Atp23 biogenesis reveals a chaperone-like folding activity of Mia40 in the IMS of mitochondria

Daniel Weckbecker¹, Sebastian Longen¹,
Jan Riemer² and Johannes M Herrmann^{1,*}

¹Division of Cell Biology, University of Kaiserslautern, Erwin-Schrödinger-Strasse 13, Kaiserslautern, Germany and ²Division of Cellular Biochemistry, University of Kaiserslautern, Erwin-Schrödinger-Strasse 13, Kaiserslautern, Germany

Mia40 is a recently identified oxidoreductase in the inter-membrane space (IMS) of mitochondria that mediates protein import in an oxidation-dependent reaction. Substrates of Mia40 that were identified so far are of simple structure and receive one or two disulphide bonds. Here we identified the protease Atp23 as a novel substrate of Mia40. Atp23 contains ten cysteine residues which are oxidized during several rounds of interaction with Mia40. In contrast to other Mia40 substrates, oxidation of Atp23 is not essential for its import; an Atp23 variant in which all ten cysteine residues were replaced by serine residues still accumulates in mitochondria in a Mia40-dependent manner. *In vitro* Mia40 can mediate the folding of wild-type Atp23 and prevents its aggregation. In these reactions, the hydrophobic substrate-binding pocket of Mia40 was found to be essential for its chaperone-like activity. Thus, Mia40 plays a much broader role in import and folding of polypeptides than previously expected and can serve as folding factor for proteins with complex disulphide patterns as well as for cysteine-free polypeptides.

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Introduction

The IMS is a cellular compartment that has received little attention so far despite its vital role in many processes, e.g. metabolism, lipid homeostasis, biogenesis of iron-sulphur clusters, aging and apoptosis. Classical ATP-dependent chaperone proteins like Hsp70 or Hsp90 were not identified in the IMS and it is unclear how IMS proteins are folded. All IMS proteins are nuclear encoded and synthesized in the cytosol. Several of these comprise internal cysteine-containing targeting sequences (so-called MISS signals) which were shown to be critical for their import into mitochondria (Milenkovic *et al*, 2009; Sideris *et al*, 2009). During or directly following translocation into the IMS, these sequences are recognized

by a hydrophobic binding groove on the oxidoreductase Mia40 and oxidized to form stable disulphide bonds (Curran *et al*, 2002; Chacinska *et al*, 2004; Naoe *et al*, 2004; Mesecke *et al*, 2005; Banci *et al*, 2009; Kawano *et al*, 2009; von der Malsburg *et al*, 2011). So far identified substrates of Mia40 are small proteins of simple helix-loop-helix structure containing one or two disulphide bonds. It was suggested that the mitochondrial disulphide relay is unable to fold and isomerize proteins of complex cysteine patterns based on the assumption that the IMS lacks proteins of complex disulphide arrangements as they are commonly found in the bacterial periplasm or the endoplasmic reticulum (Sevier and Kaiser, 2002; Tu and Weissman, 2004; Riemer *et al*, 2009; Sideris and Tokatlidis, 2010). In this study, we identified the mitochondrial protease Atp23 as a novel substrate of Mia40. Mia40 directly interacts with Atp23 and promotes its import into the IMS and its subsequent folding. During this folding reaction, Atp23 acquires five disulphide bonds. A cysteine-free variant of Atp23 is still imported into mitochondria in a Mia40-dependent reaction demonstrating that Mia40 can promote protein import in a cysteine-independent reaction. *In vivo* and *in vitro* data suggest that Mia40 stabilizes non-native Atp23 and promotes its folding in a chaperone-like manner. Whereas the hydrophobic substrate-binding pocket of Mia40 is essential for this chaperone-like function, the redox-active CPC motif of Mia40 appears to be dispensable, at least for certain substrates.

Results

Atp23 is a protease in the IMS of mitochondria

The protease Atp23 was recently identified in *Saccharomyces cerevisiae* as a factor required for processing of subunit 6 of the mitochondrial ATPase (Atp6) (Osman *et al*, 2007; Zeng *et al*, 2007). Atp6 is a mitochondria-encoded protein that is synthesized on mitochondrial ribosomes as a precursor protein; mutants lacking Atp23 accumulate the precursor of Atp6 (Figure 1A) and fail to assemble mitochondrial ATPase complexes (Figure 1B).

Atp23 is located in the IMS and associated with the inner membrane. Upon opening of the outer membrane by hypotonic swelling, Atp23 is partially inaccessible to proteinase K due to its tight association with the inner membrane (Figure 1C). Nevertheless, Atp23 is not an integral membrane protein since it lacks a hydrophobic transmembrane domain and is soluble upon carbonate extraction (Figure 1D). Atp23 was annotated as a gene encoding for a protein of 270 residues. However, we identified the fourth ATG (codon 44) in the initially annotated Atp23 sequence as the correct start codon (Supplementary Figure S1); hence, the endogenous Atp23 protein consists of 227 residues.

