

Catch me if you can! Oxidative protein trapping in the intermembrane space of mitochondria

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The intermembrane space (IMS) of mitochondria, the compartment that phylogenetically originated from the periplasm of bacteria, contains machinery to catalyze the oxidative folding of proteins (Mesecke, N., N. Terziyska, C. Kozany, F. Baumann, W. Neupert, K. Hell, and J.M. Herrmann. 2005. *Cell*. 121:1059–1069; Rissler, M., N. Wiedemann, S. Pfannschmidt, K. Gabriel, B. Guiard, N. Pfanner, and A. Chacinska. 2005. *J. Mol. Biol.* 353: 485–492; Tokatlidis, K. 2005. *Cell*. 121:965–96). This machinery introduces disulfide bonds into newly imported precursor proteins, thereby locking them in a folded conformation. Because folded proteins cannot traverse the translocase of the outer membrane, this stably traps the proteins in the mitochondria. The principle of protein oxidation in the IMS presumably has been conserved from the bacterial periplasm and has been adapted during evolution to drive the vectorial translocation of proteins from the cytosol into the mitochondria.

Introduction

Margulis' manuscript on the endosymbiotic origin of mitochondria was rejected by several journals before it was published as a book (Margulis, 1970). Despite the initial controversy, the wealth of molecular and biochemical insight that has accumulated over the last three decades clearly corroborates the close relationship of mitochondria and bacteria and leaves no doubt about the prokaryotic origin of mitochondria. In many of their biochemical and physiological properties, mitochondria still closely resemble their bacterial ancestors. Clearly, during the course of evolution, mitochondria underwent substantial changes to adapt to the specific needs of an intracellular organelle. However, the basic housekeeping functions, such as the principles of metabolic conversion, the propagation and expression of genetic information, or the synthesis and folding of proteins, are still similar to those in bacteria. In bacteria, newly

synthesized proteins achieve their correct fold by chaperones that function according to two basically distinct principles. In the cytosol, chaperones use the hydrolysis of ATP to interact with their unfolded or partially folded substrates. Two ATP-consuming chaperone systems are of outstanding importance in bacteria: the DnaK–Hsp70 and the GroEL–Hsp60 systems (for review see Hartl and Hayer-Hartl, 2002). Both have closely related orthologues in the mitochondrial matrix, and the principles of protein folding in the mitochondrial matrix closely match those in the bacterial cytosol. However, the bacterial periplasm is devoid of ATP, and protein folding is driven by the oxidation of thiol residues (for reviews see Kadokura et al., 2003; Nakamoto and Bardwell, 2004). Sulfhydryl oxidases receive electrons from newly synthesized proteins and pass them on to the electron transport chain in the inner membrane. The introduction and reshuffling of disulfide bonds thereby drives the stable folding of periplasmic proteins.

Some IMS proteins contain disulfide bridges

Until very recently, virtually nothing was known of the mechanisms by which proteins in the intermembrane space (IMS) of mitochondria are folded nor were any chaperones identified in this compartment. However, studies on the structure or biochemistry of several proteins in the IMS revealed the presence of disulfide bonds in IMS components, which were initially considered to be artifacts of aerobic oxidation during protein purification (Table I). IMS proteins for which oxidized cysteine residues have been reported are listed in Table I. In all of these proteins, the cysteine residues are highly conserved and, for those tested so far, are essential for functionality.

Recently, machinery was identified in the IMS that catalyzes protein oxidation and presumably is responsible for all of the disulfide bonds present in the IMS. It consists of two known components: a flavin adenine dinucleotide (FAD)-containing sulfhydryl oxidase named Erv1 (essential for respiration and vegetative growth) and a redox-activated import receptor named Mia40 (Fig. 1). These proteins constitute a disulfide relay system that is designed to drive the translocation of cysteine-containing proteins from the cytosol into the IMS of mitochondria.

Erv1, a conserved sulfhydryl oxidase in the IMS

Erv1 is a sulfhydryl oxidase of the IMS. The primary sequence of Erv1 does not show any recognizable similarity to that of

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Abbreviations used in this paper: FAD, flavin adenine dinucleotide; IMS, intermembrane space; TOM, translocase of the outer membrane.

