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A Disulfide Relay System in the Intermembrane Space of Mitochondria that Mediates Protein Import

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Summary

We describe here a pathway for the import of proteins into the intermembrane space (IMS) of mitochondria. Substrates of this pathway are proteins with conserved cysteine motifs, which are critical for import. After passage through the TOM channel, these proteins are covalently trapped by Mia40 via disulfide bridges. Mia40 contains cysteine residues, which are oxidized by the sulfhydryl oxidase Erv1. Depletion of Erv1 or conditions reducing Mia40 prevent protein import. We propose that Erv1 and Mia40 function as a disulfide relay system that catalyzes the import of proteins into the IMS by an oxidative folding mechanism. The existence of a disulfide exchange system in the IMS is unexpected in view of the free exchange of metabolites between IMS and cytosol via porin channels. We suggest that this process reflects the evolutionary origin of the IMS from the periplasmic space of the prokaryotic ancestors of mitochondria.

Introduction

The intermembrane space (IMS) of mitochondria, the compartment enclosed by the outer and inner membranes of the organelle, harbors many components of outstanding importance for the cell: IMS proteins function as components of the electron transport chain, enzymes of metabolic processes, or transporters for polypeptides and metal ions. In addition, a number of apoptotic factors reside in the IMS. Whereas the physiological function of certain IMS proteins was studied extensively, rather little is known about their biogenesis.

Most mitochondrial proteins are encoded in the nucleus and synthesized on cytosolic ribosomes. Proteins destined for the mitochondrial matrix typically carry mitochondrial targeting signals in the form of presequences that direct the polypeptides to and across translocation complexes present in the outer membrane, the translocase of the outer membrane (TOM) complex, and in the inner membrane, the translocase of the inner membrane (TIM)23 complex (for review, see Endo and Kohda, 2002; Jensen and Dunn, 2002; Koehler, 2004a; Pfanner and Geissler, 2001). The import of these proteins is driven by the membrane potential and by the hydrolysis of ATP in the matrix.

All IMS proteins are nuclear encoded. Proteins that end up as soluble components of the IMS are transported into mitochondria by mechanistically distinct mechanisms (Herrmann and Hell, 2005). Some IMS proteins contain matrix-targeting presequences followed by hydrophobic sorting domains (Hartl et al., 1987). These so-called bipartite presequences direct the proteins to the inner membrane before they are proteolytically removed, thereby releasing the mature parts of these proteins into the IMS (Glick et al., 1992). Bipartite presequences are characteristic for IMS proteins of relatively large molecular mass, and the translocation of these proteins is driven by the import motor of mitochondria, which is part of the TIM23 complex.

A second group of IMS proteins is characterized by a small size, usually between 7 and 16 kDa, and the presence of conserved patterns of cysteine residues. These patterns consist of one or several motifs of cysteine pairs spaced by either three or nine amino acid residues. Upon mutation of one cysteine residue in the "twin Cx_9C" motif, the yeast protein Cox17 was strongly depleted from the IMS of mitochondria and predominantly present in the cytosol (Heaton et al., 2000). Mutations in other cysteine residues like those required for binding of the copper cofactor to Cox17 did not affect the intracellular distribution of Cox17.

The import process of the "small Tim proteins" was studied in some detail. These proteins have an essential role in the transport of hydrophobic precursor proteins across the IMS (Koehler et al., 1998; Sirrenberg et al., 1998). They consist of about 80 to 110 amino acid residues and contain two characteristic Cx_3C motifs. The cysteine residues in these motifs are critical for the accumulation of these proteins in the IMS and for their stable folding (Lu et al., 2004; Lutz et al., 2003). How the stable folding is achieved in vivo is an open question, as there is evidence both for an oxidation of the cysteine residues to disulfide bridges (Curran et al., 2002; Lu et al., 2004) and for the coordination of zinc ions by the four thiol groups of the cysteine residues (Lutz et al., 2003; Sirrenberg et al., 1998). Both states may represent physiological forms of the proteins, and it was proposed that the conversion between oxidized and reduced states may depend on the redox conditions in the IMS (Curran et al., 2004; Koehler, 2004b). According to this hypothesis, the import of proteins into mitochondria may be regulated by the redox conditions, allowing the cell to adapt the proliferation of mitochondria to the physiological needs.

Recently, a component was identified that is specifically involved in the import of IMS proteins that, like Cox17 or Tim13, contain cysteine motifs (Chacinska et al., 2004; Naoe et al., 2004; Terziyska et al., 2005): Mia40 (also referred to as Tim40) is anchored via an N-terminal hydrophobic stretch to the inner membrane and exposes a large domain into the IMS. This domain is characterized by the presence of six cysteine residues, which are absolutely conserved among Mia40 homologs of fungi, plants, and animals. Mia40 is essential for viability of yeast, and depletion of Mia40 severely affects the import of small Tim proteins, of

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Cox17, and of other IMS proteins with cysteine motifs. Newly imported IMS proteins were found to form complexes with Mia40, suggesting that Mia40 might function as a receptor for these proteins. These interactions to Mia40 are transient and are detectable predominantly during the first minutes of the import reaction (Chacinska et al., 2004; Naoe et al., 2004).The function of the cysteine residues in Mia40 is unclear, but Mia40 can bind copper and zinc ions, suggesting that some of its six cysteine residues might play a role in metal binding (Terziyska et al., 2005). In addition, the cysteine residues of Mia40 may be involved in substrate binding, as the complexes of Mia40 and newly imported IMS proteins were reported to be sensitive to reducing agents (Chacinska et al., 2004).

In the present study, we have identified Erv1 as a component essential for the biogenesis of Mia40-dependent substrate proteins. Erv1 is a conserved flavin-linked sulfhydryl oxidase present in the IMS of fungi, plants, and animals (Lee et al., 2000). In *Saccharomyces cerevisiae*, the deletion of Erv1 is lethal, and *erv1* mutants are associated with a variety of phenotypes, such as aberrant mitochondrial morphology and defects in the biogenesis of iron sulfur clusters (Becher et al., 1999; Lange et al., 2001; Lisowsky, 1992). Physiological substrates of Erv1, however, were not identified so far, and the molecular basis of the defects in *erv1* mutants was not understood. Here, we present evidence that the function of Erv1 is important to maintain cysteine residues in Mia40 in an oxidized state. These cysteine residues of Mia40 are critical for its interaction with newly imported substrate proteins via intermolecular disulfide bridges. We propose the existence of a disulfide relay system as the core of the import machinery for proteins with twin Cx_3C and Cx_9C motifs in the IMS of mitochondria.

