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Review

Oxidation-driven protein import into mitochondria: Insights and blind spots ☆

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ABSTRACT

The intermembrane space of mitochondria contains a dedicated machinery for the introduction of disulfide bonds into proteins. In this case, oxidative protein folding is believed to drive the vectorial translocation of polypeptides after their synthesis in the cytosol across the mitochondrial outer membrane. Substrates of this system are recognized by a hydrophobic binding cleft of the oxidoreductase Mia40 which converts them into an oxidized stably folded conformation. Mia40 is maintained in an oxidized, active conformation by the sulfhydryl oxidase Erv1, a homodimeric flavoenzyme, which can form disulfide bonds *de novo*. Erv1 passes electrons on to cytochrome *c* and further to the respiratory chain. The components of this system, their structures and the mechanisms of disulfide bond formation were analyzed only very recently. This review discusses our knowledge about this system as well as open questions which still wait to be addressed. This article is part of a Special Issue entitled Protein translocation across or insertion into membranes.

Contents

1. Introduction

1.1. Cysteine residues have unique properties

The exceptional reactivity and chemical versatility of cysteine residues sets this amino acid clearly apart from the other 19 amino

Abbreviations: Ccs1, copper chaperone for Sod1; GSH, reduced glutathione; IMS, intermembrane space; ITS, IMS-targeting signal; MISS, mitochondria IMS-sorting signal; MTS, matrix targeting signal; Sod1, superoxide dismutase 1; TIM, translocase of the inner membrane; TOM, translocase of the outer membrane

☆ This article is part of a Special Issue entitled Protein translocation across or insertion into membranes.

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acids found in proteins. Numerous modifications of the thiol (-SH) moiety of cysteines have been identified. Examples are oxidations to sulfenic acid, sulfinic acid or sulfonic acid [1], glutathionylation [2,3], nitrosylation [4,5], and farnesylation [6]. Although many examples for these modifications were reported, we still have a very poor understanding of the extent by which cysteine residues are modified under physiological conditions. Recent proteomic data suggest that a large fraction of thiols is indeed modified *in vivo* [7]. For example, about 10% of all cysteine residues in proteins of human cells are glutathionylated under normal growth conditions; and upon treatment with the oxidizing reagent diamide up to half of all cysteine residues can be modified with glutathione molecules [7]. In addition to these modifications, cysteine residues in proteins can react with each other by oxidation to form intra- or intermolecular disulfide bonds (-SS-). These covalent interactions—if present at the right place—can considerably stabilize the structure of a protein. However, in all other cases, they potentially interfere with the fold of a protein and compromise its activity. These special features of cysteines have two consequences: First, cysteines are by far less abundant in proteins than most other amino acid residues: on average, only 2 out of 100 residues are cysteines [7,8]. Second, many of the cysteine residues found in proteins are evolutionary conserved, pointing to a specific function of these residues at the specific position. The recent development of techniques to analyse the amino acid modifications at specific sites of individual proteins or on proteome-wide scales will enable us to explore the physiological relevance of cysteine chemistry.

1.2. The formation of disulfide bonds in proteins is tightly controlled

To reduce the risk of detrimental cysteine oxidation, most cellular compartments prevent the formation of disulfide bonds. For example, in the cytosol, in the matrix of mitochondria or in the nucleus, thioredoxins and glutaredoxins together with a high concentration of reduced glutathione counteract the formation of disulfide bonds. In these compartments, only very few typically oxidation-sensing proteins are known to form disulfide bonds under physiological, healthy conditions [9,10].

In some compartments, however, the oxidation of cysteine residues is actively promoted due to the presence of oxidizing enzymes. These compartments are the periplasm of bacteria, the endoplasmic reticulum and the intermembrane space of mitochondria (IMS) [11–14]. The oxidation machinery of the latter was identified only recently and will be described in the following.

2. The mitochondrial disulfide relay—what we know

Mitochondria contain two membranes, the outer (OM) and the inner (IM) membrane, enclosing two hydrophilic compartments, the matrix and the IMS. The matrix harbours hundreds of proteins which are involved in many functions including respiration, metabolic conversions, the biogenesis of iron-sulfur clusters, or the propagation and expression of the mitochondrial genome. Almost all of these proteins are initially synthesized as precursor proteins on cytosolic ribosomes. These precursors carry aminoterminal matrix targeting signals (MTSs) which direct them into mitochondria and which are proteolytically removed in the matrix by the matrix processing peptidase [15–18]. As far as we know all matrix proteins embark on a common import route that employs the translocase of the outer membrane (TOM complex) and the translocase of the inner membrane (TIM23 complex). Translocation is driven by the membrane potential across the inner membrane and the hydrolysis of ATP in the matrix which is used by the import motor of the TIM23 complex to thread proteins into the matrix [19–22].

