Structural and Functional Roles of the Conserved Cysteine Residues of the Redox-regulated Import Receptor Mia40 in the Intermembrane Space of Mitochondria

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Oxidative folding drives the import of proteins containing twin CX_2C motifs into the intermembrane space of mitochondria. This import pathway employs a disulfide relay system whose key components are the redox-regulated import receptor Mia40 and the thiol oxidase Erv1. Mia40 contains six cysteine residues in a CPC-CX_2C-CPC arrangement in a highly conserved domain. We show that this domain is sufficient for the function of Mia40. By analysis of Mia40 cysteine mutants we demonstrate that the cysteine residues have distinct roles and are not equally important for Mia40 function. The second cysteine residue is essential for viability of yeast cells. It is required for the interaction of Mia40 with Erv1 in a disulfide intermediate and forms a redox-sensitive disulfide bond with the first cysteine residue. Both cysteine residues are required for the oxidation of the substrate, Tim10, in a reconstituted system comprised of Mia40 and Erv1. Mutants with amino acid exchanges in the third and sixth cysteine residues have severe defects in growth and in the import of intermembrane space proteins. These Mia40 variants are not tightly folded. We conclude that the cysteine residues of the twin CX_2C motif have a structural role and stabilize Mia40. In particular, the disulfide bond formed by the third and sixth cysteine residues apparently supports a conformational crucial for the function of Mia40. Furthermore, the disulfide bond in the CPC segment mediates the redox reactions with the thiol oxidase Erv1 and substrate proteins in mitochondria.

The intermembrane space (IMS) of mitochondria, the compartment between the mitochondrial outer and inner membrane, harbors many proteins that perform important functions in cellular processes, such as oxidative phosphorylation, synthesis of iron sulfur clusters, apoptosis, and transport of metabolites and proteins. Many of these proteins are characterized by a relatively small molecular mass and the presence of conserved cysteine residues arranged in twin CX_2C motifs (1–6). These cysteine residues are connected by disulfide bonds, as described for the class of small Tim proteins or the copper chaperone Cox17 (7–12).

The import of proteins with twin CX_2C motifs into mitochondria is driven by oxidative folding, which is mediated by a disulfide relay system in the IMS (13–15). The two main components of this system are the redox-regulated Mia40 protein (Tim40) and the thiol oxidase Erv1 (14, 16–22). Upon transport across the translocase of the outer membrane (TOM), the substrate proteins bind to Mia40 via disulfide bonds indicating a receptor function for Mia40 (14, 21). Subsequently, the substrate proteins are released in an oxidized state (23). Mia40 interacts with the thiol oxidase Erv1, which has the ability to oxidize Mia40 in vitro (24). In mitochondria, Erv1 is required to keep Mia40 in an oxidized state promoting further rounds of import (14). Finally, reoxidation of Erv1 appears to occur by transfer of electrons to cytochrome c and then either to the cytochrome c oxidase complex or to cytochrome c peroxidase linking the disulfide relay system to the respiratory chain (25, 26).

Mia40 was the first identified component of this import pathway (16–18, 27). In fungi, Mia40 is anchored with its N terminus into the inner membrane exposing the major part of the protein into the IMS (17, 18). This part comprises a domain with six cysteine residues forming a CPC-CX_2C-CPC-CX_2C motif which is highly conserved in all homologues of Mia40. These invariant cysteine residues play a crucial role for the function of Mia40 (17). Studies with a purified C-terminal fragment of Mia40 assigned three disulfide bonds to the oxidized state of Mia40 in vitro (24). The role of the various cysteine residues, however, is not understood at all. In particular, an analysis in vivo is required to elucidate the mechanism of Mia40 function.

We expressed in yeast a series of mutants of Mia40 to characterize the structural requirements and the role of the various cysteine residues of Mia40. Within the highly conserved C-terminal domain, the second cysteine residue is essential for viability of yeast cells. This residue is crucial for the interaction of Mia40 with Erv1 via a mixed disulfide bond and, together with the first cysteine residue, for the disulfide transfer to substrate proteins. Exchange of the first or the second cysteine residue in...
Mia40 to a serine residue renders Mia40 inactive in the oxidation of the substrate Tim10 in a reconstituted system comprised of Mia40 and Erv1. We suggest that a redox-sensitive catalytic disulfide bond between the first and the second cysteine residue and the stabilization of Mia40 by disulfide bonds made up of the cysteine residues of the twin CX$_2$C motif are crucial for the disulfide transfer-driven protein import pathway.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Plasmids**—A heterozygous deletion strain of Mia40 was generated by replacing one allele of the MIA40 gene by the HIS3 gene in the diploid yeast strain YPH501 by homologous recombination as previously described (28). Transformation of the plasmid pRS316 containing the MIA40 gene into this heterozygous deletion strain of MIA40 followed by sporulation and tetrad dissection generated a strain that carries a chromosomal deletion of MIA40 and contains the MIA40 and URA3 genes on a plasmid. To test for complementation, plasmids expressing the Mia40 mutants or wild-type Mia40 were transformed into this strain or the GAL-MIA40 strain previously described (18). Transformants were selected, and growth was analyzed on 5-fluoroorotic acid plates to counter-select against the URA3-containing MIA40 plasmid. The yeast strains harboring MIA40 gene under GAL promoter and expressing the Mia40 mutants were grown on selective lactate-containing medium supplemented with glucose or galactose. For repression of the chromosomal Mia40 allele, cells were grown in glucose-containing media for 27 h. For drop dilution tests, the strains were grown in liquid culture for 2 days, diluted to $A_{378}$ of 0.5, and then a series of 1:10 dilutions were made for each strain. From each dilution, 3.5 μl were spotted under sterile conditions on plates with the adequate medium containing 2% of glucose. Plates were incubated for 2–5 days at 30 °C.

