Topogenesis of Cytochrome Oxidase Subunit II

MECHANISMS OF PROTEIN EXPORT FROM THE MITOCHONDRIAL MATRIX*

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Cytochrome c oxidase subunit II (COXII) in yeast mitochondria is synthesized as a precursor (preCOXII) and is sorted across the inner membrane, whereby both N and C termini become exposed to the intermembrane space. We describe here how this process can be experimentally dissected into a number of distinct stages. Our results demonstrate that the translation of COXII is not obligatorily coupled to translocation. Insertion into the inner membrane and export of the N- and C-terminal domains require an energized inner membrane. The export of COXII is independent of both maturation by the Imp1p protease and assembly into the cytochrome c oxidase complex. When linked to a mitochondrial matrix-targeting sequence, the N-terminal portion of preCOXII (fused to mouse dihydrofolate reductase) can be imported into the mitochondrial matrix. Following accumulation in the matrix, this chimeric protein can become exported across the inner membrane, delivering the N terminus into the intermembrane space where it undergoes processing by the Imp1p protease. This export process displays a number of similarities to bacterial protein export and supports the view that the principles of sorting are conserved from prokaryotes to eukaryotic organelles.

In the yeast Saccharomyces cerevisiae only eight structural proteins are encoded by the mitochondrial genome (Mason and Schatz, 1973; Borst and Grivell, 1978; Tzagoloff and Meyers, 1986). The synthesis of these gene products, all integral inner membrane proteins with the exception of the ribosomal Var1 protein, has been proposed to occur concomitantly with the insertion into and translocation across the membrane. Evidence for this coupled mechanism comes from a number of independent observations. Ribosomes undergoing the synthesis of these proteins are found associated with the inner boundary membrane (André, 1965; Vignais et al., 1969; Watson, 1972). The interaction between the ribosomes and the inner membrane has been reported to be mediated by specific proteinaceous components that bind directly either to the ribosome or to the translated mRNA (Constanzo and Fox, 1990; Michaelis et al., 1991). In addition nascent polypeptide chains released from mitochondrial ribosomes with puromycin treatment are present in the inner membrane and are resistant to extraction at alkaline pH (Poyton et al., 1992; Pajic et al., 1994).

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Finally, no detectable pools of these proteins have been observed to be soluble in the matrix (Severino and Poyton, 1980; Fujiki et al., 1982; McKee and Poyton, 1984). Whether this linkage between synthesis and membrane insertion is obligatory and thus functional or whether it represents a kinetic phenomenon has not yet been clarified.

All of the membrane proteins made on mitochondrial ribosomes become inserted into the inner membrane in a manner that requires the complete translocation of hydrophilic charged segments across the lipid bilayer to the intermembrane space. Information on the mechanisms, energetic requirements, or components involved in these processes is scarce. A number of specialized factors involved in post-translational events in the assembly pathways of these proteins have been identified during the past years. These factors, such as Sco1p, ABC1, ATPase10, COX10,1 and COX11, display a strict specificity for certain complexes and appear to operate at the later stage of assembly rather than at membrane translocation (Krummeck and Rödel, 1990; Ackerman and Tzagoloff, 1990; Nobrega et al., 1990; Tzagoloff et al., 1990; Bousquet et al., 1991). No general component required for insertion and translocation of all these gene products (i.e., a translocation machinery) has been identified to date. In addition very little is known about the energetic or other requirements of protein export from the matrix, besides one study that showed that maturation of COXII is partially dependent on Δψ, but it remained unclear whether export or a post-translational step was affected (Clarkson and Poyton, 1989; Poyton et al., 1992).

We have addressed questions concerning the process of sorting and assembly of mitochondrial gene products using cytochrome oxidase subunit II (COXII) as a model system, for the following reasons. First, the COXII protein has an established and relatively simple topology; a two-membrane-spanning protein with both N- and C-terminal segments exposed to the intermembrane space (Bisson et al., 1982). The N-terminal tail of mature COXII is relatively short (27 amino acids in the case of S. cerevisiae), and the C-terminal domain is considerably longer (144 amino acids). Both termini are very hydrophilic with a strong net negative charge that must become translocated across the membrane following synthesis in the matrix. Second, COXII is synthesized as a precursor, preCOXII, that contains an N-terminal presequence (15 amino acid residues in S. cerevisiae and 12 in Neurospora crassa). PreCOXII is processed by the Imp1p protease on the external surface of the inner membrane. Hence the processing to its mature size is a convenient measure of the translocation of the N terminus across the inner membrane, where it gains access to the Imp1p protease. Finally, the accessibility of the large C-terminal