With about 50–100 proteins, the IMS of mitochondria contains a smaller number of proteins than the matrix. Nevertheless, these proteins carry out a number of important functions in particular in the transport of metabolites, proteins or lipids between both mitochondrial membranes, in the processing or assembly of mitochondrial proteins, in the communication between mitochondria and the rest of the cell or in the regulation of apoptosis. All proteins of the IMS are nuclear encoded and synthesized in the cytosol. Most of these proteins lack MTS sequences and employ diverse routes for mitochondrial import [23,24]. In many of these proteins internal targeting signals containing cysteine residues are critical for mitochondrial import. These targeting sequences were named MISS (mitochondria IMS-sorting signal) or ITS (IMS-targeting signal) and are both sufficient and necessary for the transport of proteins into the IMS [25,26]. The cysteine residues in these signals are recognized by the IMS-localized oxidoreductase Mia40 which serves as an intramitochondrial import receptor (Fig. 1). Mia40 contains a redox-active cysteine pair which is maintained in an oxidized state by the sulfhydryl oxidase Erv1. Mia40 and Erv1 constitute the mitochondrial disulfide relay system and are evolutionary conserved among plants, fungi and animals.

2.1. Substrates of the mitochondrial disulfide relay

The substrates that rely on Mia40 and Erv1 for mitochondrial import fall into several groups which will be introduced in the following.

2.1.1. Small Tim proteins (twin CX_3C proteins)

These proteins form hexameric complexes which facilitate the translocation of hydrophobic proteins across the lumen of the IMS. In mitochondria of baker's yeast, five Tim proteins were identified: Tim8, Tim9, Tim10, Tim12 and Tim13. The numbers indicate the approximate molecular weight of these small proteins. All these proteins consistently have a helix-loop-helix fold (Fig. 1A, left). Each helix contains two cysteine residues separated by three residues which gave rise to the name of twin CX_3C proteins. The helices are of antiparallel orientation and are stabilized by two parallel disulfide bonds connecting cysteine residues 1–4 and 2–3. Six small Tim proteins form heteromultimeric complexes ($[Tim9\text{-}Tim10]_3$, $[Tim8\text{-}Tim13]_3$) of about 70 kDa in which the loop regions are in close proximity to each other and the termini remain flexible similar to a jellyfish with 12 tentacles [27,28]. It was suggested that these tentacles can embrace substrate proteins to usher them across the IMS [28]. The cysteine residues are critical for the import and folding of the small Tims as well as for their assembly [25,28–31]. A mutation of the fourth cysteine residue of the human homolog of Tim8, DDP1, leads to absence of the DDP1-containing complexes causing the progressive neurodegenerative disease Mohr Tranebjaerg syndrome in affected individuals [32,33].

2.1.2. Twin CX_9C proteins

Like small Tim proteins, these proteins share a helix-loop-helix structure. Here, the cysteine residues are spaced by nine residues (Fig. 1A). The best characterized representative of this group is Cox17, a copper-binding protein that plays a role in copper transfer to cytochrome *c* oxidase [34,35]. It was proposed that the Cox17-mediated copper transfer is associated with a change of the number of disulfide bonds present in the protein [36]. However, experimental evidence is still lacking that could support a cycling of Cox17 through different redox states *in vivo*.

Besides Cox17, 13 additional proteins with twin CX_9C motifs were identified in yeast, most of which have homologs in mammals [37,38]. Many of these proteins are required for the assembly or stability of complexes of the respiratory chain, but their exact molecular function is still unknown.

2.1.3. Other substrates

In addition to these helix-loop-helix proteins, a small number of additional proteins that rely on the disulfide relay for mitochondrial