Table I. Proteins for which disulfide bonds in the IMS have been reported

Protein	Motif	Function	Reference
CCS	Other	Copper chaperone for Sod1	Lamb et al., 1999; Field et al., 2003
Cox11	Other	Assembly factor for complex IV	Banci et al., 2004
Cox12	Other	Subunit of complex IV	Tsukihara et al., 1995; Arnesano et al., 2005
Cox17	Twin Cx ₉ C	Copper chaperone	Abajian et al., 2004; Arnesano et al., 2005
Cox19	Twin Cx ₉ C	Assembly factor for complex IV	Nobrega et al., 2002; Arnesano et al., 2005
Cox23	Twin Cx ₉ C	Assembly factor for complex IV	Barros et al., 2004; Arnesano et al., 2005
Erv1	Other	Sulfhydryl oxidase	Levitan et al., 2004
Mia40	Twin Cx ₉ C	Redox-mediated receptor	Mesecke et al., 2005; Rissler et al., 2005
Rieske	Other	Subunit of complex III	Iwata et al., 1998
Qcr6	Other	Subunit of complex III	Iwata et al., 1998
Tim8	Twin Cx ₃ C	Protein import component	Curran et al., 2002b
Tim9	Twin Cx ₃ C	Protein import component	Curran et al., 2002a
Tim10	Twin Cx ₃ C	Protein import component	Curran et al., 2002a; Lu et al., 2004
Tim13	Twin Cx ₃ C	Protein import component	Curran et al., 2002b
Sco1	Other	Assembly factor for complex IV	Chinenov, 2000
Sod1	Other	Superoxide dismutase	Lamb et al., 2001; Field et al., 2003

DsbA–DsbB and Ero1, the sulfhydryl oxidases of the bacterial periplasm and ER, respectively (Sevier et al., 2001, 2005). Thus, these enzymes are either unrelated or are very distant relatives. However, sulfhydryl oxidases that share the FAD-binding domain of Erv1 are present in the ER of fungi (named Erv2 proteins) and of plants and animals (named quiescin/sulfhydryl oxidases; Coppock and Thorpe, 2006). Moreover, some viruses contain Erv1-like sulfhydryl oxidases that catalyze the oxidation of capsid proteins in the cytosol of infected host cells (Senkevich et al., 2000).

Erv1 consists of two structural segments. The N-terminal segment, which in *Saccharomyces cerevisiae* consists of 72 amino acid residues, contains an invariant CxxC motif but otherwise is hardly conserved. This segment is rich in glycine and proline residues and presumably represents a flexible, unstructured region that functions as a lever arm to bring the redox-active CxxC motif into the proximity of substrate proteins (Hofhaus et al., 2003). The C-terminal segment forms an FAD-binding domain that in *S. cerevisiae* consists of 117 amino acid residues. This domain is well conserved among Erv1-like sulfhydryl oxidases and also contains a redox-active CxxC motif (Lee et al., 2000; Wu et al., 2003; Coppock and Thorpe, 2006). Recent achievements in crystallization of the FAD-binding domains of Erv1 and Erv2 revealed a direct proximity of the isoalloxazine ring of FAD to this second CxxC motif (Gross et al., 2002; Wu et al., 2003). This suggests that this CxxC is oxidized by transfer of its electrons to the FAD cofactor. In vitro, the electrons can be further passed on to molecular oxygen, resulting in the generation of peroxide. However, this reaction is slow but strongly enhanced in the presence of oxidized cytochrome *c*, suggesting that Erv1 can transfer its electrons via cytochrome *c* to the respiratory chain (Allen et al., 2005; Farrell and Thorpe, 2005).

In baker's yeast, Erv1 is essential for viability, and mutations in the Erv1 protein lead to a wide variety of defects such as respiratory deficiency, an altered mitochondrial morphology, depletion of cytosolic iron-sulfur clusters, and the inability to import certain IMS proteins into mitochondria (Lisowsky, 1994; Becher et al., 1999; Lange et al., 2001; Chacinska et al., 2004;

Naoe et al., 2004; Terziyska et al., 2005). In addition, the mammalian Erv1 protein was proposed to function as a growth factor for hepatocytes because the addition of purified Erv1 can stimulate the regeneration of partially hepatectomized livers (for review see Pawlowski and Jura, 2006). As a result of this observation, Erv1 is also named ALR (augmenter of liver regeneration) or hepatopoietin.

