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Review

Oxidative folding: recent developments

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Abstract

Disulfide bond formation in proteins is an effective tool of both structure stabilization and redox regulation. The prokaryotic periplasm and the endoplasmic reticulum of eukaryotes were long considered as the only compartments for enzyme mediated formation of stable disulfide bonds. Recently, the mitochondrial intermembrane space has emerged as the third protein-oxidizing compartment. The classic view on the mechanism of oxidative folding in the endoplasmic reticulum has also been reshaped by new observations. Moreover, besides the structure stabilizing function, reversible disulfide bridge formation in some proteins of the endoplasmic reticulum, seems to play a regulatory role. This review briefly summarizes the present knowledge of the redox systems supporting oxidative folding, emphasizing recent developments.

Keywords: endoplasmic reticulum; intermembrane space; oxidative folding; periplasm; redox.

Introduction

Oxidative folding is a process in which oxidative posttranslational modifications are accompanied by protein folding. Although several types of oxidative protein modifications have an impact on folding, traditionally, the use of the term is restricted to disulfide bond formation. Oxidation of cysteinyl residues to disulfides can transiently occur in any subcellular compartments; however, the reducing milieu of most organelles makes the reaction reversible. Three compartments, the periplasm of prokaryotes, the lumen of the endoplasmic reticulum (ER) and the intermembrane space (IMS) of mitochondria in eukaryotes, allow the formation of stable disulfide bridges, due to their more oxidizing environment. Disulfide bonds provide increased conformational stability for these proteins, which is especially important in the extracellular space.

Oxidative folding: paraphrases on a theme

The formation of a disulfide bond follows similar principles in these compartments (Figure 1). Proteins are synthesized in a compartment where the reducing milieu maintains the cysteinyl thiols in a reduced form. Thus, proteins must be transported through a membrane to the place of the oxidative folding. Oxidation of cysteinyl residues usually is achieved by two consecutive steps. Firstly, a disulfide is transferred from an oxidoreductase to the substrate protein by thiol-disulfide exchange. Then, the electrons will be transferred by an oxidase from the reduced oxidoreductase, to an electron acceptor, mostly molecular oxygen. The reversibility of the first step also allows the isomerization of incorrect disulfides, while the second step ensures the thermodynamic feasibility of the process.

The minimal toolkit of *in vivo* oxidative protein folding comprises (a) a compartment with a relatively oxidative milieu, (b) an oxidoreductase, (c) an oxidase or oxidoreductase which tranfers the electrons to (d) a final electron acceptor. Although different compartments, enzymes and electron acceptors are involved in the three different localizations of oxidative folding, the general scheme of the electron transfer is similar. Interestingly, the participating proteins have independent origins (1), providing an example for convergent evolution.

Disulfide bond formation in prokaryotes

Most of the information on prokaryotic disulfide bond formation has been collected in *Escherichia coli*. Disulfide bond formation occurs in the periplasm, a compartment between the inner cytoplasmic membrane and the external outer membrane of Gram-negative bacteria, or the equivalent space outside the inner membrane of Gram-positive bacteria. The appropriate disulfide bonds are formed as a result of two independent, but coordinated biochemical pathways. The oxidative pathway is responsible for the *de novo* synthesis of disulfide bonds, while the isomerization pathway is responsible for the isomerization of incorrect disulfide bonds. The main elements of both pathways were mainly elucidated in the 1990s. Hence, we dispense with the detailed description of the pathways (2, 3), but try to focus and summarize new findings and approaches of the topic.

The oxidative pathway

Disulfide bonds of the extracytoplasmic proteins are introduced by the primary oxidoreductase, DsbA (Dsb, disulfide bond). Among the known enzymes that promote the exchange of thiols and disulfides, with a standard redox potential of -120 mV, DsbA is one of the strongest thiol

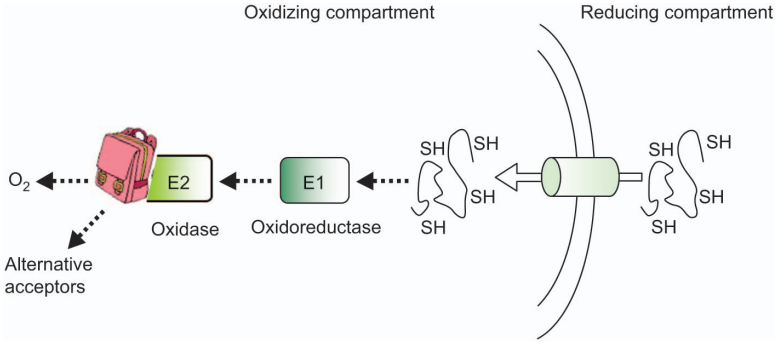

Figure 1 General scheme of the oxidative folding.
Proteins are synthesized in a reducing compartment. Having been translocated to the compartment containing the machinery of oxidative folding, electrons from cysteinyl thiols are transferred to a final acceptor via an electron transfer chain composed by oxidoreductases.

oxidants (4). Recently, two even more oxidizing DsbA homologs were described from *Neisseria meningitidis* (-80 mV) (5, 6) and from *Pseudomonas aeruginosa* (-95 mV) (7) (for comparison, the standard redox potential of thioredoxin, a small oxidoreductase enzyme containing a dithiol-disulfide active site, is -270 mV.)