To generate truncation constructs of MIA40, the promoter and the sequences coding for the mitochondrial targeting signal (MTS) and the transmembrane segment (TM) of Mia40 (cysteine residues 40 to 210) were amplified with the primers SacI-Mia40Prom and Mia40Rev210-BamHI using genomic DNA from *Saccharomyces cerevisiae* as a template. Similarly, the sequence of the MIA40 terminator (360 nucleotides after the stop codon) was obtained by polymerase chain reaction with the primers PstI-Mia40Term and Mia40 Term Rev-XhoI. These PCR products were cut with the enzymes SacI and BamHI or PstI and XhoI, respectively and subsequently ligated into the plasmid pRS314 generating pRS314-promMia40-MTS-TM-term. The plasmid pGEM4-Mia40 was used as a template for amplification of sequences coding for different amino acid segments of Mia40: amino acid residues 75–403, amino acid residues 258–403, amino acid residues 284–403, and amino acid residues 284–403. Specific primers harboring the restriction sites BamHI and PstI were used in these PCR reactions. Following digestion with the enzymes BamHI and PstI, the amplified fragments were cloned behind the sequence coding for the transmembrane segment in plasmid pRS314-promMia40-MTS-TM-term. Finally, the resulting plasmids were transformed into the Δmia40 yeast strain and tested for complementation as described above.

The MIA40 alleles encoding for the wild type and the cysteine mutants fused to a C-terminal octahistidine tag were subcloned into the plasmid pGEM4. The fragment containing the wild-type sequence was transferred from the plasmid pVTV102-Mia40-His$_6^a$ (18) to pGEM4 using the restriction sites BamHI and HindIII, generating pGEM4-Mia40-His$_6^a$. The mutant alleles mia40-C1S and mia40-C2S were subcloned in the pGEM4-Mia40-His$_6^a$ plasmid via the restriction sites SpeI and NcoI. The inserted nucleotide sequences carrying the mutations were amplified by polymerase chain reaction using the primer Mia40For571SpeI and Mia40Rev-C1S or Mia40Rev-C2S and the plasmid pVTV102-Mia40-His$_6^a$ as template. To obtain the pGEM4-Mia40-C3S-His$_6^a$ plasmid a fragment was amplified by PCR using the primers Mia40For906 and Mia40–8HIS-Rev and subcloned into pGEM4-Mia40-His$_6^a$ with NcoI and HindIII. To generate the plasmid pGEM4-Mia40-C4S-His$_6^a$ the same steps were performed using instead of the primer Mia40For906 primer C4S-Nco-Mia40. The nucleotide exchanges to obtain the mia40-C5S and mia40-C6S alleles were introduced by QuikChange® Site-directed Mutagenesis (Stratagene) using the plasmid pGEM4-Mia40-His$_6^a$ as template and the primer pairs NT5 and NT6 and NT7 and NT8, respectively. The C3,6S, the C5,6S and the C4,5S mutations in MIA40 were generated similarly using primers introducing the C6S (NT7 and NT8) or the C5S mutation (NT5 and NT6) and pGEM4-Mia40-C3S-His$_6^a$, pGEM4-Mia40-C5S-His$_6^a$ or pGEM4-Mia40-C4S-His$_6^a$ plasmids, respectively, as a template. The coding sequence of each Mia40 cysteine mutant was finally cloned from the pGEM4 plasmid into pXY142 vector using the restriction sites EcoRI and HindIII. The mia40-C1,2S mutant was obtained by PCR using pXY142-Mia40-C1S-His$_6^a$ as a template and the primers Mia40For571SpeI and NT22. The mia40-CGC mutant was amplified by PCR using primer Mia40For571SpeI and NT40. Both amplified fragments were cloned into plasmid pXY142-Mia40-His$_6^a$ using restriction sites SpeI and NcoI. The resulting plasmids were transformed into Δmia40 and GAL-MIA40 yeast strains. These strains were grown on selective medium lacking leucine and containing glucose (Δmia40) or galactose (GAL-Mia40). The plasmids for expression of the cysteine variants of Mia40 in *Escherichia coli* were cloned as described for the expression plasmid for the recombinant wild-type Mia40C (24). To generate pGEX-6P-1-Tim10, the open reading frame of Tim10 was amplified by PCR using pGEM4-Tim10 as template. The PCR fragment was cloned into the vector pGEX-6P-1 using the restriction sites BamHI and EcoRI.

