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Disulphide Bond Formation in the Intermembrane Space of Mitochondria

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Proteins of the intermembrane space (IMS) of mitochondria fulfil crucial functions in cellular processes, such as transport of proteins and metal ions, ATP production and apoptotic cell death. All IMS proteins are synthesized in the cytosol and then transported across the mitochondrial outer membrane. A subset of these proteins contains disulphide bonds. For their import into the IMS, they employ a disulphide relay system, made up of two essential proteins, Mia40/Tim40 and the flavin-dependent sulfhydryl-electron transferase Erv1. The disulphide relay system introduces disulphide bonds in substrate proteins triggering their folding. The oxidative folding traps substrates in the IMS and thereby drives their net import into the IMS. Thus, protein import is coupled to oxidative protein folding, maybe providing a first control of protein quality. Here, we review the current knowledge about the Erv1-Mia40 system and address aspects that require further consideration.

Key words: disulphide bond formation, Erv1, intermembrane space, Mia40, protein import.

Abbreviations: ALR, augmenter of liver regeneration; *At*Erv1, Erv1 from *Arabidopsis thaliana*; CTC, charge-transfer complex; Ccs1, copper chaperone for Sod1; Cyt *c*, cytochrome *c*; DTT, dithiothreitol; DsbB, disulphide bond formation protein B; ER, endoplasmic reticulum; Erv, essential for respiration and vegetative growth in yeast; GR, glutathione reductase; IMS, intermembrane space; LipDH, dihydrolipoamide dehydrogenase; Mia, mitochondrial intermembrane space import and assembly; QSOX, quiescin-sulfhydryl oxidase; Trx, thioredoxin; TrxR, thioredoxin reductase; Sod1, copper/zinc superoxide dismutase; Tim, translocase of the inner membrane; TOM, translocase of the outer membrane; twin $Cx_{3,9}C$-motif, twin Cx_3C-motif or twin Cx_9C-motif.

Mitochondria are cellular organelles derived from proteobacterial ancestors. The mitochondrial envelope is composed of the outer and the inner membrane surrounding the intermembrane space (IMS) and the matrix, respectively. To fulfil their various functions, the compartments of mitochondria contain specific sets of proteins. Thus, mitochondrial proteins have to be transported to the correct compartment followed by folding into their functional conformations. Since the mitochondrial genome encodes only a few proteins, almost all mitochondrial proteins are synthesized in the cytosol and are subsequently imported into the different mitochondrial compartments. The imported proteins contain distinct targeting signals and use specific translocation pathways to reach their compartment (*1–3*).

The IMS of mitochondria harbours a class of proteins with low molecular mass containing cysteine residues that are organized in twin Cx_3C or twin Cx_9C motifs. All known proteins with twin Cx_3C motif belong to the family of small Tim proteins which function as chaperones in the IMS during the transport of hydrophobic membrane proteins (*4–6*). The best studied member of the group with twin Cx_9C motif is the copper chaperone Cox17 which is required for the biogenesis of the cytochrome *c* oxidase (*7*). From structural analysis of the small Tim proteins and Cox17 it can be concluded that proteins with twin Cx_3C motif or twin Cx_9C motif have a simple single folding unit in common (*8*, *9*). The two $Cx_{3,9}C$ segments are juxtaposed in anti-parallel α-helices and are linked by two disulphide bonds to form a hairpin-like structure. The disulphide bonds are crucial for folding and stabilization of the structure. The presence of disulphide bonds in the IMS was quite unexpected because of its connection to the reducing cytosol in contrast to oxidizing compartments such as the endoplasmic reticulum (ER). However, many data support that the redox properties of the IMS are different from those of the cytosol and that formation of disulphide bonds in the IMS is a common principle (*4*, *6*, *10–12*). Disulphide bonds are present in several proteins located in or facing the IMS, such as the superoxide dismutase Sod1, the copper chaperone for Sod1 (Ccs1), the Rieske FeS protein and the acidic hinge protein of the cytochrome bc_1 complex, and the subunit 12 of the cytochrome *c* oxidase (*4*, *6*, *11*, *12*). It has been shown that several of the proteins with disulphide bonds employ a folding-trap mechanism for import demonstrating a novel function of disulphide bonds in protein translocation. Following transport of the unfolded polypeptide across the

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translocase of the outer membrane, the TOM complex, incorporation of disulphide bonds triggers folding of the proteins and thereby traps them in the IMS (*5*, *13*, *14*). The exergonic process drives the import of the proteins into the IMS. The coupling of protein import and oxidative folding could also reflect a mechanism of protein quality control. This novel translocation pathway uses the disulphide relay system consisting of the two essential components Mia40 and Erv1 (*14–17*).

MIA40 IS THE CENTRAL COMPONENT OF THE DISULPHIDE TRANSFER SYSTEM

Mia40/Tim40 was first identified and characterized in *S. cerevisae* (*18–20*). Homologs are also present in other fungi, amoebae, plants, and animals. As shown for *S. cerevisiae* and mammalian cells, Mia40 is essential for viability (*18–21*). In contrast, *in silico* analyses suggest that Mia40 is absent in several groups of phylogenetically very distant organisms such as kinetoplastid and apicomplexan parasites despite the presence of small Tim proteins [M.D., unpublished data, (*22*)]. Thus, the import machinery known from yeast and mammals probably underwent significant changes throughout evolution.