The variety of defects observed in Erv1 mutants might point to a wide range of different substrate proteins of Erv1 or, alternatively, to a role for Erv1 in oxidation of a factor of general relevance. The only substrate of Erv1 identified so far is the IMS protein Mia40, which indeed is a factor of general importance, as Mia40 functions as a redox-activated import receptor for IMS proteins.

Mia40, a redox-activated protein receptor in the IMS

Mia40 is ubiquitously present in the IMS of fungi, plants, and animals. All Mia40 homologues share a highly conserved domain of roughly 60 amino acid residues containing six invariant and essential cysteine residues (Chacinska et al., 2004; Naoe et al., 2004; Hofmann et al., 2005; Terziyska et al., 2005). In fungi but not in mammals or plants, this domain is tethered to the inner membrane by an N-terminal membrane anchor. This anchor is not critical for Mia40 activity and can be functionally replaced by unrelated sorting sequences that direct the conserved Mia40 domain to the IMS.

The cysteine residues in Mia40 form a characteristic CPC-Cx₉C-Cx₉C pattern. In vivo, at least some of these cysteine residues are predominantly present in an oxidized state, forming intramolecular disulfide bonds (Allen et al., 2005; Hofmann et al., 2005; Mesecke et al., 2005). The individual function of these cysteine residues is still not clear, but they have been suggested to constitute a redox-driven protein trap that is activated by Erv1-dependent oxidation and is used to import precursor proteins from the cytosol into the IMS (Mesecke et al., 2005; Tokatlidis, 2005). Erv1 directly interacts with Mia40 via disulfide bonds, and this interaction is critical for the oxidation of Mia40.

Depending on the Erv1 activity and the amount of imported protein, Mia40 cycles between oxidized and reduced states (Mesecke et al., 2005). In vitro, reduced Mia40 can coordinate metal ions like zinc and copper, and it was suggested that the reduced state of Mia40 might be stabilized in vivo by metal binding (Terziyska et al., 2005).

The Mia40-Erv1 disulfide relay system drives protein import into the IMS

Proteins of the IMS are involved in several fundamental reactions of the eukaryotic cell-like energy metabolism, the transport of metabolites, ions, and proteins, and apoptosis. All proteins of the IMS are encoded by nuclear genes and, after their synthesis on cytosolic ribosomes, need to be transported across the outer membrane of mitochondria. Some proteins of the IMS contain so-called bipartite presequences that allow import in an ATP- and membrane potential-dependent manner (for reviews see Koehler, 2004a; Herrmann and Hell, 2005). In contrast, many, if not most of the IMS proteins lack presequences or other classic mitochondrial sorting signals. Instead, these proteins contain characteristic patterns of cysteine residues that are essential for their stable accumulation in mitochondria (Hofmann et al., 2002; Roesch et al., 2002; Lutz et al., 2003). All of these cysteine-containing proteins are of low molecular mass, mostly between 6 and 14 kD. This small size might allow them to diffuse rather freely across the protein-conducting channel of the protein translocase of the outer membrane (TOM) complex (Fig. 1). After their translocation into the IMS, they interact with Mia40, forming mixed disulfides (Chacinska et al., 2004; Mesecke et al., 2005). Only the oxidized form of Mia40 is able to form these intermediates, and reduced Mia40 appears to be inactive. Upon reshuffling of the disulfide bonds from Mia40 to the imported precursor proteins, the substrate proteins are released into the IMS in an oxidized and folded state. Because folded proteins are unable to traverse the protein-conducting channel of the TOM complex, this leads to a permanent trapping of the precursors in the IMS (Lu et al., 2004). The reaction is presumably completed by reoxidation of Mia40 by Erv1, which would explain why Erv1 is required for protein import. According to this model, Erv1 and Mia40 form a disulfide relay system that facilitates vectorial protein translocation across the outer membrane by use of an oxidative folding mechanism.

