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Membrane Translocation of Mitochondrially Coded Cox2p: Distinct Requirements for Export of N and C Termini and Dependence on the Conserved Protein Oxa1p

Shichuan He and Thomas D. Fox*

Section of Genetics and Development, Cornell University, Ithaca, New York 14853-2703

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To study in vivo the export of mitochondrially synthesized protein from the matrix to the intermembrane space, we have fused a synthetic mitochondrial gene, *ARG8^m*, to the *Saccharomyces cerevisiae COX2* gene in mitochondrial DNA. The Arg8^{m}p moiety was translocated through the inner membrane when fused to the Cox2p C terminus by a mechanism dependent on topogenic information at least partially contained within the exported Cox2p C-terminal tail. The pre-Cox2p leader peptide did not signal translocation. Export of the Cox2p C-terminal tail, but not the N-terminal tail, was dependent on the inner membrane potential. The mitochondrial export system does not closely resemble the bacterial Sec translocase. However, normal translocation of both exported domains of Cox2p was defective in cells lacking the widely conserved inner membrane protein Oxa1p.

INTRODUCTION

Translocation of hydrophilic protein domains to the *trans* side of biological membranes must occur during the assembly of many integral membrane proteins, as well as during secretion. This export process has been studied in several systems, most notably the *Escherichia coli* plasma membrane and the eukaryotic endoplasmic reticulum membrane (reviewed by Corsi and Schekman, 1996; Rapoport *et al.*, 1996; Schatz and Dobberstein, 1996; Wickner and Leonard, 1996). Although translocation of nuclearly coded proteins from the cytoplasm into mitochondria has also been examined closely (reviewed by Lill *et al.*, 1996; Schatz, 1996), the export of protein domains encoded by mitochondrial DNA (mtDNA) from the matrix to the intermembrane space (IMS) has been very difficult to study (reviewed by Poyton *et al.*, 1992), owing to the lack of suitable in vitro systems and genetic tools.

The mitochondrial genome of *Saccharomyces cerevisiae* encodes eight major polypeptides, seven of which are integral inner membrane subunits of oxidative phosphorylation complexes (Tzagoloff and Myers, 1986). All of these proteins contain at least one hydrophilic tail or loop that is exported to the outside surface of the inner membrane (Poyton *et al.*, 1992). Among these, cytochrome *c* oxidase subunit II (Cox2p) is particularly interesting. Unlike most other mitochondrially encoded proteins, yeast Cox2p is made as a precursor with an N-terminal leader peptide of 15 amino acids that is removed upon translocation (Sevarino and Poyton, 1980; Pratje and Guiard, 1986). The function of the yeast pre-Cox2p leader peptide cannot be deduced by inspection since it lacks the common features of signal sequences for export in other systems (Figure 2A), and mammalian Cox2p has no leader peptide (Steffens and Buse, 1979; Anderson *et al.*, 1982). Nevertheless, in yeast the leader peptide is indispensable for Cox2p accumulation (Torello *et al.*, 1997).

The membrane topology of Cox2p is firmly established by the crystal structure of bovine cytochrome oxidase (Tsukihara *et al.*, 1996). Based on this structure, and previous studies (Poyton *et al.*, 1992), a picture of the homologous yeast Cox2p can be deduced (Figure 1). The mature yeast Cox2p has two transmembrane domains, with an N-terminal tail (N-tail) of about 26 amino acids and a C-terminal tail (C-tail) of

* Corresponding author.

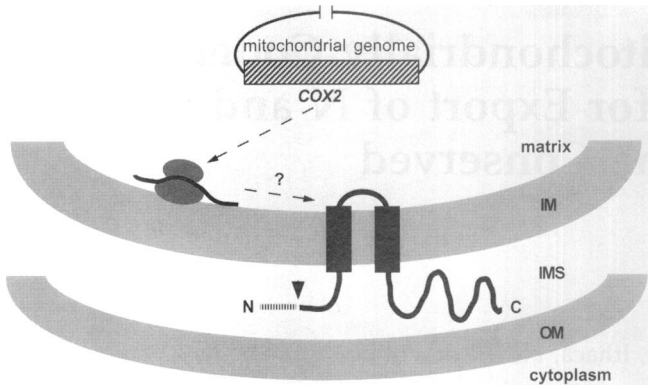

Figure 1. Topology of yeast Cox2p. The mitochondrially coded *COX2* mRNA is translated by membrane-bound organellar ribosomes and integrated into the inner membrane. The 15-amino acid leader peptide (stippled line) is cleaved after export to the IMS. Based on the crystal structure of bovine cytochrome oxidase (Tsukihara *et al.*, 1996), the exported N- and C-tails are bounded by uncharged transmembrane α-helices approximately between residues 42–61 and 82–106 (thick bars) in the inner membrane (IM).

144 amino acids on the IMS (*trans*) side of the inner membrane.

The negatively charged Cox2p N- and C-tails must be translocated across the inner membrane upon synthesis in the matrix, a process that occurs independently of cytochrome oxidase assembly (Herrmann *et al.*, 1995). However, little is known about the mechanism by which these hydrophilic domains are exported. Translocation is thought to be cotranslational (Poyton *et al.*, 1992). In *S. cerevisiae* mitochondria, translation initiation depends on mRNA-specific activators that mediate the functional interaction between mRNA 5′-untranslated leaders and mitochondrial ribosomes. Since the known activator proteins are bound to the inner membrane, it is likely that they play a role in promoting cotranslational membrane insertion (reviewed by Fox, 1996). The mitochondrial inner membrane electrochemical potential is necessary for the topogenesis of Cox2p (Clarkson and Poyton, 1989; Herrmann *et al.*, 1995). Thus far, no components of the mitochondrial export system have been identified. Indeed, the yeast genome does not encode detectable homologues of the bacterial Sec translocase that function in mitochondria (Glick and von Heijne, 1996).

Gene fusions have provided a powerful in vivo approach for studying the topogenesis of membrane proteins in bacterial and eucaryotic systems (Traxler *et al.*, 1993). To extend this approach to mitochondria, we have taken advantage of our synthetic yeast mitochondrial gene, *ARG8^m*, that directs the synthesis of a soluble enzyme within the organelle (Steele *et al.*, 1996). This gene specifies, in yeast mitochondrial genetic code, the enzyme normally encoded by the nuclear gene *ARG8*, which is synthesized in the cytoplasm and imported into the mitochondrial matrix. In this study we have inserted fusions of *ARG8^m* to *COX2* into mtDNA to direct mitochondrial translation of chimeric proteins. Analysis of the topology of these chimeric proteins and native Cox2p has allowed us to examine the function of Cox2p leader peptide, the topogenic elements within Cox2p, the effect of the inner membrane electrochemical potential on N- and C-tail export, and the involvement of the conserved inner membrane protein Oxa1p (Bonnefoy *et al.*, 1994a,b; Kermorgant *et al.*, 1997) in mitochondrial export.

