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FORUM REVIEW ARTICLE

How Proteins Form Disulfide Bonds

Matthieu Depuydt,[1,2] Joris Messens,[2–4] and Jean-Francois Collet[1,2]

Abstract

The identification of protein disulfide isomerase, almost 50 years ago, opened the way to the study of oxidative protein folding. Oxidative protein folding refers to the composite process by which a protein recovers both its native structure and its native disulfide bonds. Pathways that form disulfide bonds have now been unraveled in the bacterial periplasm (disulfide bond protein A [DsbA], DsbB, DsbC, DsbG, and DsbD), the endoplasmic reticulum (protein disulfide isomerase and Ero1), and the mitochondrial intermembrane space (Mia40 and Erv1). This review summarizes the current knowledge on disulfide bond formation in both prokaryotes and eukaryotes and highlights the major problems that remain to be solved. *Antioxid. Redox Signal.* 15, 49–66.

Introduction

The formation of a disulfide bond results from the oxidation of two cysteine thiol groups, with the concomitant release of two electrons. Disulfide bonds are important for the correct folding of many secreted proteins. In these proteins, disulfide bonds stabilize protein structure and protect the thiol group from over-oxidation. The formation of a disulfide bond is a rate-limiting step of the folding process of a protein. Therefore, although disulfides can form spontaneously *in vitro*, disulfide bond formation is a catalyzed process *in vivo* to allow the rapid folding of nascent proteins. The first catalyst of disulfide bond formation, protein disulfide isomerase (PDI), has been identified in the early sixties in the endoplasmic reticulum (ER) (49, 60). It is now clear that complex enzymatic systems catalyze disulfide bond formation in proteins not only in the ER, but also in mitochondria and in the bacterial periplasm.

Here, we review the mechanisms that govern disulfide bond formation in both prokaryotes and eukaryotes. We also summarize the current knowledge on a newly identified system whose function is to ensure that cysteine residues are not over-oxidized by reactive oxygen species (ROS) in the bacterial periplasm.

Mechanisms of Disulfide Bond Formation in Bacteria

Disulfide bond formation in Gram-negative bacteria

Disulfide bonds are introduced by disulfide bond protein A in the periplasm. The pathways of disulfide bond formation in Gram-negative bacteria have been best characterized in *Escherichia coli*. In this bacterium, disulfide bond formation takes place in the periplasm, a viscous compartment that separates the outer membrane from the inner membrane (Fig. 1A). The redox potential of the periplasm is around −165 mV (114), which makes this compartment significantly more oxidizing than the cytoplasm whose redox potential is between −260 and −280 mV (48, 66, 70).

The protein that introduces disulfide bonds into proteins secreted to the periplasm is disulfide bond protein A (DsbA) (Fig. 1A). Like most thiol-disulfide oxidoreductases, DsbA adopts a thioredoxin (Trx)-fold and presents a CXXC catalytic motif (CPHC in *E. coli* DsbA) (6, 28, 108). The two cysteine residues of the catalytic motif form a disulfide bond *in vivo* (12, 92). This disulfide is unstable (160, 163) and is rapidly transferred from DsbA to cysteine residues on unfolded polypeptide chains entering the periplasm (Fig. 1A). The structure of DsbA comprises also an α-helical domain, which structurally results in a hair curl-like domain on top of the Trx-fold (108) (Fig. 1B). This α-helical domain is inserted into the Trx-like domain and is likely to play a role in substrate binding (see below).

DsbA is a powerful oxidant. DsbA is one of the most oxidizing proteins known. *E. coli* DsbA has a redox potential of −119 mV (163), but DsbA homologs from other bacteria such as *Neisseria meningitidis* (97, 157) and *Pseudomonas aeruginosa* (141) are even more oxidizing (−80 and −95 mV, respectively). The origin of this extraordinary oxidizing power stems from intrinsic structural features of DsbA. The first cysteine of the CPHC motif (Cys30 in *E. coli* DsbA) has a low pK_a (~3) and is therefore predominantly present as a thiolate under

[1]de Duve Institute, Université catholique de Louvain, Brussels, Belgium.
[2]Brussels Center for Redox Biology, Brussels, Belgium.
[3]Department of Molecular and Cellular Interactions, Vlaams Instituut voor Biotechnologie, Brussels, Belgium.
[4]Structural Biology Brussels, Vrije Universiteit Brussel, Brussels, Belgium.