In vivo, the process is presumably more complex and requires the role of additional factors. One of these factors might be Hot13, which influences the assembly and activity of small Tim proteins in the IMS (Curran et al., 2004). Moreover, Erv1 also apparently plays a second role further downstream in the assembly of IMS proteins that is not understood (Rissler et al., 2005).

It should be stressed that this model, which is depicted in Fig. 1, is still rather speculative, and many points remain to be clarified. The presented model matches the experimental observations, but alternative mechanisms by which Erv1 and Mia40 function are also possible. For example, some substrates might be directly oxidized by Erv1, and Mia40 might then function as an analogue of a protein disulfide isomerase. It will be necessary to establish in vitro assays with purified Mia40 and Erv1 to unravel the molecular function of both components in detail.

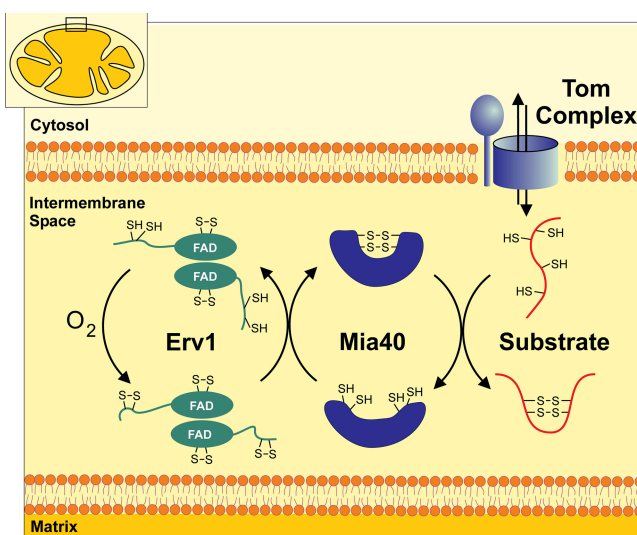


Figure 1. **Model of the Erv1-Mia40 disulfide relay.** Schematic representation of the reactions that mediate redox-driven protein import into the IMS of mitochondria. The sulfhydryl oxidase Erv1 is a dimeric FAD-binding protein that maintains an oxidized state by the use of molecular oxygen as a final electron acceptor. Erv1 directly interacts with Mia40, which functions as a redox-activated import receptor. The oxidized active state of Mia40 can interact with newly imported precursor proteins by intermolecular disulfide bonds. It has been proposed that reshuffling of the disulfide bonds releases the substrates from Mia40 in a stably folded oxidized state. Because these folded proteins cannot traverse the protein-conducting channel of the TOM complex, they remain trapped in the IMS. Alternatively, Erv1 might directly interact with some incoming substrates and pass them on to Mia40, which might function as a protein disulfide isomerase. In both cases, the Erv1-Mia40 system constitutes a folding trap that is designed to mediate the unidirectional import of proteins into the IMS of mitochondria. Reduced and oxidized thiol groups are indicated by SH and SS, respectively.

Substrates of the Mia40-Erv1 relay

The so far identified substrates of the Mia40-Erv1 relay system can be grouped into two classes that differ in their characteristic cysteine signatures. Members of the first group contain two pairs of cysteines that are spaced by three residues each; this pattern is called the twin C_{X3}C motif. Examples are the small Tim proteins, which serve as chaperones that usher hydrophobic inner membrane proteins through the hydrophilic IMS (for review see Koehler, 2004b). Mitochondria typically contain five different small Tim proteins that in fungi are called Tim8, 9, 10, 12, and 13. These proteins form hairpinlike structures in which two central antiparallel α helices are linked to each other by two parallel disulfide bonds (Allen et al., 2003; Webb et al., 2006). The small Tim proteins form hexamers in which the central twin C_{X3}C motifs contact each other (Lu et al., 2004; Webb et al., 2006). The intramolecular interactions between the cysteine residues play essential roles in complex formation, explaining why oxidation is vital for the assembly of these proteins (Allen et al., 2003; Lu et al., 2004).