Atp23 is a substrate of the mitochondrial disulphide relay

Atp23 is well conserved and homologues are found in fungi, plants and animals including human. Atp23 and its

*Corresponding author. Division of Cell Biology, University of Kaiserslautern, Erwin-Schrödinger-Strasse 13, Kaiserslautern, 67663, Germany. Tel.: +49 631 205 2406; Fax: +49 631 205 2492; E-mail: hannes.herrmann@biologie.uni-kl.de

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Figure 1 Atp23 is a protease in the IMS of mitochondria. (A) Mitochondrial translation products were radiolabelled in cycloheximide-treated wild type (WT) and $\Delta atp23$ cells and visualized by SDS-PAGE and autoradiography. In the $\Delta atp23$ cells the precursor form of Atp6 (pAtp6) accumulated and no mature Atp6 (mAtp6) was found. (B) Translation products were radiolabelled like in panel A. Then protein complexes were separated by blue native gel electrophoresis and visualized by Coomassie staining or by autoradiography. Positions of the ATPase complex (monomeric and dimeric form) are indicated. Note the absence of the ATPase complex from $\Delta atp23$ cells. (C) Wild type mitochondria (50 μ g) were incubated with isoosmotic (M, mitochondria), hypoosmotic (MP, mitoplasts) or 0.1% Triton X-100-containing buffers in the absence or presence of 100 μ g/ml proteinase K (PK) for 30 min on ice. Mitochondria and mitoplasts were pelleted by centrifugation. Proteins of Triton X-100 extracts were precipitated with trichloroacetic acid (TCA). Atp23 and proteins of the outer membrane (Tom70), the IMS (Erv1), the inner membrane (Cox2) and the matrix (Mrpl40) were detected by Western blotting. (D) Mitochondria were incubated with 0.1 M sodium carbonate and separated into a soluble fraction (S) and a membrane pellet (P). Figure source data can be found with the Supplementary data.

homologues contain ten or eleven cysteine residues mostly at conserved positions scattered across the entire sequence (Figure 2A). To assess the redox state of cysteine residues in Atp23 in whole cells, we employed an alkylation assay with the reagent methyl-(polyethylene glycol)₂₄ maleimide (mPEG). mPEG covalently binds to reduced thiols increasing the molecular mass of the protein by 1.2 kDa per moiety. When all thiols in SDS-denatured Atp23 are reduced with tris carboxyethyl phosphine (TCEP) the addition of mPEG shifts the apparent size of the protein from 28 to 40 kDa, consistent with the addition of 10 mPEG molecules (Figure 2B, lanes 1 and 2). In the absence of TCEP, however, endogenous Atp23 is not modifiable by mPEG even when denatured with SDS indicating that all cysteines are oxidized (Figure 2B, lane 3). When cells were pretreated with copper phenanthroline to induce the formation of disulphide bonds, the pattern of the mPEG-modified Atp23 protein remained unaltered (Figure 2C) whereas the pattern of Mdj1, a zinc finger domain-containing matrix protein with ten reduced cysteine residues, shifted due to the copper-induced cysteine oxidation (Figure 2D). Atp23 was also found to be oxidized when cells were grown anaerobically (Figure 2B, lanes 4 and 5) suggesting that Atp23 is not oxidized directly by molecular

oxygen but rather by the mitochondrial disulphide relay which can oxidize proteins anaerobically (Allen *et al*, 2005; Bihlmaier *et al*, 2007; Dabir *et al*, 2007). A direct function of the mitochondrial disulphide relay for Atp23 import was further supported by the observation that depletion of Mia40 or its sulphhydryl oxidase Erv1 (Fass, 2008) led to the loss of Atp23 (Figure 2E and F). Thus, Atp23 is a conserved IMS protein with five disulphide bonds that requires Mia40 and Erv1 for its biogenesis.

Atp23 contains several Mia40 binding sites

Direct interaction of Mia40 with newly imported radio-labelled Atp23 was confirmed by immunoprecipitation under non-reducing conditions or after crosslinking with difluoro dinitrobenzene (DFDNB). Both in the presence and absence of DFDNB, Mia40-specific antibodies precipitated complexes of Mia40 and Atp23 (Figure 3A). Interestingly, unlike in experiments with conventional substrates of Mia40 (Figure 3B; Naoe *et al*, 2004; Mesecke *et al*, 2005; Terziyska *et al*, 2005), multiple complexes of different sizes were recovered suggesting the binding of several Mia40 proteins per Atp23 molecule (Figure 3, arrows).

To identify the Mia40-interaction regions of Atp23, peptides of a length of 20 residues were synthesized and spotted onto three separate membranes. Two of these membranes were incubated with purified Mia40 (Figure 4A). For control, the third membrane was incubated with a purified mutant variant, Mia40^{F315,318E}, in which two phenylalanine residues in the substrate-binding region were replaced by negatively charged glutamate residues. Yeast mutants expressing this Mia40 variant instead of the endogenous Mia40 can only grow under fermentative conditions, indicating that Mia40^{F315,318E} exhibits some, although strongly reduced, activity (Kawano *et al*, 2009). Using such a peptide scan experiment, Mia40 binding sequences like the MISS sequence of Tim10 can be distinguished from other cysteine-containing sequences (Figure 4B). For Atp23, four Mia40-interaction regions were identified which represented sequences around residues 48–70, 95–110, 130–148 and 173–205, thus covering large parts of the Atp23 sequence (Figure 4A and C). Surprisingly, only some of the Mia40-interacting peptides contained cysteine residues (shown in red) or parts of predicted α -helices (shown as boxes). In the Mia40 substrates Tim9, Tim10, Tim13, and Cox17 individual cysteine residues were shown to be essential for import into the IMS (Heaton *et al*, 2000; Roesch *et al*, 2002; Lutz *et al*, 2003; Milenkovic *et al*, 2007; Sideris and Tokatlidis, 2007; Sideris *et al*, 2009) because import is mediated by one MISS signal in each of these proteins. The observation of several Mia40 interaction sequences in Atp23 inspired us to test the relevance of individual cysteine residues for Atp23 biogenesis. Surprisingly, single cysteine-to-serine mutants of Atp23 were still imported into mitochondria and were functional since respective strains processed Atp6 and grew on non-fermentative media (Figure 4D). Only combination mutants in which several cysteine residues were replaced at the same time impaired the function of Atp23 (Supplementary Figure S2A). From this we conclude that none of the ten cysteine residues by itself is essential for import and function of Atp23, consistent with the idea that several Mia40 binding sites cooperate in Atp23 import (Figure 4E).