**Isolation of Substrates with Mia40 Variants**—Radiolabeled Tim13 or Tim9 was incubated with isolated mitochondria (200 μg) from strains expressing the C-terminally His$_6^a$-tagged wild type or cysteine mutant Mia40. Mitochondria were resolubilized and solubilized in 50 μl of lysis buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole, 2 mM phenylmethylsulfonyl fluoride) containing 1% (w/v) SDS for 10 min at room temperature. Following 20-fold dilution with lysis buffer containing 0.2% Triton X-100 and a clarifying spin, mitochondrial extracts were added to 30 μl of pre-equilibrated NIHNTA-agarose beads (Qiagen). After incubation for 60 min at 4 °C, the beads were washed three times with buffer containing
10 mM imidazole. Bound proteins were eluted with SDS-PAGE sample buffer containing 300 mM imidazole. The samples were analyzed with non-reducing SDS-PAGE and autoradiography.

**Modification of Free Thiol Groups**—For modification of proteins with iodoacetamide or N-ethylmaleimide (NEM), isolated mitochondria were incubated with 80 mM iodoacetamide or 50 mM NEM in buffer (0.6 M sorbitol, 20 mM HEPES, pH 8.0) for 20 min at 25 °C. To analyze the redox-state of Mia40 or the His-tagged variants of Mia40 mitochondria were reisolated and the samples were analyzed by non-reducing SDS-PAGE and immunoblotting with antibodies against Mia40 or the octahistidine tag. M-poly-(ethylene glycol)-maleimide-5000 (PEG-Mal) (Nektar, Huntsville, AL) is a thiol-specific agent, which attaches one PEG moiety (MW = 5 kDa) to each free cysteine residue of a protein. To modify Mia40 and Mia40 variants with PEG-Mal, mitoplasts were generated by resuspension of mitochondria in 100 μl 100 mM Hepes pH 7.4. Mitoplasts were treated with or without 5 mM TCEP (Tris-(2-carboxyethyl) phosphine hydrochloride) for 3 min on ice or at 95 °C and then incubated with or without PEG-Mal for 90 min on ice. The samples were loaded on a non-reducing SDS gel, transferred onto nitrocellulose membrane and Mia40 and modified Mia40 was detected by immunodetection. Due to the additional mass of bound PEG-Mal modified proteins had a reduced mobility on SDS-PAGE gels.

**In Vitro Reconstitution Experiments**—Recombinant Mia40C, consisting of amino acid residues 284–403, and the cysteine variants of Mia40C were expressed in *E. coli* and purified as described previously (24). Tim10 was expressed in the *E. coli* strain BL21 (DE3) containing the plasmid pGEX-6P-1-Tim10 and purified essentially as described using Precision Protease instead of thrombin for proteolytic release of Tim10 (15). Erv1-His6 was expressed and purified as described (22). Tim10 was reduced with 10 mM DTT for 10 min at 95 °C. DTT was removed by gel filtration using a NAP5 column (GE Healthcare). The reduced Tim10 was incubated in the absence or presence of Mia40C or Mia40C variants and Erv1 as indicated in the figure legends. Following modification with 15 mM AMS for 1 h at 25 °C the reduced and oxidized form of Tim10 could be distinguished in a non-reducing Tricine SDS-PAGE. Both forms were detected by immunodetection with antibodies against Tim10.

**Miscellaneous**—Isolation of mitochondria from yeast cells, in vitro transcription and translation of radiolabeled preproteins and import of preproteins into isolated mitochondria were applied as published (14). Partially reduced Mia40C was generated as described previously or by incubation in the presence of 2 mM β-mercaptoethanol (24).

**RESULTS**

The Highly Conserved Domain of Mia40 Is Sufficient to Fulfill the Function of Mia40—To determine the structural features important for the function of Mia40, we generated variants of *S. cerevisiae* Mia40. These variants comprised of different parts of the hydrophilic IMS domain of Mia40, ranging from the complete IMS segment (Mia40 WT) to the shortest segment consisting of amino acid residues 284–352 that represents the conserved domain (Fig. 1A). All variants contained the N-terminal import signal and the transmembrane anchor of Mia40 to target them to the inner membrane with the hydrophilic part facing into the IMS. Plasmids encoding the variant proteins were transformed into a chromosomal *MIA40* disruption strain expressing wild-type Mia40 protein from a *URA3*-containing plasmid. The ability of the mutant proteins to functionally replace wild-type Mia40 in *vivo* was tested by plasmid shuffling. All mutant proteins were able to complement the deletion of the Mia40 protein (Fig. 1B). This shows that the domain comprising amino acid residues 284–352 is sufficient to fulfill the function of Mia40 in *vivo* when targeted to the IMS. In addition, all IMS segments rescued the deletion, when they were fused to the first 220 amino acid residues of cytochrome *b*₂, instead of the authentic targeting signal plus transmembrane anchor, to sort the variants as soluble proteins to the intermembrane space (data not shown). Thus, also in soluble form the conserved domain comprising amino acid residues 284–352 of Mia40 is sufficient to be fully active. The results are consistent with a recent study in which a C-terminal domain of Mia40 (amino acid residues 226–403) was shown to be sufficient to