MATERIALS AND METHODS

Strains, Media, and Genetic Methods

The *S. cerevisiae* strains used in this investigation are listed in Table 1. All strains are isogenic or congenic with D273–10B, except

Table 1. Yeast strains used in this study

Strain	Nuclear (mitochondrial) genotype	Reference
DL2	*MATa lys2[p$^+$]*	Folley and Fox (1991)
DFS160	*MATα ade2-101 leu2Δ ura3 arg8Δ::URA3 kar1-1 [p^0]*	Steele *et al.* (1996)
MCC125	*MATa lys2 [p$^+$ cox3-10]*	Costanzo and Fox (1993)
DFS188	*MATa lys2 leu2-3 his3Δ ura3 arg8Δ::hisG [p$^+$]*	Steele *et al.* (1996)
SH40	*MATa lys2 leu2-3 his3Δ ura3 arg8Δ::hisG [p$^+$ cox2(UTL)^a::ARG8^m]*	This study
SH34	*MATa lys2 leu2-3 his3Δ ura3 arg8Δ::hisG [p$^+$ cox2(1-16)^b::ARG8^m]*	This study
SH36	*MATa lys2 leu2-3 his3Δ ura3 arg8Δ::hisG [p$^+$ cox2(1-67)^b::ARG8^m]*	This study
SH37	*MATa lys2 leu2-3 his3Δ ura3 arg8Δ::hisG [p$^+$ cox2(1-109)^b::ARG8^m]*	This study
SH39	*MATa lys2 leu2-3 his3Δ ura3 arg8Δ::hisG [p$^+$ cox2(1-251)^b::ARG8^m]*	This study
SH131	*MATa his4-519 arg8Δ::URA3 oxa1::LEU2 [p$^+$ cox2(1-67)^b::ARG8^m]*	This study
SH132	*MATa his4-519 arg8Δ::URA3 oxa1::LEU2 [p$^+$ cox2(1-251)^b::ARG8^m]*	This study

[a] *ARG8^m* is fused to the *COX2* 5′ untranslated leader (*UTL*).

[b] The number in the parenthesis indicates the number of pre-Cox2p N-terminal amino acids that are encoded by the *COX2* DNA in the gene fusion.

DFS160. Standard genetic methods were as described (Rose *et al.*, 1988). Media and mitochondrial genetic manipulations were as described (Fox *et al.*, 1991; Mulero and Fox, 1993), except that cells were spread on plates containing 5% dextrose for microprojectile bombardment. Plasmids carrying mitochondrial gene fusions (see below) were bombarded into DFS160 and mitochondrial transformants were identified by mating to MCC125. Transformants were mated to DFS188 to integrate the gene fusions into ρ^+ mtDNA after cytoduction. Replacement of *COX2* by the gene fusions in ρ^+ mtDNA was confirmed by the resulting Arg^+ phenotypes, by Pet^- phenotypes for all but Cox2p(1–251)-$Arg8^m$p, by PCR and by DNA gel blot analysis. *OXA1* was disrupted by using a linear fragment of pNB60 (Bonnefoy *et al.*, 1994a) from G. Dujardin (Centre de Génétique Moléculaire du CNRS, France).

Gene Fusions

The synthetic mitochondrial reporter gene used in this study was a *Hin*dIII–*Bam*HI fragment of the previously described *ARG8^m* gene (Steele *et al.*, 1996; GenBank accession no. U31093), lacking coding sequence for the first 21 amino acids of preArg8p. Segments of *COX2* were generated by PCR using pJM2 (Mulero and Fox, 1993) as template. The upstream primer was CGGCCTCGAGTTTTCGGGAAGCGGGAATCC (at −315 bp relative to the *COX2* initiation codon). The downstream primers were GCGGCAAGCTTCTAACATTTTAATAAATCA (+6), GCGGCAAGCTTCATCATTCATAATGAATGTTGTTAAT (+48), GCGGCAAGCTTTTGAATATGTTATAACAATTGTAT (+201), GCGGCAAGCTTCATCACATAAATATAATAAAAT (+327), and GCGGCAAGCTTGTTCATTTAATCATTCCAA (+753). Amplified *COX2* fragments were cleaved with *Xho*I and *Hin*dIII and ligated to *ARG8^m*. The resulting fusions were inserted as *Pac*I–*Bam*HI fragments between the *COX2* flanking sequences of the mitochondrial transformation vector pHD2, which also contains a wild-type *COX3* fragment as a genetic marker (Dunstan, personal communication). All gene fusions were accurate as shown by DNA sequencing.

Mitochondrial Isolation and Subfractionation

Mitochondria were isolated from late logarithmic-phase cells grown at 29°C in complete medium containing 2% raffinose (YPRaf). They were purified by centrifugation on 5–25% Nycodenz gradients and stored when necessary as described (Glick and Pon, 1995). Mitochondria containing Cox2p(1–251)-$Arg8^m$p exhibited an abnormally light buoyant density. Mitochondria were separated into membrane and soluble fractions as described (Glick, 1995), and alkaline carbonate extractions of membranes in 0.1 M Na_2CO_3, pH 11.5, were as described (Fujiki *et al.*, 1982). Soluble fractions were precipitated in 10% trichloroacetic acid prior to SDS-PAGE. Mitochondria were converted to mitoplasts by diluting 1:10 into 20 mM K^+-HEPES, pH 7.4, and 1 mg/ml bovine serum albumin containing either 1 mM phenylmethylsulfonyl fluoride (PMSF) or 100 μg/ml proteinase K and incubated on ice for 30 min (Glick *et al.*, 1992; Glick, 1995). Control mitochondria were diluted 1:10 into 0.6 M sorbitol, 20 mM K^+-HEPES, pH 7.4, and 1 mg/ml bovine serum albumin containing either 1 mM PMSF or 100 μg/ml proteinase K and similarly incubated. Where indicated, control mitochondria were treated with proteinase K in the presence of 1% Triton X-100 or 1% octyl glucoside. Proteinase K was inactivated by adding 1 mM PMSF and samples were dissolved in SDS sample buffer plus 1 mM PMSF and boiled for 4 min prior to electrophoresis.

Translation in Isolated Mitochondria

Translation in isolated mitochondria (*"in organello"* translation) was performed as described (Poyton *et al.*, 1996), except that reactions were incubated at 29°C. Mitochondria were preincubated in translation medium with or without 40 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; or 10 mM sodium azide; not shown) for 10 min prior to addition of [^{35}S]methionine (1000 Ci/mmol) to 12 μCi/μl and further incubation for 30 min. Reactions were chased with 25 mM unlabeled methionine. Mitochondria were reisolated, washed in 0.6 mM sorbitol, 1 mM EDTA, 5 mM methionine, and 20 mM K^+-HEPES, pH 7.4, and resuspended in 0.6 M sorbitol and 20 mM K^+-HEPES, pH 7.4, prior to further processing. Labeled proteins were detected by fluorography of SDS gels treated with Amplify (Amersham, Arlington Heights, IL).

The mitochondrial inner membrane potential was monitored with the potential-sensitive fluorometric dye 3,3′-dipropylthiacarbocyanine iodide [diS-C_3(5); Sigma, St. Louis, MO; Sims *et al.*, 1974; Cyr and Douglas, 1991; Yaffe, 1991]. Excitation was at 620 nm and fluorescence was recorded at 680 nm by using an SLM 8000C Spectrofluorometer. Two milliliters of translation medium (Poyton *et al.*, 1996), omitting labeled methionine, were added to a stirred cuvette, followed by the sequential addition of 6 μM diS-C_3(5), 37.5 μg protein/ml wild-type mitochondria, 20 μM CCCP, and 0.025% β-mercaptoethanol, after each previous incubation had stabilized.