Figure 2 Atp23 is a conserved IMS protein with several disulphide bonds. (A) Comparison of the Atp23 sequences of *S. cerevisiae* (S.c.), *Drosophila melanogaster* (D.m., NP_609845), *Mus musculus* (M.m., NP_001153031) and *Homo sapiens* (H.s., NP_150592). Numbers depict the lengths of the proteins. Cysteine residues are depicted by lollipop; numbers refer to positions of cysteine residues in yeast Atp23. HExxH refers to the conserved metalloprotease motif. (B) Yeast cells were grown under aerobic or anaerobic conditions, treated with TCA and opened by vortexing with glass beads. Proteins were denatured with SDS and treated with 15 mM mPEG. The sample in lane 2 was boiled with 5 mM TCEP to reduce oxidized thiols before mPEG was added. Atp23 was visualized by Western blotting. For the sample shown in lane 5, cells were incubated with 50 mM NEM to block free thiols before they were treated with TCA, TCEP and mPEG ('inverse shift'). (C, D) The redox state of Atp23 and of the matrix protein Mdj1 were analysed as in B. For samples shown in the rightmost lanes, cells were preincubated with 0.5 mM copper phenanthroline for 15 min at 25°C prior to cell lysis to induce protein oxidation. (E) Mitochondria were isolated from wild type (WT) or Gal-Mia40 cells (Terziyska *et al*, 2005) after growth on glucose-containing (Mia40-repressing) medium for 16 h. Levels of Atp23 as well as of Mia40-dependent (Erv1, Tim10) and Mia40-independent (Ccp1, Tom70, Fum1, Mrpl36, Mrpl40) mitochondrial proteins were analysed by Western blotting. (F) Mitochondria were isolated from Gal-Erv1 cells grown in medium containing galactose (Erv1[↑]) or glucose (Erv1[↓]) as carbon sources. Cmc1 and Cox17 are Mia40-dependent proteins. Figure source data can be found with the Supplementary data.

Atp23 is imported in a Mia40-dependent but cysteine-independent manner

To further assess the relevance of the cysteine residues in Atp23 we produced a cysteine-free variant in which all ten cysteine residues had been replaced by serine residues (10CS). Surprisingly, like the wild type version of Atp23, efficient import of this protein into isolated mitochondria required Mia40 (Figure 5A) but no membrane potential across the inner membrane (Figure 5B). In contrast to Atp23, 10CS did not form covalent interactions with Mia40 during the import reaction (compare Figures 5C and 3A). However, in the presence of the crosslinker DFDNB, several complexes of Mia40 and 10CS appeared (Figure 5C, arrows) suggesting that, like with Atp23, several Mia40 proteins bind to 10CS, however, not via mixed disulphide-linked interactions. Thus, Mia40-accessible thiol groups were obviously not essential for Atp23 import. This was further supported by the observation that alkylation of free thiols with N-ethylmaleimide (NEM) did not influence the import of

Atp23 into isolated mitochondria whereas it impaired the import of the Mia40 substrate Tim9 (Figure 5D).

Next, we tested the influence of glutathione on the import of Atp23. Glutathione stimulates the import of Mia40 substrates presumably by acceleration of disulphide isomerisation reactions and liberation of non-productive import intermediates (Bien *et al*, 2010). The import of Atp23 was strongly (more than threefold) stimulated by 5–10 mM glutathione (Figure 5E and F) which represents the physiological concentration in the yeast cytosol (Ostergaard *et al*, 2004). This stimulation was much stronger than for so far studied Mia40 substrates like Tim9 (Figure 5E and F) which were stimulated about 1.5-fold (Mesecke *et al*, 2005; Bien *et al*, 2010), presumably reflecting the increased relevance of disulphide reshuffling reactions for the complex Atp23 protein. However, whereas 20 mM glutathione clearly reduced the level of Tim9 import, it did not significantly influence the import of Atp23 (Figure 5E). Since the CPC motif of a significant fraction of Mia40 is reduced at these

Figure 3 Newly imported Atp23 forms mixed disulphides with Mia40. (A) Radiolabelled Atp23 was imported into isolated mitochondria for 20 min. The sample was split. 0.2% DMSO was added to one half, 80 μ M DFDNB in DMSO to the other. Samples were denatured by boiling in 1% SDS and either directly analysed by SDS-PAGE (0.5%) or used for immunoprecipitation with Mia40 antibodies or with preimmune serum (PI). (B) For comparison, radiolabelled Tim9 was imported like in A. Mitochondria were lysed and either directly analysed by SDS-PAGE or used for immunoprecipitation with Mia40 antibodies. The material in the antibody-containing fraction (P) and the supernatant (S) is shown. Note, that only one Tim9-Mia40 complex but several mixed Atp23-Mia40 complexes are observed (arrows). Figure source data can be found with the Supplementary data.

glutathione concentrations (Bihlmaier *et al*, 2007), this is again an indication that cysteine oxidation might not be crucial for the import of Atp23 into the IMS. Glutathione had no considerable effect on the import of the 10CS mutant. It should be noted that, in the absence of glutathione, 10CS was imported with much higher efficiency than Atp23; however, in the presence of physiological glutathione concentrations, both proteins were imported with comparable efficiencies.

In contrast to Atp23 the 10CS protein was unstable in mitochondria and partially degraded in the IMS after its import (Figure 5G). Also upon expression in cells, the 10CS protein accumulated only at low levels in wild type mitochondria; however, in mutants lacking the iAAA protease Yme1 the 10CS protein was as abundant as wild type Atp23 (Figure 5H). Still, cysteine residues, though dispensable for import, are critical for Atp23 function because mutation of multiple cysteine residues of Atp23 caused a respiration-deficient phenotype even if Yme1 was absent (Supplementary Figure S2A,B). In summary, the cysteine-free 10CS mutant of Atp23 is still efficiently imported *in vivo* and *in vitro* in a Mia40-dependent manner, but partially degraded by the mitochondrial quality control system (Tatsuta and Langer, 2008) presumably due to impaired folding of the mutant protein.