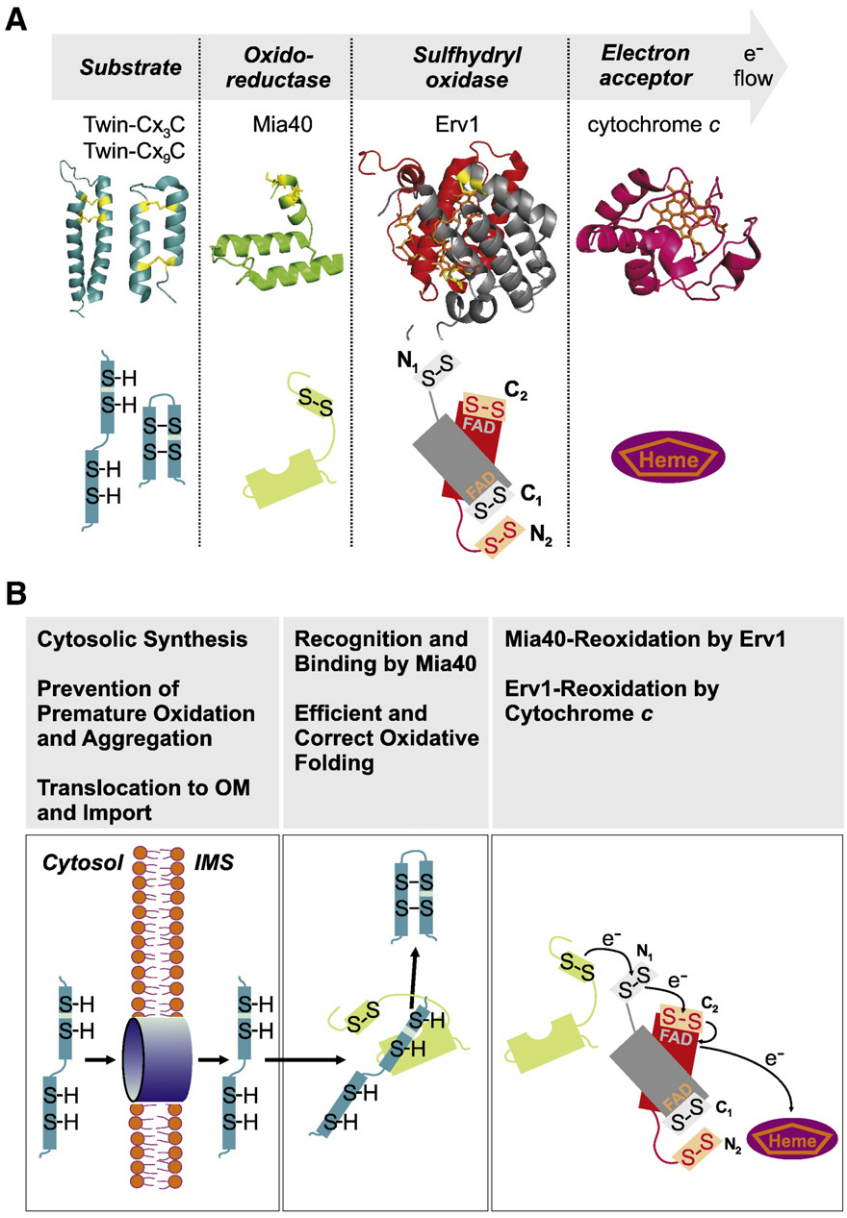

Fig. 1. Overview over the mitochondrial disulfide relay. (A) Components of the disulfide bond formation machinery in mitochondria. Depicted are the structures of Cox17 (twin CX_9C, pdb code: 1Z2G [36]), Tim9 (twin CX_3C, 3DXR [28]), Mia40 (2ZXT [42]), Erv1 (1OQC [43]) and cytochrome *c* (2PCB [89]). The respective pictograms are used in all figures throughout the manuscript. N_1, N_2, N domains; C_1, C_2, FAD domains of the Erv1 homodimer. Depicted in yellow, cysteines in disulfide bonds. (B) The import and folding of the substrate. This process can be subdivided into three steps: (1) substrate synthesis and translocation, (2) substrate oxidation by Mia40, and (3) reactivation of Mia40.

import were identified including Mia40 and Erv1 [37,39,40]. While Mia40 harbours two structural disulfide bonds in a twin CX_9C motif, Erv1 does only contain one structural disulfide in its flavodomain [41–43]. However, so far it has not been shown that the disulfide in Erv1 is indeed introduced by Mia40.

Moreover, the accumulation of copper-zinc superoxide dismutase, Sod1 and of its copper chaperone Ccs1 in the IMS depends on the disulfide relay system. Sod1 contains a structural disulfide bond which has been proposed to be introduced during interaction with its folding factor Ccs1 [44,45]. Both proteins, Sod1 and Ccs1, are dually localized in the cytosol and in the IMS. If the levels of Mia40 or Erv1 are depleted in mitochondria, the mitochondrial fractions of both proteins are severely diminished. Based on this observation it was suggested that Erv1 (and Mia40) might introduce the disulfide bond into Ccs1 which then is passed on to newly imported Sod1 protein [46,47].

2.2. Synthesis and transport of substrates to mitochondria

All nuclear encoded proteins of mitochondria are synthesized on cytosolic ribosomes and are imported posttranslationally into the organelle (Fig. 2). To pass the outer membrane substrates of the mitochondrial disulfide relay need to be in a reduced and unfolded state [48–50]. While it is unknown by which means substrates are kept unfolded in the cytosol, the binding of zinc ions might at least for some substrates stabilize the reduced state [49]. This additional stabilization of the reduced state might be necessary since the low redox potential of the disulfide bonds in twin CX_3C and twin CX_9C proteins would allow these bonds to persist even in the reducing cytosol. After import these zinc ions inhibit substrate oxidation and have to be removed. It has been proposed that the IMS-localized protein Hot13 participates in this step by removing zinc ions from imported proteins or from Mia40 [51,52].