Members of the second group of substrates are proteins containing twin C_{X9}C motifs. The best characterized representative is Cox17, a copper chaperone of cytochrome oxidase (Beers et al., 1997). Cox17 contains six conserved cysteine residues that can undergo different intramolecular disulfide interactions, thereby influencing the affinity and capacity of Cox17 for copper ions. It was suggested that redox-regulated cycling

through these different conformations drives the binding and release of copper ions (Abajian et al., 2004; Horng et al., 2004; Arnesano et al., 2005). Twin Cx₉C motifs are present in several additional IMS proteins such as Cox19, Cox23, Mdm35, Mic14 (YDR031w), and Mic17 (YMR002w), which all require Erv1 and Mia40 for their biogenesis (Gabriel et al., 2007). Interestingly, the twin Cx₉C motif of these proteins mimics the cysteine motif of Mia40; the reason for this symmetry is not known. Recently, it was shown that the import of Erv1 requires the presence of Mia40 in the IMS (Gabriel et al., 2007), suggesting that the Mia40–Erv1 relay can also be used for the import of proteins with cysteines that are not organized in twin Cx₃C or Cx₉C motifs.

More Erv1 substrates?

Many proteins with disulfide bonds do not contain twin Cx₃C and Cx₉C signatures (Table I). Still, in some of these proteins, the cysteine residues form similar patterns. For example, Cox12, which is subunit VIa of the cytochrome oxidase, contains a Cx₉C–Cx₁₀C pattern that presumably is functionally comparable with the twin Cx₉C motif. However, in other proteins like the Rieske protein or copper/zinc superoxide dismutase (Sod1), only one pair of cysteine residues exists, and the spacing and organization of these residues are not similar to that found in twin Cx₃C and Cx₉C proteins. Experimental evidence for a role of Erv1 and Mia40 in the oxidation of these proteins is still missing.

Oxidative folding despite high levels of glutathione

According to most of the cell biology textbooks, eukaryotic cells can be divided into two sections of different redox chemistry: the ER and, to some degree, also other secretory compartments are generally considered to favor the oxidation of thiol residues and thus to generate disulfide bonds between cysteine residues. In contrast, the cytosol, nucleus, and matrix of mitochondria are believed to counteract the formation of disulfide bonds by maintaining a high concentration of reduced glutathione and/or by the presence of thioredoxins (Ostergaard et al., 2004; for review see Holmgren et al., 2005). This is obviously also the case for the IMS because porin channels in the outer membrane presumably allow the free transfer of reduced glutathione. Recent studies challenged the view that disulfide bonds are limited to secretory compartments (Linke and Jakob, 2003; Paget and Buttner, 2003). The results made it clear that the simple ratio of reduced to oxidized glutathione does not determine the fate of intracellular thiol groups. Instead, the specific nature of the respective proteins and their interactions with reducing or oxidizing enzymes decide their redox states.

The proteins of the IMS might counteract their reduction by two means. First, the disulfide bonds in the IMS might be extremely stable and, thus, rather inert to glutathione reduction. The standard redox potential of Tim10 is very low, and the disulfide bridges in small Tim proteins resist even highly reducing conditions like incubation with 10 mM DTT (Curran et al., 2002a,b; Lu and Woodburn, 2005). A second mechanism to counteract reductive unfolding might be provided by the specific arrangement of cysteine residues in IMS proteins; upon reduction, these patterns might be stabilized by the binding of metal

ions like copper or zinc, which maintain the overall structure of the protein by keeping the cysteine residues in close proximity. The specific cysteine patterns providing four neighboring thiol groups are only found in IMS proteins and not in proteins of the periplasm or ER. It is conceivable that these patterns have developed specifically to promote oxidative protein folding in the presence of high concentrations of glutathione. Alternatively, it was proposed that the binding of zinc ions stabilizes nonimported precursors of small Tim proteins in the cytosol and that coordination of metal ions by the cysteine residues contributes to the import competence of IMS proteins (Lu and Woodburn, 2005).

Conclusion and perspectives

In summary, the IMS of mitochondria contains a system that catalyzes the oxidative folding of proteins to efficiently trap incoming precursors. Although the principle of oxidative protein folding is conserved from the periplasm of bacteria to the ER and IMS of eukaryotic cells, the components that mediate the reactions do not show obvious sequence homology. In the future, it will be exciting to track the phylogenetic origin and relationship of these systems to understand how they arose during evolution and from where they originated.

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