Mia40 promotes complete oxidation of Atp23 *in vitro*

Our data indicate that Mia40 is required for oxidation of Atp23 but other mitochondrial components might cooperate with Mia40 in this reaction. To test whether Mia40 is sufficient to oxidize all cysteine residues in Atp23 we developed a reconstituted folding/oxidation assay employing the catalytic

domain of Mia40 that was carried out under anaerobic conditions in a glove box (Bien *et al*, 2010). Radiolabelled Atp23 was oxidized during an incubation of 60 min (Figure 6A). Oxidation was prevented when the cysteine residues in the redox-active CPC motif of Mia40 were replaced by serine residues (SPS) (Figure 6B) even if Erv1 was added to the *in vitro* oxidation reaction (Supplementary Figure S3A). Likewise, a Mia40^{F315,318E} mutant containing negative charges in the hydrophobic substrate-binding region (Kawano *et al*, 2009) was unable to oxidize Atp23 in the *in vitro* reaction (Figure 6C and D). Thus we conclude that both the substrate-binding region and the CPC motif of Mia40 are critical for its ability to oxidize Atp23. Interestingly, a SPC variant of Mia40 still showed some residual oxidation activity in this assay (Figure 6D and Supplementary Figure S3B) consistent with the observation that such a mutant even *in vivo* can functionally replace Mia40 to some extent (Grumbt *et al*, 2007; Banci *et al*, 2009).

Mia40 serves as folding factor of Atp23

It proved to be difficult to study the folding of Atp23 and 10CS due to the aggregation-prone nature of these proteins. Even for the endogenous Atp23 and 10CS proteins that are present in *Dyme1* mitochondria, considerable fractions could be pelleted by high-speed centrifugation indicating that both proteins form aggregates in mitochondria (Figure 7A). Nevertheless, only small fractions of Atp23 and 10CS were found to be bound to Mia40 at steady state levels (Figure 7B).

Next we used recombinantly expressed and purified Atp23 and 10CS to further characterize a potential role of Mia40 in the folding of these proteins. When guanidinium hydrochloride (GdmHCl)-denatured Atp23 or 10CS were dissolved in GdmHCl-free buffer both proteins aggregated rapidly and were found in the centrifugation pellet; the addition of Mia40 reduced this tendency showing that Mia40 is able to bind and stabilize Atp23 and 10CS (Figure 7C and D, arrows). But even in the presence of Mia40 the amount of soluble Atp23 diminished with time whereas the 10CS levels in the supernatant hardly changed pointing to a continuous holding activity of Mia40 for the cysteine-free mutant. To exclude non-specific effects of the recombinant Mia40 protein on Atp23 and 10CS, we employed a Mia40 mutant in which a phenylalanine residue in the substrate-binding region was replaced by a negatively charged glutamate residue (Kawano *et al*, 2009). Interestingly, even the addition of a single negative charge into the binding pocket (Mia40^{F315E}) prevented the ability of Mia40 to stabilize Atp23 or 10CS indicating that the hydrophobic substrate-binding cleft of Mia40 is essential to prevent aggregation of Atp23 (Figure 7E and F).

To study the influence of Mia40 on the aggregation of Atp23 in more detail we developed a light scattering approach. Dilution of denatured Atp23 or 10CS into GdmHCl-free solution resulted in a rapid increase in light scattering intensity that was proportional to the protein concentration (Supplementary Figure S4A). Presence of Mia40, which itself did not show any light scattering signal (Supplementary Figure S4B), strongly reduced the aggregation of Atp23 (Figure 8A1). We estimated the concentration of Mia40 in isolated mitochondria to be about 5 μ M based on Western blotting using purified Mia40 for comparison. Since the IMS is only a small subvolume of mitochondria, the Mia40 concentration in the IMS will be considerably higher.

Figure 4 Atp23 contains several Mia40 binding sites. **(A)** Three independently spotted membranes with 70 peptides of 20 residues each covering the Atp23 sequence were incubated with purified Mia40 or Mia40^{F315,318E}. After extensive washing, the bound Mia40 was transferred onto a second set of membranes used for Western blotting with Mia40-specific antibodies. **(B)** As in A, but a 20-residue peptide matching the MISS sequence of Tim10 (LDLVTDMPFNKLVNNCYKKCI) (Milenkovic *et al*, 2009; Sideris *et al*, 2009) or a control peptide of the matrix protein Mrp10 also containing two cysteine residues (PKCAGLVTEKLSCTSESALG) were analysed. **(C)** The signals of the membranes that were incubated with Mia40 from A were quantified by densitometry. Mean values of these intensities are shown. Predicted α -helical regions in Atp23 are indicated. Peptides containing cysteine residues are shown in red. **(D)** Δ atp23 cells were transformed with an empty plasmid or with plasmids expressing the indicated Atp23 variants. Cells of these strains were lysed with glass beads and used for Western blotting revealing the presence of Atp23 and mature Atp6 (mAtp6) in all strains that expressed Atp23 variants. Asterisks depict crossreactions of the antibodies. At the bottom, tenfold serial dilutions of cultures of the respective strains were dropped onto YP plates containing glycerol as carbon source. **(E)** Model of Mia40-mediated import of Atp23. Figure source data can be found with the Supplementary data.

