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Forum Review

Redox Pathways of the Mitochondrion

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ABSTRACT

The mitochondrion houses a variety of redox pathways, utilized for protection from oxidative damage and assembly of the organelle. The glutathione/glutaredoxin and thioredoxin systems function in the mitochondrial matrix. The intermembrane space is protected from oxidative damage via superoxide dismutase and glutathione. Subunits in the cytochrome bc_1 complex utilize disulfide bonds for enzymatic activity, whereas cytochrome oxidase relies on disulfide linkages for copper acquisition. A redox pathway (Mia40p and Erv1p) mediates the import of intermembrane space proteins such as the small Tim proteins, Cox17p, and Cox19p, which have disulfide bonds. Many of the candidate proteins with disulfide bridges possess a twin CX3C motif or CX9C motif and utilize both metal binding and disulfide linkages for function. It may seem surprising that the intermembrane space has developed redox pathways, considering that the buffered environment should be reducing like the cytosol. However, the prokaryotic origin of the mitochondrion suggests that the intermembrane space may be akin to the oxidative environment of the bacterial periplasm. Although the players forming disulfide bonds are not conserved between mitochondria and prokaryotes, the mitochondrion may have maintained redox chemistry as an assembly mechanism in the intermembrane space for the import of proteins and metals and enzymatic activity. *Antioxid. Redox Signal.* 8, 813–822.

INTRODUCTION

The mitochondrion contains an outer and inner membrane that separates the matrix and intermembrane space (40). The inner membrane is impermeable to ions and solutes, and a membrane potential for oxidative phosphorylation is established. In contrast, the outer membrane contains the evolutionarily conserved porins that allow the free exchange of small molecules with a molecular mass below 5–6 kDa (6). Thus, the solute environment of the intermembrane space is thought to be similar to the cytosol, whereas the matrix redox state is dependent on the solutes and proteins transported across the inner membrane.

The redox state of protein thiols is determined by several factors. Proteins in the endoplasmic reticulum often have disulfide bonds inserted by an oxidative folding machinery, whereas cytosolic proteins are generally reduced unless they function in a regulatory or enzymatic pathway involving the reversible oxidation and reduction of vicinal thiols (49). For example, transcription factors Yap1 and OxyR form disulfide bonds during oxidative stress as a strategy to activate a transcription program of stress proteins (67). Moreover, an oxidative folding pathway in the cytosol has been devised by poxvirus so that disulfide bonds can be inserted into the virion coat; the pathway utilizes a sulfhydryl oxidase related to the endoplasmic reticulum sulfhydryl oxidase Erv2p (76). Although the cytosolic environment is reducing, disulfide bonds can be accommodated.

Recent research in mitochondrial biogenesis suggests that redox pathways function in several capacities. This review will summarize the various redox pathways of the mitochondrion. Like other compartments of the cell, the mitochondrion has a system for protecting from oxidative damage. In addition, recent studies suggest that redox pathways are important for the assembly of proteins in the intermembrane space and the respiratory complexes of the inner membrane.

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Thus, the adaptation of redox chemistry in the mitochondrion is important in a wide variety of processes.

THE REDOX BUFFERING SYSTEM OF THE MITOCHONDRION

Like other compartments in the cells, the mitochondrion has a defense system to repair oxidative damage generated by reactive oxygen species during normal aerobic metabolism or from exposure to radical-generating compounds (83). To maintain the redox balance, the mitochondrion depends on the glutathione/glutaredoxin and thioredoxin systems that utilize sulfhydryl groups (Fig. 1, Table 1) (32).

The tripeptide glutathione (γ-L-glutamyl–L-cysteinyl-glycine; GSH) is important as a cellular redox buffer because it has a low redox potential ($E'_0 = -240$ mV) and a high concentration of 13 m*M* in the cell (65). The antioxidant function of glutathione is mediated via the redox-active thiol group that becomes oxidized when GSH reduces target molecules. Two molecules of reduced GSH thus are oxidized to glutathione disulfide (GSSG).

