The Erv1–Mia40 disulfide relay system in the intermembrane space of mitochondria

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Abstract

The compartment between the outer and the inner membranes of mitochondria, the intermembrane space (IMS), harbours a variety of proteins that contain disulfide bonds. Many of these proteins possess a conserved twin Cx3C motif or twin Cx9C motif. Recently, a disulfide relay system in the IMS has been identified which consists of two essential components, the sulfhydryl oxidase Erv1 and the redox-regulated import receptor Mia40/Tim40. The disulfide relay system drives the import of these cysteine-rich proteins into the IMS of mitochondria by an oxidative folding mechanism. In order to enable Mia40 to perform the oxidation of substrate proteins, the sulfhydryl oxidase Erv1 mediates the oxidation of Mia40 in a disulfide transfer reaction. To recycle Erv1 into its oxidized form, electrons are transferred to cytochrome c connecting the disulfide relay system to the electron transport chain of mitochondria. Despite the lack of homology of the components, the disulfide relay system in the IMS resembles the oxidation system in the periplasm of bacteria presumably reflecting the evolutionary origin of the IMS from the bacterial periplasm.

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1. Introduction

Eukaryotic cells are divided in subcellular compartments, which are separated by biological membranes and thus generate and maintain selective conditions for biochemical pathways. Mitochondria are one of these compartments. They are surrounded by two membranes, the outer and the inner mitochondrial membrane, thereby constituting two aqueous subcompartments, the matrix space and the intermembrane space (IMS) between both membranes. The IMS of mitochondria harbours proteins crucial for a variety of fundamental processes of the cell. These proteins are involved in the production of energy by oxidative phosphorylation, in the transport of proteins, metal ions and metabolites, in the detoxification of harmful reaction products and in apoptotic processes.

Mitochondria are derived from prokaryotic ancestors. Despite their adaptation to the life of an organelle during evolution, many fundamental processes in mitochondria still resemble those of bacteria. The bacterial cytoplasm is a reducing compartment maintaining the cysteine residues of most proteins in their thiol state. Efficient reduction systems, the thioredoxin system and the glutathione/glutaredoxin system exist in the bacterial cytoplasm and serve to keep cysteine residues in their reduced state [1–3]. The corresponding mitochondrial compartment, the matrix space, also contains similar thioredoxin and glutathione/glutaredoxin systems to maintain its highly reducing redox state [4,5]. On the other hand, the periplasma of bacteria harbours proteins which are in an oxidized state. In this oxidizing compartment the generation of disulfide bonds is catalyzed by an oxidative folding pathway constituted by proteins of the Dsb family, DsbA and DsbB [6,7].

2. The intermembrane space of mitochondria harbours proteins containing disulfide bonds

The IMS of mitochondria, in contrast to its bacterial counterpart, the periplasm, is supposed to be reducing, since it is connected to the cytosol by the pore forming porins in the outer membrane. Because porins allow the free passage of molecules
of up to 2–6 kDa across the outer mitochondrial membrane, reduced glutathione should be able to diffuse from the cytosol to the IMS thereby generating a buffered redox environment similar to the cytosol [8]. Thus, it was rather surprising when first studies reported proteins in the IMS that contain disulfide bonds [9,10]. However, recent findings confirmed the formation of disulfide bonds as a common feature of a set of proteins present in the IMS [11–16]. Three classes of proteins containing disulfide bonds can be distinguished.

2.1. Proteins with twin Cx3C motif — the family of small Tim proteins

The small Tim proteins function as chaperones in the IMS during the transport of hydrophobic membrane proteins to the outer and inner membranes [17–21]. They are ubiquitously expressed in eukaryotes. In Saccharomyces cerevisiae, they are called Tim8, Tim9, Tim10, Tim12 and Tim13 and are characterized by a twin Cx3C motif [20]. The two Cx3C segments are juxtaposed in antiparallel α-helices and linked by two disulfide bonds to form a hairpin-like structure [16,22]. Formation of these intramolecular disulfide bonds is essential for the assembly of the small Tim proteins into hexameric complexes, such as the Tim9×Tim10 or the Tim8×Tim13 complexes [11,12,15,23].