The concentrations we used in the folding assay were 1.5 and 4.5 μ M Mia40, thus they are presumably lower than the endogenous Mia40 concentrations in the IMS. Heat-inactivated Mia40 which was used for control did not reduce aggregation of Atp23 (Supplementary Figure S4C). Likewise, Mia40^{F315E} and Mia40^{F315,318E} did not prevent aggregation of Atp23 (Figure 8A2,3) confirming that binding of Atp23 into the hydrophobic cleft of Mia40 is crucial for folding of Atp23. In contrast, the redox-active cysteine pair of Mia40 was not required for its activity in Atp23 stabilization since addition of purified Mia40^{SPS} prevented aggregation at least to the same degree as wild type Mia40 (Figure 8A4). Nevertheless, the cysteine residues in Atp23 presumably play a critical role in the folding process since the 10CS mutant was only poorly stabilized by Mia40 or Mia40^{SPS} (Figure 8A5–7).

Physiological concentrations of glutathione (5 mM GSH) did not prevent the stabilization of Atp23 by Mia40 (Figure 8A8). Addition of Mia40 had only a moderate effect on the solubility of the non-IMS protein citrate synthase (Figure 8A9) that is, for example, stabilized by members of the Hsp70 family (Silberg *et al*, 1998). In summary, these measurements indicate that Mia40 interacts with Atp23 and maintains the protein in a soluble, folding-competent conformation. In these reactions, the cysteine residues of Atp23 play a critical role and the 10CS mutant could not be efficiently stabilized by Mia40.

To follow the Mia40-dependent folding of Atp23 in the IMS, we employed a protease accessibility assay. To this end, radiolabelled Atp23 or 10CS were imported into mitochondria; then mitochondria were lysed and the extract was incubated with different concentrations of trypsin. As shown in

Figure 5 A cysteine-free mutant of Atp23 is efficiently imported by Mia40. **(A)** Radiolabelled Atp23 and 10CS were incubated with Mia40-containing or Mia40-depleted mitochondria for the times indicated. Mitochondria were treated with protease. Imported proteins were analysed by autoradiography. Western blot signals of Mia40 or the matrix protein Mrp140 are shown for control. **(B)** Radiolabelled Atp23, 10CS, Tim9 and the matrix protein Su9¹⁻⁶⁹-DHFR incubated with isolated mitochondria in the presence or absence of 0.55 µg/ml valinomycin, 4.4 µg/ml antimycin A, and 8.5 µg/ml oligomycin to dissipate the membrane potential ($\Delta\psi$) of the inner membrane. After incubation for 7 min, non-imported proteins were degraded. Samples were analysed by autoradiography. pSu9 and mSu9 refer to the precursor and mature forms of the matrix protein Su9¹⁻⁶⁹-DHFR. **(C)** Immunoprecipitation of imported 10CS as described for Figure 3. **(D)** Radiolabelled Atp23, the Mia40 substrate Tim9, and the matrix protein Su9¹⁻⁶⁹-DHFR were preincubated with 2 mM NEM or mock treated. NEM was quenched by addition of 10 mM DTT. The samples were added to an import reaction containing isolated wild type mitochondria and adjusted to the same final concentration of DTT (2.5 mM). The amount of imported protein was quantified and is shown as ratio of the values obtained from N-ethylmaleimide (NEM)-treated to untreated samples. Shown are mean values and standard deviations of three independent experiments. **(E, F)** Radiolabelled Atp23, 10CS, and Tim9 were incubated in the presence of different concentrations of glutathione for 7.5 min. The amounts of imported proteins were quantified from three independent experiments from which mean values and standard deviations were calculated. **(G)** Atp23 and 10CS were imported into mitochondria for 15 min at 25°C. The import reaction was stopped by protease treatment. Mitochondria were further incubated in ATP-containing import buffer. The amounts of imported protein after different postincubation periods were quantified from three experiments. Shown are mean values and standard deviations. **(H)** Western blot of mitochondria (50 µg) of the indicated strains in which Atp23 or 10CS were expressed from single copy plasmids. Figure source data can be found with the Supplementary data.

Figure 8B, Atp23 reached a trypsin-resistant conformation whereas 10CS, although imported by Mia40, was considerably less resistant to protease. However, it should be noted that the imported 10CS mutant was more resistant to trypsin than non-imported protein (compare lanes at 0.1 µg/ml of trypsin). This indicates that both Atp23 and 10CS are folded in the IMS; the latter is, however, less stable and more easily degradable by protease. Since the 10CS variant shows no enzymatic activity, it remains unclear whether its conformation resembles the native state of Atp23.

Discussion

Our results showed that Mia40 promotes the import and folding of Atp23. During or directly following the translocation of the reduced Atp23 precursor into the IMS, Mia40

introduces five disulphide bonds into Atp23 thereby converting it into its folded, protease-resistant conformation. Most likely Mia40 directly introduces all disulphide bonds as (i) reduced Atp23 becomes fully oxidized during incubation with Mia40 *in vitro*, (ii) Mia40 shows affinity to several sites in the Atp23 sequence, (iii) Atp23 forms high-molecular weight complexes with more than one Mia40 molecule and (iv) Atp23 is found to be fully oxidized even when cells are grown in the absence of molecular oxygen. The ability of Mia40 to oxidize proteins anaerobically was shown before for other substrates but the final electron acceptor under these conditions is unknown (Allen *et al*, 2005; Bihlmaier *et al*, 2007; Dabir *et al*, 2007).

The presence of physiological concentrations of glutathione strongly accelerates the import of Atp23 into isolated mitochondria presumably due to an improved reshuffling

Figure 6 Mia40 is sufficient for complete oxidation of Atp23 *in vitro*. (A–C) Radiolabelled Atp23 was incubated in the presence or absence of 35 μ M recombinant oxidized Mia40, Mia40^{SPS} or Mia40^{F315,318E} for the times indicated (Bien *et al*, 2010). Proteins were precipitated with TCA and denatured with SDS. Free thiols were modified with mPEG. Reduced proteins migrate slower due to the bound PEG moieties. (D) Quantification of *in vitro* oxidation experiments with the Mia40 variants indicated. Figure source data can be found with the Supplementary data.

of disulphides. Whether the import reaction *in vivo* also depends on glutathione is not known. It is conceivable that *in vivo* other intracellular reductants such as thioredoxins can be used as well, in particular since yeast cells tolerate glutathione deficiency pretty well which leads to relatively specific defects in iron homeostasis (Mühlenhoff *et al*, 2010; Kumar *et al*, 2011). Folding of a protein with 10 cysteine residues presumably requires a disulphide isomerase activity in the IMS. Whether Mia40 is directly involved in the potential isomerization is not clear but its ability to fully oxidize Atp23 *in vitro* (Figure 6A–D) strongly suggests that its role is not limited to the oxidation of thiols but that Mia40 exhibits also an isomerization activity.