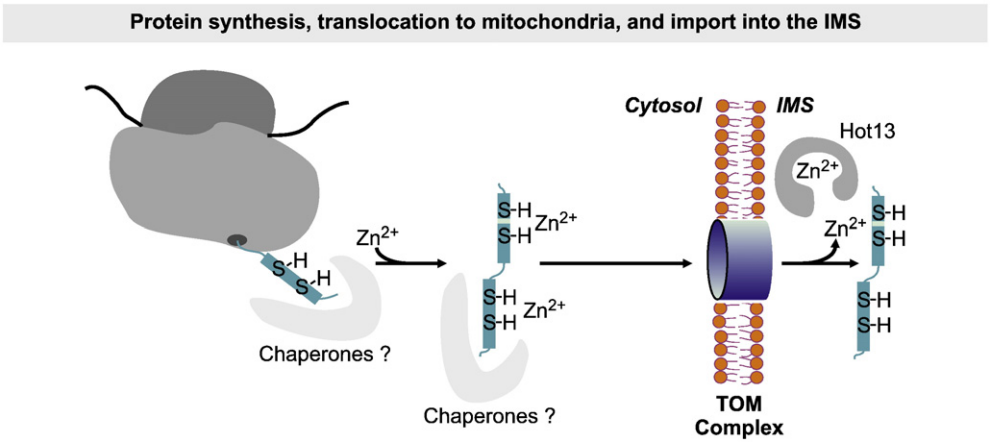

Fig. 2. Substrate synthesis and translocation into mitochondria. Substrates of the Mia40-Erv1 system are nuclear encoded and synthesized on cytosolic ribosomes. After synthesis they are kept unfolded by as yet unknown mechanisms. The binding of zinc ions presumably prevents their oxidation. Either prior or following translocation through the TOM complex zinc ions are removed. In the IMS the removal of zinc ions can be facilitated by the protein Hot13.

2.3. Protein import and substrate recognition by Mia40

Mia40 plays a central role in protein import: a diminished activity or reduced levels of Mia40 interfere with protein import into the IMS [53,54], and on the contrary, increased amounts of Mia40 lead to significantly higher import efficiencies into isolated mitochondria [40,55]. This suggests that Mia40 is the rate-limiting component of the import system. Conversely, alterations of the amounts of Erv1 do not significantly influence the import of Mia40 substrates into isolated mitochondria unless Erv1 is almost completely depleted [50,53,54,56].

The recently solved high-resolution structure of Mia40 revealed interesting insights into the mechanisms of substrate recognition [41,42]. Mia40 homologs share a highly conserved domain of about 60 residues in length comprising six invariant cysteine residues. The two N-terminal cysteines are separated by a proline forming the redox-active thiol pair that has the potential to interact covalently with substrates. This redox-active cysteine pair is C-terminally followed by a helix-loop-helix fold that resembles that of twin CX_9C proteins (Fig. 1A). The two anti-parallel helices form a fruit dish-like structure with a hydrophobic face on the inside and a hydrophilic face on the outside. The hydrophobic face is mainly formed by several surface-exposed phenylalanine residues, and is presumed to serve as substrate-binding cleft that interacts with the helix-loop-helix structures of twin CX_3C and twin CX_9C proteins via hydrophobic contacts [41,42]. Accordingly, replacement of even single residues for charged residues strongly interferes with Mia40 activity [42] (Fig. 3).

Recently, sequences in the substrates of Mia40 were identified as being crucial for mitochondrial import. In the case of Tim9, the consensus LXXXCF around the first cysteine residue serves as import signal [25], in the case of twin CX_9C proteins the consensus was aromatic-XX-hydrophobic-hydrophobic-XXC [26]. These signals were named MISS and ITS sequences, respectively, and both were suggested to provide the hydrophobic sequences that are docked onto the binding cleft of Mia40 (Fig. 3). In both cases, these signatures are in direct proximity to the critical cysteine residue which attacks the redox-sensitive CPC sequence of Mia40. Hence, the substrates of the disulfide relay system presumably are positioned by conserved signals to the oxidoreductase Mia40 which then oxidizes its substrates in a dedicated reaction scheme which is however not understood in full mechanistic detail (Fig. 4). Such a defined oxidation pathway is further supported by the observation that mutants of the small Tim proteins that lack the fourth cysteine are still bound by Mia40 but cannot be released and completely oxidized. In that case, the first cysteine (that of the MISS sequence) attacks Mia40 but cannot be connected to the fourth cysteine leading to a terminally stalled intermediate [30].

2.4. De novo formation of disulfide bonds by Erv1

Substrate oxidation converts the CPC motif of Mia40 transiently into its reduced form until it is reoxidized by the sulfhydryloxidase Erv1 [50]. Erv1 is a homodimeric protein in which each subunit consists of two domains that were referred to as N and FAD domain

