer membrane without passage through other subcompartments. Matrix proteins are imported via contact sites between both mitochondrial membranes to their functional destination. The sorting of intermembrane space proteins is more complex. Most of the proteins studied so far are first completely translocated into the matrix, interact with the heat-shock protein hsp60, and are then redirected across the inner membrane [8, 9]. The retranslocation across the inner membrane appears to be equivalent to export of proteins in bacteria. According to the endosymbiont hypothesis of mitochondrial origin, mitochondria are derived from prokaryotes. After the evolutionary endocytosis event, most genes that were originally expressed within the organelle were transferred to the nucleus. A positively charged targeting sequence directs the proteins back to mitochondria and via contact sites into the matrix. After proteolytic removal of the matrix targeting signal, the proteins follow their ancestral sorting and assembly pathway established in prokaryotes (conservative sorting).

The sorting pathways of typical inner membrane proteins, i.e. proteins that are embedded with major proteins in the inner mitochondrial membrane, are unknown. Here we report on the biogenesis of two abundant inner membrane proteins, of subunit 9 of F_0-ATPase ($F_0$9) and the ADP/ATP carrier (AAC), including characterization of the topology of mature $F_0$9 in the inner membrane. Surprisingly, we find two distinct assembly pathways: the conservative sorting pathway described for intermembrane space proteins and a non-conservative pathway.

MATERIALS AND METHODS

*Construction of the fusion protein p-$F_0$9**

Isolation of DNA fragments and oligonucleotides, plasmid preparations, ligation and transformation were performed as described previously [10–12]. A cDNA coding for subunit 9 of *Neurospora crassa* F_0-ATPase [13] cloned into a pGem3-vector was cut with *Eco*RI and treated with nuclease *Bal*31 to remove approximately 50 bp of the 3′ end of the insert including the stop codon. After treatment with Klenow polymerase, the cDNA was cut with *Hin*dIII and the *Hin*dIII-blunt fragments were ligated into a pGem3-vector containing a cDNA coding for *N. crassa* F_1-ATPase subunit β (Rassow and Neupert, unpublished results) from which a *Hin*dIII–*Bgl*II-fragment had been removed and the *Bgl*II site had been filled in with Klenow polymerase. Sequencing [14, 15] the plasmid DNA of the resulting clone, using a synthetic $F_1\beta$-specific primer (5′GGCTGTTCGAGGGAGGGTAAG3′) corresponding to nucleotides 193–213 of the antisense strand

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Abbreviations. AAC, ADP/ATP carrier; $F_0$9, F_0-ATPase subunit 9; $F_0$9*, fusion protein between the entire precursor of subunit 9 and amino acid residues 51–519 of F_1-ATPase subunit β; m-$F_0$9*, mature-sized $F_0$9*; p-$F_0$9*, precursor of $F_0$9*; GroEL, prokaryotic heat shock protein of hsp60-family; hsp60, heat-shock protein of 60 kDa in the mitochondrial matrix; mif4, yeast mutant defective in function of hsp60; MOM19, mitochondrial outer membrane protein of 19 kDa.

of the coding region of $F_1\beta$, confirmed that it contained the complete coding region for the *N. crassa* ATPase subunit 9 ligated in frame to amino acid residues 51–519 of the $F_1\beta$-coding region.

In vitro *import of precursor proteins into mitochondria*

Growth of *N. crassa* (wild-type 74A) and isolation of mitochondria by differential centrifugation were performed as described previously [16–18]. Wild-type and mutant yeast were grown and isolation of mitochondria was carried out as previously published [9, 19]. Precursors of the fusion protein p-$F_0$9*, of Fe/S-protein of the cytochrome bc_1 complex, and of AAC were synthesized in rabbit reticulocyte lysates in the presence of [^{35}S]methionine as described previously [20, 21]. Synthesis was directed by SP6-polymerase transcripts of the cDNA cloned into expression vector pGem3. Isolated mitochondria (75 µg mitochondrial protein) were incubated with 5 µl reticulocyte lysate containing [^{35}S]methionine-labeled precursor proteins and 15 µl reticulocyte lysate not containing *N. crassa* precursor proteins. 3% Bovine serum albumin, 80 mM KCl, 2.5 mM $MgCl_2$, 10 mM Mops adjusted to pH 7.2 with KOH (buffer A) [9, 13] was added to give a final volume of 100 µl. Potassium ascorbate and *N,N,N',N'*-tetramethylphenylenediamine were added as described [22]. Inhibition of the mitochondrial processing peptidase by *o*-phenanthroline and EDTA, reisolation of mitochondria and treatment of mitochondria with proteinase K or trypsin (final concentrations 20 µg/ml and 15 µg/ml, respectively) were carried out as previously described [8], except that the samples were incubated for 15 min at 0 °C. The trypsin activity was stopped by a 90-fold excess (by mass) of soybean trypsin inhibitor, and incubation was continued for 10 min at 0 °C. *In vitro* import of the AAC, treatment with proteinase K, addition of carboxyatractyloside and hydroxylapatite chromatography were performed as described [22, 23].