1The abbreviations used are: COX, cytochrome c oxidase; COXI, COXII, etc.; COX subunit I, II, etc.; preCOXII, COXII precursor; CCCP, carbonyl cyanide m-chlorophenylhydrazide; mt-Hsp70, mitochondrial heat shock protein 70; Suf9, subunit 9; DHFR, dihydrofolate reductase; PAGE, polyacrylamide gel electrophoresis.
the N- and C termini of COXII (S. cerevisiae) were raised in rabbits against the chemically synthesized peptides DSATNQEGILE and KIEAVSLPKFLE, respectively, which had been coupled to activated ovalbumin (Pierce).

For immunoprecipitation of COXII, following in vitro labeling, mitochondria were reisolated, washed in washing buffer, and lysed for 10 min at 4°C in 10 μl of 1% Triton X-100 (v/v), 1 mM phenylmethylsulfonfluoride. After an incubation for 2 min at 96°C, samples were diluted with 1 ml of 1% Triton X-100 lysis buffer (1% Triton X-100, 300 mM NaCl, 10 mM Tris/HCl, 5 mM EDTA, and 1 mM phenylmethylsulfonfluoride, pH 7.4). Immunoprecipitation of COXII was then performed using either the N- or C-terminal specific antisera, as indicated and according to published procedures (Nicholson et al., 1987).

Complex formation with mt-Hsp70 was analyzed by co-immunoprecipitation using an antibody specific for the Ssc1p, the mt-Hsp70 protein as described before (Herrmann et al., 1994b).

Sucrose Gradient Centrifugation Analysis of the Cytochrome Oxidase Complex—Mitochondria following translation in the presence of [35S]methionine were reisolated, lysed at a concentration of 0.8 mg/ml in 0.5% deoxycholate, 150 mM NaCl, 20 mM Tris/HCl, 1 mM phenylmethylsulfonfluoride, pH 7.4, and centrifuged for 15 min at 4°C with 125,000×g in a Beckman TL14 rotor. Sucrose gradient centrifugation was performed loading the extract (250 μl) on a 3 ml sucrose gradient (5–20% sucrose) in 0.1% deoxycholate, 150 mM NaCl, and 20 mM Tris/HCl, pH 7.4, and centrifugation for 5 h in a Beckman SW60 rotor at 485,000×g at 4°C. The proteins of the gradient fractions were trichloroacetic acid-precipitated, separated on SDS-PAGE, and either visualized by autoradiography or immunoblotted with antiserum against isolated cytochrome c oxidase as indicated.

Other Procedures—Mitochondrial proteins were extracted by alkaline treatment using 0.1 M Na2CO3, as described previously (Pfanner et al., 1987). Folding of DHFR was analyzed by assessing the amount of protease-resistant DHFR after lysis of mitochondria (Ostermann et al., 1990).

RESULTS

Sorting of COXII Requires a Membrane Potential across the Inner Membrane—In order to study the energetic requirements for the sorting of COXII, we analyzed in isolated mitochondria the synthesis of mitochondrially encoded proteins in the presence of various inhibitors of the mitochondrial membrane potential. The topology of the resulting newly synthesized COXII was then assessed by analyzing the morphology of newly synthesized COXII containing insertions with these inhibitors, combined with immunoprecipitation of the COXII with either N- or C-terminal specific antibodies.