![Figure 1. The conserved C-terminal domain of Mia40 is sufficient for function.](image_url)
target the protein to mitochondria and to fulfill the function of Mia40 (29).

The Conserved Cysteine Residues in Mia40 Are Not Equally Important for the Function of Mia40—Within the highly conserved domain six invariant cysteine residues are present. Mutations of pairs of neighboring cysteine residues in Mia40 have been previously shown to be lethal, but the functions of the individual cysteine residues have remained elusive (17). To study the role of the various cysteine residues, mutants of Mia40 with a C-terminal His$_8$-tag were expressed in which the cysteine residues were replaced by serine residues. Complementation of the deletion of wild-type protein by these variants was tested by plasmid shuffling as described above (Fig. 2A). Replacement of the second (C2S) and of the third cysteine residue (C3S) in Mia40 was lethal for yeast cells. A severe growth defect was observed upon change of the first cysteine residue (C1S), whereas the variants Mia40-C4S, Mia40-C5S, or Mia40-C6S were able to complement the depletion of the wild-type protein. In a next step, we characterized double cysteine mutants of Mia40 with regard to their ability to rescue the respective depletion (Fig. 2B). The mia40-C1,2S mutant was not able to rescue suggesting an important functional role of these cysteine residues (Fig. 2B). In view of the lethal phenotype of the mia40-C3S mutant, it was surprising that the mia40-C3,6S mutant was able to support growth in the absence of wild-type Mia40. The growth rate, however, was strongly reduced compared with that of wild type cells. Thus, the third cysteine residue appears not to be essential for the function of Mia40. The mia40-C5,6S mutant cells were not viable, whereas the mia40-C4,5S mutant cells showed growth rates comparable to those of wild type cells. The results indicate that only the second cysteine residue is absolutely essential for the function of the protein. In addition to the cysteine mutants, we generated a strain expressing a variant in which the proline residue between the first two cysteine residues of the CPC segment was exchanged to a glycine residue. This proline is also conserved and might be crucial for the function of the neighboring cysteine residues. However, the strain expressing the Mia40 variant containing the CGC segment grew like wild type indicating that this proline residue does not play an important role for the protein (Fig. 2C).

Because some of the mutations in Mia40 are lethal in the mia40 deletion strain and thus not applicable for further functional studies, we expressed the variants in a GAL-MIA40 strain, in which the chromosomal MIA40 gene is under control of the GAL promoter (18). Growth of these strains in the presence of glucose allows to successively deplete the wild-type cysteine residues. Functions of Conserved Cysteine Residues in Mia40

**FIGURE 2. Growth phenotypes of strains carrying Mia40 cysteine and proline mutants.** A and B, In Mia40 variants cysteine residues were replaced by serine residues. The numbers indicate the positions of the cysteine residues in the conserved CPC-C$_X$C-C$_X$C$_X$C$_X$C$_X$ motif of Mia40. Plasmids carrying these mutant alleles of MIA40 were introduced into MIA40 deletion strain (Δmia40) harboring an URA plasmid containing the wild-type MIA40 gene. The obtained strains were tested for complementation by plasmid shuffling with 5-fluoroorotic acid-containing plates. The growth of the double cysteine mutants was analyzed in a drop dilution test (B). C, the proline residue in the CPC segment of Mia40 was substituted by a glycine residue (Mia40-CGC). The proline mutant was tested as described above. D and E, the GAL-MIA40 strain carrying the MIA40 gene under control of the GAL10 promoter was transformed with plasmids expressing the indicated cysteine variants of Mia40 and a drop dilution test was made for the resulting strains. Cells were grown on medium containing 2% glucose for 3 days at 30 °C.
Mia40 and to assess the growth phenotypes of the mutants. In general, the phenotypes observed for the mutant cells reflected those obtained in the disruption background (Fig. 2, D and E). Cells harboring the Mia40-C2S or the Mia40-C3S variant protein were not viable, whereas cells expressing the Mia40-C4S or the Mia40-C5S variant protein grew similarly to the wild-type control. Cells containing the Mia40-C6S variant showed a reduction in growth and also the mutation at the first position (Mia40-C1S) led to slow growth rates (Fig. 2D). The exchange of the first two cysteine residues, as well as the exchange of the fifth and sixth cysteine residue in Mia40 was lethal for the cells. The fourth and fifth cysteine residue could be exchanged without effects on the growth rate. Again, viability of the mia40-C3,6S mutant cells was observed upon depletion of Mia40 in the GAL-MIA40 strain. In summary, mutations of the single cysteine residues in Mia40 affect growth of yeast cells to different extents. Thus, the cysteine residues within Mia40 are not equally important. This strongly supports distinct functions for the single cysteine residues.

