Biogenesis of the Essential Tim9–Tim10 Chaperone Complex of Mitochondria

SITE-SPECIFIC RECOGNITION OF CYSTEINE RESIDUES BY THE INTERMEMBRANE SPACE RECEPTOR Mia40

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Received for publication, April 19, 2007 Published, JBC Papers in Press, June 6, 2007, DOI 10.1074/jbc.M703294200

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The mitochondrial intermembrane space (IMS) contains an essential machinery for protein import and assembly (MIA). Biogenesis of IMS proteins involves a disulfide relay between precursor proteins, the cysteine-rich IMS protein Mia40 and the sulfhydryl oxidase Erv1. How precursor proteins are specifically directed to the IMS has remained unknown. Here we systematically analyzed the role of cysteine residues in the biogenesis of the essential IMS chaperone complex Tim9–Tim10. Although each of the four cysteines of Tim9, as well as of Tim10, is required for assembly of the chaperone complex, only the most amino-terminal cysteine residue of each precursor is critical for translocation across the outer membrane and interaction with Mia40. Mia40 selectively recognizes cysteine-containing IMS proteins in a site-specific manner in organello and in vitro. Our results indicate that Mia40 acts as a trans receptor in the biogenesis of mitochondrial IMS proteins.

Mitochondria must import most of their ~1,000 different proteins from the cytosol. The classical pathway of mitochondrial protein import involves cleavable amino-terminal presequences that direct precursor proteins to the translocase of the outer membrane (TOM complex) and the presequence translocase of the inner membrane (TIM23 complex) (1–7). However, a comprehensive proteomic analysis revealed that more than half of the mitochondrial proteins are synthesized without cleavable presequences (8, 9). These proteins contain internal targeting signals and use sorting mechanisms that are different from that of precursor-carrying proteins. The only point where the import pathways of cleavable and noncleavable proteins come together is the TOM complex. Immediately after passing through the TOM channel, the pathways diverge to different machineries (1, 5, 7, 10–12).

A chaperone complex of the mitochondrial intermembrane space (IMS) plays a central role in the sorting pathways of noncleavable precursor proteins (1, 5, 11–14). This hexameric Tim9–Tim10 complex is essential for cell viability (15–19). The complex guides precursors of β-barrel proteins to the sorting and assembly machinery of the outer membrane (SAM complex) (20–22), as well as the precursors of hydrophobic metabolite carriers to the carrier translocase of the inner membrane (TIM22 complex) (5, 7, 11, 23). Tim9 and Tim10 are small cysteine-rich proteins that are synthesized as noncleavable precursors on cytosolic ribosomes and imported into mitochondria.

Analysis of the biogenesis of the Tim9 and Tim10 precursors led to the identification of a novel machinery, termed the mitochondrial IMS import and assembly machinery (MIA) (24). MIA includes two essential cysteine-rich proteins that are located in the IMS, Mia40 and Erv1. Mia40 binds to IMS precursor proteins upon their translocation through the TOM complex (24–28). The interaction of Mia40 with precursor proteins, as well as with the sulfhydryl oxidase Erv1 (29), is sensitive to reductants, indicating that disulfide bonds are important for the interactions (24, 30, 31). It has been debated whether the cysteine motifs in IMS proteins are needed to coordinate metal ions or to form disulfide bonds (13, 17, 32–36). For Tim9 and Tim10, which contain four cysteine residues each, the high resolution structure of the hexameric complex demonstrated the formation of two intramolecular disulfide bonds in each subunit (19). Mia40 and Erv1 form a protein relay for disulfide formation in IMS precursor proteins by transferring disulfides from Erv1 via Mia40 to the substrate proteins (30, 31, 37). Thus, the IMS system is thought to resemble disulfide relays in the bacterial periplasm (DsbB and DsbA) (38, 39) and the endoplasmic reticulum (Ero1/Erv2 and protein-disulfide isomerase) (40–42). However, Mia40 is not homologous to bacterial or endoplasmic reticulum proteins and performs a novel function by driving protein translocation across the outer mitochondrial membrane (24–26). The molecular mechanism...
of protein targeting to Mia40 has not been elucidated. It is unknown whether Mia40 generally interacts with cysteine-rich proteins or specifically recognizes IMS-destined proteins.