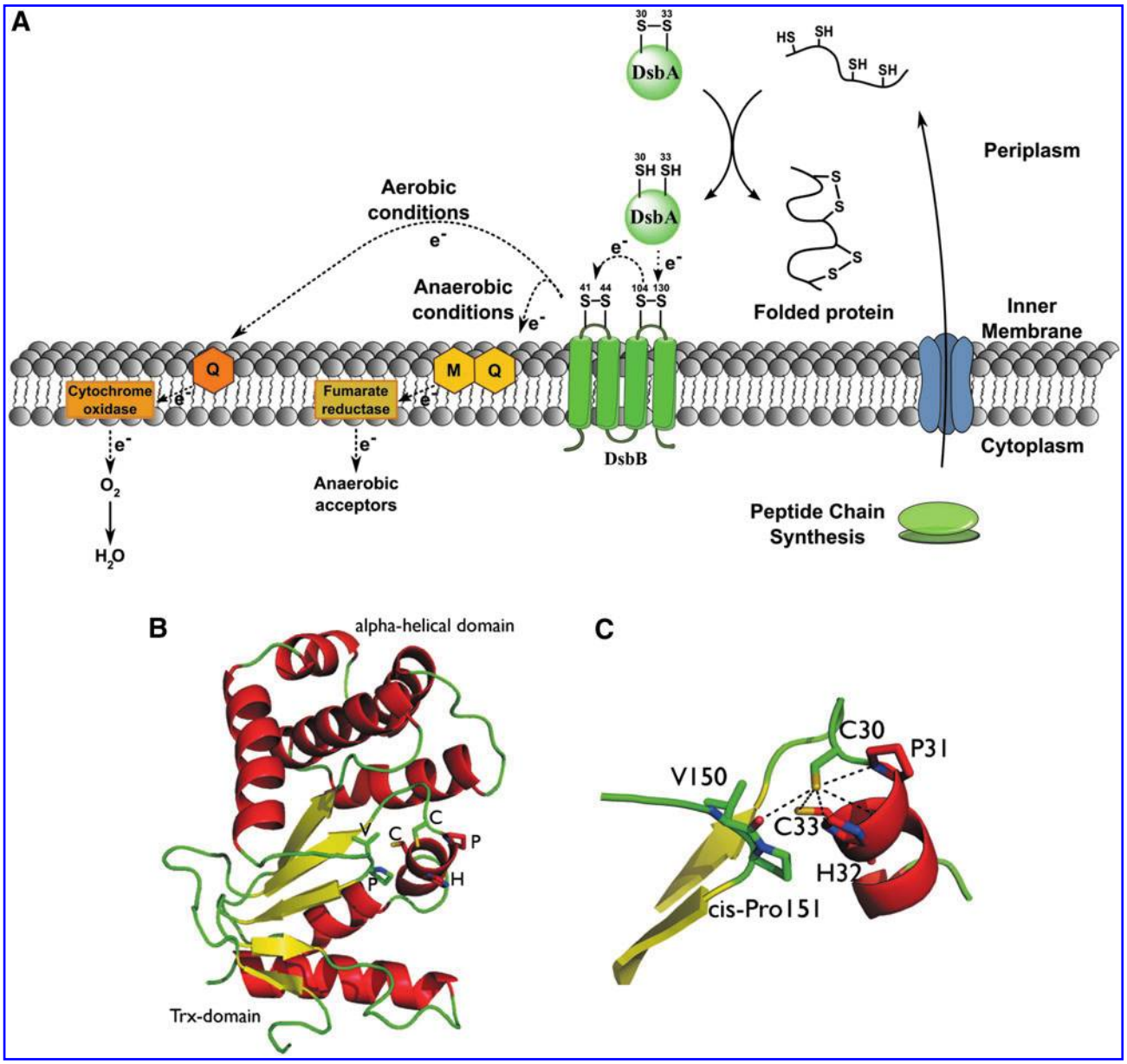

FIG. 1. Disulfide bond formation in the bacterial periplasm. (A) Proteins synthetized in the cytoplasm are secreted into the periplasm as unfolded polypeptides. Disulfide bonds are then introduced by disulfide bond protein A (DsbA), which is recycled by the inner membrane protein DsbB. Finally, e^- are shuttled from DsbB to different aerobic and anaerobic electron acceptors *via* the electron transport chain (Q, ubiquinone; MQ, menaquinone). The direction of electron flow is shown by *dotted arrows*. **(B)** Three-dimensional structure of the reduced form of *Escherichia coli* DsbA (protein database [PDB] entry code 1A2L) (57). DsbA has a thioredoxin (Trx)-fold in which an α-helical domain is inserted. **(C)** Close-up view of the active site of the reduced form of DsbA with the important residues in stick presentation. Possible hydrogen bond interactions stabilizing the thiolate anion of Cys30 are indicated by *dotted lines*. The figures were generated using MacPyMol (Delano Scientific LLC 2006). (To see this illustration in color the reader is referred to the web version of this article at www.libertonline.com/ars).

physiological conditions (118). The thiolate of Cys30 is located at the N-terminus of an α-helix whose positive dipole stabilizes the thiolate form (57) (Fig. 1B). Further, Cys30 accepts stabilizing hydrogen bonds from the CPHC active site proline, histidine, and the second cysteine (53, 57) as well as from the main chain oxygen of a valine residue located in a loop containing a *cis*-proline positioned on the opposite site of the CPHC motif (22, 47, 97, 127) (Fig. 1C). This network of hydrogen bonds between the thiolate and neighboring residues stabilizes the reduced form of DsbA (compared to the oxidized one), providing the necessary driving force for substrate oxidation by DsbA.

DsbA oxidatively folds hundreds of secreted polypeptides, including virulence factors. In *E. coli*, about 300 proteins, representing 40% of the periplasmic proteome, possess an even number of cysteine residues and therefore potentially depend on DsbA for folding (38). More than 30 DsbA sub-

strates have been identified so far using various proteomics techniques (64, 83, 100, 101, 122, 156). Noteworthy, in pathogenic strains of *E. coli* and other gram-negative bacteria, several toxins and surface proteins that are required for bacterial virulence are DsbA substrates [see the comprehensive review of Heras *et al.* for more information (63)]. Thus, DsbA appears as an attractive target for the design of drugs that lower the virulence of pathogenic strains rather than their viability.

How does DsbA interact with its substrates? Our understanding of the structural details of how DsbA recognizes its substrates has been hampered by the transient nature of the complexes formed between DsbA and its substrates. However, the recently released structure of a covalent DsbA-peptide complex (protein database entry code: 3DKS) provided some insights into substrate interactions. In the structure, the short peptide (nine residues) is bound at the interface between the α-helical and Trx domains of DsbA (124). Most of the interactions are backbone-to-backbone hydrogen bonds between the peptide and residues from a loop preceding the *cis*-proline of the Trx domain of DsbA. This type of interaction is consistent with the broad substrate specificity that allows DsbA to interact with dozens of different unfolded polypeptides entering the periplasm.

How is disulfide bond formation coordinated with protein secretion? Proteins destined for the periplasm are synthesized in the cytoplasm and present N-terminal export signals that are recognized by an export apparatus (117). The majority of them are then transported in an unfolded state across the inner membrane by the SecYEG translocon. Depending on the properties of the signal sequence, proteins are secreted cotranslationaly or post-translationaly (69, 80).

How disulfide bond formation is coordinated with the folding process of the protein in the periplasm is still not fully understood. In a recent paper, Kadokura and Beckwith tackled this problem (82) by using a DsbA mutant forming stable complexes with alkaline phosphatase (PhoA), a periplasmic protein with two consecutive disulfide bonds (84). These authors were able to follow the oxidative folding of PhoA *in vivo* the moment it was leaving the SecYEG channel. Their results indicate that disulfide bonds are preferentially introduced in a vectorial manner into PhoA molecules entering the periplasm (Fig. 2). Moreover, by fusing PhoA to different signal sequences, Kadokura and Beckwith were also able to study the influence of the mode of secretion (either post- or cotranslational) on the oxidative folding process. They found that cotranslational export of the protein favors the vectorial oxidation of the cysteine residues (82) more than post-translational export. A likely explanation to this observation is that when a protein is exported cotranslationaly, the rate of translocation is limited by the rate of protein synthesis. The polypeptide chain appears therefore more slowly in the periplasm, which favors the vectorial oxidation of the cysteine residues of the secreted polypeptide.

How is DsbA reoxidized? Following the transfer of its disulfide to substrate proteins, DsbA is released in a reduced state. It is then converted back to the oxidized state by a small membrane protein called DsbB (12), which uses the oxidizing power of ubiquinone to generate disulfide bonds *de novo* (8, 94) (Fig. 1A). Then, electrons flow from ubiquinone to cytochrome oxidases and then finally to molecular oxygen (7). Thus, disulfide bond formation is connected to the respiratory chain (7, 94). Under anaerobic conditions, DsbB passes the electrons to menaquinone and then to other final electron acceptors such as fumarate or nitrate reductases (7, 150) (Fig. 1A).