Immunochemical Procedures

Unlabeled samples corresponding to 10 μg of mitochondrial protein, unless otherwise indicated, were resolved by SDS-PAGE and blotted to membranes. Blots were probed with monoclonal anti-Cox2p (CCO6, from T.L. Mason; Pinkham *et al.*, 1994) or polyclonal serum against Arg8p (Steele *et al.*, 1996), cytochrome b_2, citrate synthase, α-ketoglutarate dehydrogenase, or Mas1p (the latter four from B. Glick and G. Schatz; Glick *et al.*, 1992). The ECL chemiluminescence detection kit (Amersham) was used to visualize immune complexes. Membranes were stripped in 500 mM NaCl, 0.5% Tween 20, and 100 mM glycine, pH 2.5, for 20 min, followed by three washes in 100 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5 (1× TBS) plus 0.1% Tween 20. Labeled proteins were immunoprecipitated after resuspension in 1% SDS and 1 mM PMSF at 95°C for 4 min and a 1:25 dilution into 1% Triton X-100, 300 mM NaCl, 10 mM K^+-HEPES, pH 7.4, 5 mM EDTA, and 1 mM PMSF. Complexes were recovered using protein A-Sepharose beads (Sigma).

RESULTS

The Pre-Cox2p Leader Peptide Does Not Signal Export but May Initiate Membrane Insertion

The pre-Cox2p N-terminal 15 amino acid leader peptide is unusual for several reasons: most yeast mitochondrially encoded proteins do not contain leader peptides, the Cox2p of metazoans does not contain a leader peptide, and the yeast pre-Cox2p leader peptide does not possess the common features of the signal sequence for protein export (von Heijne, 1988; Figure 2A). We have previously shown that deletion of the leader peptide prevents accumulation of functional Cox2p (Torello *et al.*, 1997). To examine the function of the leader peptide, a gene fusion was constructed in which the *COX2* mRNA 5′-*UTL* and sequence coding for the N-terminal 16 amino acids was fused to *ARG8^m*, a synthetic mitochondrial gene specifying a soluble protein normally imported from the cytoplasm (Steele *et al.*, 1996). The *ARG8^m* sequence used was truncated to delete the N-terminal 21 amino acids of pre-Arg8p, including the matrix cleavage site. As a control, a fusion of the *COX2* mRNA 5′-*UTL* directly to *ARG8^m* was also constructed. The chimeric genes

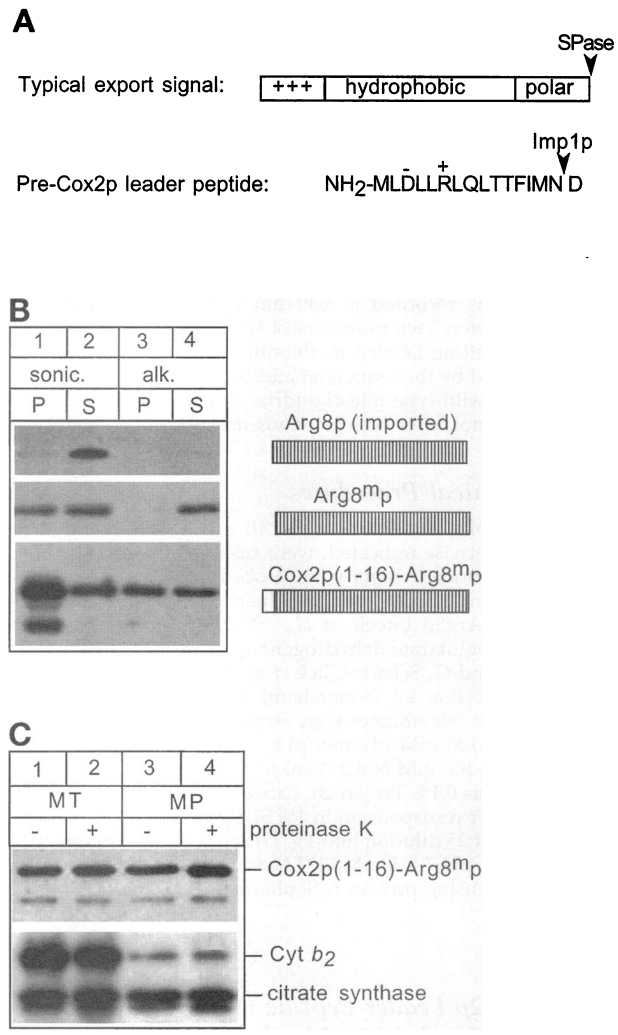

Figure 2. The pre-Cox2p leader peptide promotes membrane association of Arg8^{m}p but not translocation. (A) Typical structure of bacterial and eukaryotic export signals is compared with pre-Cox2p leader peptide sequence. + and − indicate charges. Arrowheads indicate sites of cleavage by signal peptidases (SPase) and the homologous inner membrane protease (Imp1p). (B) Purified mitochondria were sonicated and separated into membrane pellets (lane 1) and soluble supernatants (lane 2) (MATERIALS AND METHODS). Half of each membrane fraction was extracted with alkaline carbonate to separate integral proteins in the pellets (lane 3) from peripheral proteins in the supernatants (lane 4). Samples were run on 12% SDS-gels, immunoblotted, and probed with anti-Arg8p serum. Mitochondria were isolated from wild type (strain DL2) expressing the nuclear gene *ARG8* (Arg8p [imported]), strain SH40 expressing the mitochondrial gene *ARG8^m* (Arg8^{m}p), and strain SH34 expressing a mitochondrial gene encoding the fusion protein Cox2p(1–16)-Arg8^{m}p, as indicated. (C) Mitochondria (MT) expressing Cox2p(1–16)-Arg8^{m}p were converted to mitoplasts (MP) in the absence or presence of proteinase K or treated directly with protease, as indicated (MATERIALS AND METHODS). Samples were resolved by SDS-PAGE, immunoblotted, and probed with anti-Arg8p (top). The blot was then stripped and reprobed (bottom) with anti-cytochrome b_2 (Cyt b_2, an IMS marker) and anti-citrate synthase (a matrix marker).

were transformed into yeast mitochondria, replacing wild-type *COX2* by homologous recombination (Fox *et al.*, 1991). The mitochondrially expressed fusion proteins complemented the nuclear *arg8* auxotrophic phenotype.

To determine the submitochondrial location of the Arg8^{m}p moieties, mitochondria were purified from strains expressing the gene fusions, as well as from a wild-type strain containing imported Arg8p in the matrix. Purified mitochondria were fractionated to separate soluble from membrane-associated proteins. Alkaline extraction of membranes separated peripheral from integral membrane proteins. Proteins from each fraction were then separated by SDS-PAGE and probed with anti-Arg8p polyclonal serum. The wild-type imported Arg8p was a soluble protein (Figure 2B). However, a substantial proportion of the mitochondrially translated Arg8^{m}p, lacking any Cox2p sequence, was present in the membrane fraction, suggesting that the soluble protein became partially membrane-associated when translated by the membrane-bound organellar ribosomes. Nevertheless, this membrane-associated Arg8^{m}p was completely extracted from the membranes by alkaline carbonate (Figure 2B), indicating it did not insert into the inner membrane. In contrast, the mitochondrially synthesized Cox2p(1–16)-Arg8^{m}p fusion protein was predominantly associated with membranes, and more than half of this Arg8^{m}p remained in the membrane pellet following alkaline carbonate extraction (Figure 2B). These results suggest that the pre-Cox2p leader peptide portion of Cox2p(1–16)-Arg8^{m}p caused a substantial proportion of the molecules become inserted in the inner membrane.