Results

Mia40 Forms Disulfide Bridges with Newly Imported Tim13 and Cox17

Mia40 interacts transiently with newly imported Tim13 and other "small Tim proteins" following their transport across the outer membrane (Chacinska et al., 2004; Naoe et al., 2004; Terziyska et al., 2005). To further characterize the role of Mia40 in the translocation process, we imported radiolabeled Tim13 into isolated mitochondria. Following translocation of Tim13 across the outer membrane, nonimported Tim13 was removed by trypsin treatment, and the mitochondria were treated with the chemical crosslinker difluoro-dinitrobenzol (DFDNB). A fraction of the imported Tim13 was crosslinked to Mia40 as revealed by immunoprecipitation with Mia40-specific antibodies (Figure 1A, lane 3, arrowhead). The crosslink product migrated with an apparent mass of roughly 75 kDa and was absent in samples that were not treated with crosslinker (Figure 1A, lanes 4–6). Instead, a fraction of the newly imported Tim13 protein was coimmunoprecipitated with Mia40-specific antibodies despite the presence of 1% sodium dodecyl sulfate (SDS) in the lysis buffer (Figure 1A, lane 6, arrowhead). This indicated an association of Mia40 with newly imported Tim13, which is resistant to denaturing conditions. Indeed, SDS-PAGE of these immunoprecipitates under nonreducing conditions revealed an SDS-resistant complex of Mia40 and Tim13 even in the

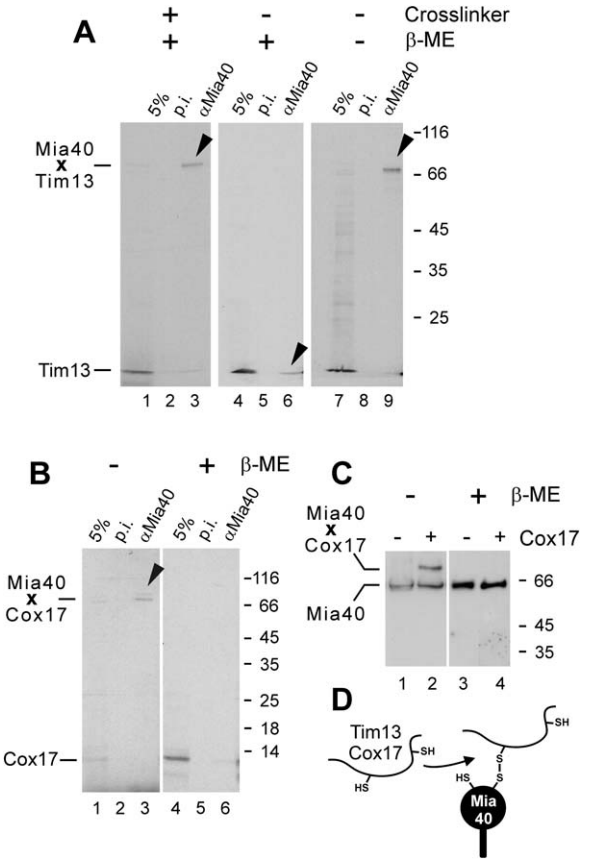

Figure 1. Mia40 Forms Disulfide Bridges with Newly Imported Tim13 and Cox17

(A) Mitochondria were incubated with radiolabeled recombinant Tim13 protein for 20 min at 25°C in the presence (lanes 1 to 3) or absence (lanes 4 to 9) of 200 μM of the crosslinker DFDNB. The crosslinker was quenched with glycine, and nonimported material was removed by trypsin treatment. The mitochondria were washed and then lysed in SDS buffer. Five percent of the resulting extracts were directly applied to SDS-PAGE. The residual lysates were used for immunoprecipitation with antibodies against Mia40 (αMia40) or with preimmune (p.i.) serum. β-mercaptoethanol (β-ME) was added to the loading buffer of samples shown in lanes 1 to 6. Proteins were resolved by SDS-PAGE and visualized by autoradiography. Positions of molecular weight standards are depicted on the right. Arrowheads indicate bands corresponding to Mia40-Tim13 adducts in lanes 3 and 9, and to Tim13 in lane 6.

(B) Recombinant radiolabeled Cox17 was imported into mitochondria. The samples were treated with trypsin and loaded onto the gel either directly or following immunoprecipitation as described in (A). The arrowhead indicates the Mia40-Cox17 adduct.

(C) Wild-type mitochondria (100 μg per sample) were incubated with Cox17 (2 μg) or mock treated for 10 min at 25°C. The samples were incubated with proteinase K (PK) on ice, washed, and dissolved in sample buffer lacking (lanes 1 and 2) or containing (lanes 3 and 4) β-mercaptoethanol. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose. Mia40 was detected by immunoblotting.

(D) Model showing the disulfide bridge between Mia40 and imported proteins.

absence of a crosslinking agent (Figure 1A, lane 9, arrowhead).

A comparable complex was observed between Mia40 and newly imported Cox17, another IMS protein that contains a conserved cysteine signature (Figure 1B). Like Tim13, Cox17 interacted with Mia40 in an SDS-resistant but β-mercaptoethanol-sensitive manner. The Cox17-Mia40 complex was also visible upon immunoblotting of extracts of mitochondria that were preincubated with purified Cox17 protein (Figure 1C). From this, we conclude that newly imported Tim13 and Cox17 form covalent interactions with Mia40 via disulfide bridges (Figure 1D). The interaction with Mia40 appears to be specific for proteins with twin Cx_3C and Cx_9C motifs, as no interactions to newly imported cytochrome *c* heme lyase were observed (see Figure S1B in the Supplemental Data available with this article online). Cytochrome *c* heme lyase contains four cysteine residues but is not a substrate of the Mia40-dependent pathway (Chacinska et al., 2004; Terziyska et al., 2005).

Import of Cox17 into Isolated Mitochondria Is Inhibited in the Presence of High Concentrations of Dithiothreitol

If the formation of a disulfide bridge is an essential step in the import process of IMS proteins, conditions counteracting the oxidation of thiol groups should interfere with protein translocation of Tim13 and Cox17. Indeed, we observed that the addition of high concentrations of dithiothreitol (DTT) to the import reaction prevented the protein import of Tim13 (Figure 2A). Likewise, high DTT concentrations blocked the import of the copper chaperone Cox17; in contrast, NADH, FAD, or the addition or removal of copper ions did not affect the import of Cox17 (Figure 2B). We titrated the effect of increasing concentrations of DTT on the import of Tim13 and Cox17 (Figure 2C). Mildly reducing conditions stimulated the import of both proteins, presumably because these proteins have to penetrate the protein-conducting channel in the TOM complex in a reduced conformation, and they undergo constant oxidation by oxygen under the conditions of the in vitro assay (Lu et al., 2004; Lutz et al., 2003). In contrast, concentrations of DTT of larger than 5 mM strongly diminished the translocation of both proteins into the IMS. Due to the unexpectedly high concentrations of DTT required to inhibit the import of IMS proteins, we missed this DTT sensitivity in our earlier study (Lutz et al., 2003). The import of preproteins into the mitochondrial matrix was not significantly affected by DTT (Figure 2C). The observed sensitivity of the import reaction toward reducing agents suggests that oxidized thiol groups play a critical role in the net transport of Cox17 and Tim13 into the IMS, probably because these proteins need to interact with Mia40 via disulfide bridges.

Depletion of Erv1 Leads to Reduced Levels of Certain IMS Proteins

The formation of disulfide bridges between cysteine residues of Mia40 and newly imported proteins points to a sulfhydryl oxidation reaction in the IMS. The IMS of mitochondria harbors a sulfhydryl oxidase, named Erv1, the substrates of which were not yet identified.