DsbB has four transmembrane α-helices, arranged in a left-handed helix bundle (73), and two small periplasmic loops that each contains two conserved redox-active cysteine residues (Cys41–Cys44 and Cys104–Cys130 in *E. coli* DsbB). DsbB has one quinone binding site (8), located in proximity to the Cys41–Cys44 cysteine pair (73).

DsbB transfers electrons from DsbA to quinones *via* a succession of thiol-disulfide exchange reactions in which the four cysteine residues of DsbB play a crucial role (Fig. 1). The first step of the reaction is the oxidation of the CXXC motif of DsbA by the Cys104–Cys130 disulfide of DsbB. Then, this disulfide

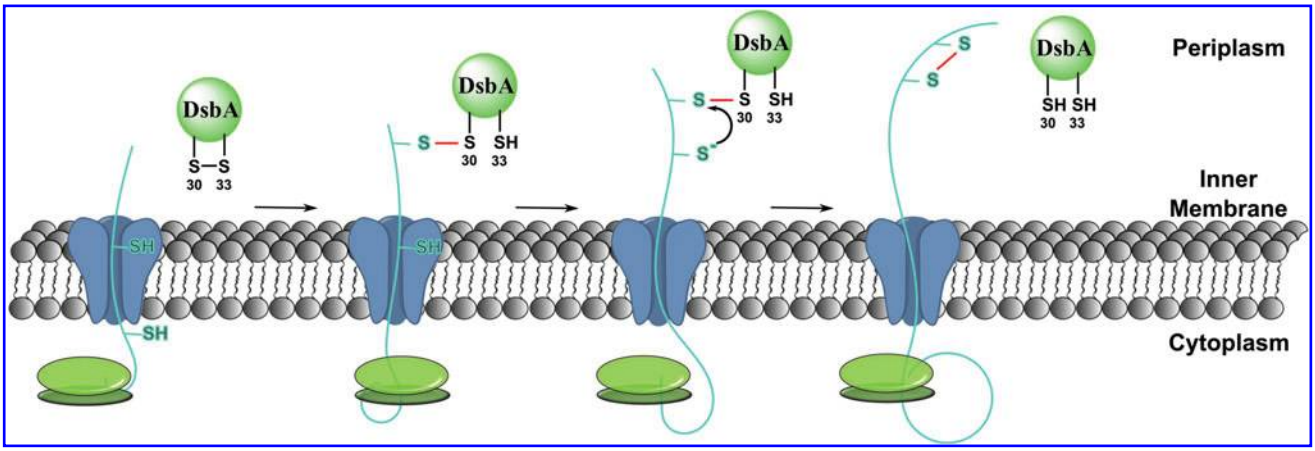

FIG. 2. Disulfide bonds are preferentially introduced in a vectorial manner by DsbA. Envelope proteins are produced in the cytoplasm and then secreted to the periplasm. Unfolded polypeptides are then translocated across the inner membrane. According to the current model, DsbA preferentially introduces disulfide bonds in a vectorial manner between cysteine residues of polypeptides entering the periplasm. (To see this illustration in color the reader is referred to the web version of this article at www.libertonline.com/ars).

is regenerated by transferring the electrons to the Cys41–Cys44 disulfide. The Cys41 and Cys44 cysteines are finally recycled back to the oxidized state by transferring the electrons to the associated quinone molecule. Ito *et al*. have thoroughly studied the mechanism of DsbB to understand how this protein generates disulfides *de novo* by quinone reduction [see references in this review and in refs. (71, 77)]. Using quantum chemistry simulation, they showed that Cys44 (75), in its thiol state, forms a charge transfer complex with the ubiquinone molecule. According to their model, the formation of this complex is followed by the formation of a covalent adduct between Cys44 and ubiquinone. A nucleophilic attack on this covalent bond by Cys41 then regenerates the Cys41–Cys44 disulfide and leads to quinone reduction.

DsbB catalyzes an energetically uphill reaction. Inaba *et al*. determined the redox potential of the Cys41–Cys44 and Cys104–Cys130 cysteine pairs to be −207 and −224 mV, respectively (74). Therefore, the oxidation of DsbA, which has a redox potential of −119 mV, is a thermodynamically unfavorable reaction. Insights into the mechanism used by DsbB to oxidize DsbA came from the recent determination of the structure of the DsbA-DsbB-ubiquinone complex (72, 73, 165). On the basis of these structural data, a model has been put forward to explain how DsbB oxidizes DsbA (73). The reaction takes off with the nucleophilic attack of Cys30 of reduced DsbA on Cys104 in the Cys104–Cys130 disulfide of DsbB, resulting in the formation of an intermediate mixed-disulfide complex between Cys30 of DsbA and Cys104 of DsbB. The formation of the Cys30–Cys104 mixed-disulfide leads to conformational changes that physically separate Cys130 of DsbB from the mixed-disulfide, preventing the backward electron flow from DsbB to DsbA and therefore driving the reaction toward the reoxidation of DsbA (73). Then, the model favors a major pathway in which Cys33 of DsbA attacks the intermolecular Cys30–Cys104 disulfide, releasing oxidized DsbA and reduced Cys104. Subsequently, Cys130 attacks the Cys41–Cys44 disulfide, which results in a Cys130–Cys41 interloop disulfide, releasing Cys44. Then, Cys104 attacks the interloop disulfide, which regenerates the Cys104–Cys130 disulfide and releases reduced Cys41. The last step of the reaction consists in the transfer of two electrons form Cys41 and Cys44 to the quinone molecule *via* the mechanism described previously. An alternative model, which is also consistent with the structural data, proposes that DsbB oxidizes DsbA using the coordinated action of the two pairs of cysteine residues of DsbB instead than by a sequential set of electron transfer reactions (81, 165). According to this model, the step following the binding of DsbA and the formation of the Cys30–Cys104 intermolecular disulfide is a rapid thiol-disulfide exchange reaction in which Cys130 of DsbB attacks Cys41 in the Cys41–Cys44 disulfide, forming the Cys41–Cys130 interloop disulfide bond and releasing reduced Cys44. In this case, it is the formation of the interloop disulfide that prevents Cys130 to attack the mixed-disulfide between DsbA and DsbB, preventing the backward reaction. The subsequent release of oxidized DsbA, the disulfide rearrangement within DsbB and quinone reduction would then occur *via* a concerted process (81, 165). In agreement with this model, it has recently been shown that quinone reduction appears to be the rate limiting step of the overall reaction cycle and that the rate of DsbA oxidation is the same as the rate of quinone reduction (151). Further experimental work is required to clearly establish the mechanism of DsbB. A particularly intriguing question is how the oxidized quinone molecule is restored after the generation of one disulfide bond.