Following translation in energized mitochondria, COXII accumulated as its mature size form, indicating that efficient export of the N terminus of COXII had taken place (Fig. 1A, lane 1). When the incubation was performed in the absence of ATP, the mature COXII was largely resistant to added protease (Fig. 1A, lane 2). Mature size COXII could be immunoprecipitated with peptide-specific antibodies raised against the N and C termini (Fig. 1, B and C, lanes 1). In these conditions, the C terminus of the maturation size COXII could not be degraded by added protease, it was exported (NcoI, CmII) and folded. This is documented by the following results. (i) When protein synthesis was inhibited by the addition of 20 μg/ml of cycloheximide, the mature COXII was sensitive to exogenously added proteases, as it does not appear to fold to a resistant conformation (cf. Fig. 3). (ii) When mitochondria were isolated in the presence of 5 mM DTT, the mature COXII was largely resistant to added protease, as it does not appear to fold to a resistant conformation (cf. Fig. 3).
31-kDa fragment could be immunoprecipitated with the anti-vitro with [35S]methionine in the presence of 1 mM valinomycin resulted in the accumulation of preCOXII (Fig. 1A). This precursor was largely resistant to digestion by protease under hypotonic swelling conditions (N_{out-Cin} topology) (Fig. 1A, lane 4). In contrast, the mature size COXII accumulated in deenergized mitochondria was sensitive to the proteasome K. The degradation of the COXII species gave rise to the above mentioned fragment, which migrated slightly faster than the cytochrome b protein and was found protected in mitoplasts (Fig. 1A, lane 4). This 31-kDa fragment could be immunoprecipitated with the anti-body specific for the C terminus (Fig. 1C, lane 2) and not with the N-terminal one (Fig. 1B, lane 2). The size of this fragment is in good agreement with that expected following proteolytic removal of the complete exported N terminus of the COXII. The protease protection of such a C-terminal fragment arises from the inhibition of export of the C-terminal domain of COXII (N_{out-Cin} topology). The presence of CCCP, azide, cyanide, and nigericin had a similar inhibitory effect on the translocation of both the N- and C-terminal tails (Fig. 1A, lanes 5–12, and Fig. 1B, and C, lanes 3–6). Quantification of these data showed that the translocation of the C terminus was more dependent on a membrane potential than that of the N terminus (Fig. 1D).

We analyzed the insertion of the transmembrane domains of the newly synthesized COXII into the inner membrane by the resistance to extraction at alkaline pH (Table I). Following translation in energized wild-type mitochondria, accumulated mature COXII behaved as an integral membrane protein; it was not extracted at alkaline pH. In the absence of a membrane potential, accumulated preCOXII was recovered in the soluble fraction following carbonate treatment. PreCOXII accumulated in mitochondria bearing a defective Imp1p protease was not extractable at alkaline pH when synthesized in the presence of a membrane potential (Table I). Therefore, the extractability at alkaline pH of the precursor form of COXII accumulated in deenergized mitochondria reflects a lack of insertion into the inner membrane. Failure to do so prevents export of the N and C termini and hence results in the accumulation of preCOXII in the matrix. Furthermore accumulation of a nontranslocated form of preCOXII demonstrates that the co-translational translation of mitochondrial encoded membrane proteins is not obligatory.

**Fig. 1.** Precursor of COXII accumulates after depletion of the membrane potential. Mitochondrial translation was performed in vitro with [35S]methionine in the presence of 1 mM valinomycin, 20 mM CCCP, 10 mM NaN_3, 10 mM KCN, 10 mM nigericin or in translation buffer alone, as indicated, for 20 min at 25 °C. Mitochondria then were either directly resuspended in SDS sample buffer (A), or converted to mitoplasts in the presence of proteinase K (100 μg/ml). Resulting mitoplasts were either directly solubilized in sample buffer (A, swelling + PK) or lysed in detergent used for immunoprecipitations with anti-serum specific for either the N terminus (B) or the C terminus of COXII (C), as described under “Materials and Methods.” All samples were analyzed by SDS-PAGE and fluorography. The resulting films were quantified by laser densitometry. The levels of proteinase K-resistant preCOXII species gave rise to the above mentioned fragment, which migrated slightly faster than the cytochrome b protein and was found protected in mitoplasts (Fig. 1A, lane 4). This 31-kDa fragment could be immunoprecipitated with the anti-
Export of COXII following synthesis in mitoplasts. Mitochondria were either mock-treated (lanes 1 and 2) or were converted to mitoplasts by hypotonic swelling (lanes 3–8) for 20 min on ice. Mitoplasts (MP) or mitochondria (M), respectively, were reisolated, resuspended in translation buffer, and preincubated for 5 min at 25°C in the absence (−Δψ, lanes 1–4) or in the presence of 1 μM valinomycin (−Δψ, lanes 5–8). Labeling was carried out as described in Fig. 1 for 20 min at 25°C. All samples were divided and were either treated with proteinase K (lanes 2, 4, 6, and 8) (under swelling conditions in the case of mitochondria; lane 2) or were mock-treated (lanes 1, 3, 5, and 7). Samples were subjected to centrifugation, and membrane pellets were lysed in SDS-sample buffer and analyzed by SDS-PAGE. Radiolabeled proteins were visualized by fluorography (A), and resulting films were quantified. The levels of preCOXII (pCOXII) and mature COXII (mCOXII) before and after protease treatment are shown (B). f indicates the mobility of the 31-kDa C-terminal COXII fragment.

