Functional Characterization of Mia40p, the Central Component of the Disulfide Relay System of the Mitochondrial Intermembrane Space

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Mia40p and Erv1p are components of a translocation pathway for the import of cysteine-rich proteins into the intermembrane space of mitochondria. We have characterized the redox behavior of Mia40p and reconstituted the disulfide transfer system of Mia40p by using recombinant functional C-terminal fragment of Mia40p, Mia40C, and Erv1p. Oxidized Mia40p contains three intramolecular disulfide bonds. One disulfide bond connects the first two cysteine residues in the CPC motif. The second and the third bonds belong to the twin CX_{9C} motif and bridge the cysteine residues of two CX_{9C} segments. In contrast to the stabilizing disulfide bonds of the twin CX_{9C} motif, the first disulfide bond was easily accessible to reducing agents. Partially reduced Mia40C generated by opening of this bond as well as fully reduced Mia40C were oxidized by Erv1p in vitro. In the course of this reaction, mixed disulfides of Mia40C and Erv1p were formed. Reoxidation of fully reduced Mia40C required the presence of the first two cysteine residues in Mia40C. However, efficient reoxidation of a Mia40C variant containing only the cysteine residues of the twin CX_{9C} motif was observed when in addition to Erv1p low amounts of wild type Mia40C were present. In the reconstituted system the thiol oxidase Erv1p was sufficient to transfer disulfide bonds to Mia40C, which then could oxidize the variant of Mia40C. In summary, we reconstituted a disulfide relay system consisting of Mia40C and Erv1p.

The pathways along which these proteins are sorted in the IMS are diverse (1–5). Precursors of proteins containing cleavable matrix targeting sequences followed by transmembrane segments are initially using the general import pathway mediated by the TIM23 (translocase of the inner membrane 23) complex until the transmembrane segment reaches the inner membrane where it undergoes translocation arrest. By a subsequent proteolytic step the mature protein is released into the IMS. On the other hand, there are many proteins of relatively small size that lack presequences but contain motifs of conserved cysteine residues, such as the twin CX_{9C} motif in the copper chaperone Cox17p and in Cox19p, Mdm35p, Mic14p, and Mic17p or the twin CX_{9C} motif in the family of small Tim proteins (6–11). Proteins with twin CX_{9C} motifs use the Mia40p-dependent translocation pathway, which consists of at least two components in the IMS, Mia40p and Erv1p (8, 12–17). Both proteins are essential for viability of yeast cells (12–14, 18–20). Depletion of Mia40p or Erv1p leads to reduced import and assembly of cysteine-rich IMS proteins.

Mia40p is present throughout the eukaryotic kingdom (12–14). In fungi, the large hydrophilic IMS domain of Mia40p is anchored to the inner membrane by an N-terminal transmembrane segment. In animals and plants, Mia40 is present as a much shorter soluble protein (21). All homologs harbor a highly conserved domain of about 60 amino acid residues. This domain contains six invariant cysteine residues in a CPC-CX_{9C}-CX_{9C} arrangement. Replacement of a cysteine pair either in the CPC or in one of the CX_{9C} segments with a pair of serine residues was lethal, indicating the crucial role of the cysteine residues for the function of Mia40p (13). Indeed, Mia40p acts as a receptor for small IMS proteins in their import reaction by forming disulfide intermediates with the IMS proteins (12–16). In their native state some of the small IMS proteins contain intramolecular disulfide bonds (10, 22–25). Although not directly shown so far, this suggests a transfer of disulfide bonds from Mia40p to the interacting IMS proteins. The generation of intramolecular disulfide bonds has been proposed to trigger folding of substrates and thereby trapping of imported substrates in the IMS (26, 27). Two redox forms of Mia40p have been detected in mitochondria, an oxidized and a reduced form. The oxidized form of Mia40p is required for the import of substrates into mitochondria (15).
Mia40p works together with Erv1p in the import pathway of small IMS proteins. Erv1p belongs to the class of FAD-containing sulfhydryl oxidases which transfer electrons from thiols to non-thiol electron acceptors and thereby generate disulfide bonds in proteins (28, 29). Indeed, thiol oxidase activity has been measured for Erv1p in vitro (30–32). Depletion of Erv1p leads to accumulation of reduced Mia40p (15). Moreover, Erv1p interacts with Mia40p via disulfide bonds (15, 16). These observations suggest that Erv1p and Mia40p form a disulfide relay system to generate intramolecular disulfide bonds in the small IMS proteins. In such a pathway, the role of Erv1p would be to transfer disulfide bonds to Mia40p to regenerate oxidized Mia40p. The pathway for the generation of disulfide bonds in the mitochondria appears to differ from the disulfide relay systems in the endoplasmic reticulum and the bacterial periplasm (33–37), because Mia40p has apparently no homologs in these compartments.