Homologs in fungi and in higher eukaryotes differ in their length of amino acid residues. The fungal proteins have a cleavable N-terminal mitochondrial targeting signal followed by a hydrophobic transmembrane segment and an extended C-terminal hydrophilic domain. These proteins are anchored with the hydrophobic segment in the inner membrane facing the IMS (*19*, *20*). In contrast, the shorter homologs in higher eukaryotes lack a cleavable mitochondrial targeting signal and the hydrophobic membrane anchor (*21*). They are soluble in the IMS. Nonetheless, human MIA40 can functionally replace Mia40 in yeast (*23*). All homologs have a highly conserved segment of about 60 amino acid residues, which is sufficient to fulfill the function of Mia40 (*24*).

In all species, this domain contains six absolutely conserved cysteine residues arranged in a CPC-segment and a twin Cx_9C-motif. Two redox forms of Mia40 can be distinguished in mitochondria: a partially reduced and an oxidized form which is the more prominent one (*15*, *24*). The molecular nature of the redox states was determined by mass spectroscopy of the purified protein. In its oxidized state, Mia40 forms three intramolecular disulphide bonds: one bond between the cysteine residues of the CPC-segment and two bonds connecting the two helices of the Cx_9C-segments (Fig. 1A) (*25*). The latter bonds are still present in the partially reduced form of Mia40, whereas the cysteine residues of the CPC-segment are present in their thiol states (Fig. 1A). In contrast to the bonds in the twin Cx_9C-motif, the disulphide bond of the CPC-segment is easily accessible to reducing agents and forms a redox-active disulphide bond. The solution structure of human MIA40 confirmed the presence of these disulphide bonds (Fig. 1B) (*26*). The folded conserved domain consists of an N-terminal α-helix with the redox-sensitive CPC-segment and an α-helical hairpin core. The anti-parallel α-helical hairpin is stabilized by the two redox-resistant disulphide bonds. In consistence with their redox properties, the structure suggests a catalytic function of the CPC cysteine pair and a structural role for the twin Cx_9C cysteine residues.

MIA40 FORMS DISULPHIDE INTERMEDIATES WITH SUBSTRATE PROTEINS

The redox-active disulphide bond of Mia40 has been shown to be required for the interaction with substrate proteins upon import into mitochondria (*25*, *26*). In consistence with a receptor function in the IMS, Mia40 specifically recognizes its substrates (*18–20*). The N-terminal cysteine residue of the first Cx_3C motif in Tim9 and Tim10 is crucial to form the disulphide bond with Mia40 (*27*, *28*). However, this might not apply to all Mia40 substrates. In Cox17, the inner pair of cysteine residues rather than the outer pair was suggested to be important to generate the disulphide intermediate (*26*).

Obviously, a cysteine residue is needed to form the covalent bond. So far, no interaction between Mia40 and substrate has been detected under reducing conditions. However, it is likely that a low affinity interaction exists. A sequence around the N-terminal cysteine residue of Tim9, consisting of nine amino acid residues and containing an important leucine residue, has been recently reported to act as mitochondrial IMS sorting signal and as substrate recognition signal of Mia40 (*29*). The leucine residue is conserved in Tim9 homologs and yeast Tim10. In addition, hydrophobic residues at this position appear to be evolutionary conserved among small Tim proteins pointing to a crucial role for hydrophobic interactions in Mia40 substrate recognition (*30*). Such a hypothesis is in accordance with the structure of MIA40 which reveals a hydrophobic surface close to the CPC-segment (Fig. 1C) (*26*). It has been proposed that the substrate protein binds to this surface allowing a cysteine residue to form a bond with the second of the CPC cysteine residues of Mia40 (*26*). Further analysis will be required to determine the molecular details of signal recognition.

MIA40 IS THE SUBSTRATE OF ERV1

To interact with substrate proteins, Mia40 has to be converted to its oxidized form, a process requiring the flavin-dependent oxidoreductase Erv1 (*15*). Erv1, which is present in all eukaryotes, is essential for viability in yeast (*31*). Like Mia40, it is located in the IMS of mitochondria (*32*). Initial studies have shown a variety of defects in the absence of functional yeast Erv1, such as impaired respiration, altered mitochondrial morphology and distribution, loss of the mitochondrial genome, and defects in the biogenesis of cytosolic FeS cluster-containing proteins (*31–33*). Later on, Erv1 turned out to act in the same pathway as Mia40 (*15–17*). Cells lacking functional Erv1 have decreased amounts of small twin $Cx_{3/9}C$ motif-containing proteins in the IMS of mitochondria as a consequence of their impaired import and assembly. Even more intriguingly, lack of Erv1 results in accumulation of the reduced non-functional form of Mia40 (*15*). Erv1 transiently interacts with Mia40 via intermolecular disulphide bonds suggesting a direct function on Mia40 (*18*, *20*). Indeed, the oxidation of Mia40

