Mba1, a Novel Component of the Mitochondrial Protein Export Machinery of the Yeast *Saccharomyces cerevisiae*

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Abstract. The biogenesis of mitochondria requires the integration of many proteins into the inner membrane from the matrix side. The inner membrane protein Oxa1 plays an important role in this process. We identified Mba1 as a second mitochondrial component that is required for efficient protein insertion. Like Oxa1, Mba1 specifically interacts both with mitochondrial translation products and with conservatively sorted, nuclear-encoded proteins during their integration into the inner membrane. Oxa1 and Mba1 overlap in function and substrate specificity, but both can act independently of each other. We conclude that Mba1 is part of the mitochondrial protein export machinery and represents the first component of a novel Oxa1-independent insertion pathway into the mitochondrial inner membrane.

Key words: mitochondria • protein translocation • Mba1 • Oxa1 • membrane insertion

Introduction

The inner membrane of mitochondria has a very high protein content and might accommodate roughly half of all mitochondrial polypeptides. A small number of these proteins are synthesized in the mitochondria, whereas the majority are synthesized in the cytosol. Import and sorting of the latter into mitochondria are achieved by translocases in the outer and inner membrane of the organelle (for review see Schatz, 1996; Neupert, 1997; Pfanner et al., 1997; Herrmann and Neupert, 2000). Although all matrix proteins appear to be imported on a single transport route, three different pathways have been identified in the past years that lead to a localization in the inner membrane. First, polytopic proteins with internal signals are transported by the translocase of the outer membrane (TOM) complex to the intermembrane space, from where they are inserted into the inner membrane by the recently identified TIM22 machinery (Sirrenberg et al., 1996; Koehler et al., 1998). Second, proteins with typical presequences can be arrested at the level of the TIM23 complex and laterally inserted into the lipid bilayer. This "stop–transfer mechanism" seems to be typical for monotypic membrane proteins whose NH_2 terminus faces the matrix (Van Loon and Schatz, 1987; Rojo et al., 1998). The third group of inner membrane proteins is completely transported into the matrix from where it reinserts into the inner membrane. Thus, domains of these proteins that are exposed to the intermembrane space have to traverse the inner membrane twice (Rojo et al., 1995). This reinsertion process resembles the membrane insertion of mitochondrially encoded proteins (Herrmann et al., 1995). Since this route seems to have evolved from the insertion process of the bacterial progenitors of mitochondria it was named the "conservative sorting" pathway (Hartl et al., 1986).

The inner membrane protein Oxa1 plays an important role in this export process. Oxa1 is conserved from bacteria to chloroplasts and mitochondria, and appears to mediate protein insertion in all of these systems (Bauer et al., 1994; Bonnefoy et al., 1994; Moore et al., 2000; Samuelson et al., 2000). Oxa1 directly interacts with insertion intermediates (Hell et al., 1997, 1998, 2001). In the absence of Oxa1, the mitochondrially encoded subunit 2 of cytochrome oxidase (Cox)[1] accumulates in the matrix and its intermembrane space domains cannot traverse the inner membrane (He and Fox, 1997; Hell et al., 1997). Similarly, in *oxa1* mutants conservatively sorted proteins like subunit 9 of the F_0F_1-ATP synthase of *Neurospora crassa* or Oxa1 itself show significant export defects and end up in the matrix after import (Hell et al., 1998). This indicates that Oxa1 represents a component of a general protein export machinery in the mitochondrial inner membrane, the OXA translocase.

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[1]*Abbreviations used in this paper:* Cox, cytochrome oxidase; DFDNB, 1,5-difluoro-2,4-dinitrobenzene; DSG, disuccinimidyl glutarate; DSP, dithiobis(succinimidylpropinate); pCox, precursor Cox.

However, there are several observations suggesting that the strict dependence of the export of Cox2 on Oxa1 may be exceptional and most inner membrane proteins can insert also in the absence of Oxa1. (a) *oxa1* deletion mutants can be rescued by reintroduction of the *OXA1* gene (Bonnefoy et al., 1994). If a functional OXA translocase would be absolutely required for the insertion of Oxa1 into the inner membrane, the Oxa1 protein synthesized in the transformed Δ*oxa1* mutant should accumulate exclusively in the matrix and therefore not be able to form a functional translocase. (b) In Δ*oxa1* strains, the F_0F_1-ATPase is still present although at reduced levels (Lemaire et al., 2000). This protein complex contains three mitochondrially encoded transmembrane subunits that obviously can insert independently of Oxa1. Deletion of the inner membrane protease Yme1 restores wild-type levels of the F_0F_1-ATPase in Δ*oxa1* mutants (Lemaire et al., 2000). Presumably, when protein degradation is reduced F_0 subunits have sufficient time to find their way into the membrane, even in the absence of Oxa1. (c) It has been shown that mutations in cytochrome c_1 make Oxa1 dispensable, and Oxa1-independent export pathways obviously have to be used in these strains (Hamel et al., 1998). These observations indicate that at least one alternative pathway for the insertion of inner membrane proteins must exist.

Here we analyzed the function of the inner membrane protein Mba1, which, together with Oxa1, was originally described as a multicopy suppressor of a mutant lacking Yta10 (Afg3), a protease in the inner membrane; loss of Mba1 was reported to cause decreased cytochrome aa_3 levels and a slow growth phenotype on nonfermentable carbon sources (Rep and Grivell, 1996; Rep et al., 1996). We show that Mba1 is required for efficient membrane insertion of several mitochondrial translation products. Moreover, Mba1 interacts physically with translocation intermediates. Our results suggest that Mba1 represents a novel component of a protein insertion machinery in yeast mitochondria that, dependent on conditions and substrates, can act either cooperatively with or independent of Oxa1.

Materials and Methods

Yeast Strains and Cell Growth

Yeast strains used in this study were isogenic to the wild-type strains W303a and YPH499 (Sikorski and Hieter, 1989). In the Δ*oxa1* strain, the complete *OXA1* reading frame was replaced by a kanamycin resistance cassette by homologous recombination of a PCR product. The temperature-sensitive *oxa1^{ts}* strain was generated by replacing the *HIS3* gene in the Δ*oxa1* strain with the *oxa1^{ts}* allele of the *pet1402* mutant (Bauer et al., 1994). To generate the Δ*mba1* strain, the complete *MBA1* reading frame was replaced by the *HIS3* gene by homologous recombination of a PCR product. The Δ*mba1* Δ*oxa1* mutant was generated by sporulation and tetrad dissection after crossing the Δ*mba1* strain with the Δ*oxa1* strain. The Δ*mba1 oxa1^{ts}* mutant was generated by sporulation after crossing the Δ*mba1* strain with a strain in which *oxa1* carried the *pet1402* mutation. The *imp1* mutant strain was a gift from E. Pratje, (University of Hamburg, Hamburg, Germany). Standard genetic manipulations were used throughout (Sherman et al., 1986). Yeast strains were cultivated at 30 or 24°C (for *oxa1^{ts}* strains) on lactate medium or on YP medium (1% yeast extract, 2% peptone) supplemented with 2% galactose and 0.5% potassium hydroxide–buffered lactate (Sherman et al., 1986; Herrmann et al., 1994a). Mitochondria were isolated as described previously (Herrmann et al., 1994a). To radiolabel mitochondrial translation products in whole cells, yeast cultures were grown to log phase in synthetic medium lacking ammonium sulfate (Sherman et al., 1986). Then 140 μg/ml cycloheximide, 60 μCi/ml [^{35}S]methionine, and 40 μg/ml of the other 19 proteinogenic amino acids were added. After incubation for 30 min at 30°C the cells were lysed by vigorous mixing with glass beads in 0.2% SDS, and the resulting extract was subjected to SDS-PAGE.