For this study, we dissected the mitochondrial import steps of the precursors of Tim9 and Tim10 and analyzed the role of individual cysteine residues of the precursors in translocation across the outer membrane, binding to Mia40 and assembly into the Tim9–Tim10 chaperone complex. We report that the cysteine residues play different roles in membrane translocation and oligomeric assembly. Translocation across the outer membrane is coupled to binding to Mia40, a process that depends on one defined cysteine residue of each precursor. Mia40 distinguishes between different cysteine residues of a precursor protein in a site-specific manner in vitro and in organello. Our findings suggest that Mia40 functions as a receptor that binds precursors via transient disulfide bonds, thereby promoting substrate entry into the IMS-specific pathway and initiating the subsequent assembly process.

EXPERIMENTAL PROCEDURES

Yeast Strains and Media—The Saccharomyces cerevisiae strain YPH499 (MATa, ade2-101, his3-D200, leu2-D1, ura3-52, trp1-D63, lys2-801) was used as wild type (43). The mutant strains mia40-4 (MATa, ade2-101, his3-D200, leu2-D1, ura3-52, trp1-D63, lys2-801, mia40::ADE2 (pFL39-FOMP2–7ts/mia40-4)) and erv1-2 (YPG-0702b; MATa, ade2-101, his3-D200, leu2-D1, ura3-52, trp1-D63, lys2-801, erv1::ADE2 (pFL39-ERV1–2ts)) were reported previously (24, 31). The wild-type strain YPH499 (MATa, ade2-101, his3-D200, leu2-D1, ura3-52, trp1-D63, lys2-801, erv1::ADE2 (pFL39-ERV1–2ts)) was generated by error-prone PCR and Mia40 beginning at residue 95 was cloned into pET10N (47) for in vitro and in vivo expression. After growth for a further 3 h, cells were harvested by centrifugation, resuspended in lysis buffer (300 mM NaCl, 50 mM NaH2PO4, 10 mg/ml RNase A, 5 mg/ml DNase I, pH 8.0), and exposed to sonication on ice. Cell lysates were spun at 12,000 × g at 4 °C for 30 min, and the supernatant fraction was used for Ni-NTA affinity chromatography. After washing with 10 column volumes of wash buffer (300 mM NaCl, 50 mM NaH2PO4, adjusted to pH 8.0), Mia40 was either eluted with elution buffer (20 mM Tris/HCl, 100 mM NaCl, 20% (v/v) glycerol, 250 mM imidazole, 50 mM iodoacetamide, pH 8.0) or factor Xa protease as per the manufacturer’s instructions (New England Biolabs).

In Vitro Binding of Proteins to Mia40—Purified Mia40 (column-immobilized or factor Xa-released) was incubated with 35S-labeled proteins in binding buffer (250 mM sucrose, 80 mM KCl, 5 mM MgCl2, 2 mM KH2PO4, 5 mM methionine, 10 mM MOPS/KOH, pH 7.2) at 4–30 °C for 4–10 min. Reactions were stopped by addition of iodoacetamide to a final concentration of 50 mM. Fractions bound to Ni-NTA columns were washed with washing buffer (30 mM imidazole, 250 mM sucrose, 80 mM KCl, 5 mM MgCl2, 2 mM KH2PO4, 5 mM methionine, 10 mM MOPS/KOH, pH 7.2), eluted with elution buffer (20 mM Tris-
HCl, 100 mM NaCl, 250 mM imidazole, pH 8.0), and analyzed by SDS-PAGE. Binding of precursors to the factor Xa-cleaved Mia40 was analyzed directly by nonreducing SDS-PAGE, followed by digital phosphorimager autoradiography (GE Healthcare).