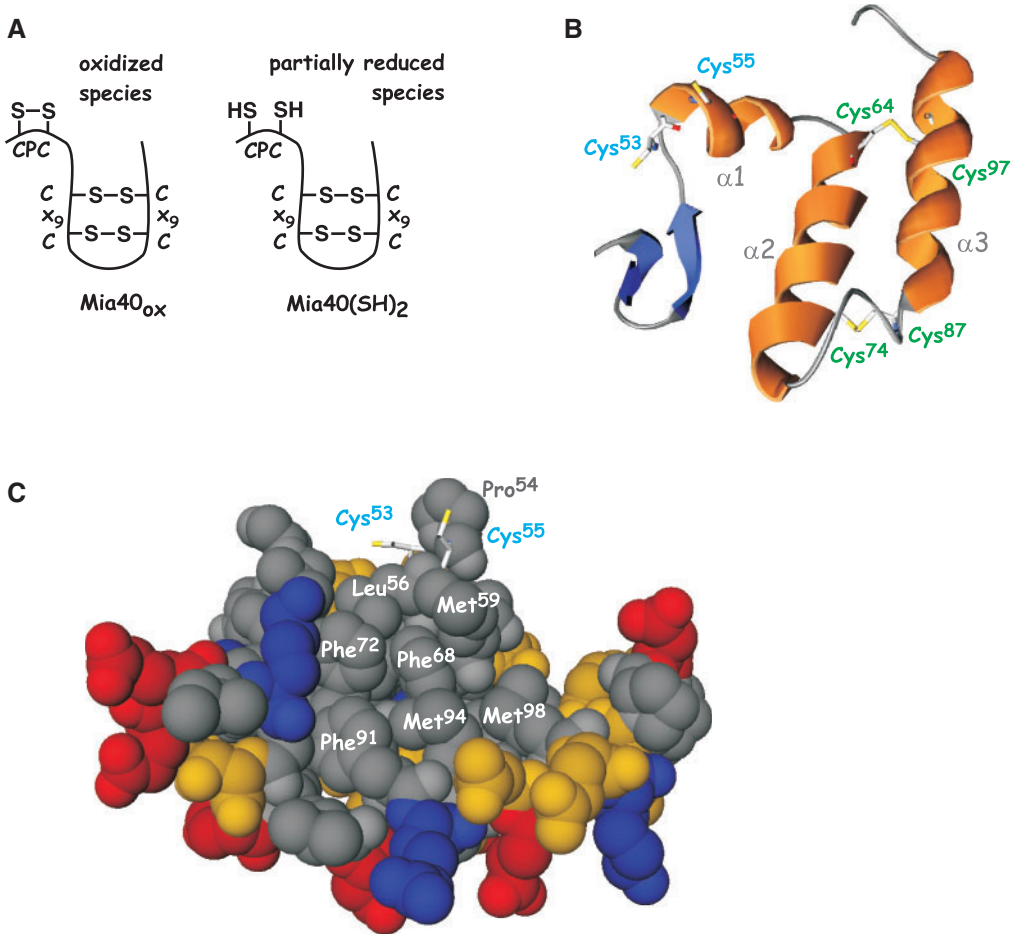

Fig. 1. **Structure and redox states of Mia40.** (A) Schematic representation of the conserved domain of Mia40 in its fully oxidized and partially reduced redox state. (B) NMR-structure of human MIA40 (see text for details). (C) A hydrophobic surface of MIA40 is probably involved in substrate binding and is in close proximity to the accessible CPC active site cysteine residues. Hydrophobic, polar and charged residues are shown in grey, yellow and red/blue, respectively. The pictures were generated using Swiss-PDB Viewer and the PDB file 2K3J (*26*).

by Erv1 was demonstrated with purified components (*25*). As observed *in vivo* and *in vitro*, Erv1 forms a disulphide intermediate with the second cysteine residue of the CPC-motif regenerating the redox-sensitive bond in Mia40 (*24*, *25*). This reconstitution proved that Mia40 is the first known physiological substrate of Erv1.

THE ERV1-MIA40 COUPLE FOLLOWS COMMON PRINCIPLES IN FLAVIN BIOCHEMISTRY

Despite significant structural differences that were previously outlined by Deborah Fass (*34*), the flavoprotein/thiol couple Erv1/Mia40 follows the same chemical principle found also in the couples thioredoxin reductase (TrxR)/thioredoxin (Trx) (*35*), glutathione reductase (GR)/glutathione (*36*) and dihydrolipoamide dehydrogenase (LipDH)/lipoamide (*37*): under physiological conditions, the flavoenzyme connects one or more thiol–disulphide exchange reactions to the oxidation or reduction of non-sulphur compounds, such as NAD(P)/H or cytochrome *c* (Cyt *c*). Thus, thiol-dependent reaction pathways can be linked to other metabolic pathways using flavoproteins as redox switches. A remarkable exception of this principle is the oxidation of protein thiol groups in the bacterial periplasm, where the membrane protein DsbB uses ubiquinone or menaquinone instead of a flavin to link thiol–disulphide exchange reactions to the electron transport chain (*38*).