Recombinant DNA Techniques

For construction of the in vitro transcription construct, *MBA1* gene was amplified from genomic yeast DNA by PCR using the primers 5′-GGGTCTAGAATGAGTGTATTAAGATC-3′ and 5′-GGGAAGCTTGCCTTAGCTTGGAGGTAAACG-3′ and subcloned into the XbaI-HindIII sites of the vector pGEM4 (Promega). For overexpression of Mba1, the *MBA1* coding region was cut out from the pGEM4 vector and subcloned into the EcoRI and HindIII sites of the expression vector pYX122 (Novagen).

Mitochondrial Subfractionation

The procedures used for subfractionation of mitochondria were described previously (Leonhard et al., 2000). Swelling and proteinase K treatment were controlled by Western blotting using cytochrome c peroxidase, Dld1, Oxa1, and Mge1 as marker proteins.

To determine the sizes of the Mba1 and Oxa1 complexes, 1 mg wild-type mitochondria was lysed in 220 μl 1% digitonin, 150 mM potassium acetate, 1 mM PMSF, and 10 mM Hepes, pH 7.4. The lysate was cleared by centrifugation at 125,000 *g* for 30 min at 4°C and loaded on a Superose 12 column (Amersham Pharmacia Biotech). Proteins in the fractions were precipitated by the addition of 12% TCA, resolved on SDS-PAGE, and analyzed by Western blotting. The signals of Mba1 and Oxa1 were quantified by densitometry.

Flotation of Integral Membrane Proteins

Proteins were synthesized in the presence of [^{35}S]methionine in isolated mitochondria for 20 min as described (Herrmann et al., 1994a). Mitochondria were reisolated and incubated for 30 min at 4°C in 200 μl 0.1 M sodium carbonate. The samples were split and proteins from one half were TCA precipitated. The other half were adjusted to 1.6 M sucrose, transferred into a 650-μl SW60 centrifugation tube (Beckman Coulter), and overlaid with 250 μl 1.4 M sucrose, 0.1 M sodium carbonate and 100 μl 0.25 M sucrose, and 0.1 M sodium carbonate. After centrifugation at 485,000 *g* for 2 h at 2°C, the floated membrane fraction was collected. Proteins were TCA precipitated, separated by SDS-PAGE, and transferred to nitrocellulose. Signals for mitochondrial translation products were detected by autoradiography and quantified using a PhosphoImaging station (BAS-1500; Fuji).

Cross-linking and Immunoprecipitation

Cross-linking analysis of mitochondrial translation products was performed essentially as described previously (Hell et al., 1998). Proteins were synthesized in the presence of [^{35}S]methionine in isolated mitochondria as described (Herrmann et al., 1994a), with the exception that BSA was omitted from the buffer and Hepes was used instead of Tris to prevent quenching of the cross-linkers. For cross-linking, 1,5-difluoro-2,4-dinitrobenzene (DFDNB) or dithiobis(succinimidylpropinate) (DSP) was added for 15–30 min.

For cross-linking of imported Oxa1p, radiolabeled Oxa1 was incubated with wild-type mitochondria in import buffer lacking BSA for 10 min at 25°C as described (Hell et al., 1997). Mitochondria were reisolated and incubated in 400 μl of 0.6 M sorbitol, 2 mM ATP, 2 mM NADH, and 20 mM Hepes, pH 7.4 in the presence of 400 μM disuccinimidyl glutarate (DSG) for 30 min.

Cross-linking was stopped by the addition of 100 mM glycine. Mitochondria were reisolated, washed, and lysed in 0.1% SDS. After a clarifying spin for 10 min at 20,000 *g*, the extract was diluted 100-fold in 1% Triton X-100, 300 mM KCl, 5 mM EDTA, 1 mM PMSF, 10 mM Tris/HCl, pH 7.4, and used for immunoprecipitation according to published procedures (Herrmann et al., 1994b).

Generation of Antisera

Antisera against the COOH terminus of Mba1 were raised in rabbits by injecting the chemically synthesized peptide CEDDAKVAIHRMK, representing amino acid residues 259–271 coupled to keyhole limpet hemocyanin (Pierce Chemical Co.). The antisera against Cox2, Cox20, Oxa1, and

Yta10 were described previously (Pajic et al., 1994; Herrmann et al., 1995, 1997; Hell et al., 2000).

Miscellaneous

Import of in vitro–synthesized proteins into isolated mitochondria (Herrmann et al., 1997) and enzymatic measurement of the activities of the bc1, Cox, and ATPase complexes were performed essentially as described previously (Hell et al., 2000).

Results

Mba1 Is a Matrix Protein Associated with the Inner Membrane

To assess the localization of Mba1, we raised an antiserum against a peptide representing amino acid residues 259–271 of Mba1. In Western blots of mitochondrial extracts, this serum specifically recognized a 24-kD band which was absent in Δ*mba1* mutant mitochondria (Fig. 1 A). The Δ*mba1* mitochondria contained wild-type levels of the proteins Oxa1 and Cox20. In contrast, the amount of Cox2 was strongly reduced (Fig. 1 A), which explains the low cytochrome aa_3 levels that are reported for this mutant (Rep and Grivell, 1996).

A mitochondrial subfractionation experiment is shown in Fig. 1 B. Mba1 was found to be resistant to added protease both in mitochondria and in mitoplasts in which the outer membrane was ruptured by hypotonic swelling (lanes 2 and 3). In contrast, the outer membrane protein Tom70 and the intermembrane space protein cytochrome b_2 (Cyt b_2) were degraded under these conditions. After detergent lysis of mitochondrial membranes, Mba1 was protease sensitive (lane 4). This indicates a localization of Mba1 in the mitochondrial matrix or in the inner membrane. Upon sonication of mitochondria, Mba1 was exclusively found in the membrane fraction (lanes 5 and 6). Even under the rigid extraction conditions of treatment with 0.1 M unbuffered carbonate, a significant fraction of Mba1 fractionated with membranes (lanes 7 and 8). A very similar behavior was reported for the overexpressed myc-tagged Mba1 (Rep and Grivell, 1996). Thus, although Mba1 does not expose protease-sensitive domains to the intermembrane space, it is tightly associated with the inner membrane. The sequence of Mba1 contains two adjacent hydrophobic stretches (amino acid residues 70–86 and 89–102) which might be embedded deeply in the membrane and lead to an extraction pattern that resembles that of the transmembrane protein Tom70.

The mitochondrial localization of Mba1 was further supported in vitro by import experiments of a ^{35}S-labeled Mba1 precursor (Fig. 1 C, pMba1). Upon incubation with isolated yeast mitochondria (lanes 2–5), a proteolytically processed mature form of Mba1 was generated (mMba1) that remained protease resistant in mitochondria and mitoplasts (lanes 3 and 4). In the absence of a membrane potential, no Mba1 was imported into mitochondria and the Mba1 precursor remained protease accessible (lanes 7–10, $-\Delta\psi$).