The Growth Defects Correlate with Defects in the Import of Substrates of Mia40—Next, we analyzed whether the mutations in Mia40 affect the import of small proteins and Erv1 into the IMS, the crucial function of Mia40 (16–18, 30, 31). To this end, mitochondria were isolated from the GAL-MIA40 strains in which endogenous Mia40 was strongly down-regulated, and the different Mia40 single and double cysteine variants were expressed with a C-terminal His$_8$ tag. Then we assessed the endogenous levels of mitochondrial proteins. The levels of substrates of the Mia40-dependent translocation pathway, such as Tim13 and Cox17, were strongly decreased in mitochondria of all mutants, which are lethal or have growth defects (Fig. 3A). In contrast, IMS proteins using other translocation pathways, such as cytochrome $b_2$, and proteins of different mitochondrial compartments were not affected. These results indicate that the reduced levels of Mia40 substrates are due to a specific effect and not to a general mitochondrial deficiency in the mutant cells.

To verify that the altered endogenous levels are caused by decreased rates of import, we incubated radiolabeled precursor proteins with mitochondria isolated from the mutant strains. Consistent with the observed reduction in the steady-state levels, the import rates of the Mia40-dependent substrates Tim13 and Erv1 were strongly reduced in the mutants in which growth was affected (Fig. 3B). Import of cytochrome c heme lyase (CCHL), an IMS protein that is not dependent on Mia40 for its transport, was not impaired in any of the Mia40 mutants. In summary, the import of small IMS proteins depends on all cysteine residues in Mia40 except the fourth and the fifth one.

FIGURE 3. Differential role of cysteine residues of Mia40 in the biogenesis of small IMS proteins. A, mitochondria were isolated from the GAL-MIA40 strains, which were transformed with the empty plasmid or plasmids expressing the indicated cysteine mutants and depleted of endogenous Mia40 for 27 h. Then mitochondria were analyzed for the levels of the indicated proteins by immunoblotting. B, import of the indicated $^{35}$S-labeled preproteins into mitochondria isolated from the strains described in A were performed. Following treatment with proteinase K, mitochondria were reisolated, and proteins were analyzed by SDS-PAGE and autoradiography.
The Variants of Mia40 Differ in Their Redox-sensitivity and Stability—In mitochondrial extracts an oxidized and a reduced form of Mia40 can be distinguished by their different mobilities upon non-reducing SDS-PAGE (14). The presence of the oxidized form is required for the import of substrates (14). So far, the molecular nature of the redox-states of wild-type Mia40 in intact mitochondria has not been analyzed. We isolated mitochondria from wild-type cells and treated them with reducing agent, DTT, at room temperature and at 95 °C to heat denature the proteins and thus allow full accessibility of the protein to reductant (Fig. 4A). Under native conditions, a slight shift in the mobility of Mia40 was observed in the presence of DTT, as previously reported for the reduced form (14). A larger decrease in mobility, however, was observed following reduction under heat denaturation. This suggests the presence of two forms of Mia40 in mitochondria: an oxidized form and a reduced form, which still contains disulfide bonds. In addition, a fully reduced form was obtained under denaturing conditions.

The redox states of the cysteine variants were analyzed to assign disulfide bonds to the various redox states in mitochondria. Mitochondria harboring the Mia40 variants were incubated with or without reducing agent under native conditions. Following thiol-trapping by modification with iodoacetamide, the redox states of the variants were analyzed using non-reducing SDS-PAGE and immunodecoration with antibodies against the C-terminal His tag. Without reducing agent all variants had a lower mobility in the SDS-PAGE than the wild-type protein suggesting oxidation of all cysteine residues in the wild-type protein (Fig. 4B). The Mia40-C1S and the Mia40-C2S variants were not sensitive to the reducing agent. In contrast, all single variants with amino acid exchanges of one of the four cysteine residues of the twin CX₉C motif showed lower mobility and were still sensitive to reducing agent. Analysis of the double cysteine variants of Mia40 confirmed these results. In the Mia40-C1,2S variant no redox-sensitive bond was found. The mutated protein had exactly the running behavior of the wild-type protein that was reduced at room temperature. On the other hand, the Mia40-C3,6S, the Mia40-C4,5S and the Mia40-C5,6S variants had a mobility lower than the partially reduced...
Functions of Conserved Cysteine Residues in Mia40