RESULTS

Cysteine Residues Are Critical for the Biogenesis Pathway of Tim9—We used blue native electrophoresis to study the import and assembly of radiolabeled Tim9 into isolated yeast mitochondria. The native gel assay of digitonin-lysed mitochondria not only separates the two mature Tim9-containing complexes Tim9–Tim10 and TIM22 but also an import intermediate of Tim9 bound to Mia40 (Fig. 1A, lane 1) (24, 31). The addition of the SH-modifying reagents N-ethylmaleimide or iodoacetamide blocked the import of Tim9 and its association with Mia40 (Fig. 1A, lanes 2 and 3), indicating that cysteine residues are crucial for formation of the Mia40 intermediate.

Mature Tim9 possesses two intramolecular disulfide bonds, one formed by the two outer cysteines (Cys-35 and Cys-59) and the other formed by the two inner cysteines (Cys-39 and Cys-55) (19, 32, 34). We asked if and which of the cysteine residues are required for the import pathway of Tim9. First, we replaced all four cysteine residues with serine residues (Fig. 1B). The resulting modified Tim9 precursor was not imported into mitochondria (Fig. 1C, lanes 1–3) (the amount of radiolabeled precursor proteins added to the import reaction was similar for each construct). Second, we generated constructs that retained two cysteines, respectively, corresponding to the disulfides...
formed in mature assembled Tim9. Upon replacement of the two outer cysteine residues with serine residues, the import and assembly of Tim9 was blocked (Fig. 1D, lanes 4–6). When the two inner cysteine residues were replaced, however, a partial import reaction was observed. Assembly to the mature Tim9–Tim10 and TIM22 complexes did not occur, but the precursor was able to form the Mia40 intermediate (Fig. 1D, lanes 7–9).

To obtain independent evidence for the interaction of Tim9 precursors with Mia40, we used denaturing electrophoresis (SDS-PAGE) under nonreducing conditions to preserve disulfide bonds. Radiolabeled Tim9 formed a high molecular mass product in wild-type mitochondria that showed a slower gel mobility in mitochondria containing Mia40 with a deca-histidine tag (Fig. 1E, lanes 1 and 2). Upon purification of Mia40_{his10} by affinity chromatography, the high molecular mass product was copurified (Fig. 1E, lane 6), demonstrating that the product contained Tim9 and Mia40. In agreement with the native gel assay, formation of the Tim9-Mia40 product was inhibited when the Tim9 precursor with replaced outer cysteine residues was used, whereas the Tim9 precursor with replacement of the two inner cysteine residues formed the Tim9-Mia40 intermediate (Fig. 1E).

To study the translocation of Tim9 across the outer mitochondrial membrane, we used a protease protection assay (24). After the import reaction, the mitochondria were treated with proteinase K to remove nonimported precursor. The mitochondria were separated by SDS-PAGE under reducing conditions, and thus all imported Tim9 molecules migrated in monomeric form (Fig. 1G). Tim9 with replaced outer cysteines was strongly inhibited in translocation to a protease-protected location, whereas Tim9 with replaced inner cysteines was imported. Thus, the result of the protease protection assay (Fig. 1G) correlated well with formation of the Mia40 intermediate (Fig. 1D and F), whereas generation of the mature Tim9–Tim10 and TIM22 complexes was blocked with each mutant Tim9 precursor (Fig. 1D). These findings suggest that the cysteine residues of Tim9 are required at various stages of the import and assembly pathway.