The formation of a disulfide bond occurs in two consecutive steps. In the first step, the deprotonated cysteine of a target protein attacks the sulfur atom of Cys30 in the Cys30-Cys33 disulfide bond of DsbA. This reaction results in the formation of an intermolecular disulfide and reduction of Cys33. In the second step, another cysteine of the target protein gets deprotonated and attacks that intermolecular disulfide. Finally, an intramolecular disulfide bond is formed in the target protein and both cysteines of DsbA become reduced (8).

Reduced DsbA gets oxidized before the next catalytic cycle by the membrane protein DsbB (9, 10). DsbB null mutants are characterized by similar (but not that serious) pleiotropic phenotypes to the DsbA null mutants. Reduced DsbB then passes the electrons towards the electron transport chain. It was demonstrated that either cytochrome *bd* or *bo* oxidase can reoxidize the DsbA-DsbB system. Their role in the electron transport is to shuttle electrons from ubiquinone to oxygen (11). Under anaerobic conditions, menaquinone is an alternative electron acceptor, getting oxidized by fumarate or nitrate reductases. The use of both ubiquinone and menaquinone as electron acceptors by DsbB, allows the DsbA-DsbB system to work under both aerobic and anaerobic conditions (12).

Bacterial VKOR, an analog of DsbB

Recent evidence shows that a homolog of vertebrate ER protein vitamin K epoxide reductase (VKOR) is also an important component of the protein disulfide bond-forming pathway in many bacteria (*Mycobacterium tuberculosis, Synechococcus* sp.). Bacterial VKOR appears to take the place of the non-homologous DsbB found in *Escherichia coli*. The VKOR homolog in *Mycobacterium tuberculosis* spans the membrane five times with four conserved cysteines facing the periplasm. The essentiality of these cysteine residues has also been demonstrated in promoting disulfide bond formation *in vivo*. Mixed disulfide between a cysteine of DsbA of *Escherichia coli*, and one of the periplasmic cysteines of the VKOR homolog has been identified as a likely intermediate in the disulfide bond-forming pathway (13). The recently described homolog of VKOR from *Synechococcus* sp., like DsbB, contains four trans-membrane segments and a quinone (14, 15). The structural similarities indicate that VKOR homologs or DsbB mediate electron transfer by a similar mechanism (16). Bacterial VKOR homologs catalyze disulfide bond formation in periplasmic proteins by cooperating with a periplasmic, thioredoxin-like redox partner. In some species, a single polypeptide chain contains both VKOR and a thioredoxin-like domain (17, 18).

The two pathways of the disulfide bridge formation in bacteria share similarities with those in eukaryotes, although the eukaryotic oxidases (Ero1p and Erv2p) are present in the ER lumen, rather than integrated into the membrane (19, 20).

The isomerization pathway

The correction of non-native disulfide bonds relies on three members of the thioredoxin family: DsbC, DsbG and DsbD (21–23). DsbC and DsbG are isomerases in which the active site must be in a reduced state to attack the incorrect disulfide bonds. Both DsbC and DsbG are kept reduced by DsbD. In DsbD mutants, neither DsbC, nor DsbG can function as an isomerase or reductase in the oxidative milieu of the periplasm. The details of the isomerization pathway were elucidated more than a decade ago and were reviewed recently (2, 3).

However, it is noteworthy that the first three physiologic substrates of DsbG were described just recently (24). All belong to the same family of L,D-transpeptidases, which are responsible for cross-linking the major outer membrane lipoprotein to the peptidoglycan of *Escherichia coli* (25). They possess a sole cysteine, essential for activity. Since they cannot form disulfides, oxidation results in sulfenic acid production, inactivating the protein. Thus, a system should exist in the periplasm that can protect single cysteine residues from oxidation. DsbG, whose negatively charged surface is

better suited to interact with folded proteins, appears to be a key player in this reducing system (24).

In a recent study, *Escherichia coli* mutants lacking DsbC were applied to investigate the isomerization pathway (26). All but one of these DsbC bypass mutants act by massively overproducing DsbA, which apparently increases the level of mixed disulfides formed between DsbA and substrate proteins. Higher levels of mixed disulfides may in turn allow cells to directly perform correct oxidation of multidisulfide containing substrates. Another mutant operates via a different mechanism. This suppressor also overproduces DsbA, although to a lesser extent, but in addition, overproduces the chaperone/protease DegP, which apparently enhances the ability of the strain to perform disulfide isomerization reactions (26). This way, both pathways mimic the eukaryotic protein disulfide isomerase (PDI), since PDI is abundant in the ER and also possesses chaperone functions (27).

Although disulfide bond formation in prokaryotes is quite well mapped, many pathogens of this group have an extended repertoire of Dsb redox proteins [DsbL, DsbI (28), SrgA (29), nmDsbA1, nmDsbA2, nmDsbA3 (30)]. There is increasing evidence of a direct link between these folding catalysts and bacterial virulence. In these bacteria, Dsb proteins contribute to virulence properties, such as adhesion, host cell manipulation and cellular spread, and are considered to be a pivotal control point, because they are essential for the generation of multiple virulence factors. Thus, drugs that target the Dsb-folding catalysts, can be suitable tools to combat infectious diseases (31).