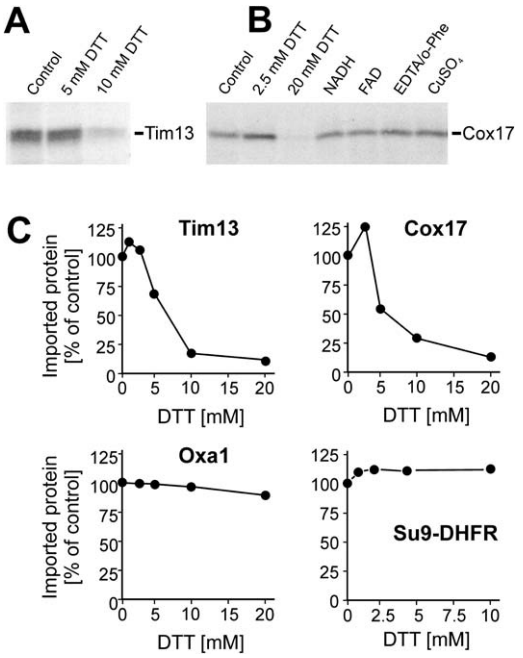

Figure 2. Import of Tim13 and Cox17 Is Sensitive to Reducing Agents

(A) Radiolabeled Tim13 was imported into isolated mitochondria for 10 min in the presence of 0, 5, or 10 mM DTT, as indicated. Nonimported material was removed by treatment with proteinase K. Imported Tim13 was visualized by autoradiography.

(B) Cox17 was imported in mitochondria in the presence of 0, 2.5, or 20 mM DTT; 2 mM NADH; 2 mM FAD; 2 mM EDTA; and orthophenanthroline (o-Phe) or 10 μM copper sulfate. Mitochondria were treated with proteinase K, and Cox17 was detected by autoradiography.

(C) Tim13, Cox17, the precursor form of Oxa1, and preSu9(1-69)-DHFR were imported into wild-type mitochondria in the presence of different concentrations of DTT. The imported proteins were detected as described in (A) and quantified by densitometry. Import efficiencies without DTT were set to 100% (control).

Erv1 is essential for viability of yeast cells, indicating that it exhibits an essential activity in the IMS. To assess a potential function of Erv1 in the biogenesis of IMS proteins, we constructed a yeast mutant that expressed Erv1 under control of the regulable *GAL10* promoter. Growth of this *GAL*-Erv1 mutant in the presence of glucose led to strongly reduced levels of Erv1 (Figure 3A). However, even after prolonged growth of cells for several days, low levels of Erv1 still remained detectable in mitochondria. These low levels were apparently sufficient to support mitochondrial function, as the cultures showed unaffected growth rates on glucose for at least 20 generations (data not shown). Depletion of Erv1 resulted in a strong reduction of other proteins of the IMS, such as Cox17, Tim13, and copper/zinc superoxide dismutase (Sod1). In contrast, IMS proteins lacking conserved cysteine signatures (cytochrome *c* heme lyase, cytochrome b_2), and proteins of the outer membrane (Tom70), the inner membrane (ATP/ADP carrier), and the matrix (Aco1) were not affected. We conclude that Erv1 is specifically required for the import and/or the stability of a group of IMS proteins. These proteins

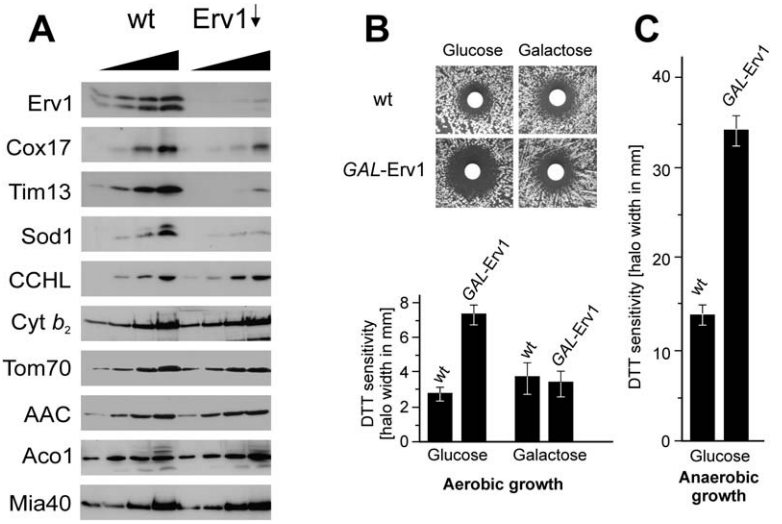

Figure 3. Depletion of Erv1 Leads to the Reduction of Certain IMS Proteins and to an Increased DTT Sensitivity of Cell Growth

(A) Wild-type (wt) and *GAL*-Erv1 cells were grown on YPGal and shifted to glucose-containing medium for 48 hr. Mitochondria were isolated from these cells. Mitochondria (12, 25, 50, and 100 μg) were applied to the gel, and the levels of various proteins were detected by immunoblotting. These proteins are located in the IMS (Erv1, Cox17, Tim13, Sod1, cytochrome *c* heme lyase [CCHL], and cytochrome b_2 [Cyt b_2]), the outer membrane (Tom70), the inner membrane (ATP/ADP carrier [AAC], Mia40), and the matrix (aconitase [Aco1]).

(B) Wild-type and *GAL*-Erv1 cells were grown to log phase in medium containing glucose or galactose as carbon sources. Equal amounts of cell culture were spread onto plates containing glucose or galactose. A filter disc was placed onto the cell lawn, which was soaked with 10 μl of 3 mM DTT. The plates were grown at 25°C for 2 (glucose) or 3 (galactose) days (upper panel). The graph shows the width of the halo (lower panel). The means and standard deviations were obtained from three plates per culture.

(C) The DTT sensitivity of the cells was assessed as in (B), but the plates were incubated in the absence of oxygen in an anaerobic chamber.

are imported into mitochondria in a Mia40-dependent manner (Chacinska et al., 2004; Naoe et al., 2004; Terziyska et al., 2005). Notably, Mia40 remains stable upon depletion of Erv1 (Figure 3A). Thus, Erv1, besides Mia40, is a second factor required for the biogenesis of these proteins.

Depletion of Erv1 Increases the Sensitivity toward Reducing Agents

A sulfhydryl oxidase activity of Erv1 was so far only shown in vitro (Lee et al., 2000). Therefore, we tested whether Erv1 can counteract the deleterious effect of membrane-permeable reductants in vivo. Wild-type and *GAL*-Erv1 cells were grown in medium containing either galactose or glucose and plated onto agar plates. A filter disc was placed onto the cell lawns, on which 10 μl of 3 M DTT was applied (Frand and Kaiser, 1998), and the plates were incubated at 24°C (Figure 3B). On glucose-containing plates, the growth of the *GAL*-Erv1 cells was inhibited in a zone that was 7.3 mm wide. This zone was significantly larger than that measured for wild-type cells (2.9 mm). In contrast, no significant difference between both strains was observed on galactose-containing plates. The sensitivity of the yeast strains toward DTT was strongly increased when the cells were grown under anaerobic conditions, suggesting that oxygen partially mitigated the effect of the reducing agent (Figure 3C). In summary, we conclude that Erv1 exhibits a sulfhydryl oxidase activity in vivo that can counteract the harmful effect of reducing agents on cell growth.