**Differential Requirement of Cysteine Residues in Targeting and Assembly of Tim9**—We asked whether both outer cysteine residues of Tim9 (Cys-35 and Cys-59) were required for translocation across the outer membrane and binding to Mia40. We thus replaced each individual cysteine residue of Tim9 with a serine residue. Each mutant precursor could not form the mature Tim9–Tim10 and TIM22 complexes (Fig. 2A). However, only the first cysteine residue Cys-35 was critical for efficient binding of the precursor to Mia40 (Fig. 2A, lanes 4–6), whereas replacement of any of the other three cysteine residues did not impair formation of the Mia40 intermediate or even enhanced it (Fig. 2A, lanes 7–15). We treated mitochondria with proteinase K after the import reaction to determine whether the mutant precursors were translocated across the outer membrane. Indeed, only replacement of the first cysteine residue impaired translocation of Tim9 into mitochondria, whereas the three other mutant precursors were imported with wild-type efficiency (Fig. 2B). A point of concern was the possibility that the replacement of cysteine residues may have resulted in misfolding or aggregation of the Tim9 precursor. Such events could alter the import competency of precursor proteins. To exclude premature folding, the precursor proteins used in this study were unfolded in 8 M urea in the presence of the reductant dithiothreitol (DTT) before the import reaction. (Additionally we observed that precursor proteins that were directly imported from reticulocyte lysates not exposed to a denaturing treatment showed the same site-specific dependence on cysteine residues as the urea unfolded precursors, indicating that misfolding of the precursors did not occur under our import conditions (data not shown).) We conclude that the first cysteine residue of Tim9 is critical for efficient binding of the precursor to Mia40, as well as translocation across the outer mitochondrial membrane.

In contrast to the first cysteine residue, replacement of the third cysteine residue (C55S) led to an increased yield of the Tim9-Mia40 intermediate (Fig. 2A, lanes 4–6 versus 10–12). These two precursors were selected for a detailed analysis. The interaction of both mutant precursors with Mia40 was largely dissociated by addition of DTT like the wild-type Tim9 precursor (Fig. 2C), indicating that formation of the Tim9-Mia40 complex required a covalent linkage via disulfide bridges with each precursor. We asked if the sulfhydryl oxidase Erv1 was required for the increased yield of Mia40-bound Tim9C55S. We used yeast mutants of ERV1 that block the formation of mature Tim9–Tim10 and TIM22 complexes, whereas the initial binding to Mia40 is still possible in case of the wild-type Tim9 precursor (Fig. 2D, lanes 13–18 compared with lanes 10–12). We conclude that the mutant precursor can still bind to Mia40 in erv1 mutant mitochondria (Fig. 2D, lanes 13–18) (31). The enhanced accumulation of Tim9C55S at Mia40 was largely prevented in both erv1 mutant mitochondria (Fig. 2D, lanes 4–9) (31). The enhanced accumulation of Tim9C55S to Mia40 is still possible in both erv1 mutant mitochondria (Fig. 2D, lanes 13–18 compared with lanes 10–12). We conclude that the mutant precursor can still bind to Mia40 in erv1 mutant mitochondria (albeit with reduced efficiency in erv1-2 mitochondria), yet the strong increase in yield of the Mia40-bound stage requires a fully functional sulfhydryl oxidase Erv1, suggesting that Erv1-promoted disulfide formation is critical for the enhanced accumulation at Mia40.

We envisaged two possibilities to explain the critical role of the first cysteine residue of Tim9. Tim9C55S was either impaired in binding to Mia40 or more rapidly released from Mia40 compared with wild-type Tim9. To differentiate between these possibilities, we used a yeast mutant of MIA40, termed mia40-4, that strongly delays the release of precursors from Mia40 (24). mia40-4 mitochondria accumulated increased amounts of wild-type Tim9, as well as Tim9C55S at Mia40 (Fig. 2E, lanes 5 and 6). However, the interaction of Tim9C55S with Mia40 was not increased in the mutant mitochondria but remained at the same low level as in wild-type mitochondria (Fig. 2E, lane 4 versus lane 1). We conclude that the first cysteine residue of Tim9 is important for efficient binding of the precursor to Mia40.

**Role of Cysteine Residues of Tim10 in Binding to Mia40 and Assembly**—We constructed four mutant forms of the precursor of Tim10 by individual replacement of each of the four cysteine residues (Fig. 1B). Upon import of the radiolabeled precursors into isolated mitochondria, blue native electrophoresis revealed that, like Tim9, each mutant precursor was blocked in assembly to the mature complexes (Fig. 3A). Replacement of the first cysteine residue strongly inhibited binding of Tim10 to...
Mia40 (Fig. 3A, lanes 4–6), whereas the other three mutant precursors bound to Mia40 (Fig. 3A, lanes 7–15). The precursors with replacement of the third or fourth cysteine residue accumulated at Mia40 in increased amounts, similar to the Tim9 precursor with replaced third cysteine residue.