The redox cycle of Mia40p, in particular the molecular basis of the redox states of Mia40p and the identity and function of its disulfide bonds, have not been determined. Likewise, the generation of disulfide bonds in Mia40p and the shuttle of disulfide bonds between Mia40p and Erv1p have not been analyzed. Here we describe the isolation and characterization of a recombinant domain of Mia40p (Mia40C) which contains all cysteine residues of complete Mia40p. This domain is catalytically functional. In the oxidized form of Mia40C, all cysteine residues are present as intramolecular disulfide bonds. The pattern of these bonds was determined by mass spectrometry. The disulfide bonds connecting two CX₃C segments stabilizes Mia40p, and the redox-sensitive disulfide bond between the first two cysteine residues has a crucial function for the disulfide exchange reactions with Erv1p and substrate proteins. In a reconstituted system the reduced form of Mia40C was oxidized in a reaction catalyzed by recombinant Erv1p. Our results demonstrate that Erv1p and its substrate Mia40p are sufficient to form a functional disulfide relay system.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Yeast Strains**—To generate pGEX-6P-1-Mia40C, amino acid residues 284–403 of Mia40p were amplified by polymerase chain reaction using the primers Mia40For850BamH1 (5′-CTCGAGATCCGTTTATAACCCAC-GACACTGG-3′) and Mia40Sc_StBglXho (5′-CTCTC- GAGAGATCTTTAAGGTTTGGATTCCTCATTC-3′) and the plasmid pGEM4-Mia40 as template. The PCR fragment was cloned into the vector pGEX-6P-1 (GE Healthcare) using the plasmid pGEM4-Mia40 as template. The resulting construct was digested with SpeI and cloned into pGEM4-Mia40-His. To generate pGEX-6P-1-Mia40C-C1,2S the plasmid pGEM4-Mia40-His-C1,2S was used as a template for amplification of a fragment with the primers Mia40For850BamH1 and Mia40Sc_StBglXho. After digestion with BamHI and XhoI, this fragment was cloned into the vector pGEX-6P-1. The strain expressing Mia40C in the wild type background was generated in the diploid strain YPH501 by replacing the nucleotides −4 to 849 of one allele of Mia40 with a GAL10 promoter-containing cassette followed by the coding sequence of eight histidine residues by homologous recombination.

**Expression and Purification of Mia40C and Erv1p from Escherichia coli**—To obtain recombinant Mia40C, E. coli XL1blue cells containing the plasmid pGEX-6P-1 expressing glutathione S-transferase (GST)-Mia40C or GST-Mia40C-C1,2S were grown in 3 liters of LB medium at 37 °C to A₆₀₀ nm of 0.5–0.8. Isopropyl-1-thio-β-D-galactopyranoside was then added to a final concentration of 1 mM, and bacterial growth was continued for 5 h. The induced cell culture was centrifuged, washed, and resuspended in 60 ml of buffer A (20 mM Tris-HCl, pH 7.0, 200 mM NaCl, 1 mM phenylmethylsulfonyl fluoride) and 0.2 mg/ml lysozyme. Cells were disrupted by sonication and centrifuged at 20,000 × g for 30 min at 4 °C. The supernatant containing glutathione S-transferase-Mia40C was applied to a column with 8 ml of glutathione-Sepharose 4B beads (GE Healthcare) equilibrated with buffer A. The column was washed with 20 volumes of buffer A. The beads were then resuspended with 30 ml of 20 mM Tris-HCl, pH 7.0, 200 mM NaCl. Precission protease was added to a final concentration of 0.05 mg/ml. After incubation for 3 h at 30 °C, the cleaved Mia40C protein was removed. The fraction removed contained Mia40C and forms of Mia40C which were linked by intermolecular disulfide bonds. To obtain a homogenous fraction of monomeric Mia40C, the protein was loaded on a Superdex 75 gel filtration column for further purification. Erv1p-His₆ was expressed in the E. coli strain BL21 (DE3) and purified as described (30).