Disulfide bond formation in mitochondria

The recently discovered presence of disulfide bonds in the mitochondrial IMS was quite unexpected because of its connection to the reducing cytosol. In contrast, the other oxidizing compartments, such as the ER and the prokaryotic periplasm, are separated from the cytosol. However, it should be taken into account that disulfide bonds are present in several proteins located in or facing to the IMS (32). Redox measurements suggest that the IMS redox state is more oxidizing (-255 mV) compared with both the cytosol (-286 mV) and mitochondrial matrices (-296 mV) (33). Furthermore, the mitochondrial IMS is not directly influenced by endogenous glutathione reductase activity. This redox environment may support the oxidative folding of proteins imported into the IMS, rather than opposing it. The investigation of the redox state of structural disulfides in several imported proteins (Erv1, Tim9, Tim10, Cox17) supports this theory, since the thiol/disulfide ratio of these proteins are lower in the IMS than in the cytosol (33). Proteins with disulfide bonds in the IMS – the potential substrates of the mitochondrial disulfide relay system – can be divided into three classes.

Small Tim proteins (twin CX3C proteins)

Five homologous small Tim proteins are present in yeast (Tim8, Tim9, Tim10, Tim12, and Tim13). These proteins are evolutionarily conserved throughout the eukaryotic kingdom. All members of the small Tim family have a molecular mass of about 10 kDa (the numbers in the name indicate the approximate molecular weight) and contain a strictly conserved twin CX3C (two cysteines separated by three other amino acid residues) zinc-finger motif (34). The terminal and central cysteine residues can form intramolecular disulfide bridges that stabilize a hairpin-like structure (Figure 2) (35, 36). Small Tim proteins are imported individually from the cytosol in a cysteine-reduced form; the oxidized (disulfide-bonded) proteins are import-incompetent (37). While keeping the protein in a reduced state is essential for its mitochondrial import, only the oxidized proteins can form the Tim complex in the mitochondrial IMS (37). The complex consists of three Tim9-Tim10 and Tim8-Tim13 heterodimers with each of the twelve subunits having two intramolecular disulfide bonds (35–37). The cysteine residues are essential for the import and folding of the small Tims as well as for their assembly (36, 38). The mutation of Cys-66 to Trp in DDP1 – the human homolog of Tim8 –

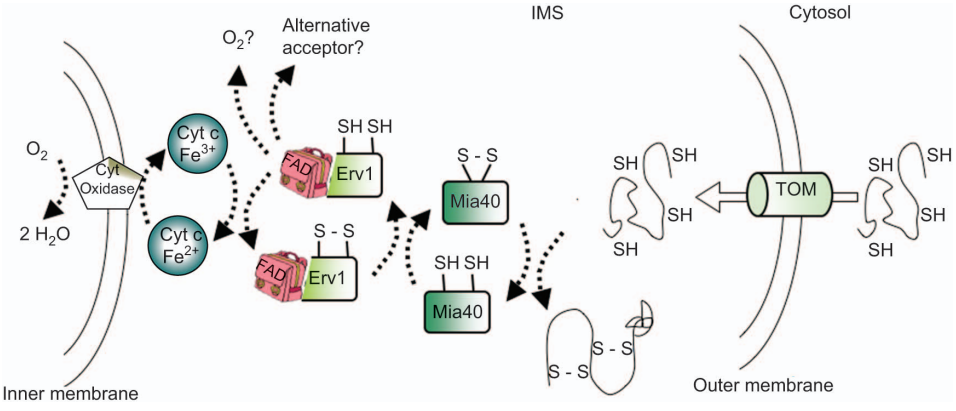

Figure 2 Oxidative folding in the mitochondrial IMS.
Proteins destined for the IMS are synthesized in the cytosol, then they are translocated through the outer mitochondrial membrane in reduced, unfolded conformation. Oxidized Mia40 recognizes and oxidizes its substrates, catalyzing the folding by disulfide bond generation. Reduced Mia40, in turn, will be reoxidized by an electron transfer chain containing Erv1, cytochrome *c* and cytochrome oxidase.

results in incorrect folding and the loss of its ability to assemble into a hetero-hexameric 70 kDa complex with human Tim13. This assembly defect of DDP1 is the molecular basis of Mohr-Tranebjaerg syndrome in patients carrying this mutation (39).

Twin CX9C proteins

The second group of IMS resident disulfide containing proteins is characterized by a twin CX9C motif (two cysteines separated by nine other amino acid residues). The best characterized protein of this group is Cox17, a copper-binding protein that plays a role in copper transfer to cytochrome c oxidase (40, 41). Besides Cox17, thirteen other proteins with twin CX9C motifs (Cmc1, Cmc2, Cmc3, Cmc4, Cox19, Cox23, Emi1, Mdm35, Mic14, Mic17, Mrp10, Pet191, Som1) were identified and shown to reside in the IMS of mitochondria and with one exception (Som1), are conserved among plants, fungi, and animals. The three-dimensional structure of Cox17 was solved by nuclear magnetic resonance spectroscopy (42, 43). The protein exhibits a helix–turn–helix fold in which the helices are connected by two parallel disulfide bonds. Secondary-structure predictions suggest similar helix–turn–helix architectures for all other potential twin Cx9C proteins (44).

Critical function of most of these proteins for the assembly or stability of respiratory chain complexes was found by testing deletion mutants of all 14 proteins. These data suggest that during the early phase of evolution of eukaryotic cells, a multitude of twin CX9C proteins developed, which exhibit largely non-redundant, critical roles for the biogenesis of enzymes of the respiratory chain in mitochondria (44).