Two Tim10 precursors were further analyzed by nonreducing SDS-PAGE. Replacement of the first cysteine residue strongly inhibited formation of the high molecular mass Tim10-Mia40 product, whereas replacement of the third cysteine residue increased formation of the product (Fig. 3B, lanes 4–9), thus confirming the findings of the blue native gel assay. Moreover, mitochondria were treated with proteinase K after the import reaction and separated by reducing SDS-PAGE. Replacement of the first cysteine residue strongly inhibited translocation of Tim10 to a protease-protected location within mitochondria (Fig. 3B, lanes 13–15). We conclude that the first cysteine residue of Tim10, as well as of Tim9, is crucial for efficient binding to Mia40 and translocation across the outer mitochondrial membrane.

The Role of Cysteines in Precursor Binding to Mia40 Does Not Depend on Outer Membrane Translocation—Different explanations were conceivable for the critical role of the first cysteine residue in binding to Mia40. (i) Tim9 and Tim10 may be translocated across the outer membrane with their amino termini first and thus Mia40 has to interact with the most amino-terminal cysteine residue. (ii) Mia40 functions as a specific receptor for IMS precursor proteins and directly recognizes the first cysteine in a site-specific manner. (iii) Factors cooperating with Mia40 are critical in recognition of the IMS precursor proteins and determine the specificity of the MIA machinery.

To address the first possibility, we established an in organello system where the IMS precursors could directly access Mia40 without translocation across the outer membrane. We generated mitoplasts by hypotonic swelling of isolated mitochondria. Thus the outer membrane is ruptured, resulting in a release of soluble IMS components and exposure of Mia40 to the external solution (21, 24). When radiolabeled Tim9 was added to the mitoplasts, the formation of mature Tim9–Tim10 and TIM22...
complexes was blocked, but the Tim9 precursor accumulated at Mia40 (Fig. 4A, lanes 4–6). Thus, mitoplasts represent a system to analyze precursor binding to Mia40 without prior translocation through the TOM complex.

We used a complete set of radiolabeled Tim9 mutant precursors with replacement of individual cysteine residues. Only the Tim9 precursor with replacement of the first cysteine residue was strongly impaired in binding to Mia40 in the mitoplast system (Fig. 4B, lanes 4–6). The other three mutant forms of Tim9 interacted with Mia40 (Fig. 4B, lanes 7–15). Similarly, the wild-type precursor of Tim10 efficiently accumulated at Mia40 in mitoplasts, whereas Tim10 with replaced first cysteine was blocked in binding to Mia40 (Fig. 4C). Thus, the critical role of the first cysteine residue of Tim9, as well as Tim10, in binding to Mia40 is observed in mitoplasts similarly to intact mitochondria. We conclude that precursor translocation across the outer membrane is not required for the site-specific interaction with Mia40, suggesting that Mia40 or associated factors specifically recognize precursor proteins.

Specific Recognition of Precursor Proteins by Purified Mia40—To address if Mia40 itself functioned as receptor for IMS precursors, we expressed a recombinant form in E. coli cells (Fig. 5A, lane 1). The cleavable amino-terminal presequence and the adjacent hydrophobic segment of Mia40 were replaced by a deca-histidine tag (the hydrophobic segment is not required for the function of Mia40 in vivo (25)). Mia40_His10 was purified from E. coli as a soluble protein by affinity chromatography (Fig. 5A, lanes 3 and 4).