Glutathione is synthesized in the cytosol in a two-step reaction catalyzed by proteins coded by *GSH1* and *GSH2* in yeast (28). In the first step, γ-glutamylcysteine synthetase (*GSH1*) catalyzes the rate-limiting step in which γ-glutamylcysteine is formed from glutamate and cysteine (63). Glutathione synthetase (*GSH2*) catalyzes the ligation of glycine with γ-glutamylcysteine. GSH is oxidized to GSSG by reactions with free radicals or in reactions catalyzed by glutathione peroxidase (encoded by *GPX1–3*) or glutathione *S*-transferase (encoded by *GTT1* and *GTT2*) (32). GSH is regenerated in an NADPH-dependent reaction catalyzed by glutathione reductase (Glr, coded by *GLR1* in yeast). Glr is a flavin-containing oxidoreductase similar to thioredoxin reductase, which uses a redox-active disulfide in its active site and NADPH for its catalytic activity (8). The *in vivo* concentration of cytosolic GSH and GSSG has been recently determined using a redox-sensitive yellow fluorescent protein (YFP) that equilibrates specifically with the glutathione redox pair and but not thioredoxin (65). At a pH of 7.0, the concentration of GSH and GSSG was measured at 13 and 0.7 m*M*, respectively. Moreover, the redox potential of the cytosol was calculated at −289 mV. This *in vivo* measurement suggests that the cytosol is more reducing than predicted on the basis of glutathione measurements with whole-cell extracts (34). Experiments relying

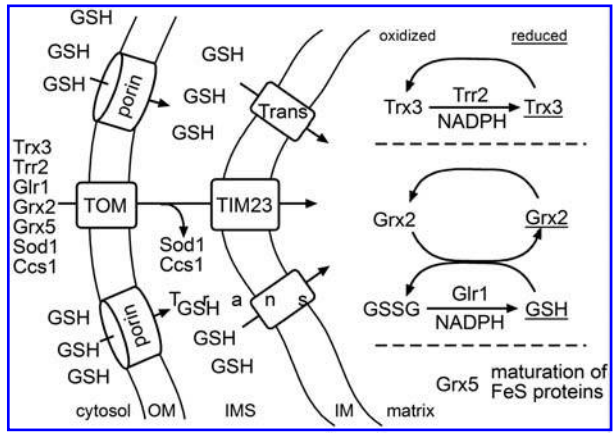

FIG. 1. Glutaredoxin and thioredoxin buffering system in the mitochondrion. Glutathione is transported across the outer membrane (OM) via porin into the intermembrane space (IMS) and across the inner membrane (IM) via a transporter (Trans) to the matrix. Glr1p, Grx2p, Grx5p, Trx3p, and Trr2p are imported via the TOM and TIM23 complexes. The glutaredoxin and thioredoxin reactions in the matrix repair oxidatively-damaged proteins. See text for details.

TABLE 1. RESIDENT MITOCHONDRIAL PROTEINS WITH DISULFIDE LINKAGES

Redox proteins	*Mitochondrial localization*	*Function*
GSH and GSSG	Matrix and IMS	Small molecule thiols
Glr1p	Matrix	Oxidative damage repair
Grx2p	Matrix	Oxidative damage repair
Grx5p	Matrix	FeS cluster protein maturation
Trx3p	Matrix	Oxidative damage repair
Trr2p	Matrix	Oxidative damage repair
Rieske FeS protein	IM oriented to IMS	Cytochrome bc_1 function
Qcr8p	IM oriented to IMS	Cytochrome bc_1 function
Sod1p	IMS	Oxidative damage repair
Ccs1p	IMS	Sod1p assembly
Sco1p	IM oriented to IMS	Cytochrome oxidase assembly
Small Tim proteins	IMS	Protein import
Cox17p	IMS	Cytochrome oxidase assembly
Cox19p (inferred)*	IMS	Cytochrome oxidase assembly
Cox23p (inferred)*	IMS	Cytochrome oxidase assembly
Mia40p	P	protein import
Erv1p	IMS	Sulfhydryl oxidase/FeS cluster export

*Inferred denotes that the disulfide linkages have not been confirmed experimentally, but have been predicted based on homology.

on whole-cell extracts may result in a shift to higher concentrations of GSSG caused by high concentrations of GSSG in the vacuole and endoplasmic reticulum (34).