Other proteins with disulfide bridges in the IMS

Cytochome *bc1* complex (complex III) of the respiratory chain also contains two proteins with disulfide bonds (45). The hinge protein Qcr6 in yeast and its mammalian homolog subunit 8 is composed of two long antiparallel α-helices. The connecting loop of Qcr6 is stabilized by a disulfide bridge, Cys101-Cys123, which is found in the same position as in subunit 8 from bovine or chicken complexes (46).

The Rieske iron-sulfur protein is one of the three essential catalytic subunits of complex III. The [2Fe-2S] cluster of the protein is held between two loops of the protein that are connected by a disulfide bridge. Even though the iron-sulfur cluster is assembled into the disulfide bridge lacking Rieske protein, the ubiquinol oxidation site is defective (47).

Copper-zinc superoxide dismutase (Sod1) is a ubiquitous superoxide anion eliminating enzyme localized in multiple cellular compartments, including the mitochondrial IMS. A highly conserved intramolecular disulfide bridge is formed between Cys-57 and Cys-146 of Sod1. This bond is necessary for Sod1 function and is very stable in the intracellular milieu, despite the highly reducing environment (48). However, Sod1 apoprotein is able to cross the outer membrane only in its metal free and reduced (thiol) state. Copper insertion and oxygen-dependent disulfide bridge formation are facilitated by the copper chaperone for Sod1, Ccs. The crystal structure of Ccs of *Saccharomyces cerevisiae* also revealed two disulfide bonds. The CXXC or CXC motifs in domains I and III of Ccs, respectively, could be involved in the mitochondrial import by the Mia40 (mitochondrial import and assembly) – essential for respiration and vegetative growth protein 1 (Erv1) system (49, 50).

In addition to the eight soluble, previously mentioned, cysteine containing accessory proteins (Cox17, Cox19, Cox23, Pet191, Cmc1-4), three similar, but membrane proteins (Cox11, Sco1, Sco2) have been identified in yeast mitochondrial IMS. All have human orthologues. Regulation of the redox state of Cox17, Sco1, and Sco2 cysteines is integral to their metallochaperone function. Redox also appears to be crucial to the regulation of a Sco-dependent mitochondrial signalling pathway that modulates the rate of copper efflux from the cell (51).

Finally, Mia40 and Erv1 should also be mentioned as the members of this group.

Mia40, primer oxidoreductase and import receptor in one protein

On one hand, the mitochondrial oxidative folding apparatus shows very little analogy with the folding apparatus localized in the bacterial periplasm or in the ER. On the other hand, a low level of similarity can be observed: both in the bacterial periplasm and in the ER exists a primer oxidoreductase (DsbA and PDI, respectively), that introduces disulfide bonds into the reduced substrate proteins. The main character of the mitochondrial disulfide formation machinery is the oxidoreductase Mia40 (mitochondrial import and assembly) (52). The molecular weight of the precursor protein is 44 kDa and 40 kDa of the mature form. Similarly to the above-mentioned bacterial and ER resident apparatuses, electrons are transferred from Mia40 to Erv1 and finally to either oxygen or cytochrome c in a cascade of oxidoreductions (Figure 2) (53–55).

Mia40 is anchored to the inner mitochondrial membrane in fungi (56), whereas Mia40 homologs of animals and plants are soluble proteins in the IMS (57, 58). The structure of Mia40 is unique in the club of known oxidoreductases of the cell. It does not have a thioredoxin domain, which is common among other known oxidoreductases, such as eukaryotic PDI and bacterial Dsb proteins. Mia40 belongs to a protein family, whose members share six conserved cysteine residues constituting a -CPC-CX9C-CX9C- motif. The oxidation-active CPC site is located in the N-terminal lid. This Mia40 unique motif is accessible to the solvent and positioned favorably for a direct and facile transfer of the disulfide bond to the substrate. The standard redox potential of the CPC disulfide bond is -200 mV (59). This way, the oxidation of substrate motifs with more reducing redox potentials, such as -340 mV [CX9C in Cox17 (60)] or -320 mV [CX3C in Tim10 (61)] is thermodynamically favored. Oxidation of the CPC site itself by the more oxidizing C-terminal CX2C pair of Erv1 [redox potential: -150 mV (54)] is also favored (59)].

Mia40, beside its oxidoreductase function, also fulfils an import-receptor role. The partially folded substrates usually

expose hydrophobic segments. The characteristic hydrophobic cleft of Mia40 can function as a substrate recognition and binding site (59), stabilizing initial non-covalent interactions that appropriately position the partially folded substrates for the formation of the first crucial mixed disulfide. Furthermore, Mia40 recognizes the substrates depending on the presence of a nine amino acid long internal targeting signal ITS (IMS-targeting signal) (62) or MISS (mitochondrial intermembrane space sorting) (63), located in different parts of the polypeptide, depending on the substrate, either near the N-terminal for the small Tims (62, 63), or near the C-terminal for Cox17 (62). In all cases, they bind to the hydrophobic cleft of Mia40 in such a way as to orient one specific cysteine of the substrate (the docking Cys) for disulfide pairing with the active site cysteine of Mia40 (62). The so-driven disulfide pairing then determines ITS α-helical formation thanks to the Mia40-substrate hydrophobic interactions. This folded segment represents the key initial folding unit that triggers complete folding of the rest of the substrate (64).

Protein disulfide isomerase activity has not been described in the intermembrane space to date. A glutathione-dependent proofreading mechanism was proposed during the oxidative protein folding in IMS (65). It seems possible that such an isomerase activity might be dispensable. Mia40 has a much greater specificity than proteins, such as PDI and DsbA and introduces specific disulfide bonds into partially folded substrates that are properly positioned on Mia40. This is the first example of such a protein and distinguishes the oxidative folding pathway of the IMS from those in the ER of eukaryotes and the periplasm of bacteria (59).