Sorting of COXII across the Inner Membrane Is Independent of Assembly into the COX Complex. We next addressed the question of whether the process of insertion and translocation of COXII over the inner membrane occurred independently of its assembly into a functional COX complex. We used mitochondria from three petite yeast strains harboring a defective COX complex. The yeast strain GR20 bears a deletion in the SCO1 gene, a protein necessary for the assembly of COXII, COXIII, and COXIV. The GR20 mutant hence does not have COX activity, as these components are not stably accumulated (Krummeck and Roedel, 1990). Another yeast strain ΔcoxIV contains a deletion in the COXIV gene, a nuclear encoded subunit of the COX complex and does not have a functional COX activity either (Dowhan et al., 1985). Finally we used a mit− strain (V234), which bears a point mutation in the COXIII gene product. No endogenous COXII was present in mitochondria from these three yeast strains, as indicated by Western blotting (results not shown).

Following translation in all of these different mitochondria, newly synthesized COXII was correctly processed to its mature size COXII (Fig. 3A). No accumulation of the C-terminal fragment characteristic of inhibition of export was observed in these mitochondria when they were subjected to hypotonic swelling in the presence of protease. We conclude, therefore, that the export process of preCOXII is independent of its assembly into the COX complex.

We further tested whether following translation in wild-type mitochondria, newly synthesized COXII was assembled into the COX complex (Fig. 3B). After labeling of translation products, a detergent extract of the mitochondria was subjected to sucrose gradient centrifugation. Endogenous COX complex peaked in fractions 5 and 6. This corresponded to a complex of ~250 kDa, the expected size for the COX complex (Fig. 3B). The newly synthesized radiolabeled mature size COXII did not assemble into the COX complex, indicating that export and assembly are two distinct events.

Import and Sorting of pSu9(1–66)preCOXII(1–74)-DHFR—To analyze COXII sorting in more detail, we constructed a
COXII protein that could be imported into mitochondria in a post-translational manner. First in the COXII gene (from N. crassa) the TGA codons specifying Trp in mitochondrial genes but stop in nuclear genes, were mutagenized to TGG, the universal Trp codon. Using this altered COXII gene, a fusion protein was constructed that consisted of the N-terminal 74 amino acid residues of preCOXII, encompassing the presequence, N-terminal tail, and first transmembrane domain (Fig. 4A). This region was fused to mouse DHFR at the C terminus, as a marker protein. This construct was cloned behind the DNA encoding the mitochondrial targeting sequence from Su9 of the N. crassa mitochondrial GrpE-homologue Mge1 (MGE). As markers for the intermembrane space-localized protease (Imp1p) and the mitochondrial matrix protease (at positions 31 and 66), to generate preCOXII(1–74)-DHFR (Fig. 4B, lane 2). This preCOXII(1–74)-DHFR was completely imported across the inner membrane and was located in the matrix as revealed by hypotonic swelling experiments (Fig. 4B, lane 3).

In the presence of added NADH, the N terminus of preCOXII(1–74)-DHFR was exported back across the inner membrane, as the COXII presequence became cleaved by the Imp1p protease (Fig. 4B, lanes 7 and 8) and the N-terminal tail was accessible to added protease under hypotonic swelling conditions (N_{out}-C_{in}, topology) (Fig. 4B, lane 9). This export event was only observed in the presence of added NADH, suggesting that the level of membrane potential required for the export process was higher than that necessary for the initial import step. In the Imp1p-defective mitochondria, processing of preCOXII(1–74)-DHFR to its mature size form was not observed, but the N terminus became exported and exposed to the intermembrane space (results not shown). Thus, in agreement with what was observed for its mitochondrialy synthesized counterpart, export of the N terminus of preCOXII and processing by Imp1p are two independent steps.