To determine whether the Cox2p(1–16)-Arg8^{m}p fusion protein was translocated across the inner membrane, we carried out protease protection experiments on mitoplasts lacking the outer membrane. Purified mitochondria were mitoplasted in the absence or presence of proteinase K and subjected to SDS-gel blot analysis using anti-Arg8p (Figure 2C). To control for effective mitoplasting, the same gel blot was stripped and probed with antibodies against cytochrome b_2, an IMS protein, and citrate synthase, a matrix protein. While the outer membrane was effectively disrupted, the fusion protein was protected from proteinase K digestion by mitoplasts. (The Arg8^{m}p moiety of our fusion proteins is very sensitive to proteinase K in mitochondria treated with Triton X-100 or octyl glucoside, as shown in Figures 5 and 7). Taken together, these results show that the yeast pre-Cox2p leader peptide functions to initiate inner membrane insertion yet is unable to signal translocation of the passenger protein across the inner membrane.

The Topogenic Elements for Cox2p N-Tail Export Lie within the N-Terminal 67 Amino Acid Residues

Immediately following the pre-Cox2p leader peptide is an exported N-tail of approximately 26 residues and the first transmembrane helix, predicted to end at residue 61. We next asked whether expression of these elements alone would lead to normal topogenesis by expressing in vivo a chimeric gene specifying a Cox2p(1–67)-Arg8^{m}p fusion protein (Figure 3A). Fractionation of mitochondria purified from this strain revealed that the fusion protein was exclusively located in the membrane fraction and was not extractable by alkaline carbonate (Figure 3B). These results suggest that the fusion protein was completely inserted into the inner membrane.

Although the size of the Cox2p(1–67)-Arg8^{m}p protein was unaffected by proteinase K treatment of mitochondria, the protein decreased in size, but not amount, after protease treatment of mitoplasts (Figure 3C). We interpret the decrease in size to indicate that the N-tail of the fusion protein was exported into the IMS, while the Arg8^{m}p moiety remained in the matrix. These results indicate that topogenesis of the N-tail is independent of the second transmembrane domain and the C-tail of the protein.

Cox2p N-Tail Export Is Independent of the Inner Membrane Potential

To determine whether Cox2p N-tail export requires the inner membrane electrochemical potential, we examined mitochondrial gene products synthesized in isolated organelles in the absence or presence of CCCP. To be sure that CCCP would dissipate the inner membrane potential in *in organello* translation medium (Poyton *et al.*, 1996), we assayed the potential-dependent uptake of the diS-C_3(5) (Sims *et al.*, 1974; Cyr and Douglas, 1991; Yaffe, 1991) by mitochondria suspended in this buffer (Figure 4; MATERIALS AND METHODS). Fluorescence of the dye was quenched by addition of mitochondria, indicating potential-dependent uptake. Addition of 20 μM CCCP dissipated the potential within 6 min, as indicated by the return of fluorescence to the level prior to addition of mitochondria. Inactivation of CCCP by the addition of β-mercaptoethanol (Yaffe, 1991) restored potential-dependent quenching.

Isolated mitochondria expressing the Cox2p(1–67)-Arg8^{m}p fusion protein were allowed to synthesize labeled organellar gene products in the absence or presence of CCCP and then converted to mitoplasts in the absence or presence of proteinase K. Labeled proteins were resolved by SDS-PAGE and visualized by fluorography (Figure 5). The labeled Cox2p(1–67)-Arg8^{m}p, whose identity was confirmed by immunoprecipitation (Figure 5, lanes 9 and 10), was shortened by protease treatment of mitoplasts regardless of whether CCCP had been added during translation or not. Identical results were obtained with sodium azide in place of CCCP (not shown). Thus, a dramatic reduction of the inner membrane potential had little if any effect on export of the N-tail.

As expected, the mitochondrially encoded ribosomal protein Var1p was protected by mitoplasts, demonstrating the integrity of the inner membrane (Var1p was degraded in the presence of Triton X-100;

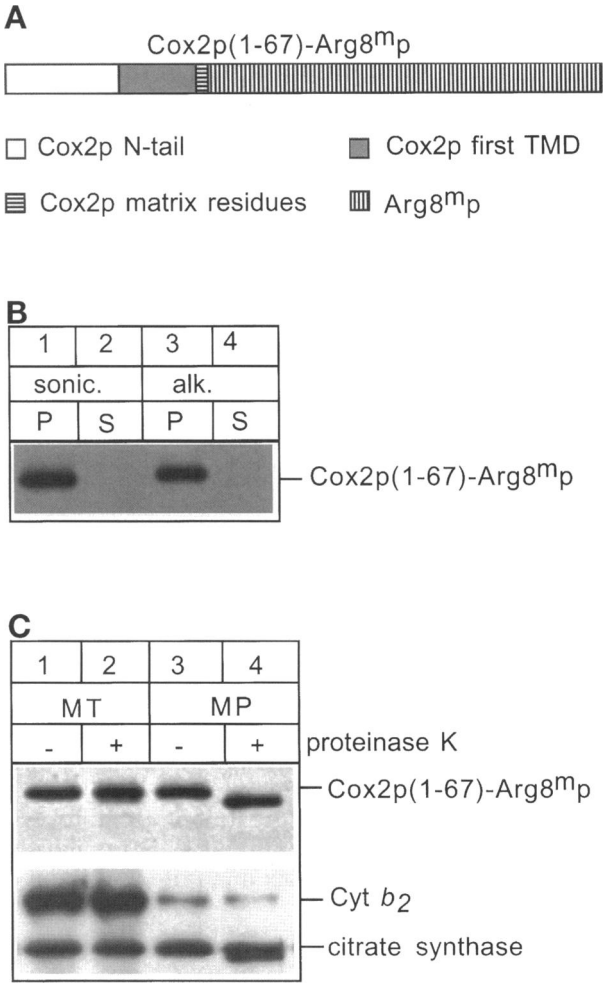

Figure 3. Cox2p(1–67)-Arg8^{m}p is inserted into the membrane with concomitant N-tail export, but the Arg8^{m}p moiety remains in the matrix. (A) The schematic structure of the fusion protein, indicating the Cox2p N-tail (roughly 41 residues including the leader peptide), first transmembrane domain (TMD), and matrix residues. The Arg8^{m}p moiety is 402 residues. (B) Mitochondria expressing Cox2p(1–67)-Arg8^{m}p (from strain SH36) were subjected to membrane fractionation and alkaline extraction, and samples were analyzed by immunoblotting with anti-Arg8p as described in the legend to Figure 2B. (C) Mitochondria (MT) expressing Cox2p(1–67)-Arg8^{m}p were converted to mitoplasts (MP) in the absence or presence of proteinase K or treated directly with protease as indicated. Samples were analyzed by immunoblotting as described in the legend to Figure 2C.

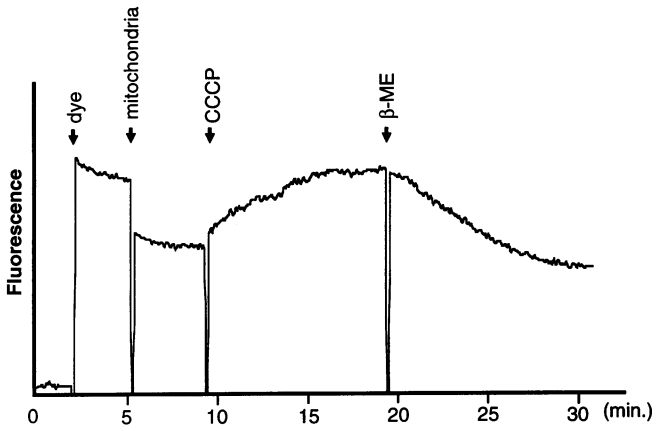

Figure 4. Disruption of the mitochondrial inner membrane potential in translation medium by CCCP. Relative fluorescence of the potential sensitive dye diS-$C_3(5)$ in translation medium was monitored as described in MATERIALS AND METHODS. Dye [diS-$C_3(5)$], wild-type mitochondria, CCCP, and β-mercaptoethanol (β-ME) were added to translation medium at the indicated times. Reduced fluorescence indicates potential sensitive uptake of dye by mitochondria. β-Mercaptoethanol inactivates the uncoupler CCCP.