Erv1, the sulfhydryl oxidase of IMS

Mia40 became reduced while it mediated the formation of disulfide bonds into the target proteins. It should be oxidized before the next catalytic cycle. This reoxidation is performed by the soluble homodimeric flavoprotein Erv1 (essential for respiration and vegetative growth) (65–68). Erv1, or its human analog ALR (augmenter of liver regeneration), is located in the IMS and belongs to the Erv1/QSOX (quiescin sulfhydryl oxidase) family of flavoproteins (69, 70). Each subunit of the Erv1 dimer consists of two domains (N and FAD domain) each with a conserved CXXC motif.

The conserved core CXXC motif (Cys-130 and Cys-133 in yeast Erv1) of FAD-domain is adjacent to the FAD cofactor (19, 71). This vicinity allows the direct transfer of electrons from the reduced core CXXC motif onto the cofactor and, subsequently, the reformation of a disulfide bond within this 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 (71, 72). This four-helix bundle also contributes to the hydrophobic interaction interface that stabilizes the Erv1 homodimer (65, 71).

The N-terminal domains of Erv1 proteins in fungi and animals also contain a second redox-active CXXC motif (Cys-30 and Cys-33 in yeast Erv1). The N domain serves as a flexible shuttle arm, mediating the electron transfer from Mia40 to the FAD domain of Erv1.

As 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. After Mia40 reoxidation, the N domain becomes reduced. It then passes electrons in an intersubunit transfer onto the CXXC motif of the FAD domain of the second subunit of the homodimer. Finally, as was mentioned above, electrons can be passed to the isoalloxazine moiety of FAD due to the close proximity of the core CXXC to the FAD cofactor (65, 73).

The connections of the disulfide relay system and the respiratory chain

Like all redox-active enzymes, Erv1 requires one or more electron acceptors. The sulfhydryl oxidases are predicted to use molecular oxygen as an electron acceptor for the oxidation of cysteine residues to protein disulfides, generating hydrogen peroxide in the process (69). In yeast mitochondria, Erv1 passes its electrons onto molecular oxygen via interaction with cytochrome c and cytochrome c oxidase. This connection to the respiratory chain increases the efficient oxidation of the relay system in mitochondria and prevents the formation of hydrogen peroxide. Thus, analogous to the system in the bacterial periplasm, the disulfide relay in the intermembrane space is connected to the electron transport chain of the inner membrane (54, 55).

As was pointed out, substrates of the mitochondrial disulfide relay system include many proteins relevant to cytochrome c oxidase biogenesis, as well as many Tim chaperones. Therefore, a defect in this pathway is likely to result in pleiotropic effects due to a defective IMS and matrix import of proteins relevant to complex IV biogenesis and a number of yet uncharacterized mitochondrial functions. This finding is supported by the recently discovered mutation in the ALR gene (74). The mutation causes an infantile mitochondrial disorder with progressive myopathy and partial combined respiratory-chain deficiency, congenital cataract, sensorineural hearing loss, and developmental delay. The consequences of the mutation at mitochondrial level were the reduction in complex I, II, and IV activity, lower cysteine-rich protein content, abnormal ultrastructural morphology of the mitochondria with enlargement of the IMS space, and accelerated time-dependent accumulation of multiple mtDNA deletions. This case highlights the role of the mitochondrial disulfide relay system (74).

Disulfide bond formation in the endoplasmic reticulum

The lumen of the ER has been the classic site of oxidative folding in eukaryotic cells. Recent observations identified new protein and non-protein components of the electron transfer chain of the oxidative folding in the ER. Moreover, a feedback regulation has been described, which prevents the development of hyperoxidizing conditions during excessive oxidative folding. Finally, oxidative folding emerged as an important regulator of Ca^{2+} handling in the ER.

New protein components in the electron relay system of the oxidative folding

The discovery of the sulfhydryl oxidase enzyme Ero1 (endoplasmic reticulum oxidoreductin) in yeast more than a decade ago and of its two mammalian isoforms (Ero1α and Ero1β) seemed to complete the picture of oxidative protein folding in the ER. According to the generally accepted scheme, PDI (and presumably some other members of the PDI family) oxidizes diverse ER substrate proteins by thiol-disulfide exchange (75). PDI is then reoxidized by Ero1, transferring electrons to molecular oxygen, generating hydrogen peroxide.

However, the recent observation, that both Ero1α and Ero1β are dispensable in viable mice (76), made the theory questionable. Although these animals are diabetic due to defects in insulin production and secretion, the other essential functions requiring disulfide bond formation remained unchallenged. Thus, a search for alternative prooxidant mechanisms has been started, unearthing well-known and new oxidants, either enzymes or low molecular weight compounds.

From the former group of candidates, several local enzymes have emerged as potential disulfide catalysts. For example, quiescin sulfhydryl oxidase (QSOX) is an effective oxidant of unfolded proteins, but a less active oxidant for PDI (77). Moreover, QSOX is localized to the Golgi apparatus (78, 79) rather than to the ER. Another ER luminal enzyme, VKOR, oxidizes PDI during the reduction of vitamin K epoxide (80); however, this activity seems to be limited to membrane bound PDI family members (81).