The Second Cysteine Residue of Mia40 Is Crucial for Formation of the Mia40-Erv1 Disulfide Intermediate—Following oxidation of substrate proteins, Mia40 is suggested to be released in a reduced form requiring reoxidation by the thiol oxidase Erv1 for further rounds of import (14). After import of substrate an enhanced amount of reduced Mia40 was indeed observed, suggesting release of Mia40 in the reduced form from the substrates (data not shown and Ref. 32). This reduced form has an identical mobility as the form with the redox-sensitive disulfide bond opened and requires interaction with the thiol oxidase Erv1 for reoxidation. The interaction of Erv1 with Mia40 was analyzed in the mutant strains. Mitochondria containing the wild-type and the mutant proteins were treated with iodoacetamide to trap disulfide intermediates. An adduct of about 85 kDa corresponding to the disulfide intermediate of Mia40 and Erv1 was observed in wild-type mitochondria (Fig. 5A). Such intermediate was virtually not detected in the mia40-C2S and the mia40-C3S mutant. Also in the mia40-C6S mutant the formation of the intermediate was strongly reduced. In contrast, the mia40-C1S, the mia40-C4S and the mia40-C5S mutants contained disulfide intermediates of Mia40 and Erv1. The second cysteine residue appears to connect Mia40 to Erv1 in the disulfide intermediate thereby allowing formation of the redox-sensitive disulfide bond in the CPC segment of Mia40 by subsequent isomerization. This conclusion is supported by the observations made in the double mutants of Mia40 (Fig. 5B). In the mia40-C1,2S mutant no Mia40-Erv1 intermediate was detected, whereas a small amount of the disulfide intermediate was present in the mia40-C3,6S mutant, in contrast to its absence in the mia40-C3S mutant. This suggests that the third cysteine residue is not absolutely essential for the formation of the disulfide bond between Mia40 and its thiol oxidase. It might stabilize together with the sixth cysteine residue a conformation which promotes efficient interaction of Erv1 and Mia40. Because a double mutation of the fourth and fifth cysteine residues does not affect the formation of the Mia40-Erv1 intermediate, these residues are not critical for the interaction. However, a combination of an amino acid exchange of the fifth and...
sixth cysteine residues was defective in the Erv1-Mia40 interaction, probably due to the absence of both stabilizing structural disulfide bonds in the twin C\(_X\)C motif. In summary, the second cysteine residue in Mia40 most likely mediates the covalent disulfide interaction between Mia40 and Erv1.

This conclusion is supported by experiments with the recombinant Mia40C variants. When the recombinant variants of the redox-sensitive disulfide bond were analyzed for their interaction with Erv1, the variant Mia40C-C1S was able to interact, whereas the Mia40C-C2S and the Mia40C-C1,2S variants were apparently not (Fig. 5C, upper panel). Notably, the amount of Mia40-Erv1 intermediate increased for the Mia40C-C1S variant, probably because a disulfide bond between the first and the second cysteine residue could not be formed. Thus, the formation of the disulfide intermediate between Mia40 and Erv1 requires the second cysteine residue of Mia40. An interaction with Erv1 was also detected for the Mia40 variants of the twin C\(_X\)C motif (Fig. 5C, lower panel). The Mia40-Erv1 adduct was decreased in its intensity for the Mia40-C6S variant and the Mia40-C3,6S variant and hardly detectable in case of the Mia40-C3S variant. This supports a role for these cysteine residues in the stabilization of a functional conformation of Mia40 important for the interaction with Erv1.