2.2. Proteins with twin Cx9C motif

Most of the proteins with twin Cx9C motif harbour a domain containing a coiled-helix-coiled-helix (CHCH) arrangement [24]. The best studied member of this group is the copper chaperone Cox17 which is required for the biogenesis of the cytochrome c oxidase [25,26]. The structural analysis of Cox17 in its copper-free apo-form confirmed the presence of two antiparallel helices containing each a Cx9C motif that are linked by two disulfide bonds [27,28]. Thus, it resembles the structural arrangement of the small Tim proteins. However, Cox17 has six conserved cysteine residues. An intramolecular disulfide isomerization reaction constitutes an isoform which binds Cu(I) [27]. Moreover, reduction of the protein affects its capacity and affinity for copper suggesting that binding and release of copper is redox-regulated [27,28].

Cox19 and Cox23 are Cox17-like proteins which are also involved in the assembly of a functional cytochrome c oxidase [29,30]. Like Cox17, both proteins contain the four cysteine residues of the twin Cx9C motif. Based on the homology to Cox17, a hairpin-like structure with two disulfide bonds is suggested, but awaits experimental evidence. The same is true for additional proteins with twin Cx9C motif in the IMS [31].

The import receptor Mia40, whose function will be discussed later on, comprises of a pattern of six conserved cysteine residues including a twin Cx9C motif. It is largely present in an oxidized state with three disulfide bonds [14,32,33].

2.3. Further proteins with disulfide bonds in the IMS

In addition, there are disulfide bonds present in proteins that do not contain a twin Cx3C motif nor a twin Cx9C motif. Cox12 is a subunit of the cytochrome c oxidase whose Cx9C–Cx9C arrangement is similar to the pattern of the former motifs forming a helical hairpin structure which is stabilized by two disulfide bonds [10,27].

The complex III of the respiratory chain which catalyzes the electron transfer from ubiquinol to cytochrome c harbours two proteins with disulfide bonds on the IMS side of the inner membrane [9,34]. The hinge protein, called Qcr6 in yeast and subunit 8 in mammalians, is composed of two long anti-parallel alpha-helices. These helices are also linked by disulfide bonds, one in case of Qcr6 and two in subunit 8. One of the three essential catalytic subunits of the complex is the Rieske FeS protein that contains a high-potential [2Fe–2S] cluster as prosthetic group. This cluster is held between two loops of the protein that are connected by a disulfide bridge. The disulfide bridge has been suggested to stabilize the FeS cluster [35]. However, other results point to a damage of the ubiquinol oxidation site in the absence of the disulfide bridge rather than to the lack of the iron–sulfur cluster in the protein [36].

The superoxide dismutase Sod1 protects the cell against oxidative damage by the superoxide anion in two compartments of the cell, in the cytosol and in the IMS, where the latter contains a small percentage of the total Sod1 found in the cell [37]. The active enzyme is a homodimer that has one intramolecular disulfide bond and one copper and one zinc ion bound per monomer [38]. Activation of Sod1 requires the copper chaperone for Sod1, Ccs1 which forms an intermolecular disulfide bond to introduce copper and the disulfide bond into Sod1 [39,40]. Moreover, the crystal structure of Ccs1 of S. cerevisiae revealed two disulfide bonds, one in a conserved Cx9C motif [41]. Like Sod1, Ccs1 shows the dual localization in cytosol and IMS [37].

Another oxidized protein appears to be Cox11. Cox11 is an assembly factor needed for the incorporation of copper into the CuB site of cytochrome c oxidase [42,43]. It has been suggested that a dimeric form of the protein might be stabilized by intermolecular disulfide bonds [44]. Sco1 is also a copper-binding protein involved in the assembly of the cytochrome c oxidase [45,46]. The IMS domain of Sco1 harbours a Cx9C motif and adopts a thioredoxin fold [47–49]. In crystallization studies the presence of a disulfide bond has been observed suggesting that the cysteine residues of the twin Cx9C motif can perform redox reactions, at least under non-physiological conditions [48].