Erv1 Counteracts the Inhibitory Effect of Reducing Agents on the Import of Cox17 and Tim13

The increased sensitivity to DTT of the Erv1-depleted mutant may point to a deleterious effect of the reducing agent in the mitochondrial IMS, which in wild-type cells is counteracted by Erv1. Therefore, we tested the influence of DTT on the import of proteins into the IMS of mitochondria isolated from wild-type and Erv1-depleted cells. In Erv1-depleted mitochondria, the translocation of both Cox17 (Figure 4A) and Tim13 (Figure 4B) was strongly inhibited in the presence of even very small concentrations of DTT. In contrast, the import of the matrix protein Oxa1 was not affected in these mutants, excluding that the protein translocation across the outer membrane is generally more sensitive to DTT in the mutant (Figure 4C).

In previous studies on the function of Erv1, a conditional mutant was employed in which the function of Erv1 can be compromised by high temperature (Lange et al., 2001; Lisowsky, 1992). This temperature-sensitive mutant (*erv1-ts*) grows without an apparent phenotype at 25°C but is unable to grow at 37°C (Figure 4D). We isolated mitochondria from this strain and from wild-type after exposure of the cells to 37°C and performed import experiments with Cox17 and Tim13. While both proteins could be efficiently imported into the mitochondria of both strains in the absence of DTT, the presence of DTT strongly diminished import of both Cox17 and Tim13 into the *erv1-ts* mitochondria (Figures 4E and 4F). From this, we conclude that Erv1 counteracts the negative effects of DTT on the import of both proteins, suggesting that Erv1 catalyzes the formation of disulfide bonds in the IMS that are critical for protein import.

Depletion of Erv1 Diminishes the Import Rates of Cox17 and Tim13

In the cell, redox equivalents are primarily provided by reduced glutathione, which is present in the yeast cytosol at concentrations of approximately 13 mM reduced glutathione (Ostergaard et al., 2004). The channel-forming porins in the outer membrane presumably allow equilibration of the glutathione levels between the cytosol and the IMS. We therefore tested the influence of glutathione on the import of Cox17 and Tim13 into isolated mitochondria. Reduced glutathione had an effect

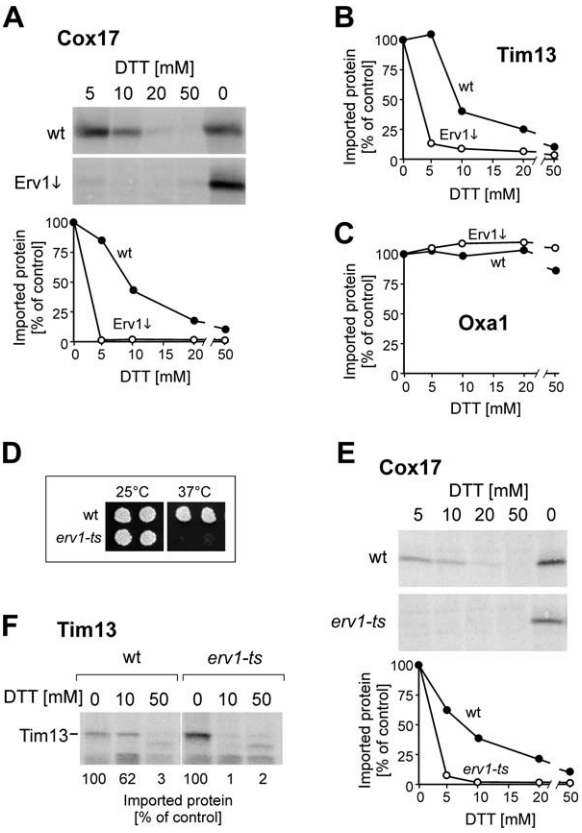

Figure 4. Depletion of Functional Erv1 Increases the Sensitivity to DTT of the Import of Cox17 and Tim13

(A–C) Cox17, Tim13, and Oxa1 were imported into wild-type (wt) and Erv1-depleted (Erv1↓) mitochondria as described in Figure 2. (Upper panel) Autoradiography. (Lower panel) Quantification of autoradiography. Import efficiencies without DTT were set to 100% (control).

(D) Wild-type and *erv1-ts* mutant cells were grown in liquid culture to logarithmic phase, spotted onto YPD plates, and incubated for 48 hr at 25°C or 37°C as indicated.

(E and F) DTT sensitivity of the import of recombinant Cox17, and of in vitro synthesized Tim13, was analyzed as in (A).

that was similar to that described for DTT (Figures 5A and 5C). Erv1 was particularly required when the import reactions were carried out under low-oxygen conditions (Figure 5A, middle and right panels). The increased sensitivity against reducing agents under anaerobic conditions was specific for IMS proteins, as the import of matrix (Su9-dihydrofolate reductase [DHFR]) and inner membrane proteins (Oxa1) was not affected even at very high concentrations of DTT (Figure 5B). The data suggest that the sulfhydryl oxidase activity of Erv1 establishes conditions in the IMS necessary for the import of Tim13 and Cox17 under physiological glutathione concentrations.

Next, we tested whether Cox17 does still form a complex with Mia40 when Erv1 is depleted. We imported recombinant Cox17 into mitochondria isolated from wild-type and Erv1-depleted cells and assessed the formation of the covalent Mia40-Cox17 complex by immunoblotting (Figure 5D). The Mia40-Cox17 complex was efficiently formed in wild-type mitochondria even in the presence of 15 mM glutathione (Figure 5D, lanes 1 and 2). In contrast, only low amounts of this complex were found after depletion of Erv1 (Figure 5D, lane 4). Thus, the function of Erv1 is critical for the efficient generation of the mixed disulfide of Mia40 with newly imported Cox17 in the IMS.

Do the in vitro import assays really reflect the in vivo situation? A fraction of the cellular Cox17 protein was reported to be present in the cytosol in wild-type cells (Glerum et al., 1996). An import defect of Cox17 caused by Erv1 depletion can be expected to affect the intracellular distribution of Cox17. Therefore, we grew wild-type and *GAL*-Erv1 mutant cells on medium containing glucose and isolated mitochondrial and cytosolic fractions. Whereas the total intracellular Cox17 level was not affected by depletion of Erv1 (Figure 5E), Cox17 was shifted from the mitochondrial fraction to the cytosolic fraction (Figure 5F). We conclude that Erv1 is required for the import of Cox17 into mitochondria in vivo.

Erv1 Interacts with Mia40

Since both Erv1 and Mia40 are required for the import of the same set of IMS proteins, we asked whether both proteins undergo a physical interaction. Sulfhydryl oxidases typically form transient disulfides with their substrates (Collet and Bardwell, 2002; Kadokura et al., 2003). We therefore tested whether Erv1 can be detected in a complex with Mia40 under nonreducing conditions. To this end, we constructed a strain that expressed, in addition to the endogenous Mia40 protein, a version of Mia40 carrying a C-terminal hexahistidinyl tag (Mia40-His_6). This protein could be isolated by affinity chromatography on NiNTA Sepharose (Figure 6A). A subpopulation of the endogenous Erv1 was copurified with Mia40-His_6, indicating that a fraction of Erv1 is associated with Mia40 (Figure 6A, arrowheads). Under the conditions of the experiment, about 5% of total Erv1 were found in association with Mia40. The Erv1-Mia40-His_6 complex was stable even in the presence of SDS, as it could be visualized by immunoblotting of eluates of the NiNTA columns with Erv1-specific serum (Figure 6B). However, when DTT was present in the lysis buffer, Erv1 was released from Mia40-His_6 and no longer found in the eluate (Figure 6C). Thus, a fraction of the Erv1 pool is associated with Mia40, presumably due to a direct linkage of both proteins by disulfide bridges.