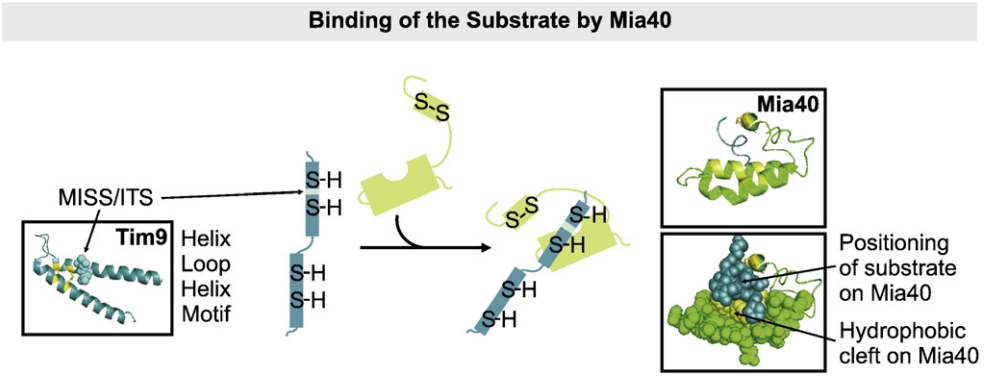

Fig. 3. Substrate recognition by Mia40 in the IMS. Reduced substrates are recognized by Mia40 by means of specific sequences around one of their conserved cysteines. A helix of the substrates is aligned in a perpendicular fashion on a hydrophobic groove of Mia40 thereby presumably positioning the cysteines of the substrate in proximity to the redox-active CPC motif of Mia40. Light green in Mia40 indicates the residues of the hydrophobic groove; light blue in the substrate indicates the amino acid residues of the MISS/ITS motif. Depicted in yellow, cysteines in disulfide bonds.

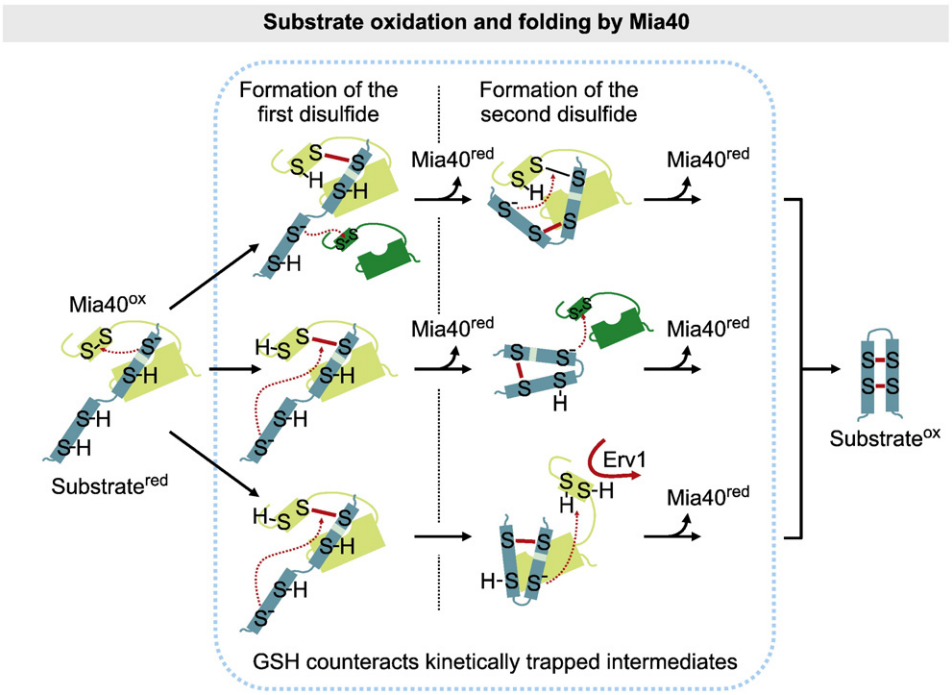

Fig. 4. Possible mechanisms of substrate oxidation. Mia40 introduces two disulfide bonds into twin CX_3C and twin CX_9C proteins. Possible reaction pathways start with the initial formation of a mixed intermolecular disulfide between Mia40 and the substrate. Theoretically, the first intramolecular disulfide bond in the substrate may be formed (1) by another Mia40 molecule (depicted in dark green; *top pathway*) (2) by the release of the first Mia40 molecule and the concomitant formation of the disulfide bond in the substrate. In this case a free semioxidized substrate molecule would be present as part of the reaction mechanism and a second Mia40 (depicted in dark green) would be required for the formation of the second disulfide in the substrate (*middle*). (3) Alternatively, the same Mia40 molecule might sequentially form both disulfides. In this case the substrate would remain non-covalently bound to Mia40 while Mia40 is reoxidized by Erv1 (*bottom*). Errors in the oxidation process can lead to the accumulation of kinetically trapped reaction intermediates which can be released in a glutathione-dependent step.

(Figs. 1A and 5). Each domain contains a conserved CXXC motif. In the C-terminal FAD domain, the redox-active isoalloxazine ring of the FAD cofactor is positioned in close proximity to the CXXC motif. The FAD is kept in place by adjacent helices 1 and 4 of a four-helix bundle that forms the core structure of Erv1 [43,57]. This four-helix bundle also contributes the hydrophobic interaction interface that stabilizes the Erv1 homodimer [43,58] (Fig. 5). No structural information could so far be obtained for the N domain, presumably due to its highly flexible nature which supports its function as shuttle arm to transfer electrons from Mia40 to the FAD domain of Erv1 [58,59].