The reduced amounts of Cox2 in Δ*mba1* mitochondria might be due either to an impaired synthesis rate or to an instability of Cox2. To differentiate between these two possibilities, mitochondrial translation products were labeled with [^{35}S]methionine in wild-type or Δ*mba1* mutant cells for 30 min. Then mitochondrial translation was

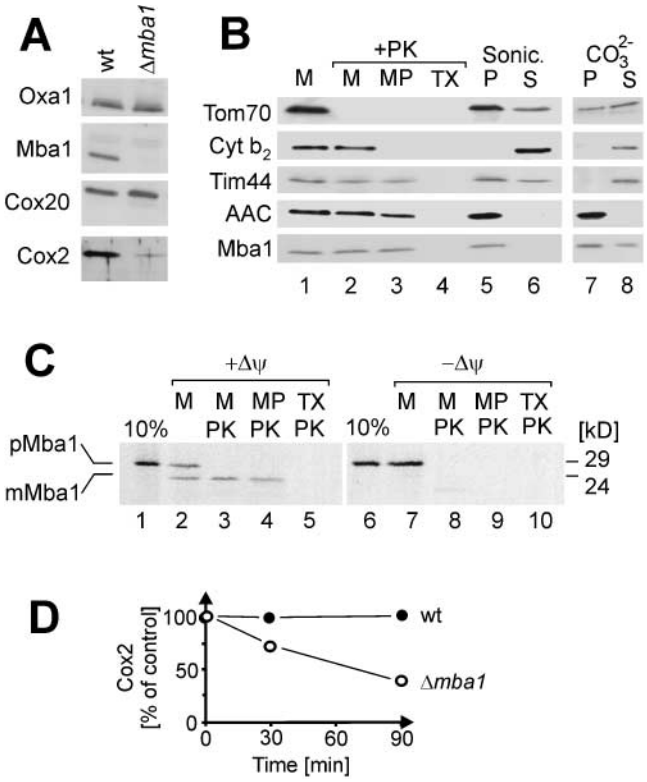


Figure 1. Localization of Mba1 on the matrix side of the mitochondrial inner membrane. (A) Western blots of extracts of wild-type (wt) and Δ*mba1* mitochondria (50 μg) using antisera against Oxa1, Mba1, Cox20, and Cox2. (B) Submitochondrial fractionation. Lane 1, mitochondria (M); lane 2, proteinase K–treated mitochondria; lane 3, proteinase K–treated mitoplasts (MP); lane 4, proteinase K–treated Triton X-100 extract of mitochondria (TX); lane 5, membrane-associated proteins (P); lane 6, soluble protein fraction after sonication of mitochondria (S); lane 7, membrane proteins; and lane 8, soluble protein fraction after carbonate extraction of mitochondria. Marker proteins for the different mitochondrial subcompartments were Tom70 for the outer membrane, cytochrome b_2 (Cyt b_2) for the intermembrane space, Tim44 for a matrix protein, and ADP/ATP carrier (AAC) for the inner membrane. (C) Import of in vitro–synthesized Mba1 precursor (pMba1) into isolated mitochondria. Mba1 precursor protein was synthesized in reticulocyte lysate and incubated for 20 min at 25°C with wild-type mitochondria. Equal aliquots were either mock treated (lanes 2 and 7), treated with proteinase K (lanes 3 and 8), converted to mitoplasts and treated with proteinase K (lanes 4 and 9), or lysed with 1% Triton X-100 before proteinase K treatment (lanes 5 and 10). For the samples shown in lanes 7–10, the membrane potential ($\Delta\psi$) was dissipated by addition of 5 μM valinomycin during the import reaction. For comparison, lanes 1 and 6 show 10% of the precursor protein used per import reaction. Proteins were separated by SDS-PAGE and transferred to nitrocellulose. Efficiencies of mitoplast formation and protease treatment were controlled by Western blotting. Mba1 signals were detected by autoradiography. (D) Newly synthesized Cox2 is unstable in Δ*mba1* mitochondria. Mitochondrial translation products were radiolabeled in wild-type and Δ*mba1* cells for 30 min. Then the cells were washed twice and reincubated in the presence of 2.5 mg/ml chloramphenicol and 5 mM cold methionine at 30°C for 0, 30, or 90 min. Proteins were separated by SDS-PAGE, and signals of radioactive Cox2 were quantified. The graph shows the amounts of Cox2 relative to the material present directly after the labeling reaction.

blocked and the cells were further incubated. After different times of this chase period, aliquots were taken and the amount of radiolabeled Cox2 was quantified (Fig. 1 D). In both strains comparable amounts of Cox2 were synthesized (data not shown). Although in wild-type cells the Cox2 level stayed constant, during the chase period Cox2 was rather unstable in the Δ*mba1* mutant and within 90 min more than half of the protein was degraded. From this we conclude that Mba1 is not required for Cox2 synthesis, but for a later step in Cox2 biogenesis.

Mba1 Is Required for Efficient Membrane Insertion of Cox2

The isolation of both Mba1 and Oxa1 in the same genetic screen (Rep and Grivell, 1996) might indicate a similar function of both proteins. Therefore, we tested whether Mba1 plays a role in the membrane insertion process of mitochondrial translation products. The mitochondrial genome of yeast encodes eight major proteins, out of which seven are integral membrane subunits of respiratory chain complexes (Borst and Grivell, 1978). One of these proteins, Cox2, is synthesized with an NH_2-terminal presequence that is removed by the Imp1 protease in the intermembrane space after translocation across the inner membrane. Therefore, the accumulation of the precursor form of Cox2 is characteristic of defects in the protein export process from the matrix and is associated with *oxa1* mutations, for example (Bauer et al., 1994; He and Fox, 1997; Hell et al., 1997). To look for an accumulation of Cox2 precursor in the matrix, we synthesized proteins in isolated wild-type or Δ*mba1* mitochondria in the presence of [^{35}S]methionine. In wild-type mitochondria no precursor of Cox2 was observed (Fig. 2 A, top). In contrast, in Δ*mba1* mitochondria a significant fraction of Cox2 remained unprocessed (Fig. 2 A, bottom). To test whether this fraction accumulated in the mitochondrial matrix, we performed the translation reaction in mitoplasts and treated them with protease to degrade translation products that expose domains into the intermembrane space (lane 4). The mature form of Cox2 was completely degraded both in the wild-type and mutant sample. In contrast, the Cox2 precursor (pCox2) formed in the Δ*mba1* mitoplasts was not degraded, indicating an export defect in the Δ*mba1* mitochondria. In addition to the Cox2 precursor, the translation products cytochrome b and Cox1 were partially protease inaccessible in Δ*mba1* mitoplasts. This suggests that Mba1 is involved in the insertion of several mitochondrially encoded proteins.

During the biogenesis of Cox2, both its NH_2 and COOH termini have to be translocated across the membrane. To assess whether the export of the COOH terminus is also affected in Δ*mba1*, mitochondrial translation products were radiolabeled in mitoplasts before an incubation without or with protease. The mitochondria were reisolated, lysed, and subjected to immunoprecipitation using antibodies against Cox2. In wild-type mitochondria only mature, protease-accessible Cox2 was detected, indicating that Cox2 was exclusively in an NH_{out} topology (Fig. 2 B, lanes 1 and 2). Depletion of the membrane potential strongly reduced the export efficiency of both the NH_2 and COOH termini resulting both in a protease-inaccessible