For Erv1, reduced Mia40 donates electrons to oxidized flavin via a disulphide relay system (*14*, *15*, *17*, *24*, *25*); the reduced flavin subsequently transfers the electrons to the electron acceptor which is probably Cyt *c* (see below) (Fig. 2A) (*16*, *39–41*). Considering the thiol/disulphide substrate, the flow of electrons in Erv1 is in the same direction as in LipDH and in the opposite direction as in TrxR and GR. FAD can be used for the transfer of single electrons as well as for the transfer of an electron pair (*e.g.* from NADPH to a disulphide in GR, TrxR and other transhydrogenases). Moreover, flavins are perfect redox switches between the transfer of two single electrons and one electron pair [*e.g.* (de)hydrogenase-electron transferases from the respiratory and the photosynthetic chain]. Erv1 probably belongs to the latter group (see below). The enzymatic mechanisms of Erv1 and other flavoproteins can be divided into two half-reactions: during the reductive half-reaction the flavin is reduced

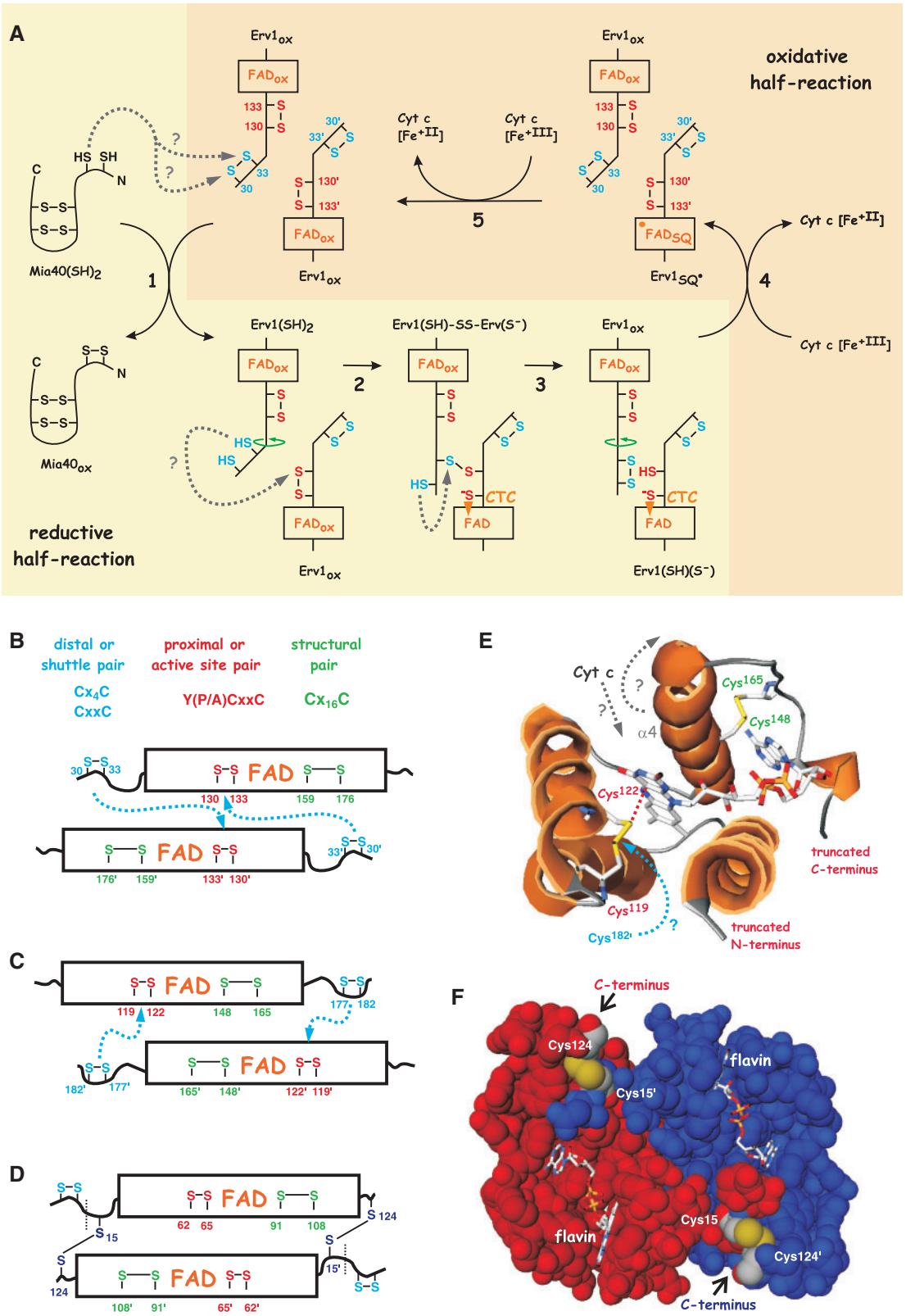

Fig. 2. **Proposed catalytic mechanism and structural aspects of Erv1/ALR.** Some aspects that need to be addressed in future studies are labelled with a question mark. For details see text. (A) Model of the reductive (steps 1–3) and oxidative (steps 4 and 5) half-reaction of homodimeric yeast Erv1. Significant structural changes due to the movement of the N-terminal tail carrying the shuttle cysteine residues are indicated by green arrows. The single electron transfers during the oxidative half-reaction and the species involved could differ among Erv-like proteins. CTC, charge–transfer complex;

and the first substrate is oxidized. During the oxidative half-reaction the reduced flavin is reoxidized and the second substrate becomes reduced. Both of the half reactions can be analysed separately by omitting one of the substrates. In the following two paragraphs a model of Erv1-catalysis is presented (Fig. 2A). Please note that most of the steps have not been experimentally confirmed yet.