Erv1 Maintains Mia40 in an Oxidized State

Mia40 contains six conserved cysteine residues. Upon reducing SDS-PAGE, Mia40 migrates as a double band indicating partial oxidation of at least one cysteine pair in the protein (Terziyska et al., 2005). To assess the redox state of Mia40 in more detail, mitochondria were incubated in the absence or presence of DTT. Then, reduced thiol groups were modified with iodoacetamide to prevent their reoxidation during electrophoresis. After separation on nonreducing SDS gels, two forms of Mia40 could be distinguished (Figure 6D), an oxidized form that migrated faster and, in the DTT-treated sample, a reduced form of lower mobility. Also, Erv1 migrated as a double band on our gel system; however,

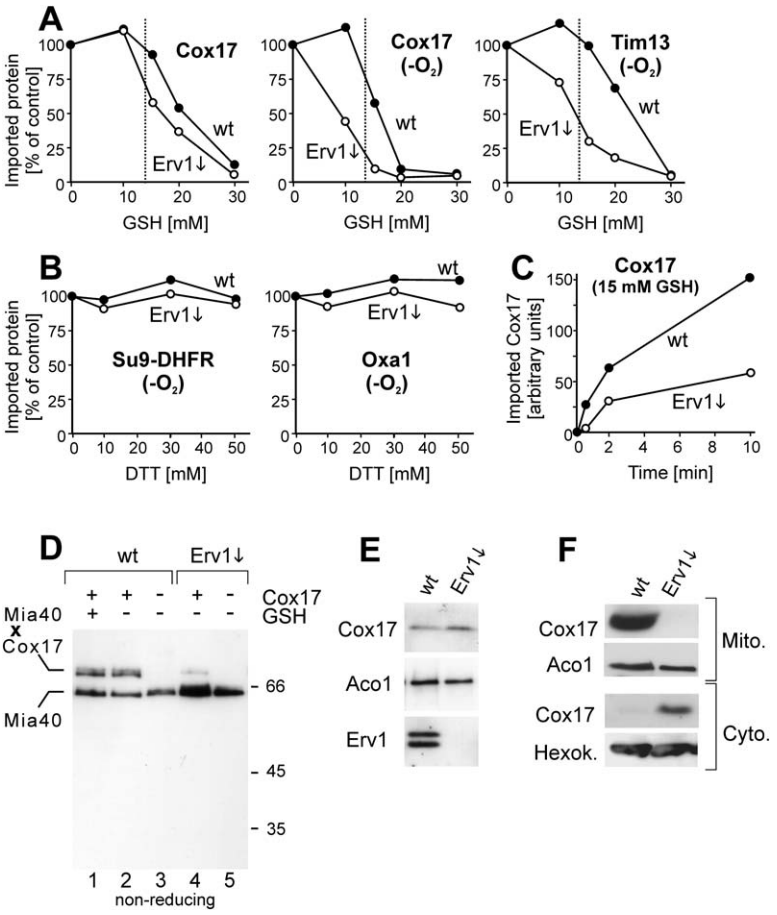

Figure 5. Depletion of Erv1 Diminishes the Import Rates of Cox17 and Tim13

(A) Cox17 (left and middle panels) and Tim13 (right panel) were incubated with wild-type and Erv1-depleted mitochondria in the presence of different concentrations of reduced glutathione (GSH). The amounts of imported proteins were assessed as described for Figure 2. The import reactions presented in the middle and the right panels were performed under microaerobic conditions in a nitrogen-filled glove bag. The broken line indicates the glutathione concentration determined for the cytosolic compartment of yeast cells (Ostergaard et al., 2004).

(B) The precursor forms of preSu9(1-69)-DHFR and Oxa1 were imported in the presence of increasing amounts of DTT under microaerobic conditions. The amounts of imported protein were quantified and expressed in comparison to the amount of protein imported without DTT.

(C) Cox17 was imported for various time periods in the presence of 15 mM glutathione under aerobic conditions. The amounts of Cox17 inaccessible to added proteinase K were quantified as described in Figure 2.

(D) Mitochondria (50 μg) from wild-type and Erv1-depleted cells were incubated with 5 μg recombinant radiolabeled Cox17 for 10 min in the absence or presence of 15 mM glutathione (GSH) as indicated. Nonimported Cox17 was removed by treatment with proteinase K. Mia40 and the Mia40-Cox17 complex were detected by immunoblotting with antibodies against Mia40 as described for Figure 1C.

(E and F) Wild-type and *GAL*-Erv1 cells were grown to logarithmic phase in glucose-containing medium. The cells were harvested, and total cell extracts (E) or mitochondrial (40 μg; Mito.) and cytosolic (200 μg; Cyto.) fractions (F) were prepared. The distribution of Cox17 was assessed by immunoblotting. Mitochondrial aconitase (Aco1), Erv1, and the cytosolic hexokinase (hexok.) served as controls.

this is apparently not due to different redox states of the protein, as both forms are present under reducing and under oxidizing conditions (Figure 6E).

We then tested the proportion of oxidized Mia40 in mitochondria incubated under different glutathione concentrations (Figure 6F). When no reduced glutathione was added, Mia40 was predominantly oxidized in wild-type mitochondria. By increasing the glutathione concentrations to up to 25 mM, most of the Mia40 protein was reduced. In the Erv1-depleted mitochondria, the redox state of Mia40 was shifted toward the reduced form. This suggests that Erv1 plays a role in counteracting the effect of glutathione. In contrast, overexpression of Erv1 increased the levels of oxidized Mia40 (Figure 6G). Apparently, Mia40 contains at least one pair of cysteine residues that can be oxidized in an Erv1-dependent reaction.

The Presence of Disulfide Bridges in the IMS Is Critical for Import of Cox17 and Tim13

Which stage of the import reaction of Cox17 and Tim13 is sensitive to reducing agents? To address this question, we preincubated mitochondria with DTT. When the DTT was washed out before the Cox17 protein was added to the mitochondria, the pretreatment had no negative effect (Figure 7A, stage A). In contrast, the presence of DTT during the incubation of mitochondria with Cox17 prevented the uptake of the protein completely (Figure 7A, stage B).

Why did the pretreatment of the mitochondria with DTT not affect the import of Cox17? The disulfide bridge in Mia40 could conceivably be regenerated before the Cox17 protein was added. To test this, we monitored the redox state of Mia40 after the import reaction by nonreducing SDS-PAGE and immunoblotting (Figure 7B). This revealed that, even when the mitochondria were pretreated with DTT, Mia40 was found in its oxidized form. In contrast, when the DTT treatment was followed by incubation with N-ethylmaleimide (NEM) to trap the thiol groups in the reduced conformation, Mia40 was found in the reduced form. Notably, when Mia40 was trapped in the reduced state, the import of Cox17 and Tim13 was almost completely blocked (Figure 7B). Thus, conditions which lock Mia40 in the reduced state prevent the import of both proteins into the IMS.