How are these disulfide bonds generated? Based on primary sequences there are no homologs of DsbA and DsbB in the IMS, the components of the oxidation system of the bacterial periplasm, the evolutionary related compartment of the IMS. Because disulfide bonds are in general not formed de novo, the presence of many oxidized proteins has suggested a protein oxidation system in the IMS, whose defect should lead to pleiotropic mitochondrial defects. Recently, such a disulfide relay system for the oxidation of thiol groups within proteins was identified in the IMS which consists of at least two proteins, the sulfhydryl oxidase Erv1 and the redox-dependent receptor protein Mia40 [14,50,51].
3. The FAD-dependent sulphydryl oxidase Erv1

The essential sulphydryl oxidase Erv1 (essential for respiration and vegetative growth), first identified in *S. cerevisiae*, is a protein with a molecular mass of 22 kDa which resides in the intermembrane space of mitochondria [52,53]. Cells harbouring a defective form of Erv1 show loss of the mitochondrial genome, impaired respiration and cell growth. In addition, they display defects in mitochondrial morphology and distribution, as well as in the biogenesis of cytosolic FeS cluster-containing proteins and in the maturation of heme and its binding to cytochrome c and cytochrome c peroxidase [52–56]. Furthermore, cells lacking functional Erv1 have decreased amounts of small proteins in the IMS of mitochondria, such as small Tim proteins, Sod1 and Cox17 [14,57,58]. These reduced protein levels are due to impaired import of these proteins into mitochondria. Moreover, the assembly of small Tim proteins is affected in a temperature-sensitive mutant of Erv1 [57]. In mammalians, the Erv1 homolog ALR (augmenter of liver regeneration) has been described as a factor promoting the regeneration of liver by an unknown mechanism [59].

Members of the Erv1 family are present in eukaryotes from fungi to plants and animals [60,61]. Together with the Erv2 proteins which are present in the endoplasmatic reticulum (ER) of fungi and some Erv1-like sulphydryl oxidases from viruses the Erv1 proteins constitute the class of Erv/ALR thiol oxidases [60–64]. The Erv/ALR thiol oxidases transfer electrons from thiol groups in their polypeptide chain to the non-thiol electron acceptor flavine adenine dinucleotide (FAD) thereby generating disulfide bonds. They are characterized by a catalytic core domain of about 100 amino acid residues which has FAD non-covalently bound in a four-helix bundle structure [65–67]. Next to the isoalloxazine ring of the bound FAD, an active site cysteine pair in a Cx2C motif is located [65–68]. In addition, there is a structural disulfide-bonded cysteine pair present in the core domain of Erv1 and Erv2. The core domain is fused to a tail segment which contains another pair of cysteine residues [65,66,69]. The position of the tail, N- or C-terminal of the core domain, and the spacing of its cysteine pairs varies between the members of the Erv/ALR family [66–70]. In *S. cerevisiae* and in human a Cx2C motif is present in the N-terminal tail, whereas in Erv1 from *Arabidopsis thaliana* the cysteine residues are located as a Cx4C motif in the C-terminal tail [71]. Based on the X-ray structures of Erv1 from *A. thaliana* and yeast Erv2 and on mutational analysis of proteins of this family, a mechanism for the oxidation of substrate proteins by Erv/ALR thiol oxidases has been proposed [65,66,69,72]. The tail segments of the oxidases containing the shuttle cysteine residues appear to be mainly unstructured indicating high flexibility for these segments [65,66]. Due to their flexibility, the tails are well suited to facilitate the disulfide transfer to substrate proteins. A dithiol–disulfide exchange between reduced substrate and the oxidized shuttle disulfide pair in the flexible tail introduces disulfide bonds in the substrates. Since the tail segments of different members of the Erv/ALR oxidases have distinct positions with respect to the core domain and selective sequence context and spacing of the cysteine residues, these segments might be used to confer substrate specificity to the various oxidases. The disulfide bond between the shuttle cysteine residues is regenerated by the transfer of electrons to the active site cysteine pair that is present in the disulfide state in the core domain. The active site cysteine pair is then reoxidized by transfer of electrons to the adjacent FAD thereby generating the disulfide bond in the Cx2C motif. FAD is recovered in its oxidized form by transfer of electrons to oxygen generating hydrogen peroxide or to cytochrome c in the case of Erv1/ALR [56,61,73,74]. Crystallographic studies of Erv1 from *A. thaliana* and rat, as well as Erv2 from yeast reveal homodimers in which the dimeric interface is made up of amino acid residues of the core domain [65–67]. In the electron transfer process, the two subunits of the dimer act together [65,66,72]. They form an intermolecular disulfide intermediate, in which the shuttle cysteine pair interacts in trans with the active site Cx2C motif of the other subunit and thereby enabling transfer of the disulfide bond. The dimeric arrangement might restrict the access to the active site cysteine residues for non-specific substrates [72]. A similar mechanism of a flexible tail with the shuttle cysteine pair might take place for the yeast Erv1, although the tail is located at the amino-terminus in this case [65,69]. However, further experimental evidence is needed, since a recent report has determined a very low redox-potential of this cysteine pair and has therefore suggested a structural role of this pair in the intermolecular dimerization rather than a redox-active catalytic function [56].