GSH passes into the mitochondrial intermembrane space through porin channels in the outer membrane and is imported into the matrix via a transporter (Fig. 1) (11, 29, 37), but GSSG is not exported from the matrix (64). Glr is coded by one gene in *S. cerevisiae* (*GLR1*) and mammals (14, 80), yet Glr1p localizes to both the cytosol (90%–95%) and the matrix (5%–10%) (66). Dual targeting is achieved utilizing alternative translational initiation sites from two in-frame start codons, which is a common theme for localizing proteins to the mitochondrial matrix and cytosol (38). The long version of Glr1p contains a mitochondrial targeting sequence, whereas the shortened version of Glr1p has a translation initiation site after the mitochondrial targeting sequence. As a result, Glr1p targeted to the mitochondrial matrix contains an additional 17 amino acids that functions as a typical N-terminal targeting sequence (66). Whether a fraction of Glr1p localizes to the mitochondrial intermembrane space has not been demonstrated.

Glutaredoxins and thioredoxins are structurally similar and have been conserved throughout evolution (32). They are small, heat-stable oxidoreductases with two conserved cysteine residues in their active sites. The first identified substrate was ribonucleotide reductase but the oxidoreductases also serve as hydrogen donors for additional metabolic enzymes that form a disulfide as part of their catalytic cycle (72). Glutaredoxins and thioredoxins participate in a wide range of cellular functions including protein folding and regulation, sulfur metabolism, repair of oxidatively-damaged proteins, and the reduction of dehydroascorbate (32). These oxidoreductases have diverged in their mechanism of regulation. NADPH and thioredoxin reductase reduce the oxidized disulfide form of thioredoxin, whereas GSH with electrons donated by NADPH reduces glutaredoxin.

The glutathione system collaborates with the glutaredoxin system. Glutaredoxins utilize GSH as an electron donor to reduce protein disulfides (through a dithiol mechanism) or glutathione–protein mixed disulfides (through a monothiol mechanism), in a coupled system with glutathione reductase and NADPH as an electron donor (30, 33). The active site of dithiol glutaredoxins is CXXC (typically CPYC), whereas monothiol glutaredoxins possess CXXX (typically CGFS) in the active site. Because of two cysteine residues in the active site, dithiol glutaredoxin can mediate reduction of protein disulfides. In contrast, the monothiol glutaredoxins utilize only the N-terminal cysteine thiol to catalyze the reduction of glutathione–protein mixed disulfides. The glutaredoxin family contains five members in yeast (GRX1–5). Dithiol redoxins Grx1p and Grx2p are homologous to bacterial and mammalian glutaredoxins, whereas Grx3p–5p are monothiol glutaredoxins. Grx1p localizes to the cytosol (25, 53), whereas Grx2p localizes to both the mitochondrial matrix and cytosol (70), using alternative initiation sites for translation as described for Glr1p. In addition, Grx3p–5p also localize to different compartments, and the mitochondrion houses Grx5p. In the mitochondrial matrix, Grx5p functions in the biogenesis of FeS clusters (73), and cells lacking Grx5p accumulate iron in the mitochondrion.

Thioredoxins also function in the general redox system of the mitochondrion. Once again, the mitochondrion contains a thioredoxin and thioredoxin reductase (69). Of the three thioredoxins, Trx1p and Trx2p localize to the cytosol (86), whereas Trx3p localizes to the mitochondrial matrix. In addition, thioredoxin reductase Trr1p localizes to the cytosol and Trr2p localizes to the mitochondrion. Mitochondrial components of the thioredoxin system contain a typical N-terminal targeting sequence (69). The mitochondrial thioredoxin system functions in protection against oxidative stress generated during respiratory metabolism. In addition, thioredoxin is a hydrogen donor in sulfate assimilation required for methionine biosynthesis (58).

The mitochondrion and cytosol thus have thioredoxin and glutaredoxin systems. In the cytosol, the redox state of the thioredoxins and glutaredoxins functions independently. In the mitochondrion, however, Glr1p and Trr2p have overlapping functions and the GSH–glutaredoxin system affects the redox state of thioredoxin (83). The redox system of the mitochondrial intermembrane space has not been studied extensively. However, it is plausible that a fraction of the cytosolic thioredoxin and glutaredoxin components may localize to the intermembrane space. As a precedence for localization to both the cytosol and intermembrane space, superoxide dismutase 1 (Sod1p) partitions to both compartments (88).