How does DsbA interact with DsbB? The determination of the DsbB–DsbA complex also shed light on the structural details of the interaction between these two proteins. In the complex, DsbB was found to bind to the so-called hydrophobic groove of DsbA (73), which is located below the redox-active cysteine residues (58). This binding site is distinct from the binding site observed in the DsbA-peptide complex (see above). Moreover, the hydrophobic interactions that take place between the hydrophobic groove of DsbA and DsbB suggest that the specificity of DsbA for DsbB is higher than for its substrates.

DsbC is a PDI that corrects DsbA's mistakes. As explained above, DsbA preferentially forms disulfides in a vectorial manner on polypeptide chains entering the periplasm. Thus, when disulfides need to be formed between cysteines that are nonconsecutive in the sequence, DsbA can introduce non-native disulfides (Fig. 3). The correction of these non-native disulfide bonds relies on a disulfide isomerization pathway whose main player is a soluble, homodimeric protein called DsbC (128, 139) (Fig. 3). Each DsbC subunit presents a catalytic domain and a dimerization domain, which are linked together by an α-helix (111). The catalytic domain of DsbC adopts a Trx-fold comprising a CXXC motif (CGYC in *E. coli* DsbC). This motif is kept reduced in the periplasm, in contrast to the CXXC motif of DsbA (79). Although the *in vivo* redox states of the catalytic cysteines of DsbC and DsbA are different, DsbC has a redox potential (−130 mV) that is similar to that of DsbA (−120 mV) (164).

DsbC is kept reduced in the periplasm, which enables this protein to correct non-native disulfides. The N-terminal Cys98 residue of the CGYC active site attacks the non-native disulfide, which results in the formation of an unstable mixed-disulfide complex between DsbC and the substrate protein. This mixed-disulfide can be resolved either by attack of another cysteine of the misfolded protein, resulting in the formation of a more stable disulfide in the substrate and the release of reduced DsbC, or by the attack of Cys101 of DsbC. In the first case, DsbC is acting as a true isomerase. In the second case, DsbC functions as a reductase, reducing the non-native disulfide and giving DsbA a new chance to form a correct disulfide. In both cases, the active site of DsbC must be in the reduced state for the enzyme to attack the non-native disulfide bond. DsbC functions as a potent isomerase *in vitro*. However, the physiological importance of the role of DsbC as an isomerase *versus* a reductase has not yet been clearly established (76). Recently, Shouldice *et al.* showed that TrxP, a protein from *Bacteroides fragilis*, which is homologous to DsbC but only exhibits a reductase activity, complements an *E. coli dsbC* deletion mutant. This indicates that reduction/oxidation of non-native disulfides can support disulfide isomerization *in vivo* (140).

The number of identified DsbC substrates is limited. The subset of secreted proteins that contain nonconsecutive disulfides and are therefore dependent on DsbC for folding is relatively small. So far, only four periplasmic proteins with