The thiol oxidase activity of Erv1 has been demonstrated in vitro using e.g. the artificial substrates lysozyme, thioredoxin and DTT [66,68,69,71]. However, there is limited knowledge about the physiological substrates. Because a variety of mitochondrial processes are affected in yeast strains defective in Erv1, Erv1 might work on substrates required in each of these different processes and/or it acts on general factors affecting many processes. The first identified substrate of Erv1, the import receptor Mia40/Tim40, is such a factor, since it is essential for the biogenesis of a variety of proteins in the IMS of mitochondria [14,57].

4. The redox-regulated import receptor Mia40

The import receptor Mia40/Tim40 (mitochondrial import and assembly) is essential for viability of cells in *S. cerevisiae* [75–78]. Its crucial function is reflected by its presence in eukaryotes from fungi to plants and animals.

Mia40 and its homologs reside in the IMS, either as soluble protein or N-terminally anchored to the inner membrane [32,76–78]. In fungi, the proteins are synthesized as preproteins with a mitochondrial matrix targeting signal (MTS) which is followed by a hydrophobic transmembrane segment. Thus, the proteins follow the general translocation pathway and are imported into mitochondria via the TOM- and the TIM23 complexes in a membrane potential-dependent manner [79–82]. The N-terminal MTS is removed by the matrix processing peptidase and the protein is laterally sorted to the inner membrane by the hydrophobic segment. Thereby, the protein is anchored to the inner membrane with its major part protruding into the IMS.
However, this membrane anchor is not crucial for the function of Mia40 [77]. In consistence, Mia40 homologs in higher eukayocytes even lack matrix targeting presequences and hydrophobic segments [32]. These proteins which are much smaller in molecular mass are soluble in the IMS of mitochondria. Native Mia40 might be in an oligomeric complex according to its behavior in gel filtration and glycerol density–gradient centrifugation experiments [32,77]. However, yeast Mia40 showed unconventional mobility in a blue native electrophoresis which did not change in the presence of reductants and strong denaturants suggesting a monomeric form [76].

All homologs have a highly conserved domain of about sixty amino acid residues with six invariant cysteine residues in common [76–78]. These cysteine residues are arranged in a CPC–Cx9C–Cx9C pattern with a characteristic twin Cx3C motif in a CHCH domain. Mia40 can adopt different redox states indicating redox switches of its cysteine residues [14,32]. Oxidized Mia40 contains three intramolecular disulfide bonds: one disulfide bond connects the first two cysteine residues in the CPC motif and two disulfide bonds bridge the Cx9C segments in the twin Cx9C motif by linkage of the distal and proximal cysteine residues [33]. In contrast to the stabilizing disulfide bonds in the twin Cx9C motif, the first disulfide bond is easily accessible to reducing agents consistent with a catalytic function of this cysteine pair in redox processes. In mitochondria, there is no indication for a complete reduction of Mia40. The reduced form of Mia40 detected has the first two cysteine residues present in their thiol state, whereas the other cysteine residues still form the two stable disulfide bonds. However, the major pool of Mia40 appears to be oxidized [14]. Moreover, metal binding might stabilize Mia40 [78]. This is consistent with the observation that reduced Mia40 has the ability to bind metal ions such as zinc and copper in vitro [78]. The functions of single cysteine residues are not known, but deletions of the first, second and third pairs of cysteine residues are lethal indicating their crucial role for the function of Mia40 [77].