**The Redox-sensitive Disulfide Bond of Mia40 Is Important for the Interaction with Substrates**—Next, we analyzed which of the cysteine residues in Mia40 are crucial for the formation of disulfide intermediates with substrate proteins. To this end, radiolabeled precursors of Tim13 were imported into mitochondria isolated from the different mutant cells. Mitochondria were lysed, and the His\(_8\)-tagged Mia40 proteins were isolated by binding to NiNTA-agarose. The formation of mixed disulfides between Tim13 and the Mia40 variants was detected by SDS-PAGE of the eluted material followed by autoradiography. The redox-sensitive disulfide bond between the first and second cysteine residue is important for the interaction with substrate, because no mixed disulfide was observed in the mia40-C1,2S mutant mitochondria (Fig. 6A). In the single mia40-C1S and mia40-C2S mutants a disulfide intermediate of Mia40 with the substrate Tim13 was still detected, albeit in reduced amounts for the Mia40-C2S mutant (Fig. 6B, upper panel). Thus, substrate proteins might be able to interact with both of the cysteine residues from the redox-sensitive disulfide bond. Other cysteine residues appear to play not a direct, but still crucial, role for this interaction. The interaction was strongly diminished in the mia40-C3,6S mutant and the mia40-C6S mutant and was virtually absent in the mia40-C3S mutant and the mia40-C5,6S mutant. In contrast, the mutation of the reducing conditions by SDS-PAGE, transferred to nitrocellulose membrane, and decorated with Erv1-specific antibodies. Different amounts of mitochondria were used to have comparable amounts of Erv1 present (25 \(\mu\)g for Mia40-WT and Mia40-C4,5S, 50 \(\mu\)g for Mia40-C1S, Mia40-C2S, Mia40-C4S and Mia40-C5S, 100 \(\mu\)g for Mia40-C6S and Mia40-C1,2S, 200 \(\mu\)g for Mia40-C3,6S and Mia40-C5,6S and 250 \(\mu\)g for Mia40-C3S). C, purified Mia40C-WT in its partially reduced form and the variants with the indicated cysteine residue exchanges (7 \(\mu\)g each and 14 \(\mu\)g for C3S) were incubated with Erv1 (7 \(\mu\)g) for 20 min at room temperature and then treated with 80 \(\mu\)m iodoacetamide. Following non-reducing SDS-PAGE samples were analyzed by immunodecoration with antibodies against Erv1 and Mia40 (upper panel) or Mia40 (lower panel). *, dimer of Mia40C; **, degradation product of Erv1.
fourth and the fifth cysteine residues, either alone or combined, did not interfere with the formation of the mixed disulfide intermediate. These cysteine residues are not as important for a stable conformation as the third and sixth cysteine residues. Similar observations were made for the interaction of another substrate protein, Tim9, with the single cysteine mutants of Mia40 (Fig. 6B, lower panel). In summary, a specific conformation of Mia40 and a redox-sensitive disulfide bond appear to be essential for the interaction with substrates.

Mia40 and Erv1 Are Required to Oxidize the Substrate Tim10 in a Reconstituted System—To analyze the role of single cysteine residues in Mia40 we reconstituted the oxidation of the substrate protein Tim10. To this end, Tim10, the functional C-terminal fragment of Mia40 and Erv1 were expressed in E. coli and purified. Reduced Tim10 was then incubated in the absence or presence of catalytic amounts of either Mia40 or Erv1 or of both. The redox state of Tim10 was determined by treatment with AMS followed by non-reducing Tricine SDS-PAGE and immunodecoration with antibodies against Tim10. As control for the mobility of the redox forms of Tim10, oxidized Tim10 (Tim10 ox., lane 1) and reduced Tim10 were treated with AMS and analyzed (lane 2 and lane 3, respectively). Tim10 red. + mod., fully reduced form of Tim10 modified with AMS. B, analysis of cysteine variants in the reconstitution assay. Reduced Tim10 was incubated with the indicated Mia40C variants and Erv1 for 210 min at 25 °C. Samples were then analyzed as in A.

The Redox-sensitive Disulfide Bond of Mia40 Is Essential for the Oxidation of Substrate—We used the reconstitution assay to determine which cysteine residues are required for the ox-

Functions of Conserved Cysteine Residues in Mia40
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dation process. The different variants of Mia40 were used in combination with Erv1 in the assay. In the presence of either the Mia40-C1S, the Mia40-C2S, or the Mia40-C1,2S variant virtually no oxidized form of Tim10 was generated (Fig. 7B). In contrast, Mia40-C3S, Mia40-C6S, and Mia40-C3,6S combined with Erv1 oxidized Tim10, albeit with reduced efficiency. These results suggest that the cysteine residues three and six are not essential for the catalytic activity of Mia40, whereas the first and second cysteine residues are crucial for its function in the disulfide transfer to substrates.

**DISCUSSION**

In this study we have analyzed the roles of the conserved cysteine residues of the redox-regulated import receptor Mia40. Based on the growth phenotypes of mutant cells and the import rates of these mutants we conclude that the first three and the sixth cysteine residues play an important role in the protein, whereas the fourth and the fifth cysteine residues are not crucial for function. This suggests distinct functions for the various cysteine residues.

All cysteine residues are present in disulfide bonds in the oxidized form of Mia40 in mitochondria. The first and second cysteine residues form a redox-sensitive bond which is opened in the reduced form in mitochondria. The results obtained for full-length Mia40 *in vivo* are consistent with the results previously reported for a purified C-terminal fragment of Mia40, which also contained one redox-sensitive bond (24). In this fragment, disulfide bonds were assigned by mass spectrometry to be present between the first and the second as well as between the third and the sixth and the fourth and the fifth cysteine residues.

The second cysteine residue in the CPC segment plays an essential role in the mechanism of Mia40 in the import of proteins with twin XP motif and twin XC motif into mitochondria. It is a constituent of the redox-sensitive bond which gets reduced upon import of substrates into mitochondria. Thus, this bond is involved in the transfer of disulfide bonds to the substrates. The Mia40-C1S mutant has a severe growth defect, but it is, in contrast to the Mia40-C2S and the Mia40-C1,2S mutant, still viable. A transfer of the redox-sensitive bond to the substrate protein is not expected to occur in the Mia40-C1S mutant, which therefore should not be able to grow. This was not observed. Apparently, a reaction is occurring that allows inefficient transfer of disulfide bonds to the substrate. Interestingly, the Mia40-C1S and Mia40-C2S variants do not show similar properties. Whereas the formation of a disulfide intermediate of the Mia40-C2S variant with Erv1 is very inefficient, the Mia40-C1S variant still interacts efficiently with Erv1. It appears possible that the formed mixed disulfide has the ability to oxidize substrate proteins or that the Erv1 recruited might be involved in the oxidation process.