**4-Acetamido-4′-maleimidylstilbene-2,2′-disulfonic Acid (AMS) Alkylation Assay**—Purified Mia40C (15–35 μM) in 20 mM Tris-HCl, pH 7.0, 40 mM NaCl was preincubated at 25 or 95 °C with 5 mM DTT or left untreated. Samples were incubated for 30–60 min at 25 °C in the presence or absence of 15–30 mM concentrations of the thiol-specific reagent AMS (Invitrogen). AMS reacts with free thiol groups, resulting in a mobility shift of the protein on SDS-PAGE due to its increase in size of 0.5 kDa per added molecule AMS. SDS-PAGE sample buffer with or without 80 mM DTT was then added, and the samples were analyzed by SDS-PAGE and staining with Coomassie Blue.

**Redox Reactions with Recombinant Proteins**—In the reconstitution experiments of the Mia40p-Erv1p disulfide transfer system, various redox forms of Mia40C were used. To generate the partially reduced Mia40p containing two free thiol groups, the protein was incubated in the presence of 5 mM DTT for 10 min at 25 °C. Then DTT was removed by gel filtration using a NAP5 column (GE Healthcare). The protein was eluted in 20 mM Tris-HCl, pH 7.0, 40 mM NaCl, and 1 mM EDTA. Fully reduced Mia40p was obtained by heat denaturation of the protein for 10 min at 95 °C in the presence of 5 mM DTT. DTT was removed as described above. In the reconstitution experiments the indicated redox form of Mia40C was mixed with Erv1p in the amounts depicted and incubated for the indicated times at room temperature. Then the redox states of Mia40C were analyzed by modification with AMS and subsequent SDS-PAGE
and staining with Coomassie Blue. The reactions were performed under microanaerobic conditions in a nitrogen-filled glove bag.

**HPLC and Mass Spectrometry**—For the mass spectrometry (MS) analysis, proteins or enzymatically digested peptides were injected on a reverse-phase chromatography column, PepMap C18 0.3/15 (LC Packings, Sunnyvale, CA), and eluted with a 35-min linear gradient of acetonitrile in water (5–50%) containing 0.05% trifluoroacetic acid with a flow rate of 4 ml/min. The MS analysis was performed on-line with a LCQ Deca XP ion-trap spectrometer equipped with an electrospray ionization source (ThermoFinnigan, San Jose, CA). The LCQ was operated in positive mode under manual control in the Tune Plus view with default parameters and automatic automatic gain control. MS/MS analysis was done to confirm the sequence of the precursor ions using low energy collision-induced dissociation with relative collision energy of 35%.

**CD Spectroscopy**—Far-ultraviolet CD measurements were performed using a Jasco J-715 spectrometer. Final CD spectra were obtained by averaging four consecutive scans, and the secondary structure content was estimated.

**RESULTS**

**Recombinant Mia40p C-terminal Domain Is Folded**—To characterize Mia40p, we purified a truncated form of Mia40p consisting of amino acid residues 284–403, Mia40C (Fig. 1, A and B). This segment of Mia40p was sufficient to confer viability to yeast cells when targeted to the intermembrane space of mitochondria lacking authentic Mia40p. It contains that part of the protein which is conserved throughout the eukaryotic kingdom (12–14). All six cysteine residues of Mia40p are present in this protein segment (Fig. 1A). We analyzed the protein by CD spectroscopy. This revealed folding of the protein with a content of 30% α-helical structure (Fig. 1C). Furthermore, folding was demonstrated by partial resistance of the purified protein against added protease. Upon treatment with trypsin a fragment of about 9.8 kDa was generated (Fig. 1D). According to analysis by mass spectrometry, this fragment consisted of amino acid residues 284–367. Thus, the tightly folded domain contains the highly conserved cysteine residues of Mia40p.