Mia40 has been identified as the central component of a pathway responsible for the import and assembly of a set of proteins into the IMS of mitochondria (Table 1) [76–78]. The substrates of this pathway are characterized by a relatively small mass of about 8–22 kDa and by the lack of typical mitochondrial targeting signals [5,76–78,83,84]. All of them have highly conserved cysteine residues. In all so far identified substrates of the Mia40-dependent translocation pathway, except for the sulfhydryl oxidase Erv1, the cysteine residues are organized in a twin Cx3C or Cx9C motif [31,83,85]. As already pointed out, the cysteine residues of these substrate proteins are connected, at least in the proteins for which structural information has been acquired, by two disulfide bonds which link two α-helices [16,22,27]. Proteins containing a twin Cx3C motif whose redox states have not yet being experimentally analyzed presumably have a similar structure with two disulfide bonds. As already discussed for Cox17, redox switches and disulfide isomerization reactions might occur in addition which would explain why metal binding to some of the proteins appear to be important for their functions [19,27,86]. Besides the already described copper chaperone Cox17 and the Cox17-like proteins Cox19 and Cox23 further substrates of the class with twin Cx9C motif include the protein Mdm35, identified as a protein crucial for mitochondrial distribution and morphology, and two proteins of unknown function, Mic14 and Mic17 [31,87]. For the import of the small Tim proteins with twin Cx3C motif, a folding-trap mechanism as driving force for the net translocation across the outer membrane has been suggested [15,86,88]. According to this model, the proteins pass the TOM complex in an unfolded reduced state. The reduced state might be stabilized in the cytosol by binding of zinc ions which could be a reason why addition of zinc ions promote the import of small Tim proteins into isolated mitochondria [89]. After passage across the TOM complex the protein is trapped in the IMS by oxidative folding [14,15,88].

The small cysteine-rich proteins require for their import Mia40 as receptor in the IMS. Mitochondria lacking functional Mia40 are selectively inhibited in the import of these proteins and have reduced endogenous levels of them as a consequence [31,76–78]. Moreover, enhanced import of these proteins is observed in mitochondria containing increased levels of Mia40 [78]. During the import process Mia40 forms a disulfide intermediate with the incoming substrate proteins [14,76]. The import of substrate proteins is inhibited in the presence of strong reducing agents which prevent formation of disulfide intermediates [14,76]. This indicates the essential role of the disulfide intermediates between Mia40 and incoming substrate proteins for their import.

After the interaction with Mia40, the small Tim proteins Tim9 and Tim10 assemble into the hexameric Tim9×Tim10 complex and the TIM22 complex [76,77]. As revealed by mutational analysis, each of the cysteine residues in the twin Cx3C– motif of the small Tim proteins are important for the assembly into the hexameric complex [15,90–93]. However, only the most amino-terminal cysteine residue is required for binding to Mia40 in a disulfide intermediate and thereby enabling transport of the protein across the outer membrane [92,93]. This cysteine residue is selectively recognized by Mia40 without the help of any other factors. So far, a specific sequence or structural motif which acts as recognition signal has not been identified in the substrates. In contrast, the other cysteine residues are crucial for the assembly into the Tim9×Tim10 complex and for the release of substrates from Mia40, rather than for binding to Mia40. Release of Tim9 from Mia40 seems to be dependent on their