Glutathione had no stimulating effect on the cysteine-free version of Atp23 which was imported by Mia40 very efficiently. Thus, Mia40 can promote the import of proteins

Figure 7 Mia40 promotes folding of Atp23 and 10CS. (A) Mitochondria from $\Delta yme1 \Delta atp23$ cells harbouring Atp23 or 10CS expression plasmids were lysed on ice in the presence of 0.5% Triton X-100. Half of the sample was directly analysed by SDS-PAGE (total, T), the other half was separated by high speed centrifugation into a pellet fraction containing proteins aggregates (P) and a supernatant (S) fraction. (B) Mitochondria from cells used in panel A were lysed with 0.1% Triton X-100. 10% of the total protein amount used served as a control. The clarified lysates were incubated with protein A sepharose beads and either preimmune serum (PI) or Mia40-specific antibodies (α Mia40). Proteins that were recovered with the beads were analysed by SDS-PAGE and Western blotting. (C) Atp23 and 10CS were purified in the presence of 6 M GdmHCl and diluted 1000-fold to a concentration of 0.25 μ M in GdmHCl-free buffer in the absence or presence of recombinant Mia40 (2.5 μ M). After the indicated time periods, aggregated proteins were pelleted (P); soluble proteins in the supernatant (S) were precipitated with TCA. (D, E) Atp23 (0.25 μ M) was incubated in the absence or presence of purified 2.5 μ M Mia40 or Mia40^{F315E} for 30 min and further treated as in C. (F) The ability of Mia40^{F315E} in stabilizing 10CS was analysed as in C. Figure source data can be found with the Supplementary data.

without binding them covalently. This ‘holding’ activity of Mia40 was also observed for denatured Atp23 and 10CS substrates which were prevented from aggregation. In these reactions, Mia40 functions similar to classical chaperones to promote folding of its client proteins (Figure 8C). The stabilizing activity on Atp23 and 10CS was also observed for the SPS mutant of Mia40 which lacked the redox-active cysteine pair. However, mutants exposing glutamate residues into the substrate-binding pocket showed no chaperone-like activity. Thus, the ability of Mia40 to interact with non-native proteins via its hydrophobic substrate-binding pocket (Curran *et al*, 2002; Chacinska *et al*, 2004; Naoe *et al*, 2004; Mesecke *et al*, 2005; Banci *et al*, 2009; Kawano *et al*, 2009; von der Malsburg *et al*, 2011) is obviously sufficient for its holdase activity and indicates that the oxidase and chaperone activity of Mia40 can be experimentally dissected.

Figure 8 Mia40 prevents aggregation of Atp23 and 10CS. (A) Recombinant Atp23, 10CS or citrate synthase were denatured in 6 M GdmHCl and diluted to a final concentration of 75 nM in a GdmHCl-free buffer in the absence or presence of 0 μM, 1.5 μM (20 ×) or 4.5 μM (60 ×) Mia40, Mia40^{F315E}, Mia40^{F315,318E}, or Mia40^{SPS}. Protein aggregation was monitored by 90° light scattering at 360 nm. The addition of high concentrations of Mia40^{F315E}, Mia40^{F315,318E}, or Mia40^{SPS} even caused an increase in the light scattering signal which presumably is due to some association of these proteins with the aggregates. Since the diameter of the aggregates has a strong influence on the light scattering signal, even a slight increase in the size of the aggregates will lead to a larger signal. (B) Radiolabelled Atp23 or 10CS were imported into wild type mitochondria at 25°C for 25 min. Non-imported protein was removed by protease treatment. Mitochondria were reisolated, washed and lysed with 0.5% Triton X-100 with the respective concentrations of trypsin. Samples were incubated for 30 min on ice. A 5%-total of the radiolabelled protein was incubated with or without 0.1 μg/ml trypsin for control. (C) Model of the import of Atp23 and 10CS by Mia40. 10CS is unstable and degraded by the iAAA protease Yme1. Figure source data can be found with the Supplementary data.

Atp23 differs from other substrates in that respect in that none of its cysteine residues was found to be essential for import. In the case of Atp23, the larger size (26.9 kDa) and the association with the inner membrane might prevent its back-translocation into the cytosol even in the absence of cysteine residues. The ability of Mia40 to bind to several regions in Atp23 might be sufficient to drive it across the outer membrane even if all cysteine residues were removed. It was shown before for the translocation systems of the endoplasmic reticulum that binding to affinity sites can promote membrane transport of polypeptide chains (Matlack *et al*, 1999). There, the Hsp70 chaperone BiP serves as molecular ratchet that drives protein translocation; translocation of

model substrates was still observed when BiP was replaced by antibodies which bind to sites on these proteins. It is not known whether endogenous substrates exist that are imported or folded by Mia40 under physiological conditions in a cysteine-independent reaction. One potential candidate for such a protein is human Ccs1 which is imported into the IMS in a Mia40-dependent reaction (Kawamata and Manfredi, 2010) although it does not contain the cysteine pair that is oxidized by Mia40 in the yeast Ccs1 protein (Klöppel *et al*, 2011). Moreover, there are a number of IMS proteins that do not contain conserved cysteine residues but the import process of most of these was not studied thus far.