**Mia40p Contains Three Disulfide Bonds with Different Sensitivity to Reducing Agents**—In mitochondria, Mia40p is mainly present in an oxidized state (15). Upon treatment with DTT, a reduced form of Mia40p was generated that displays a slower mobility than the oxidized form upon non-reducing SDS gel electrophoresis. The Mia40C protein was used to characterize the redox states of Mia40p. To this end, purified Mia40C was incubated with the agent AMS, which reacts with free thiol groups. Modification of thiol groups by AMS can be detected by SDS gel electrophoresis, since the modification results in an increase of the molecular mass of the protein by 0.5 kDa per AMS bound. An altered molecular mass of the purified Mia40C protein was not observed upon treatment with AMS (Fig. 2A, lane 2). This lack of free thiol groups points to a fully oxidized protein with three disulfide bonds. After incubation with the reducing agent DTT, a shift in the electrophoretic mobility of the Mia40C protein was observed, indicating its modification by AMS (lane 4). An even larger shift of Mia40C was detected when the protein was incubated at 95 °C in the presence of DTT before AMS treatment (lane 6). Obviously not all disulfide bonds could be reduced without heat denaturation. Partially reduced Mia40C appeared to be modified by two molecules of AMS, indicating the presence of two thiol groups and two disulfide bonds. Heat denaturation under reducing conditions generated fully reduced Mia40C. The observed mobility shift of this species was larger than expected for a completely reduced Mia40C protein modified with six AMS molecules, indicating an unusual running behavior of modified Mia40C.

We checked whether the redox states observed with the recombinant protein reflect the redox behavior of Mia40p in mitochondria. Mia40C was expressed in wild type cells, and its redox states were analyzed. In mock-treated mitochondria

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3 N. Terziyska and K. Hell, unpublished data.
most of Mia40C was not modified by AMS. After treatment with DTT, Mia40C was partially reduced and modified by AMS (Fig. 2B). In addition, heat denaturation in the presence of DTT resulted in a large shift of electrophoretic mobility indicating full reduction of Mia40p. Thus, the redox states of the protein in mitochondria are very similar to those seen with the isolated protein.

To determine the number of disulfide bonds and their redox sensitivity, we measured the molecular mass of Mia40C in the presence and absence of reducing agent and the thiol-modifying reagent NEM by mass spectrometry (Table 1). Untreated Mia40C had a measured molecular mass of 13,842.2 Da, which is 6 Da less than the calculated mass for the theoretical sequence of completely reduced Mia40. This supports the presence of all the cysteines in the oxidized state forming three disulfide bonds. Consistent with the lack of free thiols, treatment with NEM did not alter its molecular mass. A molecular mass of 13,848.8 Da corresponding to fully reduced Mia40C was observed when the sample was treated at 95 °C in the presence of DTT. Modification of this sample with NEM resulted in a mass of 14,598.8 Da (a mass difference of 750 Da to the fully reduced Mia40C), corresponding to the covalent addition of six NEM molecules (i.e., 125 Da/NEM molecule). Thus, under these conditions, Mia40C was fully reduced. In contrast, when Mia40C was treated with DTT at room temperature, Mia40C was only partially reduced. Its molecular mass of 13,844.2 Da suggests that partially reduced Mia40C contains two free thiol groups and, still, two disulfide bonds. Modification of this sample with NEM resulted in a mass of 14,094.6 Da, confirming the presence of two thiol groups.

In summary, the results demonstrate the presence of three disulfide bridges in Mia40C. Whereas one of these bonds can be easily reduced, the two others are largely resistant toward reducing conditions under non-denaturing conditions. The two resistant disulfide bonds stabilize the protein. Oxidized and partially reduced Mia40C, which still contain these disulfide bonds, were not completely digested by trypsin but, rather, degraded to the already described trypsin-resistant fragment (Fig. 2C). On the other hand, the protein was completely degraded, and no fragment was detected when these disulfide bonds were reduced at 95 °C. Treatment at 95 °C on its own does not affect the stability of the protein to digestion with trypsin. Thus, two of the disulfide bonds are crucial for stable folding of the highly conserved domain in Mia40p.