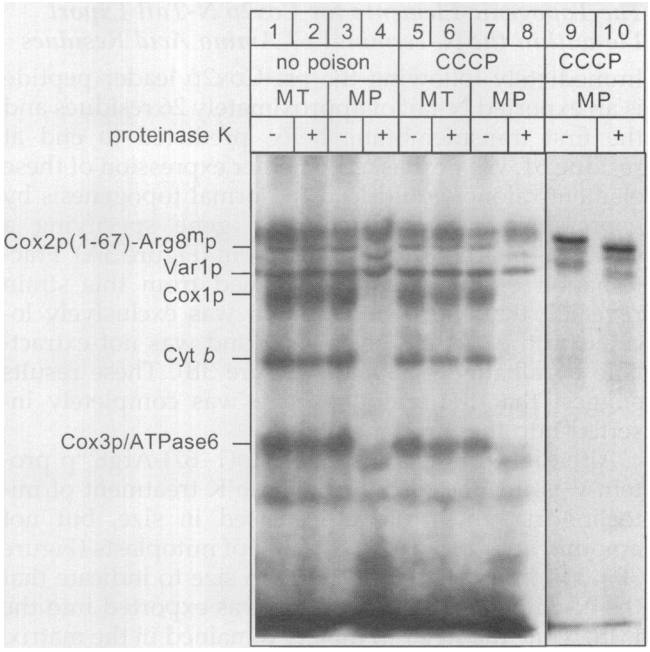

Figure 5. Export of the Cox2p(1–67)-Arg8^{m}p N-tail, labeled during translation in isolated mitochondria, is independent of the inner membrane potential. Mitochondria expressing Cox2p(1–67)-Arg8^{m}p (from strain SH36) were incubated with [^{35}S]methionine under conditions promoting *in organello* translation in the absence (lanes 1–4) or presence (lanes 5–8) of the uncoupler CCCP (MATERIALS AND METHODS). Following translation reactions, mitochondria (MT) were converted to mitoplasts (MP) in the absence or presence of proteinase K, or treated directly with protease, as indicated. The indicated labeled proteins were resolved by SDS-PAGE and visualized by fluorography [the lowest mobility band in lanes 1–8 is apparently an aggregate of ATPase9 (Herrmann *et al.*, 1994)]. Mitoplast inner membrane integrity is demonstrated by protection of Var1p, a matrix localized ribosomal protein (Var1p is sensitive to proteinase K in the presence of 1% Triton X-100, not shown). The other mitochondrial gene products have domains exposed to the IMS. The samples in lanes 9 and 10 were obtained from the fractions analyzed in lanes 7 and 8, respectively, by immunoprecipitation with anti-Arg8p, confirming the identity of Cox2p(1–67)-Arg8^{m}p.

not shown). However, the other endogenous mitochondrial gene products, whether synthesized in the absence or presence of a membrane potential, were degraded by proteinase K treatment of mitoplasts, indicating that at least some of their IMS domains had been exported from the matrix in a potential-independent manner.

The Cox2p C-Tail May Play a Role in Its Own Translocation

The 144-residue hydrophilic Cox2p C-tail is the largest domain normally exported through the inner membrane. It is highly negatively charged, with 23 acidic residues and only 9 basic residues. To ask whether its translocation was directed by upstream topogenic elements, we fused Arg8^{m}p to pre-Cox2p at a site normally located on the IMS side, three residues downstream of the second transmembrane domain (Figure 6A). Mitochondria were isolated from a strain expressing this Cox2p(1–109)-Arg8^{m}p chimera, converted to mitoplasts, and analyzed by protease protection. Protease treatment of the mitoplasts caused a slight decrease in the size of Cox2p(1–109)-Arg8^{m}p (Figure 6B, part 1), indicating that the N-tail had been exported. However, the Arg8^{m}p moiety was fully protected, indicating that the first 109 residues of pre-Cox2p could not direct translocation of the downstream passenger protein.

We next asked whether the entire pre-Cox2p protein, including its C-tail, could direct export of the soluble Arg8^{m}p passenger protein by fusing Arg8^{m}p to the Cox2p C terminus (Figure 6A). Protease protection analysis of mitochondria and mitoplasts from a strain expressing this Cox2p(1–251)-Arg8^{m}p protein revealed that the Arg8^{m}p moiety was indeed largely exported to the IMS (Figure 6B, part 3). The full-length fusion protein was accessible to exogenously added protease in mitoplasts. Furthermore, the fusion protein was apparently cleaved in vivo in the IMS since conversion of the mitochondria to mitoplasts, with or without exogenously added protease, released three Arg8^{m}p cross-reacting fragments. However, export was not complete since the Arg8^{m}p moieties of two fusion protein fragments were protected by mitoplasts. These results demonstrate that the mitochondrial export machinery is capable of translocating Arg8^{m}p as a passenger protein and suggest that the Cox2p C-tail plays a role in signaling its own export.