Instead of these long known enzymes, recent observations highlighted new Ero1 surrogates. By using a PDI mutant with a single active site cysteine, new potential PDI oxidants were identified by mass spectrometry (82). While in wild type cells, Ero1α and Ero1β were mainly complexed with the mutant PDI, peroxiredoxin 4 (PRDX4) was also identified as a partner in the mixed disulfides. The ER localization of PRDX4 was previously shown, and it was postulated as an important detoxifier of hydrogen peroxide produced by Ero1 isozymes (83, 84). However, cells derived from mice carrying gene trap mutations in both Ero1α and Ero1β genes proved to be hypersensitive to depletion of PRDX4. A possible explanation for this unexpected phenomenon is that PRDX4 can substitute for Ero1 in disulfide bond formation. Indeed, depletion of PRDX4 exacerbated the modest defects in disulfide formation seen in the Ero1α – Ero1β double knockout cells (82). The role of PRDX4 as a hydrogen peroxide dependent disulfide catalyst has been confirmed by an independent *in vitro* study showing the rapid oxidation of PRDX4 by hydrogen peroxide and a consecutive relatively slower transfer of its disulfide to PDI family proteins (85).

In wild type cells, the cooperation between Ero1s and PRDX4 on one hand, improves the efficacy of oxidative protein folding, by generating two disulfide bonds at the expense of one molecule of oxygen; on the other hand, it would solve the problem of hydrogen peroxide toxicity. However, under the experimental conditions applied by Zito et al. (82), hydrogen peroxide sources other that Ero1s should be supposed. The identification of these sources requires further studies.

PRDX4 is presumably not a single candidate as PDI oxidant. A recent study showed that two human glutathione peroxidases (GPx7 and GPx8) are ER-resident PDI peroxidases. They were able to catalyze an efficient protein refolding in the presence of PDI and peroxide. They were shown to interact with Ero1α, suggesting that they can represent a novel pathway for the productive reutilization of hydrogen peroxide produced by Ero1s during disulfide bond formation (86).

Small molecules in the oxidative protein folding

The new, verified or suggested, protein components of the oxidative protein folding bring low molecular weight compounds into the picture. Although the luminal composition of the ER is hardly known in respect to small molecules, the presence of various small electron carriers (e.g., glutathione, ascorbate, FAD, pyridine nucleotides, vitamin K, hydrogen peroxide etc.) has been demonstrated (87). Many are shown to interact with the protein components of the electron transfer chain of the oxidative protein folding in the early secretory compartment (88, 89).

Hydrogen peroxide, and other reactive oxygen species (ROS), are produced within and around the ER by local oxygenases and oxidases (e.g., cytochrome P450s, flavin-containing monooxygenases, prolyl and lysyl hydroxylases, NOX4). FAD-containing oxidases including Ero1 family members and L-gulonolactone oxidase generate one molecule of hydrogen peroxide per catalytic cycle. H_2O_2 is not only a harmful by-product leading to oxidative stress, but also a potentially useful electron acceptor that can serve the formation of disulfide bonds from cysteine thiols via a sulphenic acid intermediate. A recent *in vitro* study demonstrated that H_2O_2, added directly or generated enzymatically, was able to promote the oxidative folding of a model substrate, resulting in the efficient formation of a natively folded protein containing proper disulfide bonds. H_2O_2 also oxidizes the active site of PDI and less efficiently GSH (90). Hence, it is possible that H_2O_2 generated either by Ero1 during disulfide bond formation or by other ER flavoproteins, can be utilized in turn for the formation of further disulfide bonds. The experiments demonstrating the role of PRDX4 in hydrogen peroxide dependent disulfide bond formation (82, 85) further strengthen this view.

The contribution of glutathione to disulfide bond formation has been underestimated since the discovery of Ero1. Recent observations show that glutathione is not only an important redox buffer in the luminal compartment of the ER, but also an active player in the oxidative protein folding. Experiments in Ero1 deficient cells suggested that parallel pathways should exist for disulfide generation, which can be a glutathione disulfide (GSSG) dependent PDI oxidation (91). Thus, under normal circumstances, Ero1 and GSSG mediated oxidation of PDI family proteins are main constituents of ER redox homeostasis. *In vitro* results, gained by stopped-flow and quenched-flow techniques, confirmed these data showing that the oxidation of both human PDI and yeast Pdi1p by GSSG is an effective and fast reaction (92). On the other hand, it has been reported that the presence of the cytosol is indispensable for the proper oxidative folding.

Since the addition of GSH restored normal disulfide formation by allowing disulfide isomerization, it was concluded that cytosolic GSH and lumenal Ero1 play opposite roles in the redox control of the ER (93). In agreement with this finding, it was also reported that oxidative stress resulted in the immediate reduction of ER oxidoreductases, e.g., ERp57. The reductive force was derived from the cytosol in the form of GSH (94). These results demonstrate that glutathione plays a direct role in both disulfide bond formation and isomerization, either as GSSG or GSH, respectively. Moreover, glutathione fluxes between the cytosol and the ER lumen have an important role in the regulation of ER redox.