INTERMEMBRANE SPACE PROTEINS WITH DISULFIDE LINKAGES

An increasing list of proteins in the intermembrane space contains disulfide linkages (Table 1). Thiol linkages function in both structural and catalytic roles. Disulfide bond formation also serves as a mechanism for the import and assembly of proteins into the intermembrane space (56). It is surprising that an import pathway relying on disulfide bridges has been developed, considering the redox environment in the intermembrane space is most likely reducing. However, this import mechanism may have evolved when the progenitor mitochondrion was developing during endosymbiosis, because the intermembrane space was derived from the periplasm. It also might have developed as a sorting mechanism to earmark intermembrane space proteins from the collection streaming through the TOM complex destined for the matrix and inner and outer membranes.

Components of the cytochrome bc_1 *complex*

The Rieske FeS protein is part of the cytochrome bc_1 complex (Complex III) and is a redox protein with the highest midpoint potential of complex. A disulfide bridge placed between the two loops of the protein hold the [2Fe–2S] cluster (36). If the disulfide bridge is chemically reduced, reoxidation of the dithiol and the [2Fe–2S] cluster occurs rapidly in *Thermus thermophilus* (89), indicating that it does not require a factor for assembly. Additional structural comparisons suggest that the disulfide bridge affects the midpoint potential of the FeS cluster and may be important for stabilization of the loops (13). However, the disulfide bridge may not play a role in stabilizing the FeS cluster because other proteins such as

rubredoxins with the "Rieske-type" fold lack a disulfide bridge (48); instead van der Waals forces stabilize the two loops. Recently, Trumpower and colleagues replaced the cysteine residues with those capable of making similar van der Waals interactions (55). Elimination of the disulfide bridge did not impair stability of the FeS cluster, but instead resulted in structural changes in the environment around the FeS cluster. The structural changes affected interactions with ubiquinol and indirectly damaged the ubiquinol oxidation site (55). Thus, the disulfide linkage is requisite for activity of the cytochrome bc_1 complex.

Subunit 8 coded by *QCR8* in yeast is referred to as the "acidic/hinge" protein because 24 of its 78 residues are acidic (39). Subunit 8 is required for proper complex formation between cytochrome *c* and c_1. Structural studies have shown that Qcr8p is composed of two long alpha-helices that are connected on each end by two disulfide bonds (Cys^{24}–Cys^{68} and Cys^{40}–Cys^{54}) (35). Earlier biochemical studies support the crystal structure (57). The disulfide bridges may play a role in stability of Qcr8p.

Superoxide dismutase and the copper chaperone CCS

The copper/zinc-superoxide dismutase (*SOD1*) and its copper chaperone CCS are distributed between the intermembrane space and the cytosol in addition to the nucleus and lysosomes (15, 23). Localization of Sod1p to the intermembrane space is to presumably protect against superoxide radicals generated by the respiratory chain (88). Structurally, all eukaryotic Sod1 proteins contain one copper, one zinc, and one disulfide bond per monomer (7), which are required for activity.

The copper chaperone CCS mediates copper insertion into Sod1p by forming a transient heterodimer via a disulfide linkage (15, 44). Interestingly, Sod1p is imported into the intermembrane space in an immature form, lacking copper and zinc and a disulfide bridge (21). CCS is required for Sod1p accumulation into the intermembrane space because overexpression of CCS targeted to the intermembrane space results in elevated levels of Sod1p in the same compartment (79). The retention mechanism seems to depend on the two cysteine residues forming an intramolecular disulfide bond in Sod1p and two cysteine residues in CCS, one of which forms a transient heterodimer with Sod1p (21). Similarly, placement of the disulfide bond in cytosolic Sod1p is required for its function. A recent study shows that copper-bound CCS facilitates oxidation of cysteine residues and disulfide oxidation in Sod1p, resulting in Sod1p maturation (22). Placement of the disulfide bond in Sod1p is thus required for regulation of enzymatic activity, for prevention of misfolding, and for import into the intermembrane space.