To analyze precursor binding, purified Mia40 was bound to Ni-NTA-agarose and incubated with radiolabeled precursor of Tim9. After washing the column, the eluate was analyzed by reducing SDS-PAGE. Tim9 was found in the eluate (Fig. 5B, lane 3), whereas in the control reaction without Mia40, Tim9 did not interact with the Ni-NTA-agarose (Fig. 5B, lane 2). To determine the specificity of interaction with purified Mia40, we tested four radiolabeled proteins that contain multiple cysteine residues but are not physiological substrates of Mia40 as follows: cytochrome c heme lyase that contains four cysteine residues and has been shown to be imported into mitochondria independently of Mia40 (27); Cu,Zn superoxide dismutase that possesses two cysteine residues and depends on the specific partner protein Ccs1 for import into mitochondria (48); carboxypeptidase Y with 12 cysteine residues and protein-disulfide isomerase with 6 cysteine residues, 2 proteins located in the vacuole, and the endoplasmic reticulum, respectively (49, 50). None of these proteins specifically associated with Mia40 (Fig. 5B, lane 3), and only very weak background binding to the column material was observed (Fig. 5B, lane 2). As shown below, the precursor of Tim10 also specifically interacts with purified Mia40. Thus, the substrate specificity of recombinant Mia40 correlates well with the requirement for Mia40 in organello.

FIGURE 3. Role of cysteine residues of Tim10 in binding to Mia40 and oligomeric assembly. A, import of Tim10 (wild type) and Tim10 cysteine mutants into wild-type mitochondria. After solubilization in the presence of 50 mM iodoacetamide, the samples were analyzed by blue native electrophoresis. B, Tim10 (wild type) and Tim10 cysteine mutants were imported into mitochondria, followed by either nonreducing SDS-PAGE (lanes 1–9) or proteinase K (Prot.K) treatment and reducing SDS-PAGE (lanes 10–18). Protein bands were visualized by digital autoradiography. β-ME, β-mercaptoethanol.

FIGURE 4. Role of cysteine residues in precursor binding to Mia40 in mitoplasts. A, mitochondria and mitoplasts were incubated with 35S-labeled Tim9 (wild type) for the indicated time periods and subjected to blue native analysis. B, import of 35S-labeled Tim9 and single cysteine mutants of Tim9 into mitoplasts, followed by blue native electrophoresis. C, Tim10 was imported into mitoplasts and analyzed by blue native electrophoresis.
We then asked how efficiently purified Mia40 could bind precursor proteins and compared it directly to endogenous mitochondria-located Mia40. Radiolabeled Tim9 was either imported into mitochondria or incubated with purified Mia40. Association of Tim9 with Mia40 was determined by nonreducing SDS-PAGE. In both cases, a high molecular mass Tim9-Mia40 product was formed (Fig. 5C, lanes 1–6). The amounts of purified Mia40 and mitochondrial Mia40 used in the assay were compared by immunodecoration with Mia40-specific antibodies (Fig. 5C, lanes 7–12). Remarkably, the efficiency of Tim9 binding to recombinant Mia40 was close to that observed with Mia40 in the mitochondrial context (Fig. 5C, lanes 4–6 versus lanes 1–3). We thus have established an efficient system to study the interaction of precursor proteins with Mia40 in vitro.

We employed nonreducing SDS-PAGE to analyze the characteristics of precursor binding to purified Mia40. The high molecular mass Tim9-Mia40 product was dissociated by addition of reducing agents (Fig. 6A, lane 1), indicating that disulfide bonds are critical for the interaction like in the in organello situation. As a control we show that the high molecular mass product was neither formed in the absence of Tim9 nor in the absence of Mia40 (Fig. 6A, lanes 3 and 4). We then used the set of mutant Tim9 precursors with replacement of one or more cysteine residues. Tim9 lacking all four cysteines, as well as Tim9 lacking the two outer cysteines, did not form the Tim9-Mia40 product (Fig. 6B, lanes 3–6), whereas Tim9 lacking the two inner cysteines efficiently interacted with Mia40 (Fig. 6B, lanes 7 and 8). The Tim9 precursor proteins with replacement of individual cysteine residues interacted with Mia40 like the wild-type precursor with the exception of the first cysteine residue (Fig. 6C, lane 7). Similar findings were made with the precursor of Tim10. Mutant Tim10 lacking the first cysteine residue was blocked in forming a mixed disulfide product with purified Mia40 (Fig. 6D). Taken together, the interaction of Tim9 and Tim10 with purified Mia40 shows the identical dependence on the first cysteine residue as observed in both intact mitochondria and mitoplasts. We conclude that Mia40 recognizes precursor proteins
in a site-specific manner without a requirement for further mitochondrial factors.