Table 1  Experimentally confirmed substrates of the disulfide relay system

<table>
<thead>
<tr>
<th>Protein</th>
<th>Motif</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tim8</td>
<td>Twin Cx3C</td>
<td>Protein transport</td>
<td>[76]</td>
</tr>
<tr>
<td>Tim9</td>
<td>Twin Cx3C</td>
<td>Protein transport</td>
<td>[76,77]</td>
</tr>
<tr>
<td>Tim10</td>
<td>Twin Cx3C</td>
<td>Protein transport</td>
<td>[76–78]</td>
</tr>
<tr>
<td>Tim12</td>
<td>Twin Cx3C</td>
<td>Protein transport</td>
<td>[77]</td>
</tr>
<tr>
<td>Tim13</td>
<td>Twin Cx3C</td>
<td>Protein transport</td>
<td>[76,78]</td>
</tr>
<tr>
<td>Cox17</td>
<td>Twin Cx9C</td>
<td>Copper chaperone</td>
<td>[76,78]</td>
</tr>
<tr>
<td>Cox19</td>
<td>Twin Cx9C</td>
<td>Cytochrome oxidase assembly factor</td>
<td>[76]</td>
</tr>
<tr>
<td>Mic14</td>
<td>Twin Cx9C</td>
<td>Unknown</td>
<td>[31]</td>
</tr>
<tr>
<td>Mic17</td>
<td>Twin Cx9C</td>
<td>Unknown</td>
<td>[31]</td>
</tr>
<tr>
<td>Mdm35</td>
<td>Twin Cx9C</td>
<td>Mitochondrial distribution and morphology</td>
<td>[31]</td>
</tr>
<tr>
<td>Erv1</td>
<td>Other</td>
<td>Sulfhydryl oxidase</td>
<td>[31,85]</td>
</tr>
</tbody>
</table>
assembly in the Tim9×Tim10 complex in which the small Tim proteins contain two disulfide bonds each [11,16,76]. Thus, formation of disulfide bonds between the cysteine residues of the twin Cx3C motif appears to be needed to trigger release of small Tim proteins from Mia40 consistent with the oxidative folding-trap mechanism. Moreover, release of the oxidized proteins in a monomeric form has been recently shown for Tim8 and Tim13 [88]. As expected for an oxidative folding-trap mechanism, monomeric Tim8 is released from mitochondria when it is reduced [88]. In contrast to Tim9, dissociation of Tim8 from Mia40 appears not to be dependent on its assembly in the Tim8×Tim13 complex. This result implies that various substrates of the Mia40-dependent translocation pathway might behave differently in their import and assembly pathways. Evidence for the capability of Mia40 to transfer disulfide bonds to substrate proteins was obtained in vitro. Mia40 is able to oxidize a Mia40 variant which lacks the first two cysteine residues and thus resembles a typical substrate with a twin Cx9C motif [33].

In summary, Mia40 acts as a receptor in the IMS which selectively recognizes and binds small proteins of the IMS and thus mediates their import into the IMS. Then substrate proteins which contain disulfide bonds in their native state are released from Mia40 in the oxidized form and Mia40 is left in a reduced state.

5. Mia40 and Erv1 constitute a disulfide relay system that drives the import of proteins into the IMS

How do Mia40 and Erv1 cooperate in the biogenesis of small IMS proteins? The small IMS proteins interact with Mia40 in disulfide intermediates indicating that redox processes have to take place. Mia40 in its oxidized state is crucial for the import of IMS proteins and for the formation of the disulfide intermediates [14]. Several observations have demonstrated that Erv1 is involved in oxidation reactions of Mia40 required for the translocation of proteins into the IMS [14,33]. The absence of functional Erv1 leads to inhibition of the import of the small proteins into the IMS [14,31,57,58]. Furthermore, the import is more sensitive against reducing agents compared to the import in the presence of Erv1 which would explain why depletion of Erv1 from yeast cells results in an increased sensitivity towards reducing agents [14]. Consistent with these import defects the depletion of Erv1 affects the formation of the disulfide intermediate between Mia40 and substrate proteins [14]. Erv1 is not part of the Mia40-substrate disulfide intermediate, but rather appears to exert its function on the redox state of Mia40. In the absence of Erv1, Mia40 accumulates predominantly in the reduced form. In addition, Erv1 directly interacts with Mia40 via disulfide bonds [14,57]. These data suggested that Erv1 converts Mia40 to its oxidized form which is required for the import of small IMS proteins. The transfer of disulfide bonds from Erv1 to Mia40 has been directly demonstrated with purified proteins [33]. All three disulfide bonds were introduced in reduced Mia40 upon incubation with Erv1. Thus, Erv1 is sufficient to oxidize Mia40. To enable the interaction of these two proteins and thereby oxidation of Mia40, the first two cysteine residues of Mia40 are essential. The catalytically active disulfide bond formed by these residues appears to mediate the generation of the two structural disulfide bonds in the twin Cx9C motif of Mia40. Catalytic amounts of functional Mia40 seem to be sufficient for the oxidation of substrate proteins, when Erv1 is present to reoxidize Mia40 [33].