THE REDUCTIVE HALF-REACTION OF ERV1/ALR

Despite the structural differences and the opposite direction of the electron flow, the proposed mechanism for the reductive half-reaction of Erv1/ALR (Fig. 2A) is very similar to the oxidative half-reaction of high molecular weight TrxR (*35*).

Three crystal structures from Erv1-like proteins are available to date: Erv2 from yeast (PDB entry 1JR8) (*42*), rat ALR (1OQC) (*43*) and *Arabidopsis thaliana* Erv1 (*At*Erv1) (2HJ3) (*44*). All three proteins crystallized as head-to-tail homodimers which is in perfect agreement with previous observations on yeast Erv1 (*45*, *46*) supporting the view, that a rather poorly conserved pair of 'distal' or 'shuttle' cysteine residues from one Erv1/ARL subunit interacts with the dithiol substrate and subsequently transfers the electrons from the protein surface to a less exposed environment at the second subunit. Based on data *in vitro* and *in vivo* (*24*), the second cysteine residue in the CPC motif of Mia40 interacts with the Erv1 shuttle disulphide bond. So far, it is unclear whether the intermediate intermolecular disulphide bond is formed with Cys^{30} or Cys^{33} of Erv1 (step 1 in Fig. 2A). In general, the two shuttle cysteine residues of Erv1-like proteins are located on a presumably flexible arm either at the N-terminus (*e.g.* yeast Erv1 and ALR, Fig. 2B and D) or the C-terminus (e.g. yeast Erv2 and *At*Erv1, Fig. 2C). The number and type of amino acids separating the shuttle cysteine residues depends on the organism, *e.g.* a single glycine residue in yeast Erv2, two residues in yeast Erv1 and human ALR, and four residues in *At*Erv1. This variety is comparable with the shuttle residues in high molecular weight TrxR (*35*).

In the structures of rat ALR and *At*Erv1, the distal residues are missing or disordered, respectively, whereas in the structure of yeast Erv2 the C-terminal arm with the CGC disulphide was indeed found to be close to the redox center of the second subunit (*42–44*). This redox centre is composed of the flavin and a 'proximal' or 'active site' pair of cysteine residues in a Y(P/A)CxxC-motif (Fig. 2E) which can be reduced by DTT (*43*). The N-terminal cysteine residue in this motif, the so-called 'interchange cysteine', forms an intermediate mixed disulphide bond with one of the shuttle cysteine residues (*42*, *46*). In yeast Erv1, the interchange residue Cys^{130} could be attacked by residue $Cys^{33'}$ rather than $Cys^{30'}$ (Fig. 2A, step 2) because yeast cells carrying a mutation of Cys^{30} are still viable in contrast to Cys^{33} mutants (*46*). The more C-terminal residue at the active site, Cys^{133}, is the so called 'charge-transfer cysteine' because it can form in its reduced state a thiolate–FAD charge-transfer complex (CTC) (Fig. 2A, steps 2 and 3) which was probably stabilized in the yeast mutant $Erv1^{C130S}$. Spectra from two electron reduced mammalian ALR were interpreted to show no CTC but to reflect the formation of a semiquinone (*39*, *43*). The authors suggested that this points to a highly negative redox potential of the proximal cysteine disulphide. Accordingly, the product of step 3 in Fig. 2A could be replaced by two subunits of ALR semiquinone due to the rapid tunnelling of an electron across the dimer interface. Whether this is a special feature of ALR and whether the two active sites of the Erv1/ALR homodimer act in a concerted manner needs to be studied.

THE OXIDATIVE HALF-REACTION: IS ERV1/ALR AN ELECTRON TRANSFERASE OR AN OXIDASE?

Many flavoproteins possess a more or less pronounced dehydrogenase–oxidase activity with oxygen as electron acceptor *in vitro* (*47*, *48*). However, oxidase activities of many flavoproteins often reflect side reactions *in vitro* that can be much slower in the presence of the physiological electron acceptor.

Erv1 is commonly described as a sulfhydryl oxidase because of (i) the oxidation of dithiols in the presence of O_2 *in vitro* (*39*, *44–46*), (ii) the high sequence similarity with other members of the quiescin-sulfhydryl oxidase (QSOX) family and (iii) current models for disulphide formation in the endoplasmic reticulum which is catalysed by the flavoprotein Ero1 (*49*, *50*) and QSOX/Erv2 (*51*). The latter flavoproteins connect the oxidation of sulhydryl groups of secreted proteins and/or protein disulphide isomerase to the reduction of O_2. According to these models, one molecule of H_2O_2 is produced in the endoplasmic reticulum per disulphide bond. H_2O_2 generated by Erv1 could be removed by cytochrome *c* peroxidase (*41*). However, oxygen does not have to be the electron acceptor for homologous Erv1/ALR. Indeed, the following studies rather support the classification of Erv1 as a sulfhydryl-electron transferase and not as a sulfhydryl-oxidase (*47*, *48*; http://www.chem.qmul.ac.uk/iubmb/etp/etp1t3.html for the classification