DISCUSSION

MIA represents the most recently identified mitochondrial pathway for protein import (24–26). We report that protein targeting to the mitochondrial intermembrane space via MIA involves mechanisms that differ strongly from all previously known mitochondrial import pathways. Formation of mixed disulfides between precursor proteins and the IMS protein Mia40 represents a crucial step in translocation of precursor proteins (24, 30). We dissected the import of IMS precursor proteins into distinct steps. By systematically analyzing the role of each cysteine residue of the essential IMS proteins, Tim9 and Tim10, we found that only the most amino-terminal cysteine residue is critical for interaction with Mia40 and precursor translocation across the outer mitochondrial membrane. Each of the four cysteine residues of both precursor proteins, however, is required for the correct assembly of the mature Tim9–Tim10 complex and TIM22 complex. Thus, cysteine residues play different roles in protein targeting to Mia40 and the subsequent assembly reaction. This observation explains why previous studies, which replaced various cysteine residues in IMS precursor proteins, did not assign individual functions of cysteine residues (33–35, 51). These studies analyzed the overall import reaction of IMS proteins without dissection of import stages, and thus a distinction between Mia40 binding and assembly of mature complexes was not possible.

The critical role of the first cysteine residue of Tim9, as well as Tim10, for efficient interaction with Mia40 may be explained by different scenarios. (i) The first cysteine residue may either be required for binding to Mia40 or lack of this cysteine may cause a rapid release from Mia40. Because a mutant Mia40, which is strongly delayed in release of precursor proteins (24), did not lead to an increased interaction of precursors lacking the first cysteine with Mia40, the second possibility can be excluded, indicating that the first cysteine residue is critical for efficient binding to Mia40. (ii) It was conceivable that Mia40 must interact with the most amino-terminal cysteine residue of a precursor protein during translocation across the outer membrane, thus explaining the critical role of the first cysteine residue by the spatial position during translocation. We excluded this possibility by a mitoplast assay. In mitochondria with ruptured outer membrane, precursor proteins have direct access to Mia40 without prior translocation across the outer membrane. Under these conditions, the first cysteine residue of Tim9 and Tim10 was still required for efficient binding to Mia40, as observed in intact mitochondria, indicating a specific recognition process. (iii) To distinguish if Mia40 itself or factors cooperating with Mia40 are the determinants for the site-specific recognition of precursor proteins, we established an in vitro binding assay with purified Mia40. We observed an efficiency of precursor binding to Mia40 that was close to that of the in organello situation. The characteristics of precursor recognition observed in mitochondria were faithfully reproduced with recombinant Mia40. The interaction of precursor with Mia40 was sensitive to reductant and the first cysteine residue of Tim9, as well as of Tim10, was critical for in vitro binding. We tested several cysteine-containing precursor proteins, which are known to be independent of Mia40 in vivo (27, 48–50), and indeed none of these precursors bound to purified Mia40. Thus Mia40 alone without further mitochondrial components possesses the full specificity and ability to recognize and bind precursor proteins. We conclude that Mia40 functions as a specific receptor for IMS precursor proteins.

In summary, we have identified a novel mechanism of targeting of mitochondrial precursor proteins. Mia40 functions as a receptor on the trans side of the outer membrane. It recognizes the precursors of IMS proteins in a site-specific manner and forms transient disulfide bridges with them.

Acknowledgments—We thank Drs. Peter Rehling, Nils Wiedemann, and Chris Meisinger for discussion.

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