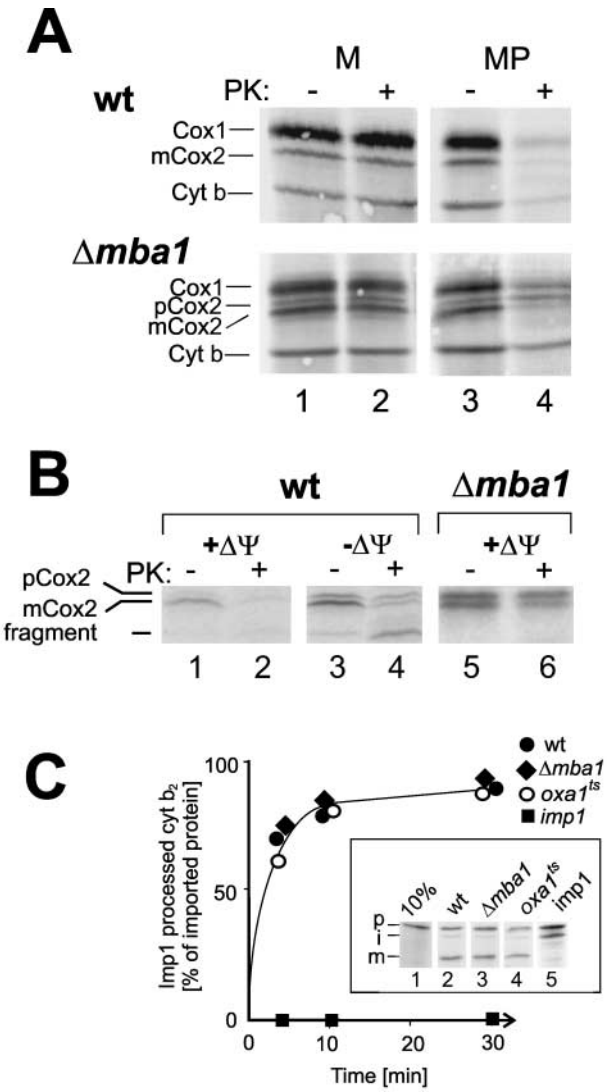


Figure 2. Mba1 is required for efficient insertion of Cox2. (A) Wild-type (wt; top) or Δ*mba1* mitochondria (bottom) were mock treated or converted to mitoplasts. Mitochondrial translation products were radiolabeled for 20 min at 25°C. The samples were split and treated without or with 50 μg/ml proteinase K (PK) as indicated. pCox2, Cox2 precursor; mCox2, mature Cox2; Cyt b, cytochrome b. (B) Translation products were radiolabeled in the presence (−Δψ, lanes 3 and 4) or absence (residual lanes) of 1 μM valinomycin in wild-type or Δ*mba1* mitoplasts and treated with or without protease K as depicted. The mitoplasts were reisolated and lysed. Radiolabeled Cox2 was visualized by autoradiography after immunoprecipitation with a COOH-terminal Cox2 antiserum. (C) Imp1 processing of cytochrome b_2 precursor. Cytochrome b_2(1-185)-DHFR precursor protein was synthesized in reticulocyte lysate and incubated for the times indicated at 25°C with wild-type, Δ*mba1*, *imp1*, or *oxa1*^{ts} mitochondria. The latter had been preexposed to 37°C to induce the phenotype. Mitochondria were then treated with 50 μg/ml proteinase K to remove nonimported protein. After SDS-PAGE and autoradiography, the fraction of Imp1 processed in relation to total imported protein was quantified by densitometry. The inset shows the signals obtained after import for 20 min in the strains indicated, and 10% of the precursor protein used for each reaction for comparison (lane 1). Complete swelling of the samples was controlled by Western blotting. p, precursor; i, intermediate; m, mature cytochrome b_2(1-185)-DHFR.

precursor form of Cox2 (N_{in}–C_{in}) and a characteristic COOH-terminal fragment which is generated from a species in N_{out}–C_{in} topology by digestion of the exposed NH_2 terminus (Fig. 2 B, lanes 3 and 4; Herrmann et al., 1995). However, in Δ*mba1* mitochondria, the COOH-terminal fragment was not generated, indicating that Mba1 is not required for translocation of the large COOH-terminal domain of Cox2 (Fig. 2 B, lanes 5 and 6).

To exclude the possibility that a decreased activity of the Imp1 protease in Δ*mba1* mitochondria may cause the observed Cox2 precursor accumulation, we analyzed the kinetics of the Imp1-dependent processing of the cytochrome b_2 presequence in wild-type and mutant mitochondria after in vitro import experiments. Cytochrome b_2 is synthesized in the cytosol with a bipartite presequence which is removed after import in two sequential steps, first by the mitochondrial-processing peptidase and then by Imp1. Both the import kinetics into the Δ*mba* mitochondria (not shown) and the kinetics of the maturation by Imp1 (Fig. 2 C) resembled that of wild-type mitochondria. This excludes a diminished Imp1 processing in the Δ*mba1* strain.

Mba1 Interacts with Mitochondrial Translation Products

Is Mba1 in direct contact with inserting polypeptides? We used chemical cross-linking reagents to answer this question, as they had been very useful to study the interaction of Oxa1 with its substrates (Hell et al., 1997, 1998). Translation products were radiolabeled with [^{35}S]methionine in isolated mitochondria. After 10 min the sample was either mock treated (Fig. 3 A, lanes 1–5) or treated with the cross-linker DFDNB (lanes 6–10). DFDNB is a lysine-specific cross-linker with a very short distance of the reactive groups (0.3 nm) that can only connect proteins that are in direct contact with each other. After 30 min the reaction was stopped by the addition of an excess of cold methionine and glycine to quench the cross-linker. Under these conditions, protein synthesis still occurred but, in addition to the completed translation products, a "smear" of uncompleted products was visible on SDS-PAGE, caused by stops of the in vitro translation process. The mitochondria were lysed and the resulting extracts were used for immunoprecipitation with antisera against Mba1 (lanes 3 and 8), Oxa1 (lanes 4 and 9), the protease Yta10 (lanes 5 and 10), or preimmune serum for control (lanes 2 and 7). No translation products were precipitated from samples without DFDNB or when preimmune serum was used. However, Mba1, like Oxa1 and Yta10, was cross-linked to polypep-