In the presence of added ATP, pSu9(1–66)preCOXII(1–74)-DHFR was imported into the mitochondria, where it accumulated as preCOXII(1–74)-DHFR in the mitochondrial matrix (Fig. 4B, lane 6). Export to the intermembrane space was not observed. This result demonstrates that the membrane potential requirements are directly for export and do not reflect an indirect requirement for matrix ATP synthesis. In the presence of both NADH and ATP, efficient export of the N-terminal tail occurred (Fig. 4B, lane 12). In addition, proteolytic degradation to a number of smaller fragments was also observed. Apparently once inserted into the membrane this fusion protein becomes a substrate for an ATP-dependent protease. As proteolytic degradation in the absence of membrane insertion (i.e. +ATP, no added NADH) was not observed (Fig. 4B, lane 6), membrane insertion precedes proteolytic degradation.

In summary, the N terminus of COXII can be imported into mitochondria in a post-translational manner, delivering it to the matrix, the site of synthesis of its mitochondrially encoded counterpart. This imported form of COXII can embark on an export event across the inner membrane, where it becomes processed by Imp1p protease.

The Import and Export Steps Are Two Independent Events—In order to address the question of whether the preCOXII(1–74)-DHFR accumulated in the matrix represented a productive sorting intermediate, we tested whether it could be chased to the exported form (N_{out}-C_{in}, topology) (Fig. 5). Radiolabeled pSu9(1–66)preCOXII(1–74)-DHFR was imported into mitochondria in the presence of NADH for 2 min at 25 °C. Samples were trypsin-treated to remove nonimported species and then subjected to a second incubation, again in the presence of NADH. After the first incubation period, the majority of the imported species was found in the mitochondrial matrix (Fig. 5B, lane 1), and only a small degree of Imp1p protease processing was observed (Fig. 5A, lane 1). A small fraction of the protein was exported after this early time point, due to the presence of the added NADH in the first reaction. Chase in the presence of NADH resulted in export from the matrix, as judged both from increased Imp1p processing and accessibility of the N-tail to added protease in mitoplasts (Fig. 5, A and B, lanes 3 and 4). This chase was complete after about 20 min, although not all exported species were processed by the Imp1p protease, again demonstrating export and maturation to be two independent events.

Both the intermediate form of Su9(1–66)preCOXII(1–74)-DHFR (processed only once by mitochondrial matrix protease, residues 1–35 removed) and the preCOXII(1–74)-DHFR species
Methods.  

(iSu9COXIIDHFR, intermediate mitochondrial matrix protease-processed Su9(1–66)preCOXII(1–74)-DHFR; pCOXIIDHFR, pre-COXII(1–74)-DHFR; mCOXII-DHFR, Imp1p-processed COXII(1–74)-DHFR).

FIG. 5. Kinetics of import, sorting, interaction with mt-Hsp70, and folding of pSu9(1–66)preCOXII(1–74)DHFR in the presence of NADH. Radiolabeled pSu9(1–66)preCOXII(1–74)-DHFR was imported into isolated mitochondria in the presence of 2 mM NADH and 0.2 mM ATP for 2 min at 25 °C and then trypsin-treated. Following the addition of soybean trypsin inhibitor, samples were then either left on ice (lanes 1) or incubated further (2. incubation) at 25 °C (lanes 2–4) for the times indicated. Samples were divided into three parts; one was mock-treated (A, mitochondria, M), and another was converted to mitoplasts in the presence proteinase K (panel B, MP + PK). The mitochondria from the third aliquot were lysed in Triton X-100 buffer, and the extracts were either co-immunoprecipitated with antisera against mt-Hsp70 (C) or preimmune serum (p.i.) (D) or were treated with proteinase K and trichloroacetic acid-precipitated to assay the amount of folded DHFR (results not shown), as described under “Materials and Methods.” *Su9COXIIDHFR, intermediate mitochondrial matrix protease-processed Su9(1–66)preCOXII(1–74)-DHFR; pCOXIIDHFR, pre-COXII(1–74)-DHFR; mCOXII-DHFR, Imp1p-processed COXII(1–74)-DHFR.