Redox-active vitamins C, E, and K have also been implicated in the mechanism of oxidative folding (87). Dehydroascorbate, the oxidized form of ascorbate, can be produced from ascorbate in the ER lumen, or can be imported into the ER (95–97). Dehydroascorbate is a well-known *in vitro* protein thiol oxidant (98), in a relatively slow non-catalyzed reaction. However, several thiol-dependent dehydroascorbate reductases have been described, including PDI (99). PDI oxidized by dehydroascorbate can generate disulfide bonds in folding proteins. The *in vivo* role of dehydroascorbate in disulfide bond formation has been proposed on the basis of results gained in a microsomal model (100). A recent re-examination of the possible role of dehydroascorbate as a folding catalyst revealed that, although the PDI-catalyzed reaction is slow, dehydroascorbate rapidly reacts with dithiols in unfolded or partially folded proteins by a PDI-independent way (101).

ROS and other low molecular weight prooxidants act as electron acceptors at different stages of the oxidative folding. It seems that they can substitute either PDI or Ero1 in the electron transfer chain. The poor representation of antioxidant enzymes, e.g., catalase and superoxide dismutase, in the ER lumen, also favors their utilization in the process of oxidative folding (87). Thus, cysteinyl thiols of the newly synthesized luminal proteins can supply, at least partially, the reducing power for the antioxidant defence of the compartment. The multiplicity, redundancy and interchangeability of the oxidizing agents ensure that the oxidative folding can work without fatal disturbances even in the absence of Ero1. A new model of oxidative folding has been forwarded in which the oxidative power is supplied by various prooxidants in a stochastic way (102). The redundancy of oxidizing agents may fit the importance of a vital process.

Regulation of Ero1 activity by intraluminal redox conditions

The luminal compartment of the ER is an oxidative environment equipped with relatively poor antioxidant defence. Thus, the oxidase activity of Ero1 constitutes a potential source of ER-derived oxidative stress. Intricate feedback mechanisms to prevent Ero1 hyperactivity have been demonstrated by recent studies both in yeast and in mammalian cells. The non-catalytic cysteines play a central role in these regulatory mechanisms. They can form regulatory disulfides, which in turn influence the catalytic activity of Ero1 depending on the local redox conditions.

It has been demonstrated that non-catalytic cysteine pairs of Ero1p (Cys90–Cys349 and Cys150–Cys295) act as redox sensors modulating the catalytic activity of the enzyme in yeast. Disulfide formation between these cysteines, e.g., in hyperoxidizing conditions during intensive oxidative folding in the ER lumen, inhibits Ero1p activity. These changes restrict the functional dynamics of the loop containing the active site shuttle cysteines (Cys100–Cys105), thereby decreasing the oxidase activity. Redox changes in the ER correlate with the thiol–disulfide conversion of the regulatory cysteinyl moieties; upon the reduction of the regulatory disulfides Ero1p is reactivated. As a result of this preventive mechanism, Ero1p activity can be down-regulated by the hyperoxidation of the ER (103).

A similar mechanism has been described in the case of human Ero1α activity. Ero1α is also regulated by non-catalytic disulfides. Their low midpoint reduction potential ensures that they are stable under the normal redox conditions of the ER lumen and are only partially reduced by protein disulfide isomerase (104). This mechanism can prevent excessive Ero1α activation and uncontrolled oxidation of protein and glutathione thiols. Moreover, a regulatory mechanism based on intramolecular disulfide switches has also been reported. Formation of a disulfide bond between the active-site Cys94 (connected to Cys99 in the active enzyme) and Cys131, results in a decreased activity of Ero1α due to the covalent blockade of the active site. Competition between substrate thiols (i.e., the thiols of the reduced protein disulfide isomerase) and Cys131 creates a regulatory mechanism, thus the activation of Ero1α is dependent on the availability of its substrate, reduced protein disulfide isomerase (105). When PDI is present in the reduced form, the thiol-disulfide exchange reaction between Ero1α and protein disulfide isomerase is unhampered and the electron flow from substrate proteins to oxygen is continuous. A recent study demonstrated the presence of a similar regulatory mechanism also in Ero1β. Ero1β oxidase activity is a subject of feedback regulation by long-range intramolecular disulfide bond formation, in which Cys130 plays a critical role. However, its regulation is less strict than that of Ero1α, which is in agreement with the stronger oxidase activity of Ero1β in conditions/cells where massive oxidizing power is required (106).

The redox regulation of the oxidative protein folding clearly shows that the proteinaceous electron relay system contains in-built mechanisms to prevent dramatic changes in the luminal redox potential of the ER; moreover, these findings nominate PDI not only as a central component of the relay system, but also as a central regulator of ER redox homeostasis.

The calcium connection

Besides the secretory proteins, the apparatus of the oxidative protein folding can oxidize ER resident proteins. In the case of reversible action, this oxidation-reduction process can play a regulatory role. An important example is the specific role of Ero1 in the regulation of calcium fluxes from/to the ER (107) and in specific subdomains of the ER (108).

Inositol 1,4,5-trisphosphate receptor type 1 (IP3R1), a ligand-operated channel mediating ER Ca^{2+}-release, is an eminent candidate for an oxidative folding-mediated regulation. The third luminal loop of IP3R1 interacts with ERp44 (109), a thioredoxin family chaperone of the early secretory pathway (110). This interaction, favored by reducing luminal conditions and by low [Ca^{2+}] in the ER, inhibits channel activity (109). The presence of reduced cysteinyl thiols in the third loop is required for the interaction. Therefore, the ERp44/IP3R1 system may act as a redox and Ca^{2+} sensor monitoring the ER environment and transmitting signals to the cytosol, accordingly. ERp44 also interacts with human Ero1 isoforms, Ero1α and Ero1β, retaining them in the early secretory pathway (111, 112). As Ero1α interacts with ERp44, which in turn inhibits IP3R1, this oxidase could control Ca^{2+} homeostasis and signaling by oxidizing the regulatory loop of IP3R1 and/or decoy ERp44 (R. Sitia, personal communication).