In summary, the data support a disulfide relay system of Mia40 and Erv1 which mediates the vectorial translocation of cysteine-rich proteins across the outer membrane of mitochondria by transfer of disulfide bonds to these proteins (Fig. 1). By shuffling of electrons to non-thiol electron acceptors disulfide bonds are generated in Erv1. These disulfide bonds are transferred to Mia40 to oxidize it and make it capable of forming disulfide intermediates with newly imported substrates proteins. Reshuffling of disulfide bonds mediates oxidative folding of the substrates and thereby their release from Mia40 in a folded state. The substrates are trapped in the IMS of mitochondria because folded proteins cannot transport across the TOM complex of the mitochondrial outer membrane. Thus the net import of the substrate proteins into the IMS is driven by the Erv1–Mia40 disulfide relay system.

It should be noted that only a model of the disulfide relay system is presented, schematically depicted in Fig. 1. The disulfide relay system might be more complex than described and further studies are required to elucidate its molecular mechanisms. In particular, the mechanisms of the function of Mia40 and the nature of its disulfide intermediates with substrate proteins and Erv1 is still speculative. Although Mia40 recognizes specifically one cysteine residue in small Tim proteins, it is not clear whether Mia40 forms one or two disulfide bonds with the substrates in the disulfide intermediates. Moreover, it remains questionable, how two disulfide bonds are introduced into the substrates with twin Cx3C and Cx9C motifs and how substrates are released from Mia40. These reactions might require additional components. In this respect, the analysis of distinct steps of the import process revealed a role of functional Erv1 for the oxidation and the assembly of small Tim proteins at a step following the formation of the Mia40-substrate disulfide intermediate [88]. Although Erv1 does not oxidize small Tim proteins in vitro [58], it appears to function not only in the oxidation of Mia40, but also in combination with Mia40 in the oxidation of substrate proteins [88]. Another candidate is the Hot13 protein, whose deletion affects the assembly and the activity of small Tim complexes [94]. Since the assembly into oligomeric complexes appears to be important for the release of some of the Tim proteins from Mia40, partially assembled subcomplexes may also play a role for the dissociation of some substrates from Mia40 [76].

In addition, the Mia40 dependent import pathway has a broader substrate spectrum of substrates than first expected, since the sulfhydryl oxidase Erv1 is a substrate of the pathway, although it does not contain a typical twin Cx9C motif (Table 1) [31,85]. It is likely that the mechanisms of import of Erv1 by Mia40 are different from those of substrates with twin Cx3C motifs and Cx9C motifs. Furthermore, it remains to be established how disulfide bonds are introduced in proteins in the IMS which do not belong to the classes of proteins with twin
Cx_3C and Cx_6C motifs. Proteins, such as Cox12 and Qcr6, which contain two alpha-helices linked by disulfide bonds and thus adopt structures similar to the typical substrates of Mia40, are presumably substrates of the disulfide relay system. Other proteins with disulfide bonds lacking these features might still employ the Mia40 dependent translocation pathway, as Erv1 does, or might require the sulfhydryl oxidase Erv1 directly for oxidation.