In Mia40C, Two Disulfide Bonds Are Formed by the Twin CX9C Motif and One by the CPC Segment—Next we determined the pattern of disulfide bridges within the protein. The largely protease-resistant domain was treated with trypsin in the presence of 2 M urea. Mass spectrometry analysis of the resulting peptides revealed two tryptic peptides of molecular masses of 2325 and 4731 Da (Table 2). The first fragment corresponds to the peptides Mia40-(313–326) and Mia40-(327–333) linked by a disulfide bond (Fig. 3A). The second fragment consisted of the disulfide-bonded peptides of Mia40(GPLGS-(284–312)) and Mia40-(334–342) (Fig. 3A). The first fragment revealed the presence of a disulfide bond between amino acid residues 317 (Cys-4) and 330 (Cys-5). The other fragment indicates a further disulfide bond between amino acid residue 340 (Cys-6) and one of the first three cysteine residues of Mia40C. Subsequent tandem mass spectrometry analysis confirmed the suggested assignment of these pep-
tides and demonstrated cysteine residue 307 (Cys-3) to be the one that forms a disulfide bond with cysteine residue 340 (Cys-6) (see supplemental Figs. S1 and S2).

To investigate the nature of the disulfide bond that can be easily reduced, digestion with trypsin was performed with partially reduced Mia40C after reaction with NEM. Mass spectrometry analysis of the resulting peptides yielded two tryptic peptides of molecular masses of 2325 and 4983 Da (Table 2). The mass difference of the latter peptide to the peptide obtained upon digestion of Mia40C reflects the modification of two cysteine residues by NEM. Subsequent tandem mass spectrometry analysis of this peptide provided sufficient information to determine the cysteine residues 296 (Cys-1) and 298 (Cys-2) unequivocally as the cysteine residues modified by NEM and to conclude that the redox-sensitive disulfide bond is formed by these two cysteines (Fig. 3B).