Proteins with the CX3C motif

The small Tim proteins (Tim8p, Tim9p, Tim10p, Tim12p, and Tim13p) contain the twin CX3C motif in which two cysteine residues are separated by three amino acids (41, 43). Spacing between each CX3C varies from 11 to 16 amino acids; this motif is unique to the small Tim proteins. This family is conserved from yeast to mammals and plants, but not found in prokaryotes (5). The small Tim proteins assemble in 70 kDa complexes in the intermembrane space and function as chaperones to guide inner membrane proteins, including those of the mitochondrial carrier family and import components Tim17p, Tim22p, and Tim23p (17–19, 52). Three subunits of Tim9p assemble with three subunits of Tim10p; similarly Tim8p assembles with Tim13p. In addition, a fraction of the Tim9p and Tim10p with Tim12p assemble with Tim22p at the inner membrane, mediating insertion of the substrate into the inner membrane. Mutations in the human homolog of Tim8p, DDP1, result in an X-linked disease deafness–dystonia syndrome (42, 74).

The cysteine residues are essential for assembly of the small Tim proteins. A missense mutation in the fourth cysteine of DDP1 results in loss of the DDP1–Timm13 complex, resulting in deafness–dystonia syndrome (74, 75). The cysteine residues may coordinate zinc and form disulfide bonds (41). Coordinating metals ions and forming disulfide bridges may be a common theme for intermembrane space import components, Sod1p, and cytochrome oxidase assembly components.

The twin CX3C motif has been proposed to coordinate Zn^{2+} by forming a zinc-finger-like structure (77). The recombinant small Tim monomers bind zinc in a 1:1 ratio (77); however, the assembled small Tim complexes do not contain zinc, but instead harbor disulfide bonds (1, 17, 18, 51). Moreover, the recombinant and endogenous complexes display the same binding properties for the membrane-spanning domains of the ADP/ATP carrier and Tim23p proteins, respectively (17, 18), suggesting that even if the complex was oxidized during purification, it was still functional. Allen *et al.* studied the folding and assembly requirements of the Tim9p–Tim10p complex in detail (1). Using trypsin digestion and mass spectrometry studies, it has been shown that Tim10p folds into a similar structure as that predicted from zinc coordination, but differs slightly in that the cysteines pair in juxtaposed disulfide bonds. The inner disulfide bond is formed by Cys^{44} and Cys^{61} and an outer pair is formed by Cys^{40} and Cys^{65}; the inner thiol linkage is crucial overall for folding and chaperone activity. Zinc might bind to the fully reduced Tim proteins, but does not promote complex formation (1).

Sco1p (synthesis of cytochrome c oxidase) contains a single CX3C motif and anchors to the inner membrane, with the bulk of the protein, including the CX3C motif, facing the intermembrane space (27, 61). Sco1p is a copper-binding protein that is required for the assembly of cytochrome oxidase, potentially playing a role in donating copper via the CX3C motif in cytochrome oxidase maturation (61). Mutation of either cysteine residue resulted in the loss of active cytochrome oxidase in yeast (71), demonstrating the importance of these residues. However, that elevated concentrations of copper did not complement loss of Sco1 function in yeast suggested possible alternative functions of Sco1p in cytochrome oxidase assembly (27). Interestingly, Sco1p shares a low similarity with peroxiredoxins and thiol:disulfide oxidoreductases with a thioredoxin fold, suggesting that Sco1p may perform a catalytic rather than copper transport role (12). Indeed, recent crystallization studies have shown that the intermembrane

space domain of hSco1p is similar to thioredoxin, with the CX3C motif positioned analogous to the CX2C in thioredoxin (3, 87). Interestingly, the photosynthetic prokaryote *R. sphaeroides*, which switches between photosynthesis and respiration depending on light and oxygen levels, encodes a Sco1p homolog PrrC of the *prrBCA* operon (20, 87). PrrC interacts with cbb_3 cytochrome *c* oxidase and inhibits activation of a histidine kinase-mediated two component signaling system composed of PrrA and PrrB. By analogy, Sco1p may function as a similar regulator, but downstream targets have not been identified (87). Sco1p thus may act as a copper-dependent redox switch.