Then, we tested whether already imported Cox17 is released upon addition of high amounts of DTT (Figure 7C). Addition of 50 mM DTT reduced the amounts of imported Cox17 in the IMS only to a limited extent. This

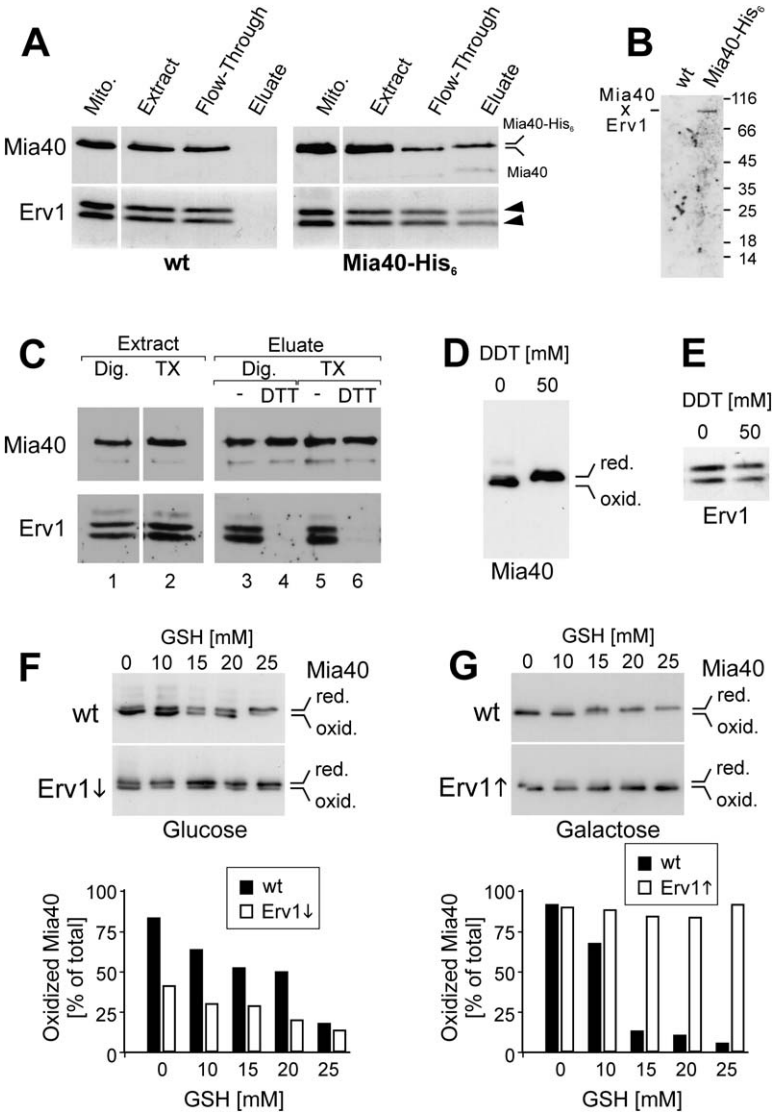

Figure 6. Erv1 Interacts with Mia40

(A) Mitochondria (500 μg) of wild-type or Mia40-His_6 cells were lysed in 250 μl 1% Triton X-100, 300 mM NaCl, 20 mM imidazole, 2 mM phenylmethylsulfonyl fluoride, 50 mM sodium phosphate (pH 8.0). The extract was cleared by centrifugation for 20 min at 125,000 × g and incubated with NiNTA Sepharose for 60 min at 4°C. The beads were washed extensively with lysis buffer, and the bound material was eluted with sample buffer containing 300 mM imidazole. Proteins in the samples indicated were resolved by SDS-PAGE under reducing conditions and analyzed by immunoblotting with antibodies against Mia40 and Erv1. Mitochondria (50 μg) were directly dissolved in sample buffer and applied to SDS-PAGE (Mito.). The tagged and untagged versions of Mia40 present in the Mia40-His_6 strain are indicated. Arrowheads depict the Erv1 protein copurified with Mia40-His_6.

(B) Extracts of wild-type and Mia40-His_6 mitochondria were prepared and incubated with NiNTA Sepharose as in (A). Proteins present in the bound fractions were resolved under nonreducing conditions by SDS-PAGE, transferred to nitrocellulose, and immunodecorated with Erv1-specific antibodies.

(C) Mia40-His_6 cells were lysed with 1% digitonin (Dig.) or 1% Triton X-100 (TX) in the absence or presence of 10 mM DTT as indicated. Mia40-His_6 was isolated on NiNTA Sepharose. Mia40 and Erv1 in the mitochondrial extract and the eluate were analyzed by immunoblotting.

(D and E) Wild-type mitochondria (50 μg) were incubated in the presence or absence of 50 mM DTT. The mitochondria were washed and incubated with 50 mM iodoacetamide for 20 min to modify reduced thiol groups. Mitochondria were reisolated and lysed in sample buffer. The proteins were resolved by SDS-PAGE under nonreducing conditions, and Mia40 (D) or Erv1 (E) were visualized by immunoblotting.

(F and G) Mitochondria were isolated from wild-type and *GAL*-Erv1 cells grown on glucose (F) or galactose (G) and incubated with reduced glutathione (GSH) of the concentrations indicated. The samples were treated with iodoacetamide, and the redox state of Mia40 was analyzed as in (D). Reduced and oxidized species of Mia40 were quantified by densitometry, and the proportion of oxidized to total Mia40 was calculated.

suggests that the DTT-sensitive stage is mainly restricted to the period of the import reaction in which Cox17 has to interact with Mia40. It should be noted that, in the case of Cox17, metal ions appeared not to be required to hold the protein in the IMS. This behavior is different from that observed for Tim13 (Lutz et al., 2003).

Discussion

We report here on the biogenesis of a class of IMS proteins that are characterized by patterns of conserved cysteine residues. Import of these proteins requires the TOM complex but is independent of TIM translocases, ATP, and the membrane potential. We show that the two essential mitochondrial proteins Erv1 and Mia40 cooperate in the import of these proteins. Mia40 in the oxidized state interacts in a covalent fashion with incoming polypeptides forming mixed disulfides. Mia40 is maintained in its oxidized state in a reaction catalyzed by the sulfhydryl oxidase Erv1. Erv1 exerts its function by direct interaction with Mia40. When the cellular level of Erv1 was downregulated, proteins that require Mia40 for import were depleted, and Mia40 accumulated in a reduced, inactive conformation.

Based on these observations, we propose a hypothetical model for the import of proteins into the IMS that relies on a disulfide relay system (Figure 7D). Newly synthesized IMS proteins traverse the TOM complex of the outer membrane in a reduced, unfolded conformation (Lu et al., 2004; Lutz et al., 2003). In the IMS, the polypeptides interact with preoxidized Mia40; by exchange of the disulfide bond, Mia40 and the incoming polypeptide form a covalently linked mixed disulfide.