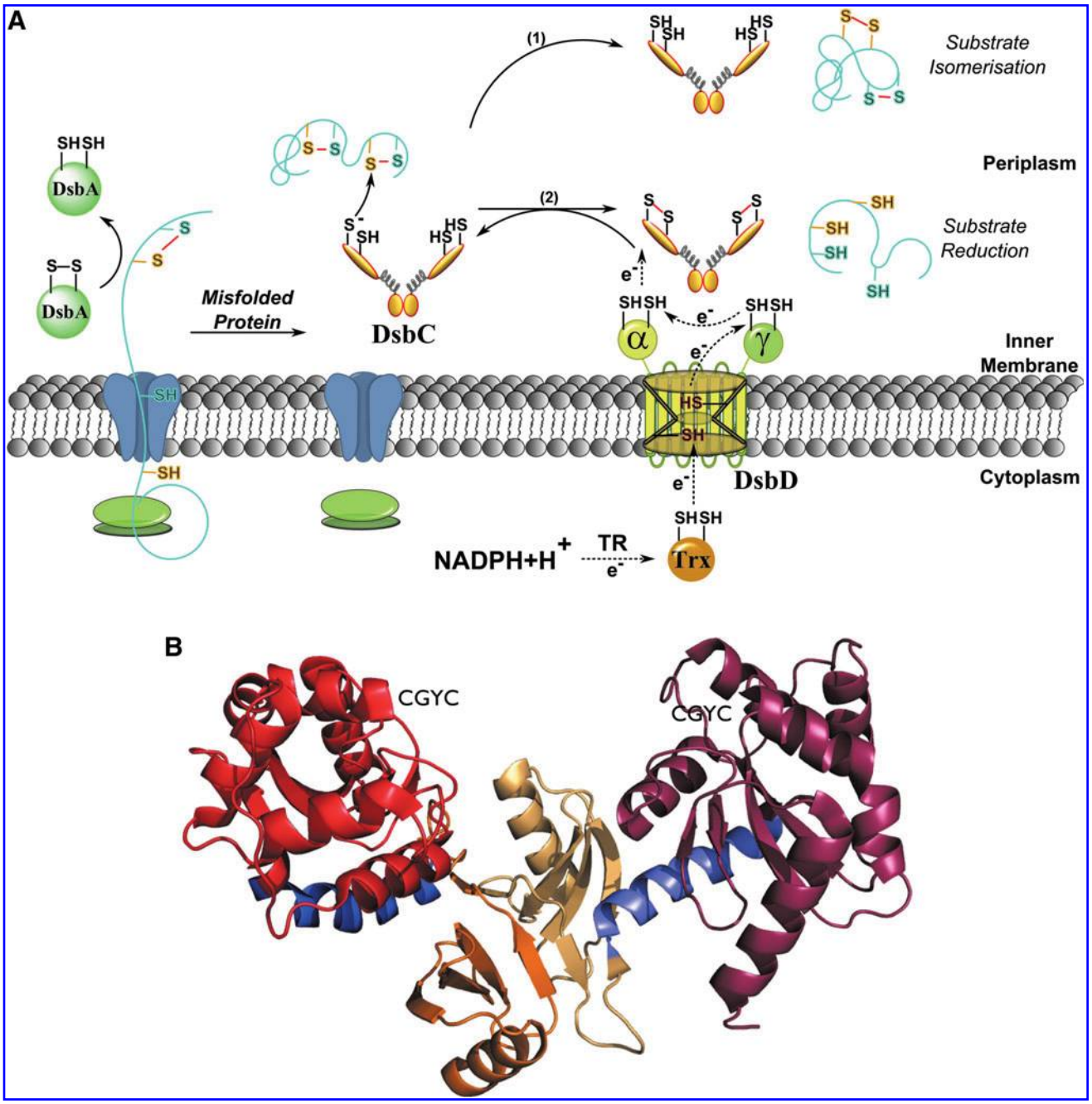

FIG. 3. Disulfide bond reduction/isomerization in the periplasm. (A) When native disulfides occur between nonconsecutive cysteines in a substrate protein, DsbA may incorrectly pair the cysteines. Incorrect disulfides are either isomerized [1] or reduced [2] by DsbC, a homodimeric soluble protein present in the periplasm. Isomerization leads to the release of the substrate with shuffled disulfides and of reduced DsbC (active) [1]. Reduction involves the (partial) unfolding of the polypetide chain and the release of oxidized DsbC (inactive) [2]. Oxidized DsbC is regenerated by the inner membrane protein DsbD. DsbD, whose transmembrane domain adopts a hour-glass structure, transfers e^- from the cytoplasmic thioredoxin reductase (TR)/Trx system to the periplasm. The direction of electron flow is shown by the *dotted arrows*. **(B)** Overall structure of *E. coli* DbC. Ribbon diagram of DsbC dimer is shown (PDB entry code 1EEJ) (110). The Trx domains are shown in red tints, the dimerization domains in orange tints, and the helical linker in blue tints. The active site residues CGYC are indicated. The figure was generated using MacPyMol (Delano Scientific LLC 2006). (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

multiple cysteine residues have been identified as DsbC substrates (14, 64, 156). Moreover, we recently showed that DsbC is also involved in the folding of LptD (34), an essential β-barrel protein that inserts lipopolysaccharides in the outer membrane. LptD has four cysteine residues that form two nonconsecutive disulfides (133). *dsbC* mutants are viable, whereas LptD is essential; this indicates that DsbA catalyzes sufficient correct disulfides in LptD for viability. This observation is consistent with our previous finding that DsbA can also correctly fold RNase I, a protein with a nonconsecutive

disulfide (114). Thus, DsbA is more specific than generally assumed, and DsbC is not always required to fold proteins with nonconsecutive disulfides.

The isomerase activity of DsbC depends on dimerization. The N-terminal dimerization domains of DsbC come together and form a V-shaped extended cleft whose inner surface is patched with uncharged and hydrophobic residues (111). This cleft is thought to be important for the binding of substrates and for the chaperone activity of the protein (23, 105, 111, 149). The dimerization of DsbC is essential for its isomerase activity as highlighted by a series of experiments that showed that fusion of the Trx-folds of DsbA or Trx to the N-terminal dimerization domain of either DsbC (136, 137) or the periplasmic chaperone FkpA (4) gives rise to chimeras that efficiently catalyze disulfide bond isomerization in the periplasm. Moreover, DsbC variants with an intact dimerization domain but keeping only one catalytic domain still display significant isomerase activity (5). This further indicates that the dimerization of DsbC leads to the formation of a substrate-binding domain that is crucial for the isomerization reaction.

How is DsbC kept reduced in the periplasm? DsbC is kept reduced in the periplasm by the inner membrane protein DsbD. DsbD is a 59 kDa protein that contains three domains: two domains, DsbDα and DsbDγ, are located in the periplasm, whereas the third domain, DsbDβ, is embedded within the membrane. Each domain of DsbD contains one pair of conserved cysteine residues that are essential for activity (145). The function of DsbD is to transfer the electrons that it receives from the cytoplasmic Trx system to the periplasmic space (Fig. 3) (86, 129). A series of *in vitro* and *in vivo* experiments revealed that DsbD transfers electrons across the membrane by a cascade of thiol-disulfide exchange reactions: electrons are transferred from Trx to the cysteine residues present in DsbDβ, then successively to the cysteines of DsbDγ and DsbDα, and then finally to the catalytic site of DsbC and/or DsbG (see below). This is a thermodynamically favorable pathway as electrons flow from a low redox potential of -270 mV (Trx) to -246 mV (DsbDβ), -241 mV (DsbDγ), -229 mV (DsbDα), -130 mV (DsbC), and/or -126 mV(DsbG) (29, 131). Noteworthy, proline residues that are located in the vicinity of the catalytic cysteines of DsbDβ have been shown to play an important role in the mechanism of DsbD (24, 26, 65).

The structures of the two periplasmic domains of DsbD have been solved separately and in complex (51, 59, 91, 146, 147). Whereas DsbDγ adopts a Trx-fold with a CXXC motif (91), DsbDα presents an immunoglobulin fold (59). An interesting observation has been reported recently regarding the CXXC motif of DsbDγ. It appears that the first cysteine of the CXXC motif of DsbDγ (Cys461) has a high pK_a value of 10.5 (109), in contrast to most nucleophilic cysteines in Trx-fold proteins that usually have low pK_a values. This makes Cys461 poorly reactive toward the disulfide of DsbDα and prevents its nonspecific oxidation. However, formation of the complex between DsbDγ and DsbDα seems to lower the pK_a of Cys461, allowing the transfer of electrons between these two domains (109, 110).

How does DsbDβ transport electrons across the membrane? Detailed structural data on the transmembrane domain of DsbD are missing, which hampers our detailed understanding of the mechanism used by DsbDβ to transfer electrons across the membrane. However, on the basis of experiments using small alkylating molecules to probe the accessibility of the catalytic cysteines of DsbDβ, Beckwith *et al.* have proposed a model for the structural organization of DsbD (24–26). According to this model, DsbD adopts an hourglass structure in which DsbDβ forms two cavities accessible to one or the other side of the membrane (Fig. 3). The membrane-embedded catalytic cysteine residues are located at the juncture of the two cavities where they are exposed to both sides of the membrane. Oxidation or reduction of these two catalytic cysteine residues does not appear to significantly affect the conformation of DsbDβ, as the accessibility of cysteines substituted for residues in four of the eight transmembrane segments is the same in both the oxidized and reduced state of DsbDβ.

Is there a futile cycle in the periplasm? The oxidation and isomerization pathways coexist in the bacterial periplasm. Both pathways involve proteins from the Trx superfamily, which raises the question of the existence of a futile cycle in the periplasm in which DsbB would oxidize DsbC and DsbD would reduce DsbA. It is now clear that both pathways are kinetically separated from each other. For instance, the apparent rate constants of DsbA oxidation by DsbB or of DsbC reduction by DsbD are above $10^6\ M^{-1}\ s^{-1}$ (52, 131). In contrast, the oxidation of DsbC by DsbB or the reduction of DsbA by DsbD is at least 10^3 to 10^7-fold slower. Structural features provide the molecular basis for those kinetic barriers. It has been shown that DsbC is protected from oxidation by DsbB by its dimeric structure (8), the orientation of the active site and the length of the α-helical linker (136).