At first glance, the third cysteine residue in Mia40 appears to be essential, because its mutation is lethal for yeast cells. However, the ability of the Mia40-C3,6S mutant to partially rescue the deletion of Mia40 and to interact with Erv1 and substrate suggests that this is not the case. Thus, the third cysteine residue presumably does not form directly disulfide bonds with substrates and/or Erv1, although this cannot be completely excluded, e.g. if there are partially redundant systems. The Mia40-C3S mutation might cause a stronger defect than the Mia40-C3,6S mutation, because it generates in the protein the free thiol group in the unpaired sixth cysteine residue. Unpaired cysteine residues might form non-native disulfide bonds in Mia40, a reaction with deleterious effects. Exchange of the sixth cysteine residue in Mia40 also causes a growth defect, but not as severe as in case of the Mia40-C3S mutant. Consistent with this result, the defects in the import of substrates and in the interaction with substrates and Erv1 are not as strong for the Mia40-C6S variant as for the Mia40-C3S mutant. The mutation of the third cysteine residue might be more deleterious, because it is closer to the redox-active first two cysteine residues or it might have a stronger tendency to form non-native disulfide bonds. Because Mia40 contains a twin CX₂C motif, it has the typical motif of substrates proteins. At least *in vitro*, a domain of Mia40 has been shown to be a substrate of Mia40 (24). Thus, the Mia40-C3S variant might be a substrate of Mia40, which cannot be released due to the lack of the partner cysteine. It would block residual functional wild-type Mia40 in the GAL-MIA40 strain. This would explain the drastic effect on substrate import and on the interactions with the redox partners. This variant might be more efficient in blocking Mia40 than other variants with exchanged cysteine residues in the twin CX₂C motif. Such hierarchy was already observed for the substrate recognition by Mia40. The first cysteine residues in the twin CX₂C motif of small Tim proteins are crucial for binding to Mia40 (33, 34).

Overlap of functions of the disulfide bonds between the third and the sixth and the fourth and fifth cysteine residue is suggested by the lethality of the mia40-C3,4S and the mia40-C5,6S mutants. These disulfide bonds are very resistant against reducing agent and appear not to be opened *in vivo* indicating a structural function in Mia40. Lack of one of these bonds destabilizes Mia40. The bond between the outer cysteine residues (C3 and C6) of the helices appears to be more important for stabilization of the structure. This bond is also closer to the catalytically active cysteine pair and might be therefore crucial for adopting a conformation allowing efficient binding of Erv1 and substrate. The lack of the bond between the fourth and the fifth cysteine residues apparently has no deleterious effect, because the outer disulfide bond in the twin CX₂C motif still efficiently links the helices. Disulfide bonds in the twin CX₂C motif between the outer and inner cysteine pairs have been also described for Cox17. Consistent with this interpretation, these disulfide bonds stabilize the Cox17 protein and are resistant against high concentrations of reducing agents (8).

We reconstituted for the first time the oxidation of a substrate protein, Tim10, by the disulfide relay system. Although there might be additional factors involved *in vivo*, Mia40 and Erv1 are necessary and sufficient to oxidize Tim10. Consistent with the mutational analysis *in vivo*, the first and second cysteine residues play a crucial role for the oxidation of Tim10. Since one disulfide bond of Mia40 is involved in the redox reactions it remains to be elucidated how two disulfide bonds are transferred to the substrate proteins. Two molecules of Mia40 may act in this process. On the other hand, it is possible that the thiol oxidase Erv1, alone or in combination with Mia40, gener-
ates disulfide bonds in the substrate proteins. A dual function of Erv1 in the oxidation of Mia40 and of substrate proteins is consistent with reports supporting a role of Erv1 in a later step of the biogenesis of small Tim proteins (21, 23). In these studies, a temperature sensitive mutant of Erv1 still allowed formation of the disulfide intermediate between Mia40 and substrate but did not promote release and assembly of oxidized substrate.

In summary, the mutational analysis in vivo together with in vitro reconstitution experiments demonstrates that the cysteine residues of Mia40 fulfill distinct functions in the protein. The first two cysteine residues form a disulfide bond which is involved in the disulfide bond transfer. The cysteine residues of the twin C(X)3C motif form disulfide bonds that stabilize Mia40. In particular the bond between the third and the sixth cysteine residues plays a crucial role in stabilizing a conformation that binds efficiently substrate and Erv1.

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