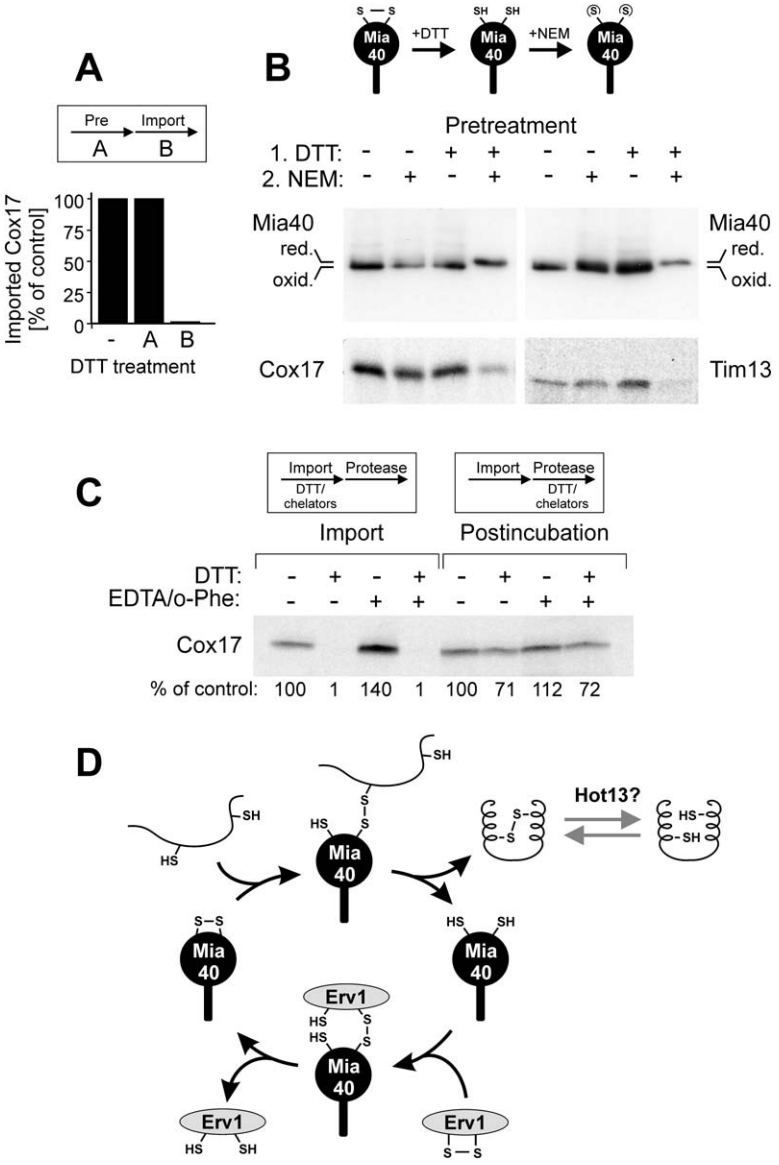

Figure 7. The Presence of Disulfide Bridges in Mia40 Is Critical for Import of Cox17 and Tim13

(A) To determine the stage in the import pathway of Cox17 and Tim13 at which disulfide bonds are critical, mitochondria were exposed either before (A) or during (B) the import reaction to 50 mM DTT (cf. upper panel). Mitochondria were preincubated in import buffer for 10 min at 25°C in the absence or the presence (sample "A") of DTT. Mitochondria were reisolated and resuspended again in import buffer. DTT was added to sample "B," and 0.2 μg radiolabeled Cox17 was added to both samples. After 10 min at 25°C, mitochondria were again reisolated and then resuspended in 600 mM sorbitol, HEPES/KOH (pH 7.4) containing 50 μg/ml proteinase K. After incubation for 20 min, mitochondria were isolated and washed. The amounts of imported Cox17 were assessed by SDS-PAGE, autoradiography, and densitometry.

(B) A mitochondrial suspension was split into four aliquots, two of which were incubated in the absence and two of which were incubated in the presence of 10 mM DTT for 10 min. In order to modify reduced thiol groups, 50 mM NEM was added to one of each type of aliquots. After 10 min, mitochondria were reisolated and split into two halves, which were incubated in import buffer with either recombinant Cox17 or Tim13. Nonimported material was removed by protease treatment. The mitochondria were washed, reisolated, and dissolved in sample buffer lacking β-mercaptoethanol. One-half of the sample buffer was directly loaded on a gel to detect reduced and oxidized species of Mia40. The other half was reduced by addition of β-mercaptoethanol, resolved by SDS-PAGE, and autoradiographed to detect imported Cox17 and Tim13.

(C) Left part ("Import"): Recombinant Cox17 was imported into wild-type mitochondria in the presence or absence of 50 mM DTT or 2 mM EDTA and 2 mM o-phenanthroline as indicated. The mitochondria were reisolated, washed, and treated with proteinase K. Right part ("Postincubation"): Cox17 was imported in the absence of DTT and chelators. DTT and chelators were then added as indicated during the postincubation of the samples with protease. The amounts of imported Cox17 were quantified and expressed in relation to the amounts of protein imported in the absence of DTT and chelators.

(D) Model for Erv1-dependent formation of oxidized Mia40, which has the capacity to bind newly imported proteins via disulfide bridges. See text for details.

Finally, the polypeptide is released upon further isomerization of the disulfide bridge. Reduced Mia40 is reoxidized by Erv1, a process that is essential to promote further rounds of import. In the absence of Erv1 activity, Mia40 accumulates in a reduced, i.e., inactive, conformation. Our model implies that the import reaction is accompanied by a flow of electrons from imported proteins to Mia40 and then from the reduced Mia40 to oxidized Erv1. Erv1 then transfers the electrons via its flavin cofactor to an unknown electron acceptor, perhaps the electron transport chain of oxidative phosphorylation. This reaction in the end would support trapping and oxidative folding of substrate proteins, a mechanism previously suggested by Toklatlidis and coworkers (Lu et al., 2004).

According to our model, the proteins would initially be released in an oxidized state. This is in agreement with observations of small Tim proteins forming disulfide bridges following translocation into the IMS (Koehler, 2004b; Lu et al., 2004). On the other hand, the cysteine residues of Tim proteins were also reported to be present in a reduced, zinc bound conformation. This seeming contradiction might be due to a more complex redox regulation of IMS proteins. Noteworthy, a potential candidate for a factor that reduces small Tim proteins in the IMS, named "Helper Of Tim" or Hot13, was recently identified (Curran et al., 2004). This IMS protein is required to maintain small Tim complexes in an active conformation. Interestingly, Hot13 counteracted the deleterious effects that oxidative agents have on

small Tim proteins, suggesting that Hot13 exhibits a reducing function on small Tim proteins. Thus, factors of opposing redox activity might initially oxidize (Erv1 and Mia40) and then reduce (Hot13) newly imported IMS proteins and thereby control the redox state of IMS proteins. On the other hand, it is conceivable that certain proteins are released from Mia40 directly in a reduced state. Such a hypothetical reaction may depend on the redox potential of such proteins. Studies with purified factors to analyze their individual redox potentials will help in the future to unravel the precise mechanism by which proteins are released from Mia40.