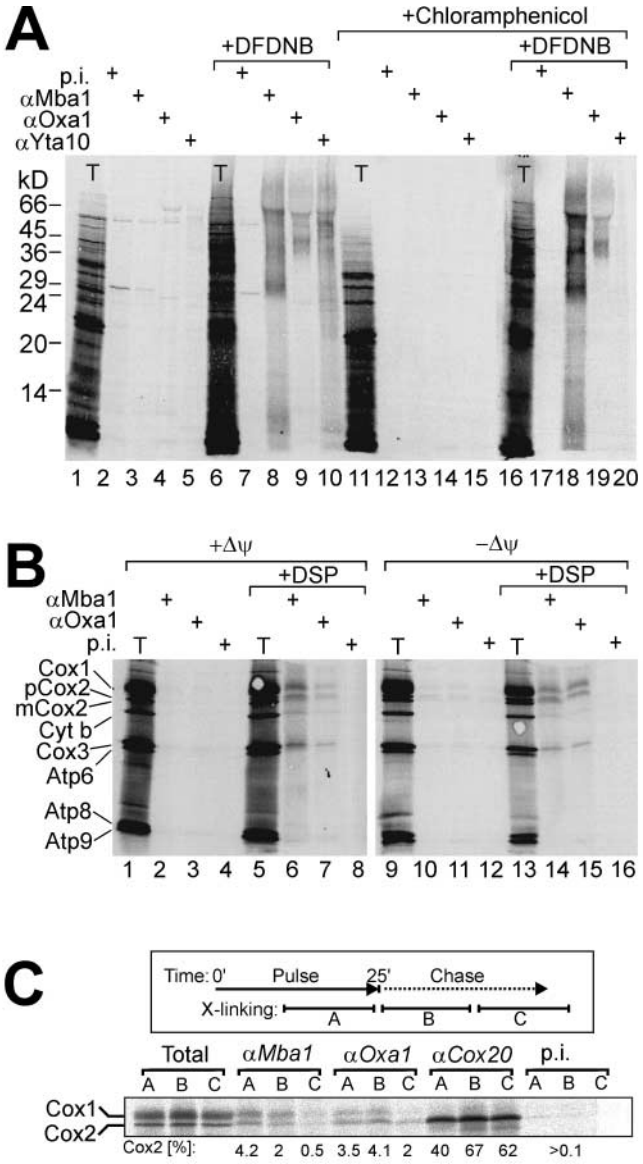


Figure 3. Mba1 is in direct proximity to mitochondrial translation intermediates. (A) Mitochondrial translation products were radiolabeled for 10 min at 25°C. The reaction was split and treated without (lanes 1–10) or with (lanes 11–20) 2 mg/ml chloramphenicol. After 1 min the samples were either mock treated (lanes 1–5 and 11–15) or treated with 400 μM DFDNB (other lanes) for 20 min. Cross-linking was quenched by the addition of 100 mM glycine. The mitochondria were reisolated, washed, and incubated in 1% SDS for 30 s at 96°C. The extract was centrifuged for 5 min at 15,000 *g* and either directly applied to SDS-PAGE (T, total) or used for immunoprecipitation with preimmune serum (p.i.) or antiserum against Mba1, Oxa1, and Yta10 as indicated. (B) Mitochondrial translation products were radiolabeled for 15 min in the absence (+Δψ, lanes 1–8) or the presence (−Δψ, lanes 9–16) of 1 μM valinomycin. Then the samples were mock treated (lanes 1–4 and 9–12) or treated with 200 μM DSP (other lanes) for 30 min. Cross-linking and translation were stopped by addition of glycine and unlabeled methionine. Mitochondria were reisolated, washed, and further treated as outlined in A. Lanes labeled T show 10% of the extract used for immunoprecipitation. (C) Transient interaction of Mba1 and Oxa1 with newly synthesized Cox2. Mitochondrial translation products were radiolabeled in three separate reactions for 25 min (Pulse). The labeling was stopped by the addition of 25 μg/ml puromycin and 5 mM methionine, and the samples were further incubated for 30 min (Chase). During this procedure 400 μM of the cross-linker DSP was present for 15 min and then quenched with 100 μM glycine. As depicted in the insert, sample A was cross-linked during the labeling period, B directly afterwards, and C 15 min after the beginning of the chase reaction. The mitochondria were reisolated, lysed, and either directly applied to SDS-PAGE (Total) or used for immunoprecipitation with preimmune serum (p.i.) or antiserum against Mba1, Oxa1, and Cox20 as indicated. Lanes labeled "Total" show 10% of the extract used for immunoprecipitation. The amounts of immunoprecipitated Cox2 were quantified and are shown as a percentage of total Cox2.

tides of a wide size range, indicating the interaction of all three proteins with uncompleted translation products.

These cross-linked polypeptides might represent nascent chains that are bound to ribosomes or uncompleted translation products that are prone to protein degradation. To test this we blocked the translation reaction with chloramphenicol before DFDNB was added (lanes 11–20). Chloramphenicol leads to an arrest of protein synthesis and nascent chains remain firmly associated with the ribosomes. Under these conditions, no cross-linking of translation products to Yta10 was found, indicating that this protease may preferentially bind to uncompleted polypeptides which are no longer bound to ribosomes. In contrast, an increase of the cross-linking efficiency to Mba1 was observed, suggesting that Mba1 interacts with translation products predominantly during their synthesis. This is further indicated by the wide size range of the cross-link products, which is typical for cross-linked nascent chains and which would not be expected if cross-linking occurred only to completely synthesized and inserted proteins.

Which proteins are substrates of Mba1? To answer this question we used the cleavable cross-linker DSP. During protein synthesis in isolated mitochondria, rather low amounts of DSP were added so that translation was allowed to continue during the cross-linking reaction, leading to the completion of the synthesis of cross-linked adducts (Hell et al., 1998). The mitochondria were lysed, and the extracts were used for immunoprecipitation with antisera against Mba1 and Oxa1 or with preimmune serum. Then the cross-links were cleaved and the samples were applied to SDS-PAGE. Four out of the eight translation products could be specifically pulled down together with Mba1 and Oxa1: subunits 1, 2, and 3 of the cytochrome c oxidase and, with lower efficiency, cytochrome b (Fig. 3 B, lanes 6 and 7). Thus, these proteins are in close proximity to Mba1 and Oxa1 at some stage of their synthesis. The other translation products do not interact with Mba1 and Oxa1, or do not expose lysine residues which would allow cross-linking. A similar result was obtained when the membrane potential was depleted during synthesis (lanes 9–16). Thus, the membrane potential is not required for the interaction of mitochondrial translation products with Mba1 and Oxa1.

At which time point of the Cox2 biogenesis does the interaction to Mba1 and Oxa1 occur? To address this question translation products were radiolabeled in wild-type mitochondria for 25 min in three parallel reactions. Then the labeling reaction was stopped by addition of puromycin and an excess of cold methionine and the mitochondria were further incubated ("chase"). The cleavable cross-linker DSP was present for 15 min either during the labeling period (A) or at an early (B) or late (C) stage of the chase period as depicted in Fig. 3 C. Then the mitochondria were reisolated, lysed, and subjected to immunoprecipitation with sera against Mba1, Oxa1, Cox20, or preimmune serum. Cross-links to Mba1 and Oxa1 were mainly formed during or directly after the labeling period. This indicates a transient interaction of Cox2 with both Mba1 and Oxa1 very early in the Cox2 biogenesis. In contrast, the cross-link efficiency to Cox20 was much higher and was maximal at later time points. Cox20 was reported to represent a chaperoning factor that forms a stable interaction with unassembled Cox2 (Hell et al., 2000).

Mba1 Facilitates Export of Nuclear-encoded Proteins

Efficient membrane insertion of Cox2 appears to depend on the recruitment of the ribosomes to the inner membrane (Sanchirico et al., 1998) which may allow a cotranslational translocation process. If Mba1 is required to recruit ribosomes to the inner membrane, it should be dispensable for a posttranslational export of the Cox2 NH_2 terminus. To test this we performed import experiments with the in vitro–synthesized fusion protein pSu9(1-66)Cox2(1-74)-DHFR, comprising a mitochondrial targeting signal followed by the first 74 amino acid residues of Cox2 and mouse dihydrofolate reductase (Fig. 4 A). This protein is imported into the matrix where the mitochondrial targeting signal is removed so that the NH_2 terminus of Cox2 can be inserted into the inner membrane (Herrmann et al., 1995). Upon insertion, the NH_2-terminal 39 amino acid residues are exposed to the intermembrane space, which leads to the generation of a characteristic fragment after protease treatment of mitoplasts. This allows the quantification of the posttranslational insertion of the NH_2 terminus of Cox2. Both Mba1 and functional Oxa1 were required for efficient insertion of Cox2(1-74)-DHFR (Fig. 4 A). This suggests that, similar to Oxa1, Mba1 plays a role in the protein export process from the mitochondrial matrix.

Is Mba1 also involved in the insertion of nuclear-encoded proteins? Oxa1 has been used as a model protein to analyze the export of nuclear-encoded proteins from the matrix into the inner membrane (Herrmann et al., 1997). After import into the mitochondrial matrix, Oxa1 is initially protected against protease, even after opening of the outer membrane. In contrast, after export of the NH_2 terminus of Oxa1 protease, treatment of mitoplasts leads to a characteristic 27-kD fragment. To analyze the export efficiency in various strains, radiolabeled Oxa1 was imported into isolated mitochondria for 10 min. Then mitochondria were reisolated, converted into mitoplasts, treated with protease, and the ratio of protease accessible (i.e., inserted) to total protein was determined (Fig. 4 B). Complete opening of the intermembrane space was controlled by Western blotting. In wild-type mitochondria, 61% of Oxa1 was inserted. The insertion was slightly reduced in Δ*mba1* and *oxa1*^{ts} mitochondria (the mitochondria were not pretreated at 37°C, and thus the temperature-sensitive phenotype was not induced). However, Oxa1 was inserted with significantly reduced efficiency in mitochondria of a Δ*mba1 oxa1*^{ts} double mutant (Fig. 4 B). This indicates that even at permissive conditions Oxa1^{ts} function depends on the presence of Mba1. Thus, in the background of this partially impaired Oxa1 protein, Mba1 is required for the insertion of a nuclear-encoded protein, which indicates that Oxa1 and Mba1 cooperate in the process of Oxa1 insertion.