DsbC can function independently of DsbD. We recently found that the simultaneous absence of DsbA and DsbC severely impairs the integrity of the cell envelope and affects the global protein content of the periplasm (156). Unexpectedly, we found that strains lacking both DsbA and DsbD, the protein that is responsible for keeping DsbC active as an isomerase, do not share these characteristics. Our results suggest therefore that DsbC is able to cooperate with DsbA in a DsbD-independent manner to ensure the correct folding of proteins secreted to the cell envelope. One possibility is that DsbC is able to cycle from the reduced to the oxidized state upon substrate oxidation and substrate reduction, respectively: when DsbC gets oxidized upon reduction of a nonnative disulfide, it could be recycled back to the reduced form by transferring its disulfide to a reduced protein (156).

What is the role of glutathione in the bacterial periplasm? Reduced glutathione (GSH) is a ubiquitous compound that serves as the main redox buffer in the cell (21). Although it has been known for some time that *E. coli* cells secrete glutathione (114), the presence of this redox buffer in the periplasm was only recently demonstrated. Beckwith *et al.* artificially directed a normally cytosolic glutaredoxin to the periplasm (44). In that context, the exported glutaredoxin supported disulfide bond formation independently of DsbB. However, the oxidizing activity of the exported glutaredoxin required the presence of the glutathione biosynthetic pathway, which supports the presence of glutathione in the peri-

plasm at functionally significant levels. This is consistent with our previous observation that alterations in glutathione biosynthesis increase the redox potential of the periplasm (114). In addition to glutathione, L-cysteine has recently been shown to function as a hydrogen peroxide (H_2O_2) scavenger in the periplasm (120). Both glutathione and L-cysteine are therefore likely to contribute to the global redox equilibrium of this extracytoplasmic compartment.

Disulfide bond formation in Gram-positive bacteria

In Gram-positive bacteria, disulfide bond formation has been best studied in *Bacillus subtilis.* This bacterium has a set of proteins called bacillus disulfide bond (Bdb), reminiscent of the *E. coli* Dsb proteins.

BdbD, the functional equivalent of DsbA, introduces the disulfide bonds. BdbD is anchored to the cytoplasmic membrane and adopts a Trx-fold containing a CPSC catalytic motif. The redox potential of BdbD is −75 mV, which is remarkably oxidizing (30). The structure of BdbD revealed the unexpected presence of a calcium-binding site, which influences the redox properties of the protein. Calcium depletion decreases the redox potential of BdbD by ~20 mV. So far, the only identified substrate of this potent oxidase is the protein ComGC, which is involved in natural competence development (112).

BdbD is reoxidized by BdbC, which belongs to the same bicistronic operon (17, 112). BdbC has a sequence identity of ~40% with DsbB and the protein is predicted to have the same topology. BdbC also presents two extracytoplasmic loops containing a pair of catalytic cysteine residues. The *B. subtilis* strain 168 possesses another DsbB homolog, BdbB, which directly forms two disulfide bonds in the antimicrobial peptide sublancin168 (96).

A reduction pathway has also been described in *B. subtilis*. This pathway involves CcdA, a membrane protein that transports reducing equivalents across the inner membrane. CcdA corresponds to the membranous β domain of DsbD and lacks the periplasmic DsbDα and DsbDγ domains (135). CcdA delivers electrons to two membrane-anchored proteins, ResA and StoA, which are involved in cytochrome c (Cyt c) maturation and the formation of the spore cortex, respectively (42, 43).

In *B. subtilis*, the conserved protein YphP might have isomerase activity (36). YphP is structurally related to Trx, but possesses a CXC motif instead of the canonical CXXC motif. However, the subcellular localization of YphP has not been determined and further evidence is required to confirm that it functions as an isomerase *in vivo*.

Vitamin K epoxide reductase as a substitute for DsbB. Bioinformatic searches revealed that some bacteria possess DsbA homologs but lack a homolog of DsbB. This led to the identification of a new protein with DsbB-like activity, which is present in the genome of several bacteria, including actinobacteria, cyanobacteria, and aerobic δ-proteobacteria. This protein is the bacterial homolog of the vitamin K epoxide reductase (VKOR) (38). Vertebrate VKOR catalyzes the reduction of vitamin K, a quinone. This reaction is one of the steps in the set of enzymatic reactions that leads to blood coagulation in humans (102, 121). VKOR is a membrane protein with four conserved catalytic cysteine residues, two of which are in a CXXC motif (50, 130). Expression of *Mycobacterium tuberculosis* VKOR in *E. coli* restores the phenotype of *dsbB* mutants and the complementation is dependent on DsbA, indicating that VKOR is functionally equivalent to DsbB (38, 39). Recently, the structure of a VKOR homolog from *Synechococcus* sp. has been solved in complex with a redox partner presenting a Trx-like fold. Remarkably, the overall structure of VKOR is similar to the structure of DsbB, the catalytic core of VKOR appearing as a four-transmembrane helix bundle that surrounds a quinone (103).

Prediction of disulfide bond formation by counting the number of cysteine residues

The study of disulfide bond formation has been restricted so far to a relatively small number of bacteria, despite the huge diversity of bacterial species. To evaluate the capacity for disulfide bond formation of the hundreds of bacteria for which the genome is sequenced, Beckwith *et al*. used a bioinformatic approach based on counting the number of cysteine residues in proteins that enabled them to make predictions about disulfide bond formation across bacterial species (38). In this method, a bias for even numbers of cysteine residues is considered as an indicator of disulfide bond formation. In *E. coli* for instance, this bias is clear when we compare the number of cysteine residues between periplasmic and cytoplasmic proteins. The number of cysteines in periplasmic proteins is mostly even, whereas in cytoplasmic proteins the number is indifferent (38). This is probably due to the presence of the cytoplasmic Trx system that keeps cytoplasmic proteins in their reduced form. Daniels *et al*. applied a similar approach to address the problem of disulfide bond formation in Gram-positive bacteria (32). They found that in aerobic Actinobacteria, secreted proteins present cysteines in an even-biased pattern, which is indicative of disulfide bond formation. In contrast, aerobic Firmicutes tend to exclude cysteine residues from their cytoplasmic and exported proteome. Those bacteria therefore tolerate growth under reducing conditions. In the case of anaerobic Firmicutes, which grow in the absence oxygen, no bias is observed. Since overoxidation is not a problem under anaerobic conditions, there is indeed no need for a biased cysteine content.