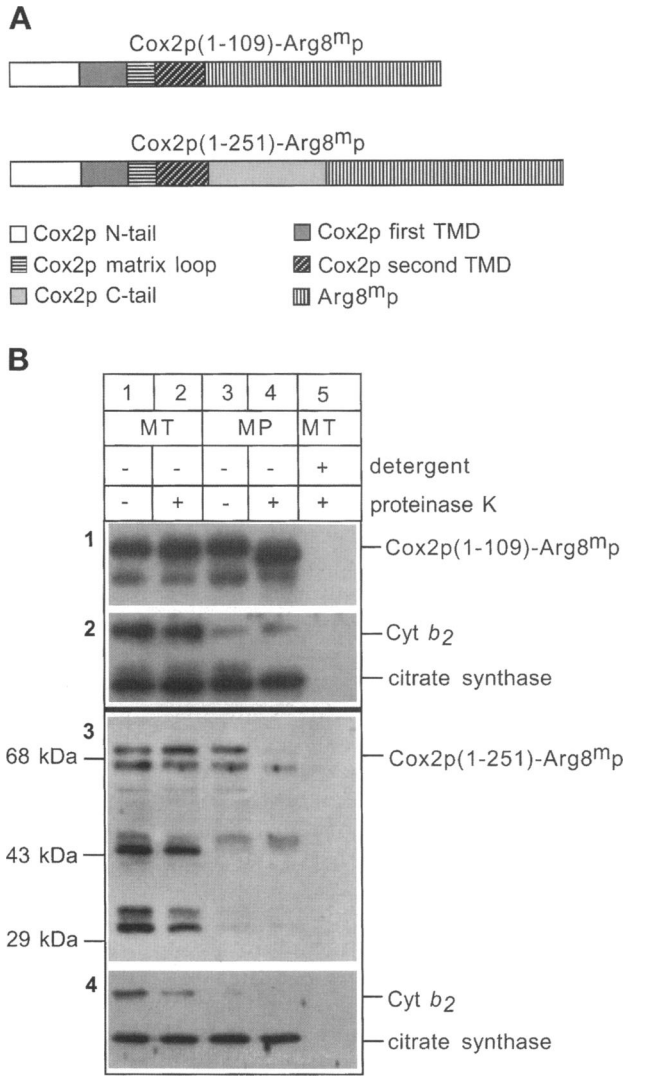

Figure 6. Translocation of the Arg8^{m}p passenger protein across the inner membrane depends on the Cox2p C-tail. (A) Schematic structures of the Cox2p(1–109)-Arg8^{m}p and Cox2p(1–251)-Arg8^{m}p fusion proteins, which lack and contain, respectively, the exported Cox2p C-tail upstream of the 402-residue Arg8^{m}p moiety. (B) Mitochondria (MT) expressing Cox2p(1–109)-Arg8^{m}p (parts 1 and 2; from strain SH37) or Cox2p(1–251)-Arg8^{m}p (parts 3 and 4; from strain SH39) were converted to mitoplasts (MP) in the absence or presence of proteinase K or treated directly with protease in the absence or presence of 1% Triton X-100, as indicated. Samples were analyzed by immunoblotting as described in the legend to Figure 2C. The experiment of part 1 reveals shortening of Cox2p(1–109)-Arg8^{m}p by addition of protease to mitoplasts, indicating N-tail export only. The experiment shown in part 3 reveals that the intact Cox2p(1–251)-Arg8^{m}p is accessible to protease in mitoplasts, indicating translocation of the Arg8^{m}p moiety to the IMS (see text).

Translocation of the Cox2p C-Tail Requires an Inner Membrane Electrochemical Potential

To determine whether Cox2p C-tail export requires the inner membrane electrochemical potential, we isolated mitochondria from wild-type cells and allowed them to synthesize labeled organellar gene products in the absence or presence of CCCP. The labeled mitochondria were then converted to mitoplasts in the absence or presence of proteinase K. Radiolabeled Cox2p was detected by immunoprecipitation with a monoclonal antiserum specific for an epitope in the C-tail of Cox2p followed by SDS-PAGE and fluorography (MATERIALS AND METHODS). When translation was conducted in the absence of CCCP, Cox2p was highly susceptible to proteinase K in mitoplasts (Figure 7), indicating that the C-tail was exported. However, when translation was conducted in the presence of CCCP to dissipate the inner membrane potential, an immunoreactive C-tail fragment of Cox2p was protected from proteinase K added to the mitoplasts (Figure 7). Identical results were obtained when sodium azide was used in place of CCCP (not shown). We interpret the generation of the shorter fragment by proteinase K as confirmation of the potential-independent translocation of the N-tail (see above), while protection of the C-tail demonstrates that the membrane potential plays a major role in driving translocation of this acidic domain across the inner membrane.

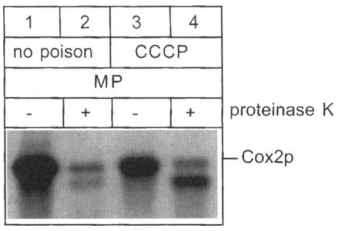

Figure 7. Export of the wild-type Cox2p C-tail, labeled during translation in isolated mitochondria, depends upon the inner membrane potential. Wild-type mitochondria (from strain DL2) were incubated with [^{35}S]methionine under conditions promoting *in organello* translation in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of the uncoupler CCCP and converted to mitoplasts (MP) in the absence or presence of proteinase K, as indicated. The mitoplasts were solubilized and subjected to immunoprecipitation with an anti-Cox2p monoclonal antibody (MATERIALS AND METHODS). This antibody reacts with Cox2p(1–251)-Arg8^{m}p but not Cox2p(1–109)-Arg8^{m}p (our unpublished data) and is thus C-tail-specific. Immunoprecipitates were resolved by SDS-PAGE and visualized by fluorography. Mature Cox2p is indicated. The shorter fragment represents the protected C-tail after digestion of the exported N-tail.

Oxa1p Is Required for Export of the Cox2p N- and C-Tails

Oxa1p is a nuclearly encoded mitochondrial integral inner membrane protein (Kermorgant *et al.*, 1997). Mutants lacking Oxa1p fail to process the pre-Cox2p leader peptide (Bauer *et al.*, 1994) and are unable to assemble cytochrome oxidase (Bonnefoy *et al.*, 1994a).

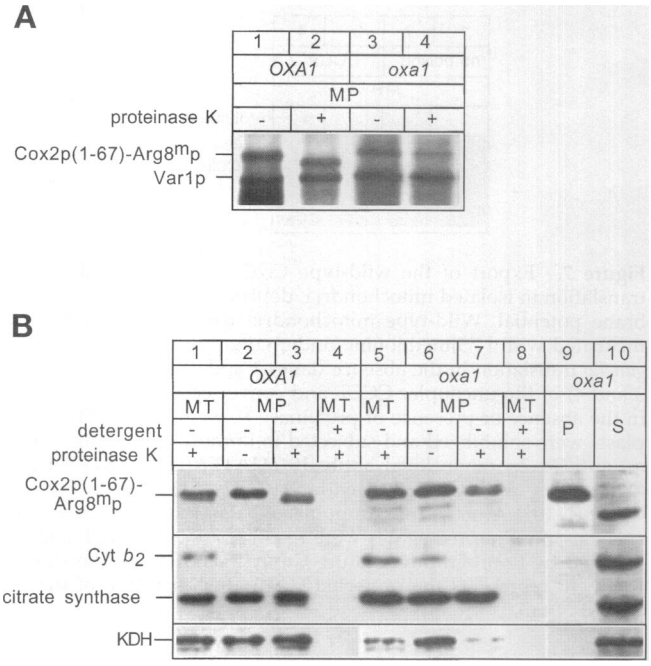

Figure 8. Oxa1p is required for export of the Cox2p N-tail. (A) Mitochondria expressing Cox2p(1–67)-Arg8^{m}p were isolated from *OXA1* (SH36) and *oxa1* (SH131) strains and incubated with [^{35}S]methionine under conditions promoting *in organello* translation. Following translation reactions, the mitochondria were converted to mitoplasts (MP) in the absence or presence of proteinase K, as indicated, and resolved by SDS-PAGE. To compensate for reduced labeling in the mutant mitochondria, the amount of protein loaded in lanes 3 and 4 was twice that in lanes 1 and 2. Labeled Cox2p(1–67)-Arg8^{m}p and Var1p were detected by fluorography. (B) Mitochondria (MT) expressing Cox2p(1–67)-Arg8^{m}p were isolated from *OXA1* (lanes 1–4) and *oxa1* (lanes 5–8) strains and treated with protease K in the absence or presence of 1% octyl glucoside, as indicated. Mitochondria were also converted to mitoplasts (MP) in the absence or presence of proteinase K, as indicated, following a 10-min mock translation reaction at room temperature and associated washes (MATERIALS AND METHODS). We have observed that the inner membranes of mitochondria lacking Oxa1p are fragile and can be partially stabilized by this modification to the standard mitoplasting procedure. Samples were analyzed by immunoblotting as described in the legend to Figure 2C. The *oxa1* mutation reduces the steady-state level of Cox2p-Arg8^{m}p fusion proteins. We therefore loaded more protein (2.5-fold) in lanes 5–8 (25 μg) than in lanes 1–4 and increased exposure times to achieve comparable band intensities for Cox2p-Arg8^{m}p fusion proteins. Partial permeability of inner membranes lacking Oxa1p was revealed by degradation of the matrix marker α-ketoglutarate dehydrogenase (KDH), which is more sensitive to proteinase K than citrate synthase (Glick *et al.*, 1992). Fusion proteins inside mitoplasts from *oxa1* mutants were also partially degraded by proteinase K. Mitochondria from the *oxa1* strain were extracted with alkaline carbonate to separate integral proteins in the pellets (lane 9) from peripheral proteins in the supernatant (lane 10). The anti-Arg8p cross-reacting band in lane 10 may be a fragment of the fusion protein but its identity has not been established.