To confirm this, a mutant variant of Mia40C in which the cysteine residues Cys-1 and Cys-2 were exchanged to serine residues, Mia40C-C1S/C2S, was expressed in bacteria and purified. This variant was folded in a similar manner as Mia40C, as indicated by its CD spectrum (Fig. 1). Its measured molecular mass of 13,812.3 Da was consistent with an oxidized protein whose disulfide bond between the cysteine residues Cys-1 and Cys-2 was opened with DTT. The redox state of Mia40C was then analyzed by AMS treatment and SDS gel electrophoresis. The treatment with Erv1p prevented modification of Mia40C by AMS, indicating its reoxidation (Fig. 4A). This reaction was dependent on Erv1p, since control reactions with buffer or with reduced and alkylated Erv1p did not lead to reoxidation. Similar observations were made with fully reduced Mia40C. In this case the oxidized form of Mia40C was also recovered after incubation with Erv1p but not after incubation with FAD or buffer (Fig. 4B). Thus, the thiol oxidase Erv1p generates disulfide bonds in Mia40C.
FIGURE 3. Mass spectrometry analysis of the pattern of disulfide bonds in Mia40C. A, electrospray ionization-MS spectra of two tryptic peptide fractions of the oxidized form of Mia40C. Purified Mia40C was digested by incubation with 1/10 of trypsin (w/w) in 2 M urea in 0.1 M ammonium carbonate at 37 °C overnight. The tryptic peptides obtained were separated by reverse-phase HPLC and analyzed online by mass spectrometry. The sample contained two abundant peptides with masses of 2325 Da (upper panel, detected as 3$^+$ and 2$^+$ ions at $m/z$ 776 and 1163) and 4731 Da (lower panel, detected as 4$^+$ and 3$^+$ ions at $m/z$ 1183 and 1578) that correspond to peptides 313–326 and 327–333 and GPLGS-(284–312+334–342), respectively, as indicated. The tag of amino acid residues GPLGS results from the processing site of the prodroin protease and from the restriction site used for cloning of the fusion protein. m/z, mass-to-charge ratio. B, MS/MS fragmentation spectrum of the triply charged ion at $m/z$ 1661 obtained after trypsin digestion of partially reduced Mia40C which was treated with NEM. The partially reduced Mia40C modified by NEM was treated and analyzed as described in A. The tryptic peptide with a mass of 4983 Da that corresponds to peptide GPLGS-(284–312+334–342) was detected as 3$^+$ and 4$^+$ ions at $m/z$ 1661 and 1246, respectively (not shown). The fragments that were detected are indicated above the peptide sequence for N-terminal ions and below for C-terminal ions. C, redox states of purified Mia40C-C1,2S was analyzed by modification with AMS. The protein was treated and analyzed as described in Fig. 2A. D, schematic representation of the redox states and of the disulfide bonds of Mia40C. The cysteine residues of Mia40C are labeled according to their order in Mia40p; that is, the first residue as C1 (amino acid position 296), the second C2 (amino acid 298), the third C3 (amino acid 307), the fourth C4 (amino acid 317), the fifth C5 (amino acid 330), and the sixth C6 (amino acid 340).
This reaction requires the formation of disulfide-bonded intermediates consisting of Erv1p and Mia40C. To trap such intermediates, samples containing partially reduced Mia40C and Erv1p were incubated then treated with iodoacetamide and subjected to SDS gel electrophoresis. A protein species was detected which corresponded in size to an adduct of Mia40C and Erv1p (Fig. 4C). Immunodecoration with antibodies against Mia40p as well as Erv1p revealed the presence of both proteins in this species. As expected for a disulfide intermediate, this adduct was not detectable in the presence of DTT (data not shown). In partially reduced Mia40C, the residues Cys-1 and Cys-2 were present as free thiols and they, therefore, were likely involved in the interaction with Erv1p. Indeed, interaction of Erv1p with the variant Mia40C-C1,2S that lacks these cysteine residues was not observed (Fig. 4C).

In conclusion, Mia40C and Erv1p can form a mixed disulfide intermediate, and thereby, Erv1p can oxidize Mia40C and catalyze the formation of disulfide bonds between Cys-1 and Cys-2 in Mia40C. We, therefore, have successfully reconstituted the reaction of transfer of disulfide bonds from Erv1p to Mia40C.

Mia40C Can Oxidize the Mia40C Variant—We asked whether the mutant variant Mia40C-C1,2S can be oxidized by Erv1p despite the lack of disulfide intermediates. In contrast to wild type, the Mia40C variant after incubation with Erv1p was still modified by AMS, indicating the absence of oxidized Mia40C variant (Fig. 5A). Thus, the redox-sensitive cysteine residues Cys-1 and Cys-2 in Mia40C are crucial for the transfer of disulfide bonds from Erv1p to Mia40C and, hence, for the formation of the disulfide bonds in Mia40C. In the absence of Cys-1 and Cys-2, Mia40C is not a substrate for Erv1p. On the other hand, Mia40C-C1,2S still contains the twin CX,C motif, a characteristic of substrates of Mia40p. Is Mia40C able to oxidize the Mia40C variant? Equal amounts of reduced Mia40C-C1,2S variant and the oxidized form of wild type Mia40C were incubated, and the redox states of both proteins were analyzed by modification with AMS and subsequent SDS-gel electrophoresis. Upon incubation with wild type Mia40C, the Mia40C-C1,2S variant was oxidized, as indicated by almost complete absence of the fully AMS-modified species of the variant (Fig. 5B). Obviously, the Mia40C variant was oxidized, and most of the wild type Mia40C was partially reduced. An intermolecular isomerization reaction between the twin CX,C motifs to transfer the disulfide bonds from the wild type to the variant is unlikely, because wild type Mia40C in which the CPC motif was reduced was not able to reoxidize the variant of Mia40C in the assay (Fig. 5C).