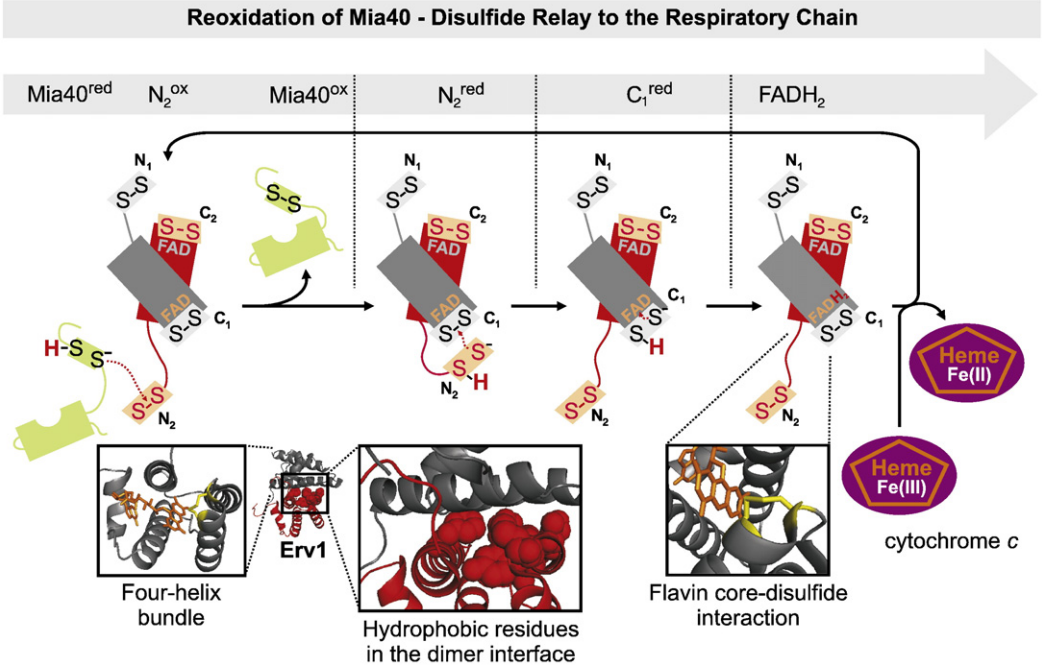

Fig. 5. The electron transfer from Mia40 to cytochrome *c*. Reduced Mia40 passes electrons onto the N domain of Erv1 thereby becoming oxidized again. Subsequently, electrons are passed on in an intersubunit transfer from the N domain to the FAD domain and further via the FAD cofactor onto cytochrome *c*. Depicted in yellow, cysteines in disulfide bonds.

The reoxidation of Mia40 is driven by a cascade of electron transfer reactions within Erv1 that have only recently been identified [58,59] (Fig. 5). In a first step a cysteine residue of the reduced CPC motif of Mia40 forms a mixed disulfide with a cysteine residue in the initially oxidized CXXC motif in the N domain of Erv1. This interaction presumably takes place between the second cysteine in the Mia40 CPC motif and the second cysteine of the CXXC motif in the N domain of Erv1 since only these cysteine residues are essential for the transfer reaction [41,58,60,61]. The specific recognition of Mia40 by the unstructured N domain suggests that Mia40 and the N domain form a well adapted interaction couple.

After Mia40 reoxidation the N domain is in a reduced state. It then passes electrons in an intersubunit transfer onto the CXXC motif of the FAD domain of the second subunit of the homodimer [58]. This electron transfer probably constitutes the specificity step in the oxidation reaction performed by Erv1 since the core CXXC can only be efficiently accessed by the N domain or small reductants like DTT, but not by other proteins or the reductants TCEP and reduced glutathione (GSH) [58,59,62]. Due to the positioning of the core CXXC in close proximity to the FAD cofactor electrons can finally be passed to the isoalloxazine moiety of FAD.

2.5. Electron transfer from Erv1

At least *in vitro* the reduced flavin cofactor in Erv1 can be reoxidized by various means. First, a direct reaction with molecular oxygen results in the transfer of two electrons and the subsequent release of hydrogen peroxide from Erv1 [59,62–65]. Secondly, Erv1 can also interact with oxidized cytochrome *c* which significantly accelerates the oxidation of Mia40 *in vivo* and *in vitro* [56,58,64–66]. Subsequently, cytochrome *c* can be oxidized by cytochrome *c* oxidase of the respiratory chain [58,65] or, at least in fungi, by the IMS component cytochrome *c* peroxidase [63]. Either way, channelling of the electrons through cytochrome *c* leads to the final production of water instead of hydrogen peroxide. At present it is still unclear which of the presented pathways is predominant *in vivo*. Support for a critical function of the respiratory chain comes from the fact that the loss of cytochrome *c* oxidase activity in mutants or upon cyanide treatment prevents efficient oxidation of Mia40 [65].