Proteins with the twin CX9C motif

Like the twin CX3C motif, another set of intermembrane space proteins contain the twin CX9C motif in which two cysteine residues are separated by nine amino acids. This group includes Cox17p (2), Cox19p (62), and Cox23p (4) that are involved in cytochrome oxidase assembly. In addition, Mia40p, which is essential for the import of the small Tim proteins and Cox17p and Cox19p, contains this motif (10, 61, 80).

Cox17p was identified in a genetic screen for components required for cytochrome oxidase maturation (26). Yeast strains lacking *COX17* are respiratory deficient, but respiration can be restored by the addition of copper to cells, suggesting that Cox17p, like Sco1p, might function as a copper chaperone (26). Structural studies have shown that copper binding depends on the oxidation state of the cysteine residues (2, 68). In the fully reduced state, Cox17p binds 4 Cu(I) ions, but in the oxidized state with two or three disulfides, Cox17p does not bind Cu(I) ions. Two disulfide bonds (Cys^{36}–Cys^{47} and Cys^{26}–Cys^{57}) link two antiparallel α-helices, keeping them closely positioned. Upon reduction, the two α-helices spread apart to accommodate Cu(I) ions (2). The disulfide bonds are not essential for physiological function, suggesting a role in structural stabilization. Cox17p contains two additional cysteine residues, Cys^{23} and Cys^{24}. These cysteine residues do not form disulfide linkages but instead are important for Cu(I) ligation and Cox17p function (2).

Structurally, the two α-helices of Cox17p are preceded by two coiled-coil regions. This motif is described as a coiled-coil–helix–coiled-coil–helix (CHCH) domain (85). Additional mitochondrial proteins with a CHCH domain include Cox12p (84), Cox19p (62), and Cox23p (4), which are involved in copper insertion into cytochrome oxidase (9). These proteins most likely contain similar disulfide bonds. The coiled-coil regions may be important for oligomerization (2). For example, Cox12p may serve as a docking site for Cox17p, facilitating copper transfer to cytochrome oxidase.

Mia40p/Tim40p is an essential intermembrane space protein, tethered to the inner membrane, and mediates the import of the small Tim proteins, Cox17p and Cox19p (10, 60, 81). Mia40p also contains the twin CX9C motif, and recombinant Mia40p binds copper in addition to zinc (81), suggesting that it might have a structure similar to Cox17p. Mia40p interacts transiently with imported substrates via a disulfide bond. Specifically, Tim13p lacking the cysteine residues was not imported and did not bind to Mia40p (81).

Erv1p, a sulfhydryl oxidase of the intermembrane space

Erv1p, a sulfhydryl oxidase in the intermembrane space, is essential for viability and related to Erv2p of the endoplasmic reticulum (46). In addition, the human homolog ALR (augmenter of liver regeneration) suggests that Erv1p plays a critical role in organ processes (24). Like the small Tim proteins and Mia40p, Erv1p is conserved in plants, fungi, and animals, but absent in prokaryotes (31, 50). The N-terminal regions of Erv1p and Erv2p are distinct, whereas the C-terminal domain of Erv1p and Erv2p has the redox-active center and the FAD-binding domain and shares a 30% similarity (78).

Early studies suggested that Erv1p functioned in an oxidative folding pathway, but the substrates were not identified (46). Erv1p contains six cysteine residues. The cysteine residues of the C-terminus function with FAD. Specifically the Cys^{130} and Cys^{133} disulfide bridge is part of the redox center, whereas the Cys^{159}–Cys^{176} pair stabilizes the FAD-binding domain (31). In contrast, the N-terminal Cys^{30}–Cys^{33} pair mediates dimerization of yeast Erv1p. Dimer formation seems essential for function because Erv1p of plants lacks the N-terminal cysteine residues, but dimerization is promoted by cysteine residues in the C-terminus (31, 47).

The first mitochondrial function for Erv1p was described by Lill and colleagues, showing that Erv1p functions in the export of FeS clusters from the mitochondrion (45). A yeast strain defective in Erv1p function showed decreased incorporation of ^{55}Fe into cytosolic FeS proteins Lue1p and Rli1p, but assembly of mitochondrial FeS proteins was not affected. They postulated that Erv1p functions after the putative FeS cluster exporter Atm1p of the inner membrane and facilitates maturation of cytosolic proteins with FeS clusters. A recent study suggests that Erv1p also mediates assembly of disulfide bonds in Mia40p and plays a role in import of intermembrane space proteins (56). Thus, Erv1p may function as a general sulfhydryl oxidase in the intermembrane space.