To ask whether Oxa1p could be required for export, we disrupted *OXA1* in strains expressing the Cox2p(1–67)-Arg8^{m}p and Cox2p(1–251)-Arg8^{m}p fusion proteins from mtDNA.

To study export of the N-tail we first allowed isolated mitochondria from wild type and an *oxa1* mutant to synthesize labeled Cox2p(1–67)-Arg8^{m}p and then converted them to mitoplasts in the absence or presence of proteinase K (Figure 8A). The fusion protein was largely protected from destruction by the protease in both wild-type and *oxa1* mitoplasts. However, although the fusion protein in wild-type mitoplasts was shortened, indicating export of the N-tail (see above), the fusion protein in the *oxa1* mitoplasts was not. This result strongly suggests that Oxa1p is necessary for normal N-tail translocation.

We also examined immunologically the Cox2p(1–67)-Arg8^{m}p accumulated in mitochondria of growing *oxa1* mutant cells (Figure 8B). (The *oxa1* mutant contained substantially less of both fusion proteins than wild type: we compensated for this by increasing protein loadings and exposure times as indicated in the legend to Figure 8.) In contrast to wild type, the *oxa1* mitoplasts protected a substantial portion of the fusion protein from shortening by proteinase K (upper band of the doublet in lane 7), indicating that N-tail export was impaired. However, much of the protected fusion protein in the *oxa1* mitoplasts was shortened by the protease treatment. This result may indicate that N-tail export, normal or abnormal, can occur in the mutant mitochondria by an Oxa1p-independent mechanism that is too slow to be detected in the labeling experiment of Figure 8A. The full-length Cox2p(1–67)-Arg8^{m}p was apparently inserted into membranes in the *oxa1* mutant, since the protein remained membrane bound after alkaline extraction of whole mitochondria (Figure 8B).

To ask whether the *oxa1* mutation also impaired C-tail export, we isolated mitochondria expressing Cox2p(1–251)-Arg8^{m}p and examined the fusion protein immunologically (Figure 9). The *oxa1* mitochondria contained full-length Cox2p (1–251)-Arg8^{m}p and a series of fragments largely distinct from those present in *OXA1* organelles. In further contrast to wild type, conversion to mitoplasts failed to release the fragments from *oxa1* mitochondria or expose them to digestion by added proteinase K. These results indicate that export of the Arg8^{m}p moiety fused to the Cox2p C-tail is dependent on Oxa1p. Apparently, the fusion protein was attacked by endogenous proteases when trapped on the matrix side of the inner membrane, producing the novel fragments. The full-length Cox2p(1–251)-Arg8^{m}p and the longer fragments were apparently inserted into the membrane since they resisted extraction by alkaline carbonate, unlike the shorter fragments (Figure 9).

Taken together, these data suggest that Oxa1p is required for normal translocation of the Cox2p N- and C-tails, at a step following the initiation of pre-Cox2p membrane insertion. Oxa1p is not required for import of proteins from the cytoplasm and, hence, not re-

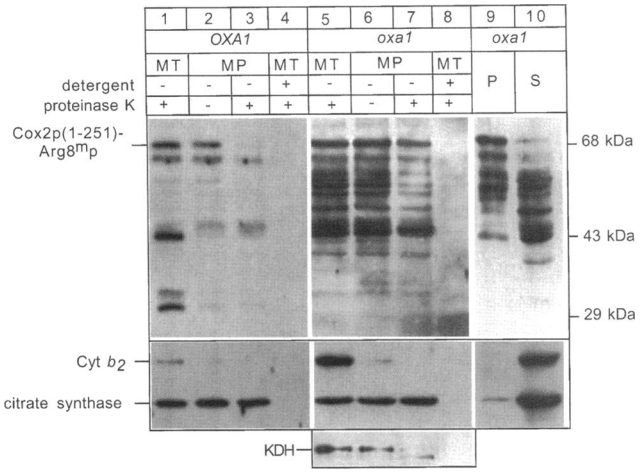

Figure 9. Oxa1p is required for export of the Cox2p C-tail. Mitochondria (MT) expressing Cox2p(1–251)-Arg8^{m}p were isolated from *OXA1* (lanes 1–4; SH39) and *oxa1* (lanes 5–8; SH132) strains and converted to mitoplasts (MP) by the standard procedure in the absence or presence of proteinase K, as indicated. (Lanes 1–4 are identical to Figure 6B, parts 3 and 4, lanes 2–5. They are presented again for comparison.) Mitochondria from the *oxa1* mutant were treated with proteinase K in the presence of 1% octyl glucoside (lane 8). Samples were analyzed by immunoblotting as described in the legend to Figures 2C and 8B. Intact mitochondria from the *oxa1* strain were extracted with alkaline carbonate to separate integral proteins in the pellets (lane 9) from peripheral proteins in the supernatant (lane 10).

quired to maintain an inner membrane electrochemical potential, since the matrix localization of citrate synthase, α-ketoglutarate dehydrogenase (Figure 8) and Mas1p (not shown) were not affected in the *oxa1* mutants.

DISCUSSION

Mitochondrial genomes encode a handful of inner membrane proteins that are topologically equivalent to plasma membrane proteins of bacteria, their distant ancestors. The mechanisms by which the IMS domains of mitochondrial gene products are translocated across the inner membrane have resisted detailed analysis, largely owing to the fact that in vitro studies are hampered by our inability to translate mitochondrial mRNAs in homologous mRNA-dependent in vitro systems. Herein, we have described an in vivo approach to these mechanisms, by using the soluble passenger protein Arg8^{m}p, encoded by a synthetic DNA sequence, *ARG8^m*. We have incorporated this gene into the yeast mitochondrial genome so that Arg8^{m}p is fused to various portions of Cox2p and translated within the organelle. Our results reveal that the mechanisms promoting translocation of IMS domains of Cox2p through the inner membrane do not closely resemble those of the well-characterized bacterial Sec translocase (Wickner and Leonard, 1996). However, they do depend on the function of a nuclear gene, *OXA1*, that encodes a mitochondrial integral inner membrane protein and is widely conserved among bacteria and higher eukaryotes (Bonnefoy *et al.*, 1994a,b; Kermorgant *et al.*, 1997).

Most exported N-tails of *E. coli* inner membrane proteins are not preceded by leader peptides (reviewed by Dalbey *et al.*, 1995). Instead, the first transmembrane domain of such proteins serves as a signal-anchor sequence. In contrast, *S. cerevisiae* pre-Cox2p contains a 15-amino acid N-terminal leader peptide. This leader peptide does not resemble signal sequences in bacterial or eukaryotic systems (Figure 2A), although it is removed from the precursor by an inner membrane protease complex (IMP) whose subunits are homologous to signal peptidase, following translocation to the IMS (Pratje *et al.*, 1983; Behrens *et al.*, 1991; Schneider *et al.*, 1991; Nunnari *et al.*, 1993). This leader peptide is not highly conserved insofar as it is absent from vertebrate forms of Cox2p. Nevertheless, it plays a critical role in accumulation of functional Cox2p in yeast (Torello *et al.*, 1997). Our results indicate that this 15-residue sequence may initiate insertion into the membrane, since attaching it to Arg8^{m}p [in the Cox2p(1–16)-Arg8^{m}p fusion] causes the protein to become firmly associated with the mitochondrial inner membrane (Figure 2). However, the pre-Cox2p leader peptide did not direct translocation of the downstream passenger protein across the inner membrane. Thus, while apparently necessary for export, the yeast leader peptide is not sufficient to fully engage a translocation machine. Clearly, mammalian mitochondria either initiate Cox2p translocation by a different mechanism or utilize a similar signal contained within the mature protein.