FIG. 6. Export of preCOXII(1–74)-DHFR is strongly dependent on a membrane potential. pSu9(1–66)preCOXII(1–74)-DHFR was imported into mitochondria in the absence of added NADH for 5 min at 25 °C. Following trypsin treatment, mitochondria were further incubated for 20 min at 25 °C either in the presence of increasing concentrations of NADH (A) or in the presence of 2 mM NADH together with either no further additions, or 1 μM valinomycin, 20 μM CCCP, 1 μM azide, or 20 μM oligomycin 40 units/ml apyrase, as indicated (B). Then the samples were divided; one-half of each was subjected to swelling in the presence of proteinase K, and the other half was mock-treated. Samples were analyzed by SDS-PAGE and fluorography, and the resulting films were then quantified. The levels of exported COXII(1–74)-DHFR (N terminus protease accessible in mitoplasts) (●) and Imp1p-matured COXII(1–74)-DHFR (■) are shown as a percentage of total imported (trypsin-resistant) pSu9(1–66)preCOXII(1–74)-DHFR. COXII-DHFR, COXII(1–74)-DHFR.

(residues 1–66 removed), which accumulated in the matrix after early time points of import were present in a complex with mt-Hsp70 as shown by co-immunoprecipitation (Fig. 5C, lanes 1 and 2). The DHFR domain of these imported species was tightly folded (results not shown), suggesting that the interaction of mt-Hsp70 had occurred with the Su9 and/or the pre-COXII part of the protein. Chase to the exported form was accompanied by a release from mt-Hsp70 (Fig. 5D, lanes 1 and 2). It is tempting to speculate that mt-Hsp70, by binding to these domains of the protein, prevents its subsequent aggregation and maintains the protein in a competent conformation necessary for the further membrane translocation event.

The chase of matrix-localized form to the exported one requires an energized inner membrane, as is shown by the following experiment (Fig. 6A). pSu9(1–66)preCOXII(1–74)-DHFR was initially imported into mitochondria in the absence of added NADH. Following trypsin treatment, chase of the matrix-accumulated species to the exported form and its subsequent maturation by Imp1p protease were related to the membrane potential. CCCP and azide were more effective inhibitors than valinomycin. Export was, however, only weakly inhibited by prior depletion of the matrix of ATP (Fig. 6B). As this matrix-localized species was no longer complexed to mt-Hsp70 after the first incubation (results not shown), matrix ATP appears only to be required for release from mt-Hsp70 and not at later stages of the export of the N-terminal tail.

In summary we demonstrate that a fusion protein encompassing the first transmembrane domain of preCOXII can be imported into mitochondria. This protein does not become arrested at the level of the inner membrane upon import in a stop-transfer manner but is completely imported into the matrix despite its hydrophobicity. Upon accumulation in the matrix this protein has the ability to access and embark on an export pathway very similar or identical to that of its mitochondrially encoded counterpart. It becomes exported in a membrane potential-dependent manner across the inner membrane, where it undergoes processing by the Imp1p peptidase. The import and export steps can be dissected from each other due to the strict dependence of the export process on a high membrane potential requirement.
Characterization of Mitochondrial Protein Export

DISCUSSION

This study provides novel insights into the mechanism of inner membrane insertion and translocation of mitochondrially encoded proteins. We have characterized this process using cytochrome oxidase subunit II (COXII) as a model protein (Fig. 7). COXII is synthesized as a precursor, preCOXII, in the mitochondrial matrix and undergoes an insertion into and across the inner membrane, which results in the complete translocation of both the N and C termini across the membrane into the intermembrane space (N\textsubscript{out}-C\textsubscript{out} topology). The process of export of these hydrophilic domains has been experimentally dissected here (Fig. 7). In the absence of a proton motive force (ΔΨH\textsuperscript{+}) across the inner membrane, correct sorting of newly synthesized COXII is inhibited. In this case accumulation of a matrix-localized preCOXII (N\textsubscript{in}-C\textsubscript{in} topology) and of a mature size species whose C-terminal domain remained in the matrix (N\textsubscript{out}-C\textsubscript{in}) were observed. Thus we conclude that the translocation of both termini is supported by a membrane potential. The translocation of the C-terminal domain displayed a requirement for a higher membrane potential. Whether this is because the C terminus is significantly longer or more negatively charged than the N terminus, or a combination of both, awaits further investigation. Accumulation of preCOXII in the absence of a ΔψH\textsuperscript{+} as non-membrane-integrated species, speaks for a requirement of the membrane potential also at the step of insertion of the transmembrane domains of COXII into the inner membrane. Both the Δψ and ΔpH components of the ΔWH\textsuperscript{+} appear to be supporting the export process, as all inhibitors tested interfered with the translocation of both termini. Thus we conclude the membrane potential does not directly influence the processing of preCOXII to the mature species by Imp1p, but rather affects the preceding step of export from the matrix.