A NEW IMPORT PATHWAY FOR THE SMALL INTERMEMBRANE SPACE PROTEINS

Erv1p functions with Mia40p in a new protein import pathway for intermembrane space proteins (Fig. 2) (10, 56, 60, 81, 82). While the molecular details remain to be elucidated, Mia40p forms transient disulfide bonds with the imported substrates. The small Tim proteins, Cox17p and Cox19p, are imported across the TOM complex in an unfolded reduced state (Fig. 2, Step 1) (51, 54). To trap the proteins in the intermembrane space, oxidized Mia40p forms a transient disulfide bond with the substrate (Fig. 2, Step 2). The presence of reducing agents decreases the abundance of a Mia40-substrate intermediate, whereas the presence of copper stabilizes this interaction (10, 81). The substrate is released by an unknown mechanism that may involve rearrangement of disulfide bonds and subsequently acquires a

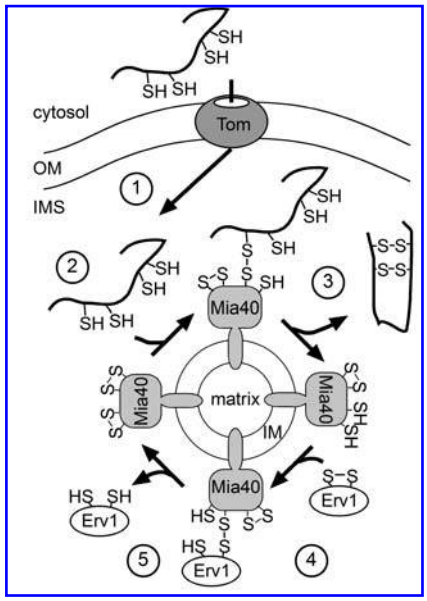

FIG. 2. Import pathway utilized by the small Tim proteins, Cox17p and Cox19p. The substrates are imported in an unfolded, reduced state across the outer membrane (OM) (*Step 1*). Oxidized Mia40p serves as the import component. The precursor binds to Mia40p transiently through a mixed disulfide bond (*Step 2*). The precursor is released and folds into a mature protein in the intermembrane space (IMS) (*Step 3*). Reduced thiol groups on Mia40p are oxidized by the sulfhydryl oxidase, Erv1p (*Steps 4 and 5*). Detailed mechanistic steps have not been elucidated, including the terminal electron acceptor required for Erv1p regeneration and additional proteins that may participate in the import and assembly pathways.

mature conformation. Mia40p is then reoxidized by the sulfhydryl oxidase Erv1p (Fig. 2, Steps 4 and 5) (56). Approximately 10% of the Erv1p pool interacts with Mis40p under nonreducing conditions. However, the specific amino acids, presumably cysteine residues, mediating the interaction have not been identified.

This model suggests that electrons are transferred from the imported proteins to Erv1p via Mia40p. Erv1p transfers the electrons through the flavin cofactor to an unknown electron acceptor. In prokaryotes, the electron acceptor is often the electron transport chain (59), which Erv1p also could utilize. However, yeast cells lacking the mitochondrial genome and, as a result, oxidative phosphorylation and electron transport, still import proteins; this suggests that an alternative electron acceptor also may receive the transferred electrons. Future experiments will be required to identify additional players and determine the mechanistic details in this exciting new import pathway.

IMPORT OF CARRIER PROTEINS IS REDOX REGULATED

The mechanism by which the small Tim proteins escort a hydrophobic precursor from the outer membrane to the inner membrane has been investigated in detail (16). The small Tim proteins form chaperone-like complexes but the transfer of substrate does not require an energy source such as ATP, and the small Tim proteins are not similar to typical chaperones such as the Hsp60 and Hsp70 families (17, 18, 43). Preliminary studies on the import of the carrier proteins suggest that the TIM22 pathway may be redox regulated (Fig. 3) (16). From *in organello* import assays, a translocation intermediate consisting of the carrier protein and the Tim9p–Tim10p complex accumulated in the intermembrane space in the presence of oxidant (Fig. 3, Stage 1). When subsequently treated with reductant, the carrier was "chased" into the inner membrane (Fig. 3, Stage 2). The small Tim complex may be disassembled by the presence of reductant, resulting in transfer of the substrate to the insertion complex. Alternatively, the carriers may have increasing binding affinity for components such as Tim22p in the 300 kDa insertion complex. The disassembled small Tim proteins may be recycled and reassembled for another round of translocation (Fig. 3, Stage 3).