Mechanisms of Disulfide Bond Formation in Eukaryotes

Disulfide bond formation in the ER

PDI functions as an oxidase and an isomerase. The ER, the first organelle of the secretory pathway, serves as a folding factory for proteins that will either be secreted by the cell or inserted into membranes (143). In the ER, secretory proteins reach their native conformation and are covalently modified by the formation of disulfide bonds or by the addition of carbohydrates or glycosylphosphatidylinositol. Numerous molecular chaperones, glycosylating enzymes, and protein folding catalysts are present in the ER to guarantee the quality of the folding process (107, 143). Among those proteins, the PDI, present in the ER in high concentrations, is the key enzyme that introduces disulfide bonds into folding proteins. In contrast to prokaryotes, where distinct proteins are involved in thiol oxidation and disulfide isomerization, PDI is a multifunctional protein that catalyzes disulfide bond formation as

well as disulfide bond reduction and isomerization (60) (Fig. 4A). Like DsbA and DsbC, the activity of PDI depends on catalytic cysteine residues arranged in CXXC motifs (CGHC in PDI). When the cysteine residues are oxidized, PDI reacts with folding proteins and electrons are transferred from the reduced substrate proteins to the CGHC motif. When the cysteine residues of PDI are reduced, PDI has the possibility to react with non-native disulfides to form a mixed-disulfide complex. As previously discussed for DsbC, the mixed-disulfide can be resolved by attack of the second cysteine of the CGHC motif, releasing PDI oxidized and the non-native disulfide reduced. Alternatively, another thiolate of the substrate might attack the mixed-disulfide, which results in the reshuffling of the disulfide within the substrate protein, whereas the redox state of PDI remains unchanged.

Other oxidoreductases sharing homology with PDI are present in the ER lumen. The number of those proteins varies from species to species. For example, whereas five different PDI family members are present in yeast, the human ER harbors 20 of them. Although several of those proteins have now been characterized *in vitro*, their physiological functions are still poorly defined. The properties and activities of PDI homologs have been reviewed elsewhere and will not be described here (27, 60).

Structural and redox properties of PDI. PDI has four distinct domains (named *a*, *b*, *b′*, and *a′*) as well as an acidic C-terminal extension and an interdomain linker inserted between domains *b′* and *a′* (19 residues in human PDI). The four *a*, *b*, *b′*, and *a′* domains present a Trx-fold. However, only the N-terminal *a* and C-terminal *a′* domains possess a CXXC catalytic motif and are therefore active (40) (Fig. 4B).

The structure of the full-length mammalian protein is not known, although the structures of individual domains (88–90, 119) and of other members of the human PDI family are available (11, 37, 159). The structure of the full-length PDI from yeast, a protein that is 29% identical to the human protein and shares the same overall domain architecture, has been solved recently (153). Yeast PDI appears as an asymmetric twisted-U in which the catalytic *a* and *a′* domains face each other (Fig. 4B). The interior of the U-shaped PDI is lined with hydrophobic residues, which are most likely involved in peptide binding (33). The *b′* domain seems to provide the

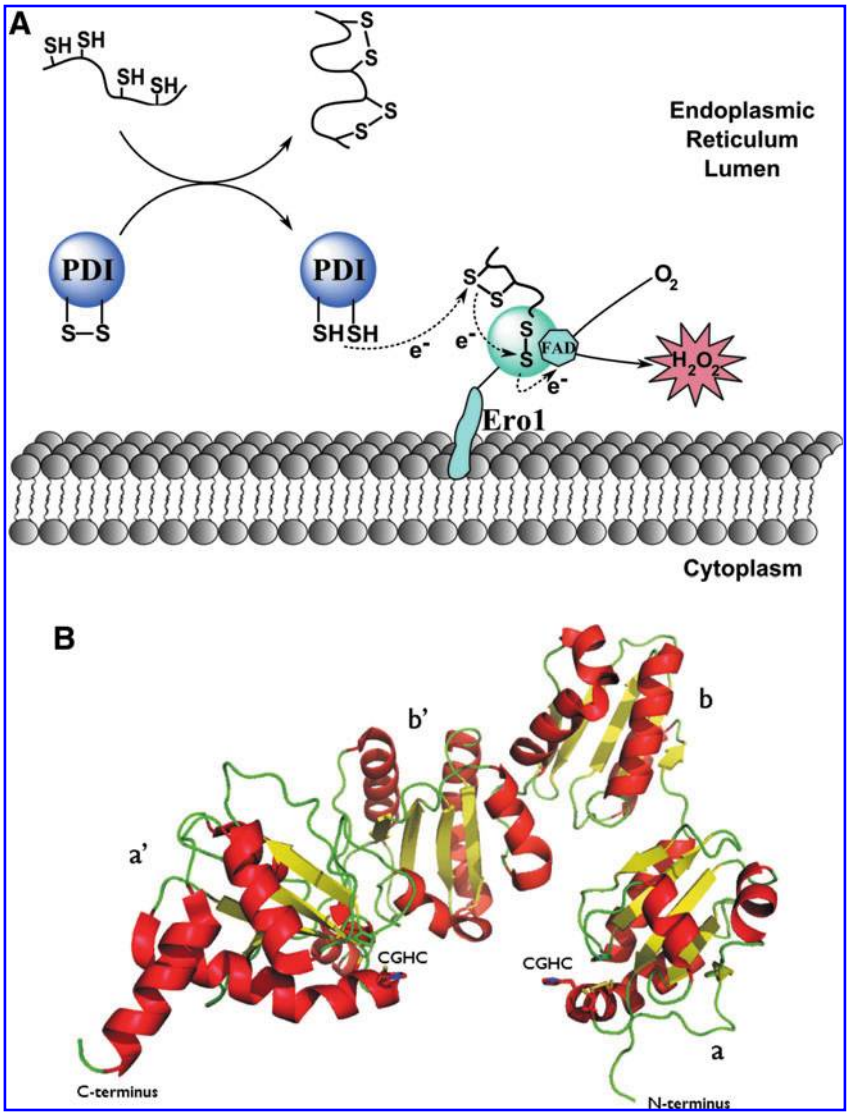

FIG. 4. Disulfide bond formation in the endoplasmic reticulum by protein disulfide isomerase (PDI). (A) In the endoplasmic reticulum, disulfide bonds are introduced by PDI. PDI is then reoxidized by the flavoprotein Ero1, which transfers the e^- to molecular oxygen, generating hydrogen peroxide. The direction of electron flow is shown by the *dotted arrows*. **(B)** Overall structure of yeast PDI. Ribbon diagram of PDI with the *a*, *b*, *b′*, and *a′* domains is shown (PDB entry code 2B5E) (153). The active site residues are in stick presentation. The figures were generated using MacPyMol (Delano Scientific LLC 2006). (To see this illustration in color the reader is referred to the web version of this article at www.libertonline.com/ars).

principal binding site (93), but the other domains of PDI have also been implicated (60). In addition to its catalytic activity, PDI functions also as a chaperone, being able to assist the refolding of proteins with or without disulfide bonds (158). The CXXC catalytic motifs are not required for this chaperone activity (125).