Correct sorting of the N terminus of COXII was observed when a COXII-DHFR chimeric protein was imported into the mitochondria, a process facilitated by a matrix-targeting signal fused in front of the COXII sequence. The entire protein was accumulated in the matrix and then could be exported to an N\textsubscript{out}-C\textsubscript{in} topology, probably along the same sorting pathway as the authentic COXII, as demonstrated by Imp1p processing and similar energetic requirements. In this respect it is interesting to note that in leguminous plants COXII is encoded in the nucleus. The gene encodes a mitochondrial targeting signal that is separated from the COXII open reading frame by an intron (Nugent and Palmer, 1991; Covello and Gray, 1992). Thus in these plants sorting of COXII in a post-translational manner is obligatory. Together these observations substantiate the conclusion that the process of mitochondrial protein export is not necessarily coupled to protein synthesis. Since, however, pools of mitochondrially synthesized membrane proteins in the matrix are normally not observed, both processes are probably closely coordinated under physiological conditions. A tight control of translation and translocation could serve to enhance both the kinetics and efficiency of export.

Export of the N terminus of COXII was found to occur independently of export of the C terminus. How far the insertion of the first transmembrane domain is coupled to that of the cleavable presequence of COXII is not clear presently. The function of this presequence is unknown; interestingly, it is not present on all COXII proteins sequenced, e.g. bovine and human (Stefans et al., 1979; Chomyn et al., 1981). Preliminary results have shown that in the absence of the transmembrane domain, translocation of the N-terminal domain into the intermembrane space and Imp1p processing can occur, but inefficiently. Thus the presequence together with the N-terminal tail may have some targeting function; however, it is clearly enhanced when the transmembrane domain is present, which may function to stabilize the protein in the lipid bilayer.

In addition export of COXII was observed to occur independently of its assembly into a functional COX complex. A similar observation was also made for COXII and COXIII (results not shown). Thus these newly synthesized proteins are not directly inserted from the matrix into their final functional locations. As this process of export requires the translocation of sometimes highly charged domains, this event may be mediated by a specific channel in the inner membrane. The question of how such a putative channel is composed and whether it is used by all the mitochondrially encoded membrane proteins awaits further analysis. A possible candidate for a component of such a channel could be the recently described OXA1p/pet1402 gene product, a multispanning inner membrane protein. Deletion of this gene or mutations in it, result in a petite phenotype and interestingly in accumulation of uncleaved preCOXII (Bauer et al., 1994; Bonnefoy et al., 1994).

Finally, several aspects of the COXII sorting process resemble protein export in prokaryotes. Export of both termini of mitochondrial COXII requires a membrane potential across the inner membrane. In bacteria a number of reports have demonstrated that the export of both N and C-terminal domains are supported by a ΔψH\textsuperscript{+} across the plasma membrane (Schiebel et al., 1991; Whitley et al., 1994). Furthermore, both the exported N and C tails of mitochondrial and bacterial COXII are negatively charged, while the matrix loop (i.e. nontranslocated segment) between the two transmembrane domains is positively charged. As shown in the bacterial system, positively charged amino acids flanking transmembrane segments tend to be more prevalent in the cytoplasmic than in periplasmic space ("positive-inside" rule) (von Heijne, 1989; Boyd and Beckwith, 1990; Dalbey, 1990). We propose that these positive charges flanking the transmembrane domains of mitochondrial COXII serve to retain this segment in the matrix and thereby determine the orientation of the membrane insertion process. Moreover, the Imp1p protease responsible for the maturation of

2 J. M. Herrmann, unpublished results.
preCOXII is homologous to the bacterial leader peptidase. The similarity of these two proteases indicates conservation of at least one component of the bacterial secretory machinery during the evolution of mitochondria from their prokaryotic ancestors. Most importantly we demonstrate here that a COXII-DHFR derivative, when imported into mitochondria, accumulates initially in the matrix in such a manner that it is competent to embark on this export pathway. Thus we show that a bacterial type of export pathway exists in mitochondria, and this pathway can be accessed by nuclearly encoded proteins following their import into the mitochondrial matrix.

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