Classical chaperone systems were not identified in the IMS. Small Tim proteins were reported to serve as chaperones; however, they are limited to the binding of preproteins that are in transit in the IMS (Koehler, 2004). Moreover, the iAAA protease Yme1 exhibits a chaperone-like activity and presumably plays an ATP-driven role as a foldase in the IMS (Leonhard *et al*, 1999; Rainey *et al*, 2006). It was shown before that Mia40 can induce the formation of helical structures in simple helix-loop-helix proteins while they are covalently bound to Mia40 (Banci *et al*, 2010). Our data show that the role of Mia40 in protein folding is not limited to the oxidative folding of these simple proteins, nor is the presence of cysteine residues a prerequisite for Mia40 substrates. Thus, the substrate spectrum of Mia40 is much broader than previously expected. The role of Mia40 in the folding of Atp23 is reminiscent to that of other oxidoreductases such as protein disulphide isomerase or DsbA which serve as folding factors for a large variety of proteins (Bardwell *et al*, 1991; Tu *et al*, 2000; Tsai *et al*, 2001; Bukau *et al*, 2006). The mitochondrial IMS developed from the bacteria periplasm during evolution of the eukaryotic cell. Due to the absence of ATP from the periplasm, protein folding in this compartment does not rely on ATP-hydrolysing chaperones but rather on a set of different folding factors that stabilize non-native proteins and promote their folding (Merdanovic *et al*, 2011). Studies in the future will have to test a potential general role of Mia40 as folding factor for IMS proteins.

Materials and methods

Yeast strains and plasmids

All yeast strains used in this study were based on the wild type strain W303-1A (Sherman *et al*, 1986) except for a regulatable Mia40 strain which was based on YPH499 (Mesecke *et al*, 2005). The Δ atp23 and Δ atp23 Δ yme1 strains were described before (Osman *et al*, 2007; Potting *et al*, 2010). Mia40 and Erv1 were depleted using strains in which the corresponding genes were controlled by a *GAL10* promoter (Mesecke *et al*, 2005). All strains were either grown on lactate medium containing 0.1% galactose, synthetic lactate medium containing 2% lactate and 0.1% galactose or a 1% yeast extract, 2% peptone (YP) medium containing 2% of galactose, glucose or glycerol (Altmann *et al*, 2007).

To generate atp23 mutants the Atp23 sequence was cloned into pGEM4 (Promega) vector. Point mutations were introduced using the QuikChange site-directed mutagenesis kit from Stratagene. The resulting pGEM4 constructs were digested with EcoRI and SalI; inserts were cloned into pYX142 for expression in yeast. The mutations were confirmed by sequencing.

Solubility assay with endogenous A3tp23 and 10CS

100 μ g of mitochondria of cells expressing Atp23 or 10CS were lysed in 0.5% Triton X-100, 150 mM NaCl, 5 mM EDTA, 20 mM Tris pH 7.4. After a clarifying spin, half of the extract was taken as total and subjected to TCA precipitation. Aggregates were pelleted from the residual extract by centrifugation for 30 min at 140 000 g. Proteins of the supernatant were precipitated with TCA.

In vitro oxidation of radiolabelled Atp23 and in vivo redox state of Atp23

Atp23 was synthesized and radiolabelled *in vitro* with reticulocyte lysate from Promega (Pelham and Jackson, 1976). For *in vitro* oxidation experiments proteins were synthesized under anaerobic conditions and in the presence of 1 mM DTT. The Atp23 lysate was diluted hundredfold and incubated with the indicated recombinantly purified proteins under anaerobic conditions in an oxygen-deprived glove box. At the indicated time points the reaction was stopped by addition of TCA to a final concentration of 12%. After precipitation the proteins were resuspended in modification buffer

(2% SDS, 80 mM Tris-HCl pH 7.0, 0.05% bromocresol purple) containing 15 mM mPEG and incubated for 1 h at 25°C in the dark. The samples were subsequently boiled for 1 min at 96°C and analysed by SDS-PAGE and autoradiography.

For determination of the *in vivo* redox state cells were grown on YPD at 30°C in the presence or absence of oxygen. Cells were ruptured by vortexing with glass beads in 10% TCA. As a control cells were incubated in 0.5 mM copper phenanthroline for 15 min at 25°C prior to cell lysis to induce protein oxidation. After TCA precipitation of the proteins the pellets were resuspended in modification buffer. 15 mM mPEG were added to the samples. Two controls were prepared: Both were treated with 5 mM TCEP and boiled for 10 min at 96°C. mPEG was subsequently added to one control (final concentration: 15 mM), an equivalent amount of DMSO was added to the other control. All samples and the controls were incubated for 1 h at 25°C in the dark, boiled for 1 min at 96°C and analysed by SDS-PAGE and Western blotting against the His₆-tag of Atp23.

Import of radiolabelled proteins into isolated mitochondria

Import reactions were essentially performed as described previously (Mesecke *et al*, 2005) with the exception that the import buffer did not contain β -mercaptoethanol but 10 mM creatine phosphate, 100 μ g/ml creatine kinase, 2 mM malate, and 2 mM succinate. If the import reaction was followed by a TCA precipitation no BSA was added to the import buffer.