6. The disulfide relay system is connected to the respiratory chain of mitochondria

Upon generation of disulfide bonds in substrate proteins the disulfide relay system shuttles electrons to FAD tightly bound to Erv1. In order to allow continuous reoxidation of Mia40 and thus permanent catalysis of disulfide bond generation by the disulfide relay system, the reduced Erv1 needs to be reoxidized (Fig. 1). Flavin-dependent sulfhydryl oxidases use molecular oxygen as electron acceptor [61]. Indeed, Erv1 transfers electrons in vitro to oxygen thereby producing hydrogen peroxide [56,68,73,74]. However, cytochrome c has been proven to be a 100-fold better electron acceptor of ALR, the human Erv1, than oxygen [73]. This led to the suggestion that Erv1 shuttles electrons in vivo to cytochrome c [58,73]. Further studies confirmed the competition of cytochrome c with molecular oxygen for the uptake of electrons from recombinant Erv1[56,74]. In addition, Erv1 directly interacts with cytochrome c in mitochondria as well as in vitro [56]. Cytochrome c shuttles electrons from the cytochrome bc_1 complex to the cytochrome oxidase of the respiratory chain. Thus, the reduction of cytochrome c by Erv1 links the disulfide relay system to the respiratory chain, since reduced cytochrome c can feed the electrons into the cytochrome c oxidase to generate water from molecular oxygen. Indeed, the absence of cytochrome c affects the reoxidation of Mia40 in mitochondria and therefore the import of proteins into the IMS of mitochondria [74]. Moreover, the manipulation of the redox state of cytochrome c by inhibitors or mutants of the respiratory chain has an influence on the redox state of Mia40. Block of the cytochrome c oxidase activity increases the amount of reduced cytochrome c and thus reduced Mia40, whereas inhibition of the cytochrome bc_1 complex accumulates oxidized cytochrome c which then efficiently oxidizes Mia40. These effects on Mia40 suggest that the disulfide relay system shuttles electrons from Erv1 to oxidized cytochrome c.
Interestingly, cytochrome c can also be reoxidized by the cytochrome c peroxidase, Ccp1, which thereby functions as electron acceptor of Erv1 [56]. In this case, the required oxidized form of Ccp1 is generated by reduction of hydrogen peroxide to water. Hydrogen peroxide can be produced by the transfer of electrons from Erv1 to oxygen directly. Thus, Ccp1 has the ability to link the two electron accepting pathways of Erv1. Both pathways depend on the presence of oxygen. However, under anaerobic conditions yeast cells are viable suggesting another, so far unknown, final electron acceptor for cytochrome c [56,58]. This connection of the disulfide relay system to the respiratory chain has been suggested to increase efficient oxidation of the relay system and to prevent the formation of deleterious hydrogen peroxide in the IMS [74].

7. The disulfide relay system of the IMS has similarities to the protein oxidation systems in the bacterial periplasm and the endoplasmic reticulum of eukaryotes

In the bacterial periplasm, two components are important for oxidation of protein thiols, DsbA and DsbB (for review see [6,7]). The soluble protein DsbA interacts with substrate proteins introducing disulfide bonds by uptake of electrons which are further transferred to the membrane embedded protein DsbB. DsbB shuttles the electrons then via quinones to the electron transport chain and finally to molecular oxygen under aerobic conditions or to other electron acceptors under anaerobic conditions. In the ER, the FAD-binding protein Ero1 oxidizes the protein disulfide isomerase PDI which then introduces disulfide bonds into substrate proteins (for review see [95,96]). In fungi, there is a second oxidation pathway for the generation of disulfide bonds in the ER. In this pathway, the flavin-dependent thiol oxidase Erv2 can transfer disulfide bonds to PDI. As already described, Erv1 is homologous to Erv2, but not to Ero1 or the bacterial DsbB. Furthermore, Mia40 lacks any sequence and structural similarity to the thioredoxin-like proteins PDI and DsbA. However, the mitochondrial relay system appears to share basic principles with the systems in the ER and the periplasm. First, there is a relay system of two components to transfer disulfide bonds to substrate proteins. Second, intramolecular dithiol–disulfide exchange reactions are employed in the Erv/ALR thiol oxidases, in Ero1 and in DsbB to shuttle electrons to the non-thiol electron acceptors. Moreover, the secondary structure of a four-helix bundle as catalytic core appears to be a motif present in the Erv/ALR oxidases, as well as in Ero1 and DsbB, although DsbB is not a FAD containing thiol oxidase [60,65,66,97,98]. Third, all disulfide relay systems can use oxygen as final electron acceptor and, with the exception of Erv2, appear to be able to utilize alternative final acceptors under anaerobic conditions. In case of the mitochondria and the bacterial periplasm, the disulfide relay systems share the connection to the electron transport chains in the membranes, even though it is a 2-electron-transfer to quinones in the periplasm and a single electron transfer to cytochrome c in mitochondria. In the future, it will be interesting to compare the properties and mechanisms of these disulfide relay systems and to analyze their evolutionary origin.

8. Concluding remarks

There is clearly an oxidative pathway present in the IMS of mitochondria which has been adapted to drive the import of a set of proteins with conserved cysteine motifs into the IMS by an oxidative folding-trap mechanism. As pointed out, the oxidative pathway employs a disulfide relay system between the sulfhydryl oxidase Erv1 and the redox-dependent import receptor Mia40. In the future, the detailed mechanisms of this pathway have to be unraveled, in particular the single steps of the disulfide transfer and the nature of the disulfide intermediates, as well as a role of additional components. In this respect, it will be valuable to analyze and dissect the process in the reconstituted system with purified components.

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