Topogenic information necessary for export of the Cox2p N-tail and insertion of the first transmembrane helix is present in the first 67 amino acids of pre-Cox2p containing these two domains, plus the leader peptide and a short segment of the matrix loop (Figure 3). In this regard, Cox2p N-tail export resembles that of the 100-residue *E. coli* ProW N-tail, which depends only on sequences upstream of the first intracellular loop (Whitley *et al.*, 1994).

Bacterial N-tail export is generally not dependent on the Sec translocase machinery (Whitley *et al.*, 1994; Dalbey *et al.*, 1995). This is probably also true for Cox2p since yeast mitochondria apparently lack an homologous Sec translocase (Glick and von Heijne, 1996). The export of negatively charged *E. coli* N-tails, such as that of ProW, depends on the inner membrane potential, suggesting that it may be facilitated by an electrophoretic mechanism (Andersson and von Heijne, 1994; Whitley *et al.*, 1994; Cao *et al.*, 1995). However, this is apparently not the case for the negatively charged (−4) N-tail of Cox2p.

We found that export of the Cox2p(1–67)-Arg8^{m}p N-tail did not depend on the inner membrane electrochemical potential when the protein was translated within isolated mitochondria. The N-tail of the fusion protein, synthesized in the presence of the uncoupler CCCP (or sodium azide), was fully accessible to exogenously added proteinase K after removal of the outer membrane (Figure 5). Indeed, at least some IMS domains of several other mitochondrially coded membrane proteins were also accessible to protease under these conditions. Previous workers have examined the potential dependence of wild-type pre-Cox2p leader–peptide cleavage by endogenous IMP, during synthesis in isolated mitochondria, and found that pre-Cox2p processing was only partially inhibited by uncouplers (Clarkson and Poyton, 1989; Herrmann *et al.*, 1995). Thus, those data are consistent with the view that N-tail export can occur in the absence of a membrane potential. The previously observed partial inhibition of leader–peptide cleavage by uncouplers could be due to differences in behavior between wild-type pre-Cox2p and our fusion protein, partial inhibition of IMP itself, or other unknown factors. Posttranslational Cox2p N-tail export has also been examined following import into isolated mitochondria of an in vitro-synthesized fusion protein (Herrmann *et al.*, 1995). In this case, export was highly dependent on the membrane potential. The fact that cotranslational N-tail export occurs in the absence of a membrane potential while posttranslational export is highly potential-dependent suggests that membrane-bound translation could normally play a role in driving translocation across the inner membrane of mitochondrially coded IMS domains.

Export of the large (144 residue) acidic Cox2p C-tail is highly dependent on the inner membrane electrochemical potential when the wild-type protein is translated within isolated mitochondria (Herrmann *et al.*, 1995; Figure 7). We have not fully characterized the topogenic elements required for export of the Cox2p C-tail. However, the two transmembrane domains of Cox2p present in the Cox2p(1–109)-Arg8^{m}p fusion protein are not sufficient to direct export of the Arg8^{m}p moiety attached at the point where the Cox2p C-tail would normally enter the IMS (Figure 6). This behavior contrasts with that of fusions to *E. coli* leader peptidase, which has a membrane topology similar to that of Cox2p. In that case, an alkaline phosphatase moiety fused after the second transmembrane domain was efficiently translocated (San Millan *et al.*, 1989). The export of the leader peptidase C-terminal domain is a Sec-dependent reaction (Lee *et al.*, 1992).

Yeast mitochondria are capable of translocating the Arg8^{m}p passenger protein through the inner membrane when it is fused to the C terminus of Cox2p in the Cox2p(1–251)-Arg8^{m}p fusion protein (Figure 6). Taken together with the lack of export when Arg8^{m}p was substituted for the Cox2p C-tail, this result suggests that at least some topogenic information necessary for export of the C-tail is contained within the C-tail itself and that this information can cause export of the downstream passenger protein. Clearly, given the potential dependence of C-tail export, the acidic character of this domain is likely to play a role in causing its export.

The Arg8^{m}p moiety is large (402 residues) and hydrophilic. The fact that this moiety can pass across the mitochondrial inner membrane upon synthesis in the matrix strongly argues for the existence of a mitochondrial protein-export machinery. Our passenger-protein export system should be a valuable tool in screening, and possibly selecting, for mutations affecting components of this translocase.

oxa1 mutants fail to process the leader peptide of pre-Cox2p (Bauer *et al.*, 1994). This phenotype is distinct from that of *imp1* and *imp2* mutations affecting the IMP protease complex (Pratje *et al.*, 1983; Behrens *et al.*, 1991; Schneider *et al.*, 1991; Nunnari *et al.*, 1993) in that *oxa1* mutations completely block assembly of the cytochrome oxidase complex (Bonnefoy *et al.*, 1994a) but not processing of the nuclearly coded precursors of cytochromes b_2 and c_1 (Bauer *et al.*, 1994). Oxa1p is an integral mitochondrial membrane protein (Kermorgant *et al.*, 1997), with several predicted membrane spanning domains (Bauer *et al.*, 1994; Bonnefoy *et al.*, 1994a). The function of Oxa1p is conserved among eukaryotes, since the human homologue partially complements the respiratory defect of yeast *oxa1* mutants (Bonnefoy *et al.*, 1994b).

Yeast strains lacking Oxa1p exhibited defects in the export of the Cox2p N-tail of Cox2p(1–67)-Arg8^{m}p, and of the C-terminal Arg8^{m}p moiety of Cox2p(1–251)-Arg8^{m}p. The defects in translocation appear to be specific for export since all nuclearly coded mitochondrial proteins examined (citrate synthase, α-ketoglutarate dehydrogenase, Mas1p, and cytochrome b_2) were correctly processed and localized in the *oxa1* mutants. The *oxa1* mutation did not prevent membrane association of the mitochondrial gene products since neither full-length fusion protein could be extracted from membranes with alkaline carbonate. Our results suggest that Oxa1p could be a component of the mitochondrial export machinery that acts after pre-Cox2p initiates membrane insertion. This idea accounts for the presence of a functional Oxa1p homologue in mammals, which lack a Cox2p leader peptide. The data do not distinguish whether C-tail export depends directly on the function of Oxa1p or is blocked in the *oxa1* mutant as a consequence of defective N-tail export. It is interesting to note that *oxa1* mutants are also severely deficient in the mitochondrial ATP synthase (Altamura *et al.*, 1996), two of whose mitochondrially coded subunits are predicted to have exported N-tails (Nagley, 1988). However, it

must be noted that we have not demonstrated a direct role for Oxa1p in the translocation process.

Several bacterial species, including *E. coli*, have clear Oxa1p homologues of unknown function (Bonnefoy *et al.*, 1994a). In *Bacillus subtilis*, the homologous gene *spoIIIJ* appears to play a role in signal transduction across the membranes separating the mother cell and developing prespore (Errington *et al.*, 1992). Our data on the dependence of mitochondrial protein export on Oxa1p suggest the possibility that these bacterial homologues may play a role in promoting Sec-independent membrane translocation in bacteria.

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