Other proteins (indicated with "?" in Fig. 3) most likely facilitate import of the carrier proteins, particularly recycling of the small Tim proteins. Hot13p (Helper of Tim, 13 kDa) was identified as a protein that bound to the small Tim proteins and facilitated assembly of the 70 kDa small Tim complexes (16). Hot13p is a zinc-ring finger protein localized to the intermembrane space. Mitochondria lacking Hot13p contained decreased levels of the small Tim proteins. Moreover,

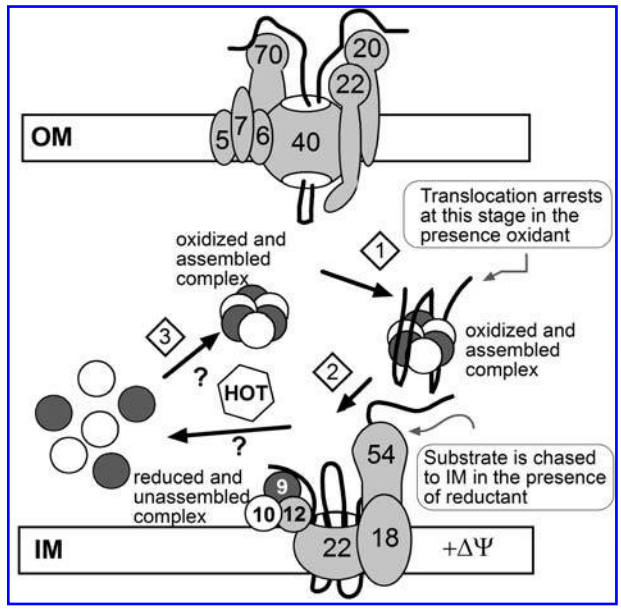

FIG. 3. The import pathway for carrier proteins is redox regulated. In the assembled complex, the small Tim proteins are oxidized and bind to substrate crossing the TOM complex. In the presence of oxidant, a translocation intermediate bound to the small Tim complexes can be arrested in the intermembrane space (*Stage 1*). In the presence of reductant, the translocation intermediate is subsequently "chased" and the substrate inserts into the inner membrane (*Stage 2*). The small Tim proteins are reassembled for another round of import, which may be facilitated by Hot13p (HOT; *Stage 3*). Detailed mechanistic steps have not been elucidated and additional proteins may participate in this pathway.

the import of the small Tim proteins was not defective, suggesting that Hot13p was required after Mia40p (89). However, the small Tim proteins were not assembled into 70 kDa complexes efficiently. Hot13p therefore may be a new factor that facilitates recycling of the small Tim proteins during import of the carrier proteins. As with the Mia40p–Erv1p import pathway, studies are required to identify additional components and to understand the import mechanism.

CONCLUSIONS

These studies suggest that the mitochondrion is home to a large collection of proteins with disulfide bonds. In the matrix and intermembrane space, these pathways are important for the repair of oxidatively-damaged proteins. Additionally, redox chemistry is used as an import and assembly mechanism for proteins in the intermembrane space. Although the intermembrane space redox potential is reducing, this compartment seems to have developed mechanisms to insert and maintain disulfide bonds in proteins. Future studies should increase our understanding of how disulfide linkages are maintained and how thiol chemistry is used for assembly pathways in this compartment.

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ABBREVIATIONS

COX, cytochrome oxidase; FeS, iron sulfur (cluster); Glr, glutathione reductase; Grx, glutaredoxin; GSH, glutathione; GSSG, oxidized glutathione; IM, inner membrane; IMS, intermembrane space; OM, outer membrane; Sod, superoxide dismutase; TIM, translocase of the inner membrane; TOM, translocase of the outer membrane; Trx, thioredoxin; YFP, yellow fluorescent protein.

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