Next we used chemical cross-linking to test whether Mba1 physically interacts with the imported Oxa1 during its insertion (Fig. 4 C). After import of Oxa1, mitochondria were incubated with the cross-linker DSG, lysed, and the resulting extract was used for immunoprecipitation with Mba1-specific or preimmune serum. In the presence

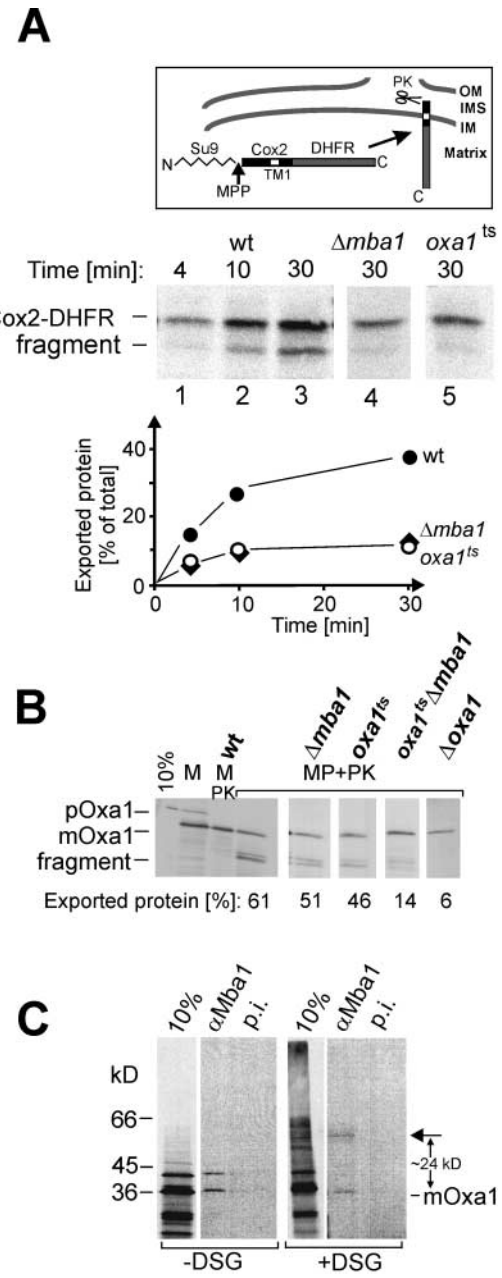


Figure 4. Mba1 interacts with nuclear-encoded proteins. (A) Posttranslational insertion of the Cox2 NH_2 terminus. In the inset, the export of the Cox2 NH_2 terminus after import of pSu9(1-66)pCox2(1-74)-DHFR is depicted. N, NH_2 terminus; C, COOH terminus; OM, outer membrane; IMS, intermembrane space; IM, inner membrane; MPP, cleavage site of the mitochondrial processing peptidase; Su9, subunit 9 presequence; TM1, first transmembrane domain of Cox2. (Middle and bottom panels) Wild-type, Δ*mba1*, and *oxa1*^{ts} mitochondria were pretreated for 10 min at 37°C and incubated with pSu9(1-66)pCox2(1-74)-DHFR for 4, 10, and 30 min at 25°C. The mitochondria were converted into mitoplasts and treated with proteinase K (50 μg/ml) to digest the NH_2 terminus of inserted Cox2. Lanes 1–3 show the generation of the fragment in wild-type mitochondria; lanes 4 and 5 show the 30 min reactions of Δ*mba1* and *oxa1*^{ts} mitochondria, respectively. The graph shows a quantification of the fraction of inserted Cox2 relative to total imported protein at various times of import. The numbers were corrected for the methionine residues contained in the different Cox2 species. (B) Oxa1 precursor (pOxa1) was synthesized in reticulocyte lysate and incubated for 20 min at 25°C with wild-type, Δ*mba1*, *oxa1*^{ts}, *oxa1*^{ts} Δ*mba1*, or Δ*oxa1* mitochondria. Mitochondria were then converted to mitoplasts (MP) and treated with 50 μg/ml proteinase K (PK) as indicated to convert inserted Oxa1 into a characteristic fragment. The ratio of fragment to total imported Oxa1 was determined by densitometry. (C) Radiolabeled Oxa1 precursor was imported into wild-type mitochondria for 10 min. Then the reaction was split and one half was mock treated, and the other treated with 100 μM DSG for 20 min. The mitochondria were reisolated, lysed, and either directly analyzed by SDS-PAGE (10%) or used for immunoprecipitation with Mba1 antiserum or with preimmune serum. The cross-link product specifically precipitated with Mba1 serum is indicated by an arrow.

of the DSG a cross-link product of ~60 kD was specifically pulled down with the Mba1 serum. This corresponds to a size shift of ~24 kD, as would be expected for a cross-link to Mba1.

Mba1 Can Function Independently of Oxa1

What is the mode of cooperation of Mba1 and Oxa1? Both complexes may either work in parallel or have overlapping but independent functions. Alternatively, they might function sequentially. For example, Mba1 might pass on a substrate protein to the OXA complex. We used the advantage of yeast genetics to differentiate between both possibilities. If Mba1 would function upstream or downstream of Oxa1, the phenotype of a Δ*oxa1* Δ*mba1* double and a Δ*oxa1* single mutant should be similar. In contrast, we observed severe synthetic growth defects of the double mutant even on glucose (Fig. 5 A): this strain hardly grew on glucose at 24°C and grew slowly at 30°C (Fig. 5 A, top). This synthetic effect indicates that Mba1 can function independently of Oxa1 and can facilitate protein insertion on a pathway that works in parallel to the Oxa1 route. A similar strong growth defect was observed when *MBA1* was deleted in an *oxa1*^{ts} background at restrictive conditions (37°C). Even at permissive conditions (24°C) this mutant was unable to grow on glycerol (Fig. 5 A, bottom), although the steady state levels of Oxa1 were unchanged (not shown). In addition, a complete block of the Cox2 processing was observed in the *oxa1*^{ts} Δ*mba1* strain even at 24°C (not shown). Thus, the *oxa1*^{ts} strain requires Mba1 for Cox2 export and respiration competence even at the permissive temperature. This suggests that Oxa1^{ts} is partially defective at all temperatures, but that Mba1 can compensate for the defects at lower temperatures.

Mba1 Is Not Part of the OXA Complex

Oxa1 has been reported to be part of an oligomeric complex (Hell et al., 1998). Is Mba1 a subunit of this complex? No cross-links of endogenous Mba1 and Oxa1 could be detected and Mba1 was not coimmunoprecipitated with Oxa1 or vice versa, even when mitochondria were lysed with the very mild detergent digitonin (not shown). Both proteins did not cofractionate upon gel filtration chromatography, as shown in Fig. 5 B. Mba1 is part of a 200-kD complex that is slightly but distinctly smaller than the OXA complex (250–300 kD). In addition, deletion of Mba1 did not affect the levels of Oxa1, which might be expected if both proteins are subunits of one complex (Fig. 1 A). This also excludes that the effects observed in the