Are catalytic amounts of the oxidized form of wild type Mia40C able to mediate oxidation of the cysteines of the twin CX,C motifs? Without further addition of Erv1p, almost all of the variant Mia40C-C1,2S stayed reduced (Fig. 5D, lane 7). However, the addition of the thiol oxidase Erv1p resulted in efficient oxidation of the Mia40C variant (Fig. 5D, lanes 8 and 9). Erv1p apparently regenerated the oxidized form of wild type Mia40C and, thus, allowed further rounds of oxidation. This oxidation process clearly works via the catalytic amounts of Mia40C, since Erv1p on its own did not reoxidize the Mia40C variant (Fig. 5D, lanes 5 and 6). We conclude that Mia40p has the potential to oxidize substrates with twin CX,C motifs, such as the Mia40C-C1,2S variant. Thus, the Erv1p–Mia40p disulfide relay system is able to shuttle disulfide bonds to a substrate in a reconstituted system.

**DISCUSSION**

In this study we have characterized the Erv1p-Mia40p disulfide relay system of mitochondria using an *in vitro* system reconstituted from its isolated components. In the oxidized
small IMS proteins are transported across the translocase of the outer membrane in an unfolded reduced state into the IMS, where they interact with the oxidized form of Mia40p. The first disulfide bond in Mia40p mediates the formation of a mixed disulfide intermediate in which one of its cysteine residues is linked to a cysteine residue of the substrates with twin CXC motif. In an isomerization reaction the disulfide bond is transferred to the substrate, and Mia40p is released in its partially reduced form containing the residues Cys-1 and Cys-2 in their thiol state. In the course of the reaction a second disulfide bond appears to be generated in the substrates. This process might also involve Mia40p and Erv1p. Subsequently, the partially reduced Mia40p interacts with Erv1p, forming a mixed disulfide. Mia40p transfers electrons to Erv1p, resulting in the formation of a disulfide bond between Cys-1 and Cys-2 of Mia40p and, thus, of fully oxidized Mia40p. The disulfide bond in Erv1p is recovered by electron transfer to the FAD bound to Erv1p. Oxidized FAD appears to be recovered by the transfer of electrons to cytochrome c (17, 38). In the future the detailed analysis of reaction intermediates will be important to refine the model.

A disulfide transfer from the catalytic disulfide bond of Mia40p to substrate proteins is consistent with previously reported observations. In mitochondria, thiol modification of the cysteine residues of the reduced form of Mia40p was shown to block the import of Mia40p substrates into the IMS of mitochondria (15). As described in this study, the cysteine residues Cys-1 and Cys-2 are the ones that are in their thiol state in the reduced form of Mia40p in mitochondria. Moreover, Mia40p forms specific disulfide intermediates with substrates in the import reaction (8, 15, 16, 39). It has been reported for the small Tim proteins, Tim9p and Tim10p, that the first cysteine residues, but not the others of the twin CXC motif, are crucial for the formation of the mixed disulfide intermediate with Mia40p (40, 41). This specificity suggests a disulfide bond between this first cysteine residue and Mia40p in the disulfide intermediate consistent with the transfer of one disulfide bond. The CPC segment in Mia40p might be favorable for the efficient transfer of disulfide bonds to substrate proteins, since the close arrangement of cysteine residues is probably the reason for the generation of a relatively unstable disulfide bond. Studies with pep...
tides have shown that a CXC arrangement creates relatively unstable disulfide bonds (42). A similar CGC arrangement is present in the Env2 protein, which transfers disulfide bonds to substrates in the endoplasmic reticulum (43).

The cysteine residues of the twin C\textsubscript{X}C motif of Mia40p are present in a coiled-coil-helix-coiled-helix domain which is also found in a variety of other mitochondrial proteins, such as Cox23p, Cox19p, Cox17p, Mic14p, Mic17p, and Mdm35p (6–9, 44, 45). Consistent with our data, the NMR structure of Cox17p showed two interhelical disulfide bonds between the helices of its twin C\textsubscript{X}C motif (25, 46). These disulfide bonds stabilize the two helices in an antiparallel fold. Similar to the bonds in Mia40p, they are stable under mild reducing conditions. The stabilization by disulfide bonds might be a general feature of the class of proteins with coiled-coil-helix-coiled-helix domain containing a twin C\textsubscript{X}C motif. We suggest a structural function of the disulfide bonds of the twin C\textsubscript{X}C motif for Mia40p, but other functions cannot be completely excluded.