Subfractionation of mitochondria

Digitonin treatment of mitochondria was carried out as follows: mitochondria were resuspended in 250 mM sucrose, 1 mM EDTA, 10 mM Mops, 100 mM KCl, adjusted to pH 7.2 with KOH (buffer B) [8] and digitonin was added from four-fold-concentrated stock-solutions in buffer B to the final concentrations indicated in the figures. The final concentration of mitochondrial protein/sample was 5 mg/ml. After incubation for 4 min at 0 °C the samples were diluted 10-fold with buffer A or B. Aliquots corresponding to 25 µg mitochondrial protein were centrifuged for 10 min at $25000 \times g$. Enzyme activity of adenylate kinase in pellets and supernatants was determined as described previously [24]. Aliquots corresponding to 25 µg mitochondrial protein were treated with proteinase K or trypsin, the membranes were reisolated, dissolved in Laemmli buffer [25] and subjected to gel electrophoresis.

Other methods

Determination of protein concentration, SDS/polyacrylamide gel electrophoresis, fluorography of gels and quantitation of fluorographs by densitometry were performed according to published procedures [8, 25–27].

RESULTS

Conservative sorting of $F_0$9

$F_0$9 of *N. crassa* and higher eukaryotes is synthesized in the cytosol as a precursor protein (p-$F_0$9) with a positively charged amino-terminal presequence [16, 28]. After translocation into mitochondria, the presequence is proteolytically removed and $F_0$9 is assembled into the ATPase complex [29].

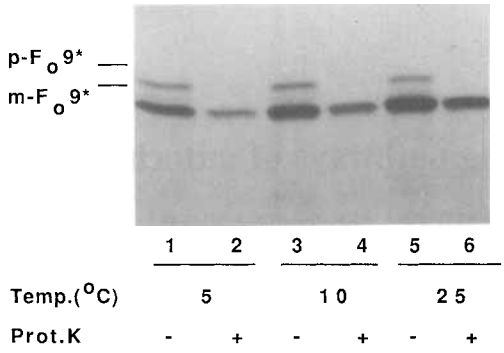

Fig. 1. *Accumulation of $F_0$9* in translocation contact sites.* Reticulocyte lysates containing ^{35}S-labeled precursor of $F_0$9* (p-$F_0$9*) and isolated *N. crassa* mitochondria were incubated in the presence of ascorbate *N,N,N',N'*-tetramethylphenylenediamine for 15 min at the indicated temperatures (Temp.) as described in Materials and Methods. The samples were halved and one half was treated with proteinase K (Prot. K). The reisolated mitochondria were analyzed by SDS/PAGE. A fluorograph of the dried gel is shown. m-$F_0$9*, mature-sized $F_0$9*

Proof of the presence of sorting signals in p-$F_0$9 that direct the protein via the conservative pathway [8, 9] would involve demonstration of the following properties: translocation via contact sites between both mitochondrial membranes into the matrix, interaction with hsp60, and retranslocation of the transport intermediate accumulated in the matrix into the inner membrane to its correct topological orientation. However, a detailled analysis of import and topogenesis of p-$F_0$9 was rendered difficult by several characteristics of the protein. p-$F_0$9 is a short precursor protein, the mature portion has a high endogenous protease resistance [29] and has a strong tendency to aggregate with itself and with hydrophobic surfaces. The assembled protein is almost completely buried in the inner mitochondrial membrane and its topology is difficult to assess [30, 31]. In order to overcome these experimental problems, we constructed a hybrid protein consisting of the complete precursor of *N. crassa* $F_0$9 to whose carboxyl-terminus was fused the mature portion of F_1-ATPase subunit β. The resulting chimeric protein was termed $F_0$9*.

We asked if p-$F_0$9* is transported via translocation contact sites, the major site for import of mitochondrial precursor proteins [18, 32, 33]. Fig. 1 demonstrates that, after import at low temperature, $F_0$9* was trapped in a two-membrane-spanning fashion. The amino-terminal presequence was cleaved by the processing peptidase in the mitochondrial matrix [27] (Fig. 1, lanes 1 and 2), whereas the precursor was still accessible to externally added protease (Fig. 1, lane 2) under conditions where the protease did not penetrate through the outer membrane [8, 9, 32]. $F_0$9* was thus accumulated in translocation contact sites. At a higher temperature, $F_0$9* was completely translocated into mitochondria and thereby protected against externally added protease (Fig. 1, lane 6).