2.6. The energetics of the system

Recent *in vitro* reconstitution studies revealed mechanistic insights into the mitochondrial disulfide relay. These studies defined the minimal set of factors required for disulfide bond formation as well as the flow of electrons in this system. Moreover, the electrochemical potentials of all components as well as of several substrates were determined allowing a thermodynamic assessment of mitochondrial disulfide bond formation (Fig. 6).

Both disulfide bonds of the twin CX_9C and twin CX_3C motifs have electrochemical potentials between −310 and −340 mV [49,64,67–69]. Given the redox potential of the yeast IMS of about −255 mV [70], these disulfide bonds are presumably very stable in the IMS in living cells. The potential of the CPC redox-active motif in Mia40 (−290 mV) is more oxidizing than those of substrate disulfides [42].

The redox potentials in the redox-active CXXC motifs of the N and FAD domains of Erv1 and of the FAD cofactor are −320, −150 and −215 mV, respectively [63], [66]. Thus, the disulfide bond in the N domain should be surprisingly stable and initially this was attributed to a potential structural role of this domain. However, recent studies clearly demonstrated the role of the N domain in electron shuttling [58]. Although the redox potential of the redox-active cysteine pair in the FAD domain would theoretically allow a reaction with reduced glutathione, the architecture of Erv1 disfavours this reaction, thereby preventing the risk of futile cycles [58,59,71]. Instead, the FAD domain appears to be perfectly adapted to the interaction with the cysteine pair of the N domain in order to promote the efficient electron transfer through the components of the disulfide relay.

From the FAD in Erv1 electrons are finally shuttled onto cytochrome *c* and from there to the respiratory chain or directly onto molecular oxygen [63,65,66]. Due to the very oxidizing redox potential of cytochrome *c* and oxygen (the O_2/H_2O as well as the O_2/H_2O_2 couple) the overall oxidation reaction becomes irreversible.

3. The mitochondrial disulfide relay—what we still do not know

While detailed insights into different aspects of mitochondrial disulfide bond formation were gained in recent years numerous aspects remain to be addressed. Four of these will be discussed in the following: (1) the targeting of substrates to mitochondria, (2) the spatial organization of disulfide bond formation, (3) isomerisation processes in the IMS, and (4) the regulation of the disulfide relay under physiological conditions.

3.1. Targeting of Mia40 substrates to mitochondria

Receptor subunits of the TOM complex interact with MTS sequences of precursors destined to the mitochondrial matrix [72–75]. Targeting is facilitated by cytosolic chaperones of the Hsp70 and Hsp90 families which directly contribute to receptor binding [76]. A similar high-affinity binding to TOM subunits was not observed with substrates of the mitochondrial disulfide relay system like Tim13 [77]. Nevertheless, when mitochondria were simultaneously incubated with Tim13 and chemical amounts of a matrix protein, the import efficiency of Tim13 was significantly reduced suggesting that Tim13 shares its import route in part with matrix proteins, most likely while translocating through the TOM pore across the outer membrane [77].

If not via recognition by TOM receptors, how might the substrates of Mia40 become targeted to mitochondria? The co-purification of certain cytosolic mRNAs with mitochondria led to the hypothesis that the recognition of specific mRNAs to the mitochondrial surface contributes to the targeting of ribosomes that synthesize mitochondrial proteins [78,79]. The mRNAs of Mia40 substrates that were analyzed in these studies (Cmc3, Cox17, Cox23 and Pet191) did however not show a preferential fractionation with mitochondria but rather behaved like mRNAs of cytosolic proteins. Thus, the early steps in protein translocation to the IMS are still elusive and will need to be analyzed in the future.

3.2. Spatial organization of the machinery

Substrates of the mitochondrial disulfide relay have been found to covalently interact with Mia40 which therefore was initially termed the "import receptor" of the system [50]. However, it remains unclear whether Mia40 interacts during or after translocation with its substrates. An interaction with substrates in transit might contribute to their vectorial translocation across the OM. We still lack information on the machinery *in vivo*, in particular the spatial arrangement of its components. Interestingly, a ternary complex between Erv1, Mia40 and a substrate molecule was recently observed [80]. Such a complex appears perfectly suited to efficiently promote multiple rounds of substrate oxidation in the twin CX_3C and twin CX_9C proteins. During the shuttling of electrons in this complex substrates might remain associated with the hydrophobic cleft of Mia40 while both disulfide bonds are formed. This also should improve the oxidation kinetics because it would require fewer binding and release steps than alternative oxidation mechanisms (Fig. 4). The specific orientation of the two parallel disulfide bonds in substrates of Mia40 might be a consequence of this mechanism since the CPC motif should be able to form both disulfides without changing the orientation of the substrate in the binding cleft.

It furthermore remains unclear how the transfer of electrons from Erv1 to cytochrome *c* is coordinated. This is a challenging step as it represents a two-electron to one-electron transfer conversion in which harmful single-electron radical intermediates could be generated.