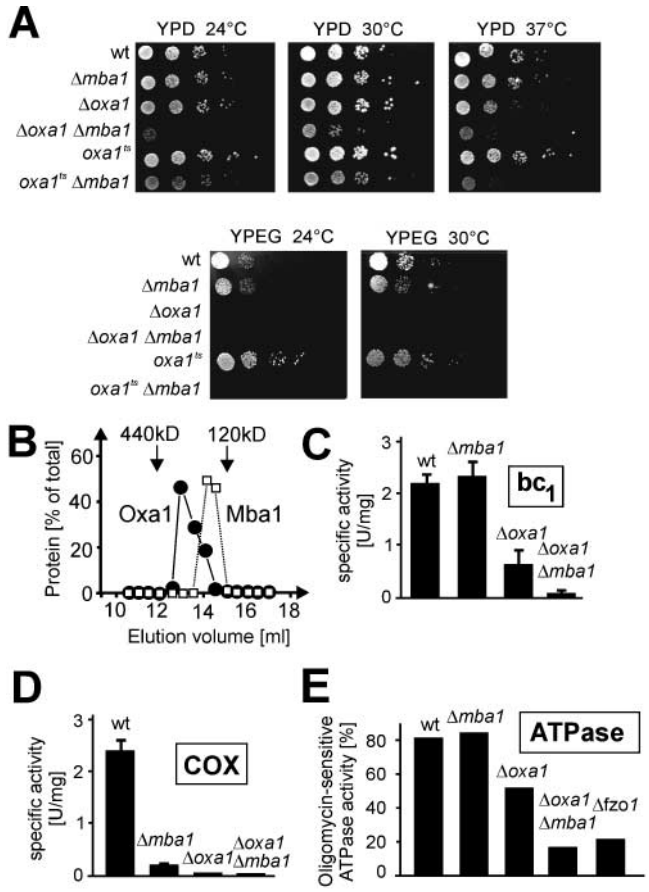


Figure 5. Mba1 can function independently of Oxa1. (A) Mutations in *mba1* and *oxa1* cause synthetic growth defects. The strains indicated were grown on YPD medium to log phase and serial 10-fold dilutions of the cultures were spotted on YP plates containing 2% glucose (YPD; top) or 2% glycerol and 2% ethanol (YPEG; bottom). The plates were incubated at the temperatures indicated for 2 (top) or 4 d (bottom). (B) Oxa1 and Mba1 do not cofractionate upon gel filtration. A digitonin extract of wild-type mitochondria was separated on a Superose 12 column. Resulting fractions were analyzed by Western blotting and the signals for Oxa1 and Mba1 were quantified. (C and D) The activities of the bc1 and the Cox complex are decreased in *oxa1* and *mba1* mutants. Enzyme activities of wild-type, Δ*mba1*, Δ*oxa1*, and Δ*oxa1* Δ*mba1* mitochondria were measured in three experiments. Error bars show the standard deviation. (E) ATPase activities of wild-type, Δ*mba1*, Δ*oxa1*, Δ*oxa1* Δ*mba1*, and Δ*fzo1* were measured in the presence or absence of oligomycin. The percentage of oligomycin-sensitive ATPase activity is shown. The *rho*^{0} mutant Δ*fzo1* was used as a control for mitochondria lacking functional F_0-ATPase.

Δ*mba1* strain were caused by reduced steady state levels of Oxa1. In summary, these experiments indicate that Mba1 and Oxa1 are not stably interacting with each other. However, a transient interaction of both components cannot be excluded.

mba1 and oxa1 Mutations Cause Synthetic Defects of Respiratory Chain Enzymes

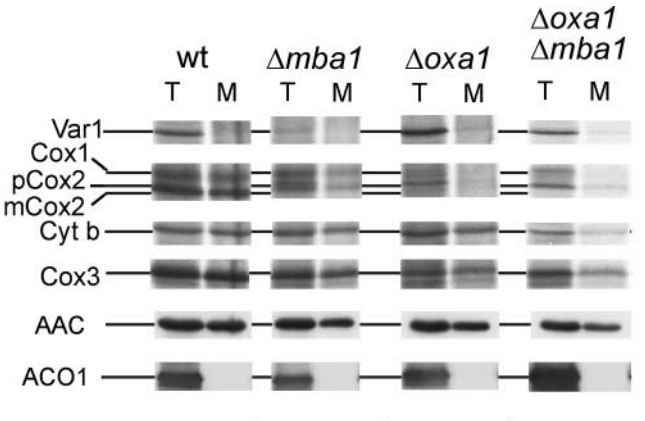
To analyze the nature of the synthetic growth phenotypes observed for *mba1* and *oxa1* mutants, we measured the levels of activity of the bc_1 and the Cox complexes of the respiratory chain in single and double deletion strains (Fig. 5, C and D). The absence of Mba1 alone led to a reduction in Cox activity by >90%, but did not affect bc_1 activity. Δ*oxa1* mitochondria still contained ~30% of the wild-type bc_1 activity, yet almost no activity was detectable in the double deletion mutant. Defects in the biogenesis of the F_0 part of the F_0F_1-ATPase cause a strong reduction of the percentage of oligomycin-sensitive mitochondrial ATPase activity (Schatz, 1968). As shown in Fig. 5 E, deletion of both *MBA1* and *OXA1* reduced the level of oligomycin-sensitive ATPase to ~20%, which is similar to amounts found in *rho*^{0} strains, e.g., Δ*fzo1* (Rapaport et al., 1998), which do not contain any functional F_0-ATPase (Ackerman and Tzagoloff, 1990). Thus, in the presence of Oxa1, Mba1 is dispensable for the biogenesis of the bc_1 and ATPase complex, but Mba1 is absolutely required if Oxa1 is absent.

mba1 and oxa1 Mutations Cause Synthetic Defects in Protein Export

Next we determined the efficiency of membrane integration in the different mutants. Mitochondrial translation products were radiolabeled for 20 min. Then a fraction containing integral membrane proteins were isolated by carbonate extraction and flotation and compared with the total amount of synthesized proteins (Fig. 6). The membrane insertion of cytochrome b was significantly reduced in the absence of Mba1 and almost completely abolished in Δ*mba1* Δ*oxa1* mitochondria. The insertion of Cox1 and Cox3 was also impaired in Δ*mba1* mitochondria. This fractionation protocol probably underestimates the defects in the mutants since it does not allow verification of a correct topology and functionality of the membrane-associated proteins.

Mba1 Can Partially Compensate for the Loss of Oxa1

To further analyze this Oxa1-independent function of Mba1, we asked whether an interaction of Mba1 with mitochondrial translation products can be observed in the absence of Oxa1. We performed chemical cross-linking using either wild-type, Δ*oxa1*, or Δ*imp1* mitochondria (Fig. 7 A). Translation products were labeled in isolated mitochondria in the presence of [^{35}S]methionine. After incubation with the cleavable cross-linker DSP (lanes 5–11) or mock treatment (lanes 1–4), the mitochondria were lysed and cross-linked adducts to Mba1 or Oxa1 were isolated by immunoprecipitation. Then the cross-links were broken and the samples were analyzed by SDS-PAGE and autoradiography. In wild-type mitochondria, Cox1, Cox2, and Cox3 were specifically cross-linked to both Mba1 and Oxa1. In the absence of Oxa1, Mba1 still interacted with Cox3, indicating that Oxa1 is not required for this interaction. The levels of Cox1 synthesized in Δ*oxa1* mitochondria are strongly reduced, so that its interaction to Mba1 cannot be assessed. However, Cox2 is efficiently synthesized, but no cross-linking to Mba1 was observed. Thus, Oxa1 is dispensable for the interaction of Mba1 with Cox3, but is required for the contact of Mba1 to Cox2. It is unlikely that this is due to the Cox2 processing defect in Δ*oxa1* mitochondria since the cross-linking efficiency of Cox2 to Mba1 was unaffected in Δ*imp1* mitochondria that also accumulate Cox2 precursor (Fig. 7 A, bottom).



pCox2	-	3%	9%	2%
mCox2	75%	57%	-	-
Cyt b	98%	57%	88%	8%
Cox3	98%	65%	73%	52%

Figure 6. *oxa1* and *mba1* mutants show synthetic protein insertion defects. Translation products were radiolabeled for 20 min at 25°C in mitochondria of the strains indicated. Mitochondria were reisolated and treated with 0.1 M Na_2CO_3. Samples were split. Proteins were either directly precipitated with TCA (T, total) or after flotation through a sucrose gradient (M, membranes), separated by SDS-PAGE, and transferred to nitrocellulose. Var1, Cox2, cytochrome b, and Cox3 signals were detected by autoradiography, quantified, and the proportions of floatable material are indicated. For control, the soluble protein Aco1 and the membrane protein ATP/ADP carrier (AAC) were detected by Western blotting.