Mature $F_0$9 contains two hydrophobic stretches each of them of sufficient length to span the inner membrane once [30, 31]. The topological arrangement of $F_0$9 in the membrane, especially the sidedness of the carboxyl-terminus and the amino-terminus, was unknown. A comparison with the homologous ATPase subunit c of bacteria would suggest that both

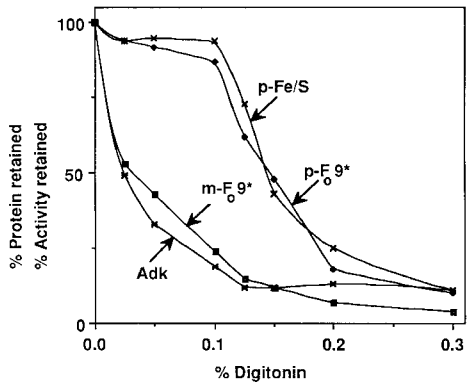

Fig. 2. *Accumulation of the precursor of F_o9* in the mitochondrial matrix.* Reticulocyte lysates containing precursors of F_o9* and Fe/S-protein (p-Fe/S) were incubated with isolated mitochondria of *N. crassa* in the presence of ascorbate, *N,N,N′,N′*-tetramethylphenylenediamine, EDTA and *o*-phenanthroline for 25 min at 25 °C as described in Materials and Methods. In a second sample p-F_o9* was imported in the absence of chelating reagents. After import, the samples were combined. Mitochondria were reisolated and resuspended in buffer B and aliquots, each corresponding to 75 µg mitochondrial protein, were treated with different concentrations of digitonin. Following dilution with buffer B and removal of aliquots corresponding to 25 µg mitochondrial protein for determination of enzyme activity of adenylate kinase (Adk), the remaining parts of the samples were treated with proteinase K, separated into pellets and supernatants and analyzed by SDS/PAGE, fluorography and laser densitometry. Shown is the fraction of radiolabeled protein or enzyme activity retained in the pellet. p-Fe/S is used as marker for the matrix space [8]

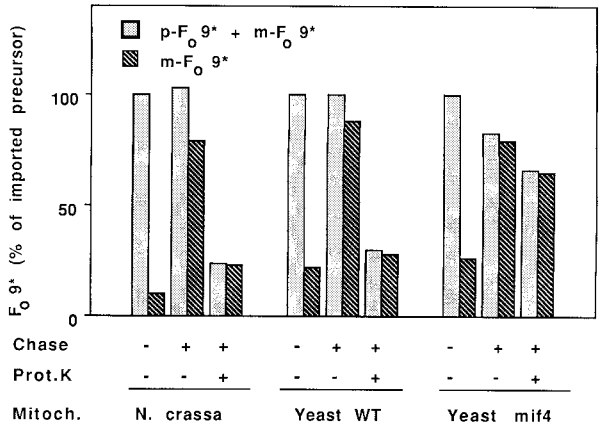

Fig. 3. *Retranslocation of F_o9* from the mitochondrial matrix into the inner membrane.* Precursor of F_o9* was imported in the presence of EDTA and *o*-phenanthroline into mitochondria of *N. crassa*, yeast wild-type (WT) and yeast mif4 as described in the legend of Fig. 2. The reisolated mitochondria were resuspended in buffer B and treated with 0.05% digitonin followed by a treatment with trypsin. Aliquots corresponding to 25 µg mitochondrial protein were withdrawn for SDS/PAGE (−chase). The remaining parts of the samples were incubated for 25 min at 25 °C in the presence of 2 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM NADH and 2 mM ATP (+ chase). The samples were halved and half was treated with proteinase K (Prot. K). Aliquots corresponding to 25 µg mitochondrial protein were subjected to SDS/PAGE, fluorography and laser densitometry

termini face the intermembrane space side [30, 31, 34]. Our studies on the topology of imported F_o9* are in agreement with this prediction. Fractionation of mitochondria with digitonin (Fig. 2) allowed a differential and successive extraction of the mitochondrial intermembrane space and matrix [8, 9]. Processed F_o9* (m-F_o9*) was accessible to added protease as soon as the intermembrane space was opened (as evidenced by release of the marker protein adenylate kinase; Fig. 2). The carboxyl-terminal extension was digested by the protease whereas the polypeptide portion corresponding to authentic F_o9 remained unaffected [29]. This provides evidence that the carboxyl-terminus of mitochondrial F_o9 is facing the intermembrane space side of the inner membrane. A fusion protein similar to F_o9* but without the second hydrophobic sequence of the mature F_o9 was also translocated to the inner membrane; the carboxyl-terminal extension was only accessible to added protease when the matrix space had been opened (K. Mahlke, N. Pfanner and W. Neupert, unpublished results), suggesting that the portion between the two hydrophobic sequences of F_o9 is directed towards the matrix space. The amino-terminus most likely faces the intermembrane space side.