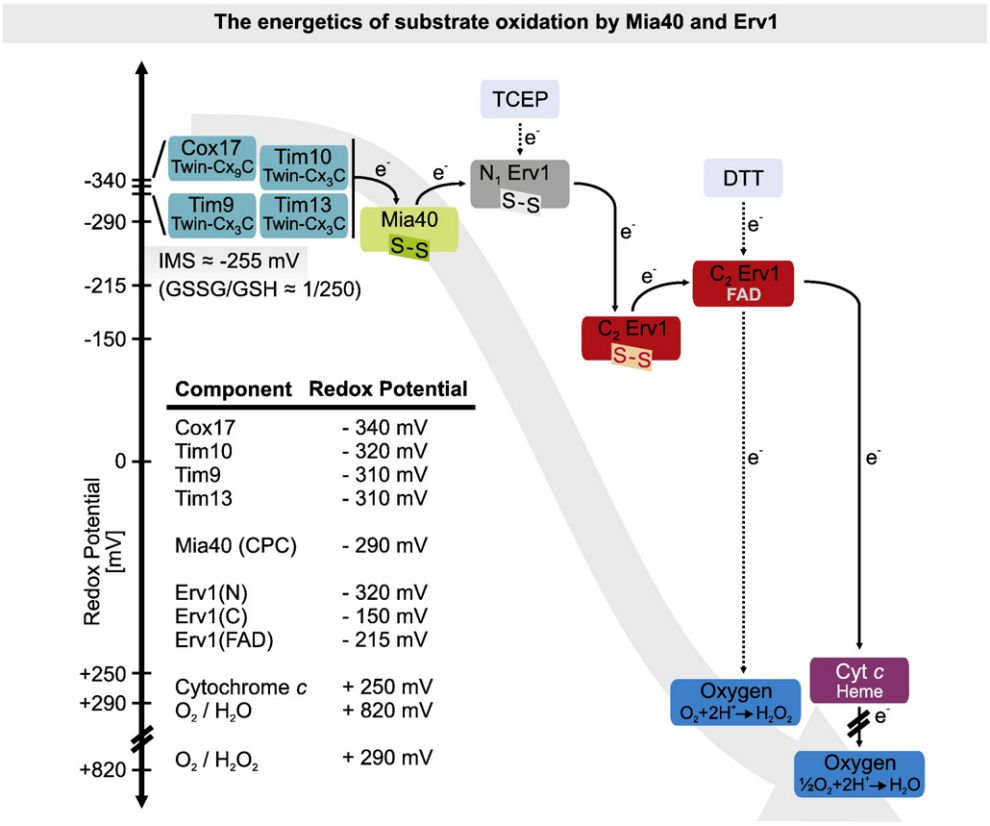

Fig. 6. The energetics of the mitochondrial disulfide relay. The redox potentials for the different players of the disulfide relay are shown on a linear scale. The differences in these potentials suggest that the exchange reaction between substrate, Mia40 and the N domain of Erv1 are in principle reversible. However, the coupling to the reduction of oxygen drives the electron flow vectorially from substrate proteins through the components of the system. For further details, see text.

3.3. Keeping substrate oxidation on track—isomerisation reactions

The well-characterized machineries that introduce disulfide bonds in the ER and the periplasm are known to form non-productive disulfide bonds as side products which are corrected by dedicated isomerisation machineries. These systems require electrons that are drawn from the cytosol, either via a specific trans-membrane protein shuttle system (DsbD in the periplasm) or by transport of GSH across the membrane (ER) [12,81,82].

The recent reconstitution of the mitochondrial disulfide relay indicated that also this system, at least *in vitro*, is prone to form kinetically trapped intermediates. GSH can counteract the accumulation of these unfavourable species and thereby considerably improves the performance of this system [58]. It remains to be shown to which extend such intermediates are formed *in vivo* and whether enzymes like glutaredoxins or thioredoxins contribute to these reduction/isomerization steps.

It is still unclear whether glutathione freely diffuses across the outer membrane via porins. Recently, it was demonstrated that the redox buffers of the cytosol and the IMS are distinct from each other with the IMS being more oxidizing [70]. The IMS might also contain enzymes to open unwanted disulfide bonds. One candidate for such a component is Cyc2, a flavoprotein that was suggested to maintain the cysteine residues in apocytochrome *c* reduced to allow the incorporation of its heme cofactor [83].

3.4. Regulation of protein oxidation under physiological conditions

In the ER, the oxidative folding machinery is regulated on the transcriptional as well as on the protein level [84–88]. This allows a flexible and fast adaptation to different physiological requirements thereby on the one hand preventing hyperoxidizing conditions in the ER lumen and on the other hand providing a sufficient oxidative capacity. It has to be analyzed in the future, whether similar regulation mechanisms exist for the mitochondrial system.

Most studies on mitochondrial disulfide bond formation were performed with crude mitochondrial extracts or with *in vitro* reconstituted systems. Therefore, many aspects of oxidative protein folding in living cells remain unclear. *In vivo*, additional proteins or locally variable oxygen concentrations might modulate the kinetics of the system. It will be exciting to address these aspects in the future in yeast as well as in mammalian cells and tissues.

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