It should be pointed out that this model is in agreement with the folding trap hypothesis, according to which proteins are finally locked in the IMS due to their stable folding (Lu et al., 2004; Lutz et al., 2003). After dissociation of imported proteins from Mia40, the acquisition or maintenance of a folded conformation is still required to prevent the release of the polypeptide back into the cytosol. In some cases, such folded conformations might be stabilized by disulfide bonds formed by the Mia40-Erv1 disulfide relay system, consistent with a suggested trapping mechanism by oxidative folding (Lu et al., 2004). In other cases, in particular for proteins that are present in the reduced form, the stabilization might also require the binding of cofactors or the assembly with complex partners (Lutz et al., 2003). The individual properties of each IMS protein might influence the specific impact of cofactors, disulfide bridges, and binding partners, explaining why chelators and reducing agents affect these proteins to different degrees. The observation that Mia40 is able to bind zinc and copper ions (Terziyska et al., 2005) is conceivable with a potential role of this protein in the cofactor acquisition and folding of proteins in the IMS. Such a function of Mia40 would be comparable to that of the copper chaperone of Sod1 (CCS). This copper binding protein forms a transient disulfide bridge with the apoform of Sod1; after the transfer of the copper ion from CCS to Sod1, the intermolecular disulfide bridge is converted into an intramolecular disulfide bridge in Sod1, resulting in the release of Sod1 in a copper-containing oxidized form (Field et al., 2003; Lamb et al., 2001).

The observed oxidation of a cysteine pair in Mia40 by the sulfhydryl oxidase Erv1 is highly reminiscent of redox processes in the endoplasmic reticulum and the periplasm of bacteria (Hiniker and Bardwell, 2004; Kadokura et al., 2003; Sevier and Kaiser, 2002). The endoplasmic reticulum harbors a homolog of Erv1, named Erv2 (Gerber et al., 2001). Like Erv1, Erv2 is a flavoprotein that exhibits sulfhydryl oxidase activity (Gross et al., 2002; Sevier et al., 2001). It oxidizes the protein disulfide isomerase PDI, which receives the electrons from substrate proteins and catalyzes their oxidative folding. Similarly, in the bacterial periplasm the membrane protein DsbB oxidizes newly synthesized polypeptides via the soluble protein DsbA. Our observations suggest that Erv1 plays a role comparable to that of Erv2 and DsbB. It oxidizes Mia40, which then can trap newly imported proteins in the IMS via disulfide bridges. Interestingly, in contrast to the cytosol, where cysteine residues almost exclusively remain reduced, the IMS harbors a number of proteins for which the presence of disulfide bridges was reported, such as Cox11 (Banci et al., 2004), Erv1 (Levitan et al., 2004), Rieske iron sulfur protein (Iwata et al., 1996), Qcr8 (Iwata et al., 1996), CCS (Field et al., 2003), Sod1 (Field et al., 2003), small Tim proteins (Curran et al., 2002; Lu et al., 2004), and Sco1 (Williams et al., 2005).

The existence of a disulfide relay system in the IMS is rather surprising in view of the reducing environment of the cytosol to which the IMS is connected via porin channels. A rational explanation may come from the evolutionary origin of mitochondria from endosymbiotic prokaryotes. It is conceivable that during evolution the process of thiol oxidation was conserved from the periplasm of bacteria to the IMS of recent mitochondria.

Experimental Procedures

Plasmids and Yeast Strains

For construction of plasmids and strains, see the Supplemental Data. Yeast strains were grown in liquid lactate medium (Herrmann et al., 1994) in the presence of 0.1% glucose or galactose in order to repress or induce the *GAL10* promoter or on plates consisting of 1% yeast extract, 2% peptone, 1% agar, and 2% of either glucose or galactose. The *erv1-ts* strain was grown at 25°C and shifted to 37°C 15 hr prior to the isolation of mitochondria. For anaerobic growth, plates were incubated in anaerobic chambers (GENbox anaer, BioMérieux, Marcy l'Etoile, France).

Protein Import into Isolated Mitochondria

Mitochondria were isolated as described (Herrmann et al., 1994). Radiolabeled precursor proteins were synthesized in vitro in the presence of [^{35}S] methionine in reticulocyte lysate according to the protocol of the manufacturer (Promega) or purified from *E. coli*. Recombinant Tim13 was purified as described before (Lutz et al., 2003). For purification of Cox17, see the Supplemental Data. Typically 0.2 μg of purified denatured Cox17 or Tim13 was used per import reaction. Import reactions into isolated yeast mitochondria (100 μg) were carried out in 0.6 M sorbitol, 0.1 mg/ml BSA, 2 mM potassium phosphate, 2 mM β-mercaptoethanol, 50 mM HEPES-KOH (pH 7.4) at 25°C if not indicated otherwise. Import reactions of matrix-targeted proteins were supplemented with 2 mM ATP and 2 mM NADH. Import was stopped by diluting the reaction tenfold in ice-cold 0.6 M sorbitol, 20 mM HEPES-KOH (pH 7.4) with or without 50 μg/ml proteinase K or trypsin. Signals of radiolabeled proteins were detected by autoradiography on Biomax MR-1 films (Kodak, Rochester, NY) and quantified using a Pharmacia Image Scanner with Image Master 1D Elite software package.

For import experiments under oxygen-deprived conditions, the buffers used were degassed for 30 min using a PC 2001 Vario diaphragm vacuum pump (Vacuubrand, Wertheim, Germany) and then purged with nitrogen gas for 2 min. The import reactions were carried out under nitrogen atmosphere in air-tight glove bags.

Crosslinking and Immunoprecipitation

For crosslinking following import of radiolabeled proteins, the import reactions were incubated for 20 min at 25°C with 200 μM DFDNB or mock treated for control. Crosslinking was stopped by the addition of 100 mM glycine (pH 8.0). Mitochondria were reisolated, washed, and lysed in 1% SDS, 300 mM NaCl, and 1 mM EDTA. After a clarifying spin for 30 min at 47,000 × g, the extract was diluted 20-fold in 0.2% Triton X-100, 150 mM NaCl, 20 mM sodium phosphate (pH 7.5) and used for immunoprecipitation according to published procedures (Herrmann et al., 2001). The extract was added to 25 μl of protein A Sepharose (Amersham Biosciences) and 4–6 μl of rabbit antisera or 20 μg of purified antibody. After incubation for 2 hr at 4°C, the beads were washed twice in Triton buffer and once in phosphate-buffered saline. Precipitated proteins were eluted with sample buffer containing 1% SDS.

Miscellaneous

Antisera against Cox17 and Erv1 were generated by injecting rabbits with purified GST-Cox17 and GST-Erv1, respectively. To pre-

vent artificial oxidation of the cysteine residues of Mia40 during SDS-PAGE, mitochondria were incubated with 50 mM iodoacetamide in 50 mM Tris (pH 8.0) before they were reisolated and dissolved in sample buffer. The halo assay to assess the DTT sensitivity of yeast strains was carried out as described (Frand and Kaiser, 1998). For isolation of yeast cytosol, cells were converted to spheroplasts by treatment with zymolyase and ruptured in a glass homogenizer as described (Herrmann et al., 1994), and the resulting debris was cleared by ultracentrifugation for 20 min at 100,000 × g.

Supplemental Data

The Supplemental Data include Supplemental Experimental Procedures and one supplemental figure and can be found with this article online at http://www.cell.com/cgi/content/full/121/7/1059/DC1/.

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