When the metal-dependent processing peptidase in the matrix [27] was inhibited by chelating agents [35], F_o9* was still imported into mitochondria and accumulated as the non-processed precursor form p-F_o9* (Figs 2 and 3). p-F_o9* was located inside the inner membrane barrier with its entire hydrophilic extension as it was accessible to added protease only when the matrix space was opened (Fig. 2). Upon reactivation of the processing peptidase by divalent cations, p-F_o9* was processed to m-F_o9* (Fig. 3, chase). Import and processing occurred with the same efficiency in mitochondria from *N. crassa* and yeast. Although F_o9 is synthesized within mitochondria in yeast (and plants), *N. crassa* F_o9 can be imported and correctly processed by yeast mitochondria suggesting a high degree of conservation of import mechanisms [36]. The correct topological arrangement of m-F_o9* in the inner membrane is demonstrated by its accessibility to added proteinase K in mitochondria with opened intermembrane space (Fig. 3, *N. crassa* and yeast wild-type). m-F_o9* thus became retranslocated from the matrix into the inner membrane.

We recently described that the heat-shock protein hsp60 in the mitochondrial matrix is required for assembly of proteins imported into mitochondria [19]. Intermembrane space proteins that follow the conservative sorting pathway via the matrix space [8, 9] interact with hsp60 prior to retranslocation across the inner membrane [19, 37]. This retranslocation is inhibited in a temperature-sensitive yeast mutant (mif4) where hsp60 in the mitochondrial matrix is non-functional [19]. In mitochondria from mif4 cells grown at non-permissive temperature, precursor proteins are imported into the matrix and are processed by processing peptidase; assembly into multisubunit complexes and retranslocation of proteins to the intermembrane space, however, are impaired. Fig. 3 shows that while p-F_o9* is efficiently imported and processed by mif4 mitochondria it is not retranslocated to its correct orientation in the inner membrane, but remains located inside the inner membrane barrier. This suggests that functional hsp60 is required for the assembly pathway of F_o9*.

In summary, F_o9* is translocated via contact sites into the mitochondrial matrix. Functional hsp60 is required for retranslocation of F_o9* into the inner membrane. The import pathway of F_o9* thus fulfills all criteria established for conservative sorting of proteins. The sorting signal for retranslocation into the inner membrane appears to reside in the mature portion of F_o9 as hybrid proteins that contain the complete presequence of the F_o9 precursor but not the mature protein

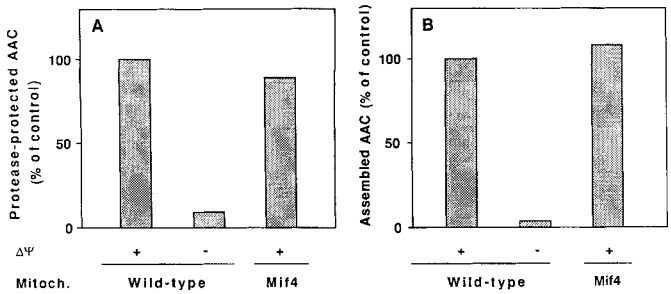

Fig. 4. *Assembly of ADP/ATP carrier does not require functional hsp60.* Reticulocyte lysates containing ^{35}S-labeled precursor of AAC was incubated with isolated mitochondria from yeast wild-type and yeast mif4 for 20 min at 25 °C as described in Materials and Methods. Carboxyatractyloside was added and incubation was continued for 5 min at 25 °C. For incubation in the absence of a membrane potential, the import mixture received valinomycin at a final concentration of 1 μM. The samples were treated with proteinase K. Mitochondria (Mitoch.) were reisolated and analyzed by SDS/PAGE, fluorography and laser densitometry (A). Assembled AAC was assessed by hydroxylapatite chromatography [23] (B)

part all end up as soluble proteins in the mitochondrial matrix [37] (K. Mahlke, N. Pfanner and W. Neupert, unpublished results). Consistent with this hypothesis is the fact that $F_0$9 from yeast and plants and bacterial ATPase subunit c are all synthesized without an amino-terminal peptide extension [38–40].