This putative mechanism is supported by indirect observations. Ca^{2+} release from the ER is known to be involved in ER stress dependent apoptosis. Recent results showed that in ER stressed cells, Ero1α was induced by the proapoptotic transcription factor CHOP, and siRNA knockdown of Ero1α suppressed apoptosis (113). IP3-induced calcium release, increased during ER stress, was also blocked by siRNA-mediated silencing of Ero1α or IP3R1 and by loss-of-function mutations in Ero1α or CHOP. Thus, the CHOP mediated pathway provokes calcium-dependent apoptosis through an Ero1α-IP3R pathway. On the basis of these findings, a specialized subdomain of the ER, the mitochondria-associated membrane (MAM) has been forwarded as a possible site for Ero1α-regulated calcium fluxes. MAM facilitates direct calcium fluxes between the ER and mitochondria. It was hypothesized that Ero1α can be enriched in this specific domain of the ER. In fact, it was found that Ero1α is almost exclusively localized to the MAM in normoxic conditions. However, the localization was strictly dependent on redox conditions; chemical reductants released Ero1α from the MAM (108A). The calcium uptake into the ER can be also modulated by luminal redox conditions: it was reported that the luminal oxidoreductase ERp57 modulates the redox state of intraluminal thiols in SERCA 2b by a Ca^{2+}-dependent way (114).

The physiological significance of these events is underlined by a recent study on Ero1 and calcium homeostasis, which reported that altered Ero1 activity affects cardiac functions via intracellular calcium fluxes (115). Adult cardiomyocytes from the mouse line with loss of function insertion mutations of Ero1α presented reduced peak amplitudes of calcium transients. The decreased Ero1α activity blunted cardiomyocyte inotropic response to adrenergic stimulation and sensitized mice to adrenergic blockade.

Outlook

Studies on the mechanism of oxidative folding in the bacterial periplasm and eukaryotic ER and IMS have revealed the backbone of the participating electron transfer systems. However, the regulation of these machineries and the details of the redox environment surrounding them remained to be clarified.

Although the main proteinaceous components of the oxidative folding have been presumably identified in all three compartments, recent observations on the involvement of PRDX4 and PDI peroxidases in ER localized disulfide formation showed that additional players can still emerge. Erv1 in IMS might also have other possible substrates to be identified. Beside Dsbs, *Escherichia coli* possesses many other proteins that can affect protein folding in the periplasm. Moreover, recent reports indicate that other bacteria evolved different and multiple mechanisms for the generation of disulfide bonds. Bacterial proteins and toxins containing disulfides are often determinants of pathogenecity upon bacterial infections. Thus, the exploration of similarities and differences in bacterial disulfide generating systems is important for the identification of possible targets of antibiotic therapy.

The redox environment of these compartments, i.e., the periplasm, the IMS and the ER lumen, is only partially due to methodological difficulties. Redox sensors that are selective towards various redox-active compounds (e.g., glutathione, ascorbate, pyridine nucleotides) and can be applied in cellular systems are wanted. Moreover, components of the subcellular redoxomes (transporters and oxidoreductases) should be identified. Alternative electron acceptors in hypoxic conditions should also be searched for.

Recent findings, with respect to the regulation of oxidative folding in the ER, highlighted the presence of regulatory disulfides. Similar regulatory mechanisms can be predicted in the case of non-catalytic disulfides in the IMS. Furthermore, enzymes of the oxidative folding can contribute to the thiol-disulfide based redox regulation of the enzymes/transporters/channels of the ER and IMS. The list of these regulated proteins will surely be enlarged.

The structural and functional connection between the ER and mitochondria (e.g., by MAM) suggests a possible cooperation of the two organelles in oxidative folding. A carrier-mediated ER-to-mitochondria electron transfer via the MAM or the cytosol could utilize electrons by an energetically advantageous manner.

Last, but not least, our knowledge is limited on the physiological and pathological relevance of these systems, particularly in the IMS. In the case of ER, the involvement of oxidative folding in organelle stress and in the consecutive adaptive unfolded protein response is more or less obvious.

Highlights

- Evidence has been presented that a homolog of vertebrate ER protein VKOR is also an important component of the protein disulfide bond forming pathway in many bacteria.
- Bacterial Dsb proteins have emerged as possible targets for antibiotic treatment.

- The redox state of IMS is more oxidizing than the redox state of cytosol or mitochondrial matrix, which may support the oxidative folding of IMS imported proteins.
- Electrons are transferred from the cysteines of mitochondrial proteins to Erv1 via the oxidoreductase Mia40 and finally to either oxygen or cytochrome c.
- Substrates of the mitochondrial disulfide relay system include proteins relevant to cytochrome c oxidase biogenesis and Tim chaperones; therefore a defect in this pathway results in pleiotropic effects.
- Additional components (PRDX4, PDI peroxidases) of the electron relay system of oxidative protein folding have been described in the ER.
- Recent observations highlight the ancillary role of small molecular weight proooxidants (hydrogen peroxide, dehydroascorbate) in the oxidative folding in the ER.
- Feedback regulatory mechanisms have been reported in the ER, which can prevent the disadvantageous outcome of hyperoxidizing conditions.
- The enzymes of oxidative folding create connections between ER calcium fluxes and luminal redox conditions.

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