•FAD_{SQ}, FAD semiquinone. (B–D) Comparison of disulphide bond patterns in yeast Erv1 (B), *At*Erv1 (C) and mammalian ALR (D). Intermolecular interactions between the two subunits during electron transfer are labelled with arrows. The long and the short form of ALR lacking the distal cysteine residues are distinguished by a dotted line. The intermolecular disulphide clamp is shown in purple. (E) Structure of one *At*Erv1 subunit with FAD and disulphide bonds highlighted. The dotted line indicates a distance of 3.5 Å between the S_γ atom of the cysteine residue and the C-4a atom of the flavin where a CTC could be formed after reduction. The C-terminal tail of the protein was not resolved in the structure. Residue $Cys^{182'}$ might be better suited for interaction with the interchange residue Cys^{119}, in analogy to the structure of Erv2 (*42*). A putative structural change at the C-terminus of helix α4 improving the accessibility of the *si* side of the flavin and therefore the transfer of electrons to Cyt *c* is labelled as well. (D and F) In contrast to yeast Erv1 and *At*Erv1, an intermolecular disulphide clamp is formed between less conserved cysteine residues in the short form of ALR (*43*). Both residues are too far away to directly interact with the flavin and their function remains to be studied. Pictures of *At*Erv1 (E) and rat ALR (F) were generated using Swiss-PDB Viewer and the PDB files 1OQC (*43*) and 2HJ3 (*44*).

of flavoproteins): (i) Farrell and Thorpe clearly showed that reduced ALR prefers two molecules of Cyt *c* [Fe^{+III}] over one molecule of O_2 *in vitro* (Fig. 2A, steps 4 and 5). Considering the K_m^{app} values and the concentrations of Cyt *c* and O_2 in the IMS the difference becomes even more significant (*39*). (ii) Accordingly, Cyt *c* was also shown to be a good electron acceptor for yeast Erv1 *in vitro* and *in vivo* linking Erv1 oxidation to the respiratory chain (*16*, *40*, *41*). (iii) Since Cyt *c* was pulled down with Erv1 (*41*), and since the Cyt *c* reductase activity of ALR could not be inhibited by the addition of superoxide dismutase (*39*), a rather strong and direct interaction between Erv1/ALR and Cyt *c* seems to be likely. Because the *si* side of the flavin is quite shielded in the crystal structures, we hypothesize that the C-terminal part of helix α4 might undergo structural changes to allow substrate binding and electron transfer (Fig. 2E). Alternatively, electrons might tunnel to Cyt *c* or might be transferred via radical sulphur species. (iv) Dehydrogenase-electron transferases often form semiquinone radicals of at least moderate stability (*48*). In consistence with a function as an electron transferase a stable semiquinone was observed for the short form of ALR during aerobic turnover of DTT (*39*). In contrast, the flavin semiquinone was found to disappear quite rapidly in case of *At*Erv1 (*52*).

Based on the available data, the term Mia40- or sulfhydryl-cytochrome *c* reductase (EC 1.8.2) seems to be better suited to describe yeast Erv1 and mammalian ALR than the term sulfhydryl oxidase (EC 1.8.3). Considering the positions, structures, redox potentials and reactivities of the shuttle cysteine residues and the other redox couples, there are probably significant differences between Erv1-like proteins from different species and alternative electron donors and acceptors are likely to be discovered.

DO DISULPHIDE BONDS OF ERV1/ALR HAVE REGULATORY FUNCTIONS?

A third disulphide bond between two well conserved cysteine residues in a $Cx_{16}C$-motif of the ALR/Erv1-domain is located close to the adenine moiety of FAD and is roughly 15 Å away from the active site (Fig. 2B–E). This disulphide bond was detected in all three crystal structures and has been classified as a 'structural disulphide' bond (*42–44*). The more C-terminal cysteine residue of this couple is clearly solvent accessible in the structures. The disulphide bond is conserved in Erv1-homologs from many eukaryotes including kinetoplastid but not apicomplexan parasites (M.D., unpublished data). Mutations of the disulphide bond in yeast Erv1 and *At*Erv1 affected their functions (*46*, *52*). A regulatory and/or allosteric function of the structural disulphide remains to be studied.

In addition, an intermolecular disulphide bond we would like to term 'disulphide clamp' was observed in the crystal structure of rat ALR lacking the N-terminal shuttle cysteine residues (*43*). The bond was also found in solution but is not involved in catalysis of artificial low molecular mass substrates probably due to its distance to the active site (Fig. 2F) (*39*). The disulphide clamp is formed between a single cysteine residue in a CP-motif in front of the ALR/Erv1-domain of one subunit and a single cysteine residue close to the C-terminus of the other subunit (Fig. 2D). The first cysteine residue is conserved among Erv1-homologs from many eukaryotes including kinetoplastid but not apicomplexan parasites, whereas the second residue is less conserved but can be also found, *e.g.* in *Drosophila*, *Caenorhabditis* and *Neurospora* (M.D., unpublished data). Both cysteine residues are missing in yeast Erv1 which seems to be rather an exception. A regulatory or structural function of the disulphide clamp, such as stabilizing the homodimer or anchoring the end of the flexible arm, cannot be ruled out and needs to be addressed.