Assembly of AAC does not require functional hsp60

Are all mitochondrial inner membrane proteins sorted according to the conservative pathway? Previous studies showed that the translocation of AAC from the outer membrane into the inner membrane and the assembly into the dimeric form did not require ATP [13]. Interaction of proteins with hsp60, however, involves an ATP-dependent step [37]. Import and assembly of AAC, a protein that probably does not have a prokaryotic equivalent [41], might thus occur independently of hsp60. To test this directly, we imported *in vitro* synthesized precursor of AAC into mitochondria isolated from wild-type and mif4 yeast cells (Fig. 4A). Assembly of AAC was analyzed by binding of the specific inhibitor carboxyatractyloside to the dimeric form and passage of this assembled AAC over hydroxylapatite. Non-assembled precursor forms of AAC do not bind carboxyatractyloside and are retained on hydroxylapatite columns [22, 23, 42]. Assembly of AAC occurred with the same efficiency in mif4 mitochondria as in wild-type mitochondria (Fig. 4B), confirming that functional hsp60 is not required. AAC apparently uses a non-conservative mechanism for sorting to the mitochondrial inner membrane.

DISCUSSION

We describe two different pathways for sorting of mitochondrial inner membrane proteins: conservative and non-conservative sorting.

$F_0$9 of *N. crassa* and higher eukaryotes is directed by an amino-terminal presequence from the cytosol via the receptor MOM19 (mitochondrial outer membrane protein of 19 kDa) [43] and contact sites into the mitochondrial matrix. Upon proteolytic removal of the presequence, $F_0$9 interacts with hsp60 in the mitochondrial matrix and is retranslocated into the inner membrane. We speculate that the two latter steps are used in a similar fashion by $F_0$9 synthesized within the mitochondria of yeast and plants and were conserved during the evolution of mitochondria from their prokaryotic ancestors. This implies that the homologous ATPase subunit c of bacteria might interact with GroEL, the bacterial homologue to hsp60 [44]; indeed, GroEL has been shown to be involved in the assembly of a number of proteins [45–49]. The conservative sorting of $F_0$9 shares many properties with the conservative sorting of precursor proteins to the mitochondrial intermembrane space, such as cytochromes b_2 and c_1. The major difference is represented by the location of the sorting signal in the precursor proteins. Whereas the signal apparently resides in the mature portion of $F_0$9, the second half of the presequence of cytochromes b_2 and c_1 functions as the matrix export signal [6, 9].

Originally it was proposed that hydrophobic sequences of 20 or more amino acid residues, e.g. in the presequences of cytochromes b_2 and c_1 or in the mature part of $F_0$9, stop the transfer of proteins across the inner membrane during import at contact sites [50, 51]. As shown here and in earlier work [9], the mitochondrial translocation machinery apparently allows the transfer of proteins with long hydrophobic segments across two membranes into the matrix. However, a stop transfer mechanism may be important in the re-export (re-insertion) pathway into the mitochondrial inner membrane that shares characteristics with protein export in prokaryotes and protein translocation into the endoplasmic reticulum [2, 6, 52]. The hydrophobic sequences of $F_0$9 appear to be anchored in the inner membrane and could function together as re-export signals that are functionally equivalent to non-cleavable signal sequences in secretory proteins. In contrast, the interaction of the hydrophobic part of the presequences of cytochromes b_2 and c_1 with the membrane represents an intermediate stage that is followed by proteolytic cleavage to the mature-sized proteins on the intermembrane-space side of the inner membrane [6].

ADP/ATP carrier, the most abundant mitochondrial protein, probably does not have a prokaryotic equivalent [41]. It was probably introduced into the mitochondria during or after the evolutionary endocytosis event. The biogenesis of AAC shows a number of peculiarities. The cytoplasmically synthesized precursor protein contains several internal signal sequences [53, 54] but none at the amino-terminal end. It uses the 72-kDa mitochondrial outer membrane protein MOM72 as a surface receptor [55], whereas precursor proteins carrying an amino-terminal presequence employ MOM19 as a receptor [43]. Finally, sorting and assembly of AAC does not involve the ATP-dependent folding machinery containing hsp60 [37] in the mitochondrial matrix (non-conservative sorting). The example of AAC might suggest that several new mechanisms evolved for mitochondrial proteins introduced by the eukaryotic cell. Among them is a new sorting pathway not involving hsp60. The intramitochondrial sorting of the uncoupling protein, a nuclear-encoded inner membrane protein homologous to AAC [56], might follow a similar pathway [57]. Translocation through mitochondrial contact sites, however, appears to represent a common step in both conservative and non-conservative sorting pathways [8, 9, 22].

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