In the reconstituted system the thiol oxidase Erv1p is sufficient to generate the disulfide bonds in Mia40p. This is consistent with observations made in vivo. Depletion of Erv1p affects the redox state of Mia40p, and Erv1p interacts covalently with Mia40p (15, 16). The results presented here demonstrate that Mia40p is a substrate of Erv1p but that Erv1p was only able to oxidize Mia40C when the first two cysteine residues of Mia40C were present. Erv1p appears to form the redox-sensitive disulfide bond between the residues Cys-1 and Cys-2, which is then used to generate further disulfide bonds in substrates with twin C\textsubscript{X}C motif. This hypothesis is supported by several observations. Erv1p did not directly oxidize the Mia40p variant containing only the twin C\textsubscript{X}C motif. In addition, depletion of Erv1p from cells led to accumulation of Mia40p, which had the residues Cys-1 and Cys-2 present in the reduced state (15). Moreover, Tokatlidis and co-workers (17) have reported that Erv1p did not directly oxidize small Tim proteins, substrates of the Mia40p translocation pathway containing twin C\textsubscript{X}C motifs. Mia40p might not be the only substrate of Erv1p in the IMS of mitochondria, since there are, in addition to proteins with twin C\textsubscript{X}C motifs, other proteins containing disulfide bonds. Examples are the Rieske Fe/S protein of the respiratory chain, the assembly factor Cox11p of the cytochrome oxidase, and the superoxide dismutase Sod1p and its copper chaperone Ccs1p (47–50).

The copper chaperone Cox17p does not only have the two disulfide bonds in the twin C\textsubscript{X}C motif in common with Mia40p. It also has two additional conserved cysteine residues and can form a labile third disulfide bond under oxidizing conditions (25, 46). However, in contrast to the redox state of Mia40p, there is no evidence for the presence of a fully oxidized form of Cox17p in vivo, and the spacing of the additional cysteine residues is different. The partially reduced form of Cox17p containing two disulfide bonds could bind copper upon an intramolecular isomerization reaction of one disulfide bond (25). When Mia40p was purified in the presence of copper and zinc ions under reducing conditions, it had the ability to bind copper and zinc (14). Future studies will have to elucidate whether metal ions play a role for the function of Mia40p. Metal ions might stabilize the partially reduced form of Mia40p as observed e.g. for the reduced form of small Tim proteins (51).

The results of this study propose that the biogenesis of Mia40p itself is dependent on the disulfide transfer system in the IMS. In yeast, the import of Mia40p into mitochondria does not require this system since Mia40p is synthesized with an N-terminal presequence and anchored into the inner membrane (12–14). However, folding and oxidation of the conserved domain of Mia40p occurs in the IMS. In higher eukaryotes, Mia40 does not contain a presequence and most likely uses the disulfide relay system for import into the IMS of mitochondria (12–14, 21). The results presented suggest that Mia40p introduces the disulfide bonds in the twin C\textsubscript{X}C motif of newly imported Mia40p. This triggers folding of imported Mia40p and, thereby, trapping and stabilization of the protein. The redox-sensitive disulfide bond between Cys-1 and Cys-2 of Mia40p is generated by the disulfide transfer from Erv1p to obtain oxidized Mia40p. In agreement with such a mechanism, expression of human Mia40 in yeast mitochondria is strongly impaired in the case of variants with mutations of the cysteine residues in the twin C\textsubscript{X}C motif (21).

In conclusion, the reconstitution of the disulfide transfer from Erv1p via Mia40p to a protein with twin C\textsubscript{X}C motif demonstrates the presence of a disulfide relay system in the IMS of mitochondria. Future studies will have to analyze the molecular mechanisms of the various steps involved, such as trapping of substrates, formation of the disulfide bonds in the substrates, and reoxidation of Mia40p by Erv1p.

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Functional Characterization of Mia40p

37470 JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 282 • NUMBER 52 • DECEMBER 28, 2007

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