ERV1 AND MIA40 COOPERATE IN OXIDATIVE FOLDING IN THE IMS

The disulphide relay system of Mia40 and Erv1 couples the import and the folding of IMS proteins containing disulphide bonds (Fig. 3). Current data suggest a model of a 'linear disulphide transfer pathway' (*14*, *15*, *24*, *26*). According to this model, disulphide bonds are introduced in substrate proteins by Mia40 which transfers electrons to oxidized Erv1 and then to an electron acceptor, such as Cyt *c* (*39–41*). Indeed, steps of the process could be reconstituted (*24–26*). Reduced proteins with a twin Cx_9C-motif were oxidized by Mia40 *in vitro* (*25*, *26*). This reaction requires the presence of the redox-sensitive bond of Mia40 in its oxidized form, whereas the structural bonds of the twin Cx_9C-motif in Mia40 are not involved. Catalytic amounts of Mia40 are not sufficient to oxidize substrate proteins. Thus, Mia40 does not function as a thiol oxidase generating disulphide bonds by transfer of electrons to oxygen. Together with Erv1, on the other hand, catalytic amounts of Mia40 oxidize substrate proteins with twin $Cx_{3,9}C$-motif (*24*, *25*). In consistence with a 'linear disulphide transfer pathway', Mia40 is absolutely needed, since Erv1 alone does not form disulphide bonds in the substrates *in vitro* (*24*).

In substrates with twin $Cx_{3,9}C$-motif the two disulphide bonds appear to be introduced in a coordinated manner (*24*, *25*, *28*, *53*). How the second disulphide bond is formed, is an open question. There are several possible mechanisms: first, it has been suggested that the second disulphide bond can be introduced *in vitro* by oxygen in the presence of Mia40 (*26*). However, the role of oxygen *in vivo* is not clear. Since yeast cells grow anaerobically, and since it is unlikely that oxygen produced within the cell is sufficient, oxygen seems not to be essential for the disulphide bond formation. Second, Mia40 might catalyse incorporation of both disulphide bonds. Thus, two cycles of oxidation by Mia40 would function in a coordinated manner. Third, reduced small Tim proteins can be oxidized by glutathione, even at GSH/GSSG ratios found in the cytosol (*54*). Thus, the second bond in substrates might be formed by oxidized glutathione, while the substrate interacts with Mia40. Fourth, recent data might point to a pathway, we like to term 'combined disulphide transfer pathway' meaning that Erv1 might introduce disulphide bonds not only in Mia40, but also in substrate proteins bound to Mia40.