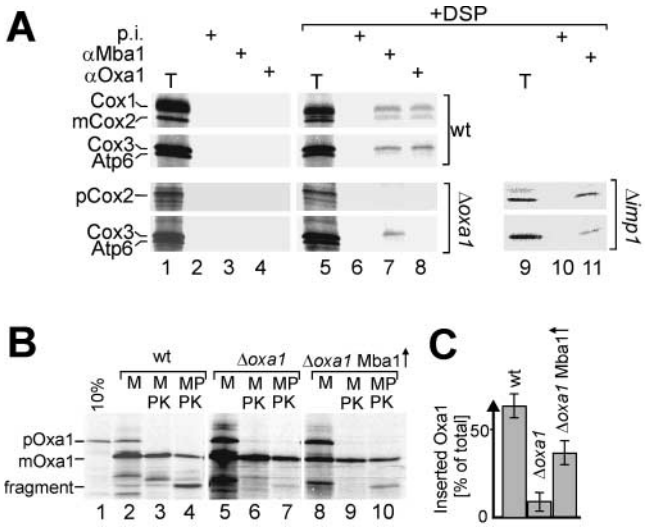


Figure 7. Mba1 can function in the absence of Oxa1. (A) Translation reactions in wild-type, Δ*oxa1*, or Δ*imp1* mitochondria were incubated with the cleavable cross-linker DSP and analyzed by immunoprecipitation as described in the legend to Fig. 3 B. mCox2, mature Cox2. (B) ^{35}S-labeled Oxa1 precursor was imported for 15 min at 25°C into wild-type, Δ*oxa1*, and Δ*oxa1*Mba1↑ mitochondria. The samples were divided into three parts. Mitochondria (M) were then either mock treated, treated with proteinase K (PK), or converted to mitoplasts (MP) and proteinase K treated. Lane 1 shows 10% of the preprotein used per reaction. (C) The ratio of protease-accessible Oxa1 to total imported Oxa1 was quantified from four independent experiments and depicted in the diagram. Error bars show the standard deviation ($n = 4$).

To directly prove an Oxa1-independent function of Mba1, we tested whether overexpression of Mba1 allows the loss of Oxa1. Expression of Mba1 under control of the strong *TPI1* promoter did not suppress the growth defect of a Δ*oxa1* mutant on glycerol, indicating that Mba1 overexpression does not make Oxa1 dispensable. However, overexpression of Mba1 clearly restored the competence of the mitochondria to integrate newly imported Oxa1 into the inner membrane (Fig. 7, B and C). In vitro–synthesized Oxa1 precursor was imported into wild-type, Δ*oxa1*, or Δ*oxa1*Mba1↑ mitochondria. The mitochondria were reisolated, converted to mitoplasts, and protease treated (lanes 4, 7, and 10). After SDS-PAGE and autoradiography the ratio of protease-accessible to total imported Oxa1 was quantified as depicted in Fig. 7 C. In Δ*oxa1*Mba1↑ mitochondria, 31 ± 6% of the imported Oxa1 NH_2 termini had reached the intermembrane space compared with 7 ± 5% in Δ*oxa1* and 62 ± 6% in wild-type mitochondria. This indicates that, at least in case of this nuclear-encoded protein, the overexpression of Mba1 can partially compensate for the loss of Oxa1.

Discussion

The mitochondrial inner membrane belongs to the protein-richest membranes of the eukaryotic cell. It accommodates a large number of different integral membrane proteins which, typically assembled in multiprotein complexes, perform a variety of functions, like substrate transport or generation of ATP. Upon growth of mitochondria, these proteins have to be efficiently inserted into the inner membrane and the correct topology of each single transmembrane segment must be achieved. Many of these proteins are inserted from the matrix side and intermembrane space domains of these proteins have to be exported across the inner membrane. The OXA complex plays an important role in this insertion (or export) process. However, there may exist other additional components which act together with Oxa1 or independent of Oxa1 to facilitate the membrane insertion of proteins.

We have identified Mba1 as one such other component that is in contact with insertion intermediates as they integrate into the lipid bilayer. Like Oxa1, Mba1 is required for efficient protein insertion of both mitochondrially and nuclear-encoded proteins. Mba1 and Oxa1 overlap not only in substrate specificity, but most likely also in their function. In the presence of Oxa1, Mba1 is largely dispensable, indicating that Oxa1 can function independently of Mba1. In contrast, Mba1 is unable to replace Oxa1 completely even upon overexpression, but restores the defective insertion of the Oxa1 precursor in a Δ*oxa1* mutant. This partial suppression may be explained by the observation that Mba1 needs Oxa1 to interact with Cox2, whereas its interaction with Cox3 is independent of Oxa1.

Is Cox2 the only protein that exclusively depends on Oxa1 function? It has been shown that mutations in Cox2 suppress the growth defects observed in *oxa1*^{ts} mutants (Meyer et al., 1997), and therefore the integration of other

proteins may not be strictly dependent on Oxa1 function. We showed that the absence of both Mba1 and Oxa1 cause a strong growth defect even on glucose. This synthetic defect points not only to an Oxa1-independent function of Mba1, but suggests that both components can act in parallel pathways which, if both are blocked, lead to severe problems in the biogenesis of mitochondria. The Δ*oxa1* Δ*mba1* double mutant is associated with an almost complete inactivation of both the respiratory chain and the F_0F_1-ATPase, which is known to lead to a reduced growth even on glucose probably due to a dissipation of cellular ATP levels by the uncoupled F_1-ATPase (Lai-Zhang et al., 1999).

What is the molecular function of Mba1? Translocation pathways use typically three different types of components: (a) receptors that make contact to the substrates, (b) pore forming proteins that mediate the translocation, and (c) chaperones that assist translocation intermediates or completely translocated proteins until they attain their folded and assembled state. It is not trivial to analyze the precise function of these components, and even in the case of Oxa1, which has now been studied for several years, the exact molecular role is not clear. The localization of Mba1 at the matrix side of the inner membrane and the following observations seem best compatible with a receptor function of Mba1: (a) Mba1 interacts with nascent chains during their synthesis on mitochondrial ribosomes; (b) this interaction is transient and restricted to an early stage in the biogenesis of substrate proteins; (c) a conditional *Oxa1*^{ts} mutant protein loses function completely in a Δ*mba1* background, indicating that Mba1 cooperates with the Oxa1 translocase; and (d) overexpression of Mba1 can partially suppress the defects of Δ*oxa1* mitochondria, and thus Mba1 can improve protein insertion on an Oxa1-independent pathway. Hence, Mba1 might serve as a substrate receptor that delivers proteins both to Oxa1 and to a translocase in parallel. In the absence of Mba1, low local substrate concentrations at the inner membrane might abolish the function of the partially defective Oxa1^{ts} protein. On the other hand, increased Mba1 concentrations might improve the recruitment of substrates to a so far uncharacterized translocase, resulting in the observed suppression of defects of Δ*oxa1* mitochondria.

Interestingly, the transcription of *MBA1* is closely coregulated with that of genes encoding subunits of the mitochondrial ribosome. In a comparison with 300 expression profiles, 7 out of the 9 best correlating transcripts to *MBA1* were for mitochondrial ribosomal proteins: MRPS9, MRPL24, MRPL6, YDR115w (L34), MRP21, MRPS28, and MRPL9 showed correlation coefficients higher than 0.7 (Hughes et al., 2000). This coregulation of ribosomes and Mba1 may ensure an adequate insertion capacity for the level of proteins produced in mitochondria.

Together with Oxa1, Mba1 was first isolated as a protein that upon overexpression suppressed mutations of the inner membrane protease Yta10 (Afg3). What might be the reason for this interaction? It was shown recently that the degradation of membrane proteins by inner membrane proteases requires the active extraction of transmembrane segments (Leonhard et al., 2000). How this extraction or "deinsertion" is achieved is unclear, but it is conceivable that insertion machineries play a crucial role in this process. Increased levels of these machineries in the suppressor strains might improve degradation in the *yta10* mutants, perhaps by allowing degradation by the Yta10 homologue Yme1 which faces the intermembrane space.

How many insertion pathways into the inner membrane do exist? In an elegant genetic screen, He and Fox (1999) isolated Pnt1 as a component required for efficient insertion of the Cox2 COOH terminus. Loss of Pnt1 caused only minor growth defects and it was proposed that it might overlap in function with Oxa1 (He and Fox, 1999). Another candidate for a component involved in protein insertion is the Oxa1 homologue Cox18. It will be a major challenge for the future to further characterize the mode of function and interaction of these various components on a molecular level.

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