For coimmunoprecipitation the crosslinker DFDNB (final concentration: 80 μ M) or DMSO were added to the samples which were then incubated for 20 min at 25°C. Crosslinking was stopped by addition of 100 mM glycine and incubation for another 3 min. NEM (final concentration: 50 mM) was added to all samples to trap intermolecular disulphide bonds. Samples were centrifuged at 25,000 $\times$ g for 10 min at 4°C. The pellets were resuspended in 100 μ l lysis buffer I (50 mM NaPhosphate buffer pH 8.0, 300 mM NaCl, 1% SDS, 2 mM PMSF) and boiled for 5 min at 96°C. Then, 1.4 ml of lysis buffer II (0.2% Triton X-100, 50 mM NaPhosphate buffer pH 8.0, 300 mM NaCl, 2 mM PMSF) were added, the samples were split into two halves and incubated for 10 min at 4°C on a shaker followed by a clarifying spin (25 000 g, 10 min, 4°C). To one half of the samples protein A-sepharose beads were added that had been preincubated with Mia40-specific antibodies for 2 h at 4°C. Beads that were added to the other half had been incubated with pre-immune (PI) serum. After overnight incubation at 4°C, beads were washed 3 times with lysis buffer II, resuspended in non-reducing SDS sample buffer, boiled for 5 min at 96°C and incubated for another 5 min at 30°C. Samples were analysed by SDS-PAGE and autoradiography.

Protein purification

Erv1 as well as the soluble C-terminal domains of Mia40, Mia40^{F315E}, Mia40^{F315,318E}, Mia40^{SPC} and Mia40^{SPS} were expressed from the vectors pET24a(+) (Erv1) or pGEX-6p-1 (Mia40 variants), respectively, and purified as described previously (Grumbt *et al*, 2007). The Mia40 proteins were eluted with precession protease. Erv1 was dialyzed in 50 mM NaPhosphate buffer pH 8.0 containing 50 mM NaCl. Protein concentrations were determined by measuring the absorption at 280 nm with a Jasco V-650 spectrophotometer.

Atp23 and 10CS were purified from Rosetta 2(DE3) cells containing a pET21a(+) vector to express the respective proteins with a hexahistidine tag. The cells were grown overnight at 37°C in LB medium containing 100 μ g/ml ampicillin and 35 μ g/ml chloramphenicol. On the next morning the cells were diluted to an OD₆₀₀ of 0.2 in a final volume of 100 ml. At an OD₆₀₀ of 0.7 protein expression was induced by addition of 1 mM isopropyl-1-thio- β -D-galactopyranoside (IPTG), and the cells were further grown at 30°C for 3 h. Then, the cells were harvested by centrifugation, resuspended in 5 ml lysis buffer (100 mM NaH₂PO₄, 10 mM Tris, 6 mM GdmHCl, 300 mM NaCl, 10 mM imidazole, pH adjusted to 8.0 with NaOH) and stirred overnight at RT. The lysate was centrifuged at 10 000 g for 30 min at RT. 250 μ l equilibrated Ni-NTA beads were added to the supernatant and incubated for 1 h at 25°C. Beads were washed with a 20-fold volume of washing buffer (100 mM NaH₂PO₄, 10 mM Tris, 6 mM GdmHCl, 300 mM NaCl, 20 mM imidazole, pH adjusted to 8.0 with NaOH). Proteins were finally eluted with elution buffer containing 100 mM NaH₂PO₄, 10 mM Tris, 6 mM GdmHCl, 150 mM NaCl (pH adjusted to 7.4 with NaOH) and 50-300 mM imidazole.

Fractions were analysed by SDS-PAGE. Those fractions containing Atp23 or 10S, respectively, were pooled and stored at -20°C . The proteins were subjected to solubility and light scattering assays.

Solubility assay with Atp23 and 10CS purified from *E. coli*

Recombinantly purified Atp23 or 10CS were diluted to a final concentration of $0.25\ \mu\text{M}$ in a buffer containing $50\ \text{mM}$ NaPhosphate buffer pH 6.0, $150\ \text{mM}$ NaF, and 20% glycerol in the presence or absence of a 10-fold excess ($2.5\ \mu\text{M}$) of recombinantly purified Mia40 variants. Samples were incubated for the indicated time periods at 30°C and subsequently centrifuged at $30\,000\text{g}$ for 15 min at 4°C . Pellets were resuspended in reducing SDS-sample buffer. Supernatants were subjected to TCA precipitation. The precipitates were resuspended in reducing SDS-sample buffer and together with the other samples analysed by SDS-PAGE, Western blotting and Ponceau S or Coomassie staining.

Light scattering

Citrate synthase (from Sigma-Aldrich, denatured overnight at 4°C in $6\ \text{M}$ GdmHCl and $40\ \text{mM}$ HEPES pH 7.4), Atp23, and 10CS were diluted to a final concentration of $75\ \text{nM}$ in $40\ \text{mM}$ HEPES pH 7.4 containing different concentrations of Mia40 variants. Light scattering was measured at $360\ \text{nm}$ with a Jasco FP-6500 spectrofluorometer. Samples were stirred and held at 30°C during measurement. Mia40 boiled for 5 min in the presence of $5\ \text{mM}$ TCEP and 0.1% SDS served as a negative control.

Dot blot assay

Peptides of Atp23 with a length of 20 amino acids each were synthesized on a cellulose membrane as described previously (Frank and Overwin, 1996; Kramer and Schneider-Mergener, 1998). The amino acid frame was shifted by 3 amino acids from one spot to the next. The peptide spots covered the whole amino

acid sequence of Atp23. The membrane was incubated with methanol for 2 min at room temperature, subsequently washed 2 min with H_2O and equilibrated in binding buffer ($20\ \text{mM}$ Tris-HCl pH 7.0, $200\ \text{mM}$ NaCl) for 20 min. After this, the membrane was incubated for 3 h with $0.5\ \mu\text{M}$ of recombinantly purified Mia40 dissolved in binding buffer. The membrane was washed twice for 10 min with binding buffer, twice with TBS ($10\ \text{mM}$ Tris-HCl pH 7.4, $150\ \text{mM}$ NaCl) and subjected to immunoblotting against Mia40.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contribution: DW and SL carried out the experiments. All authors discussed the results and designed the experimental approaches. JMH wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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