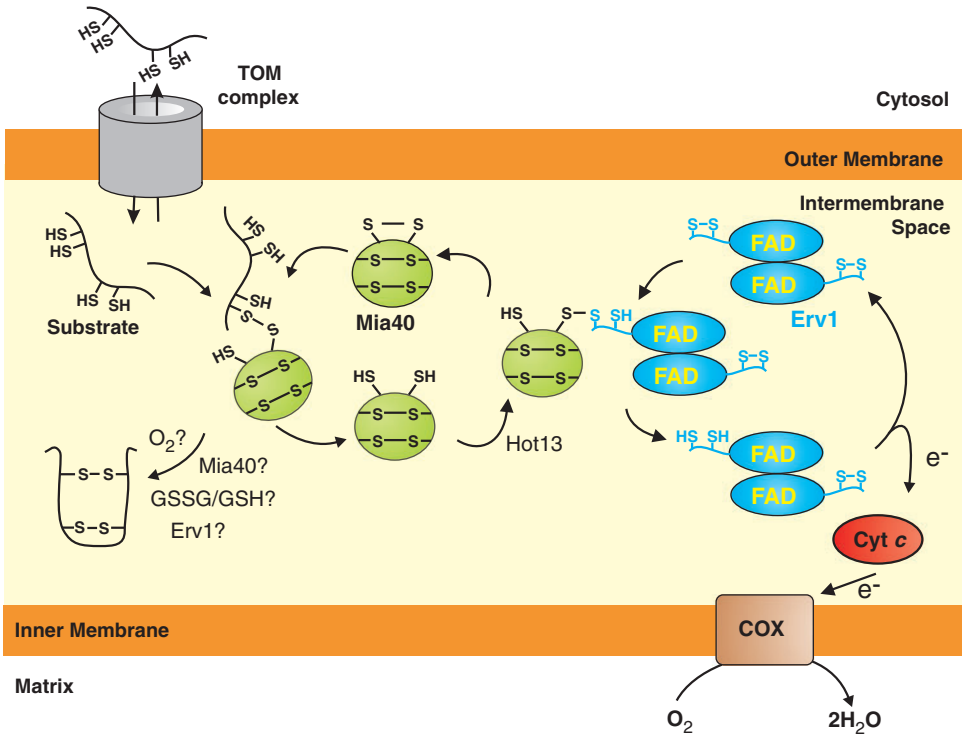

Fig. 3. **Model of the Erv1-Mia40 disulphide relay system that drives import of proteins into the IMS.** Substrate proteins containing twin Cx_3C or twin Cx_9C motifs cross the TOM complex in a reduced and unfolded state. Substrates then form a transient disulphide intermediate with Mia40 followed by their release in the oxidized form. The oxidation triggers folding of the substrates which are trapped in the IMS, since the folded proteins cannot pass the TOM complex. It is still unknown, how the second disulphide bond in the oxidized substrate is generated. A second Mia40, glutathione, oxygen or Erv1 may play a role for the oxidation (see text). Following substrate release the redox-active disulphide bond in Mia40 is reduced. The oxidized form of Mia40 is regenerated by the FAD binding protein Erv1. Reduced Erv1 subsequently shuttles electrons (e^-) to Cyt *c*. These redox reactions of Erv1 are described in detail in the text and in Fig. 2. Cyt *c* transfers the electrons via Cyt *c* oxidase (COX) to the final electron acceptor, oxygen. For reoxidation of Erv1 and Cyt *c* an alternative pathway via molecular oxygen and Cyt *c* peroxidase has been reported (*41*).

This hypothesis is supported by the analysis of temperature-sensitive mutants of Erv1, which still allowed the formation of the Mia40-substrate disulphide intermediate, but inhibited the release of oxidized substrates (*55*). Furthermore, a ternary complex of Mia40, imported substrate, and Erv1 was detected in limited amounts in mitochondria (*53*). It has been suggested that Erv1 promotes oxidation of substrates while both Erv1 and substrate are associated with Mia40 (*53*). However, the physiological role and the exact composition of this complex are not known to date.

The import pathway of IMS proteins is presumably affected by additional factors. Zinc ions have been reported to play a role for the import of proteins into the IMS (*18*, *56–58*). They might prevent oxidation of small Tim proteins in the cytosol and thereby keep them in the import-competent reduced state (*57*). On the other hand, zinc ions stabilize the partially reduced form of Mia40 and inhibit the activity of Erv1 (*57*, *58*). Thus, they may inhibit the activity of the disulphide transfer system. Interestingly, there is a conserved zinc-binding protein, Hot13, in the IMS which is crucial for the biogenesis of small Tim proteins (*59*). Hot13 has been reported to physically interact with Mia40 and might improve the Erv1-dependent oxidation of Mia40 and thus the import of small Tim proteins by counteracting inhibitory effects of zinc ions (*58*).

Considering the mechanism of the Erv1-Mia40 system, it is noteworthy that the system also mediates import of Erv1 and Ccs1, the copper chaperone for Sod1 (*60–63*). Ccs1, a complex protein consisting of three domains, lacks a twin $Cx_{3,9}C$-motif (*64*, *65*). Thus, the function of the Erv1-Mia40 system is not restricted to twin $Cx_{3,9}C$-motif containing proteins indicating a broader substrate specificity. Because of the covalent interaction of Ccs1 with Mia40 via disulphide bonds, it appears likely that the Ccs1 protein is oxidized by the Erv1-Mia40 system. The distribution of Sod1 between the cytosol and the IMS is also affected by the disulphide transfer system, probably via Ccs1, which promotes mitochondrial import of Sod1 (*62*, *63*). Since the disulphide relay system is coupled to the respiratory chain, the distribution of Ccs1 and Sod1 might be regulated by the activity of the respiratory chain and the metabolic state of mitochondria (*62*, *63*). Indeed, inhibition of complex III of the respiratory chain increased Sod1 level in mitochondria in a Ccs1-dependent manner (*63*). Such regulation might allow efficient protection against oxidative stress.

The identification of different kind of substrates might suggest mechanistic variability of the disulphide relay

system for the oxidation of substrates. In summary, future studies will have to elucidate the molecular mechanisms of substrate oxidation, in particular the mechanism of coordinated transfer of two disulphide bonds.

CONCLUSION

The identification of a disulphide relay system in the IMS of mitochondria has opened an exciting area of research. The system is the first mitochondrial translocation pathway with a covalent interaction between a component of the pathway and the imported protein. It appears to share common principles with the disulphide forming systems in the endoplasmic reticulum and the periplasm of prokaryotes (*38*, *49*, *50*): the generation of disulphide bonds by the transfer of electrons to a non-thiol electron acceptor and the subsequent transfer of disulphide bonds to substrate proteins. However, there are many differences, which may reflect the more reducing environment in the IMS compared to those in the ER and the periplasm. The disulphide bonds of the IMS proteins with twin $Cx_{3,9}C$-motif seem to be very stable and have a highly negative redox potential. The two disulphide bonds are presumably introduced in a coupled, highly specific reaction. Thus, there might be no need for an isomerase activity in the IMS as present in the ER and the periplasm, although such an activity cannot be excluded to date. In contrast to its counterparts in the other systems, Mia40 does not contain a thioredoxin domain and forms relatively stable disulphide intermediates with substrate proteins and Erv1. It will be important to find out whether these features contribute to accuracy and specificity. We are still at the beginning to unravel the function, mechanism, regulation and specificity of the Erv1-Mia40 system. Future studies will have to address these points and to dissect the single steps of the mechanism of disulphide bond formation in the IMS.

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CONFLICT OF INTEREST

None declared.

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