The redox potential of the active sites of PDI is about −180 mV (106). The first cysteine of the CGHC catalytic motif has a low pK_a value (estimated between 4.4 and 6.7) (61, 85, 95, 132), which enables this residue to participate in thiol-disulfide exchange reactions. Like in DsbA, the low pK_a value of the nucleophilic cysteine results from interactions with residues that are adjacent in the structure, such as the histidines that lie between the catalytic cysteines of domains a and a′.

How is PDI reoxidized? When functioning as an oxidase, PDI transfers a disulfide from its catalytic site to the folding polypeptide chains. The cysteine residues of PDI are then reoxidized by Ero1, a protein associated with the luminal face of the ER membrane (46, 154) (Fig. 4A). Whereas yeast and invertebrates contain a single *Ero1* gene, genomes of vertebrates, including mammals, usually have two *Ero1* paralogs.

Ero1 is a flavoenzyme that uses flavin adenine dinucleotide (FAD) to transfer electrons from PDI to molecular oxygen, generating H_2O_2 *in vitro* (67) (Fig. 4A). It is generally assumed that H_2O_2 is produced also *in vivo*, although this has not been demonstrated.

Ero1 has four essential cysteine residues. Two of them, present in a CXXC motif, are in proximity to the FAD cofactor, which is cradled by four antiparallel α-helices (54). Together with the FAD, these cysteines constitute the catalytic site of Ero1. The other two cysteine residues are located on a flexible loop that is exposed on the surface of the protein and lacks significant secondary structure (54). Reminiscent of the mechanisms that have been described above for the Dsb proteins, the transfer of electrons from PDI to oxygen involves a succession of thiol–disulfide exchange reactions between several couples of redox-active cysteine residues. First, the cysteines that are present on the flexible loop of Ero1 are reduced by PDI. Then, electrons are transferred to the active site cysteines of the CXXC motif, further to FAD and then finally to molecular oxygen, with the concomitant production of H_2O_2 (55).

Is the activity of PDI regulated by the redox conditions of the ER? In addition to the four cysteine residues involved in electron transfer, Ero1 possesses another two pairs of cysteines that have been proposed to regulate its activity by controlling the motion of the flexible loop on which the shuttle cysteine residues are located (138). When the redox conditions within the ER become too oxidizing, the regulatory cysteines are oxidized. With oxidized regulatory cysteines, the movements of the flexible loop appear to be restricted, preventing electron transfer from PDI to the catalytic center of Ero1. Under more reducing conditions, the regulatory cysteines are reduced, which likely allows the loop to shuttle electrons from PDI to the CXXC active site and to the FAD cofactor. Thus, in function of the redox environment of the ER, these additional cysteines of Ero1 fine-tune the redox activity of PDI. Regulation of Ero1 activity by formation of these regulatory disulfides has been demonstrated for the enzyme from yeast (138) and for one of the human paralogs (3, 9). This mechanism is probably required to ensure that the redox potential of the ER is optimal for disulfide bond formation and for controlling the levels of harmful H_2O_2 production by Ero1.

What is the role of glutathione in the ER? The lumen of the ER contains significant concentrations of oxidized glutathione (GSSG). In this compartment, the ratio of GSH to GSSG is generally considered to be 3–1, whereas it is about 100–1 in the cytoplasm (70). Knowing that the total concentration of glutathione in the ER is about 9 mM (13), the calculated redox potential of this compartment is approximately −190 mV. *In vitro* experiments have shown that these redox conditions are optimal for disulfide bond formation, which led to the hypothesis that GSSG was directly involved in disulfide bond formation in the ER by oxidizing folding proteins. However, with the discovery of Ero1, which provides the oxidizing equivalents required for PDI to function as an oxidase, the role of GSSG became obscure, and whether GSSG plays a direct role in disulfide bond formation or not is still a matter of debate. Recent data, however, revealed that GSH is involved in the control of the redox homeostasis of the ER by modulating the activity of Ero1 *via* the reduction of the regulatory disulfides (138). However, as GSH is a poor *in vitro* reductant of Ero1, it is likely that GSH influences Ero1 activity indirectly. A possibility is that GSH reduces another ER oxidoreductase, possibly PDI, which in turn would reduce the regulatory disulfides of Ero1. GSH was also shown to play a role in the isomerization of non-native disulfides bonds that form in proteins (20, 78, 115). Here also, although a direct involvement of GSH in the reduction of non-native disulfides is possible, it seems that GSH acts primarily by reducing PDI and/or other ER oxidoreductases.

Disulfide bond formation in mitochondria

On the basis of the endosymbiotic theory, it had been postulated that disulfide bond formation would occur in the intermembrane space of mitochondria (IMS), a compartment that is topologically equivalent to the bacterial periplasm. This hypothesis seemed unlikely since porins present in the outer membrane of mitochondria allow the passage of glutathione and other small molecules, which suggested that the redox potential of the IMS would be similar to that of the cytosol. However, it is now clear that an oxidative folding pathway is present in the IMS (19, 113, 116). Noteworthy, a recent study (68) determined that the redox potential of the IMS is more oxidizing (−255 mV) than the surrounding cytosol and the mitochondrial matrix (−290 mV).

Mia40, a unique oxidase. The central player of the mitochondrial disulfide formation machinery is the oxidase Mia40 (19, 116) (Fig. 5A). Mia40, which is soluble in mammals and plants and membrane-anchored in fungi, contains an essential N-terminal CPC active site motif as well as two CX_9C motifs that play a structural role. The redox potential of the CPC motif is −200 mV (10). In contrast to proteins from the Trx family, it is the second cysteine of the active site motif that is involved in the formation of a mixed disulfide bond with the substrate (10). The structure of the protein presents a lid where the CPC motif is located as well as a core domain where the twin CX_9C motifs form a double-disulfide-linked hairpin structure (10, 87) (Fig. 5B). The lid folds onto the core domain

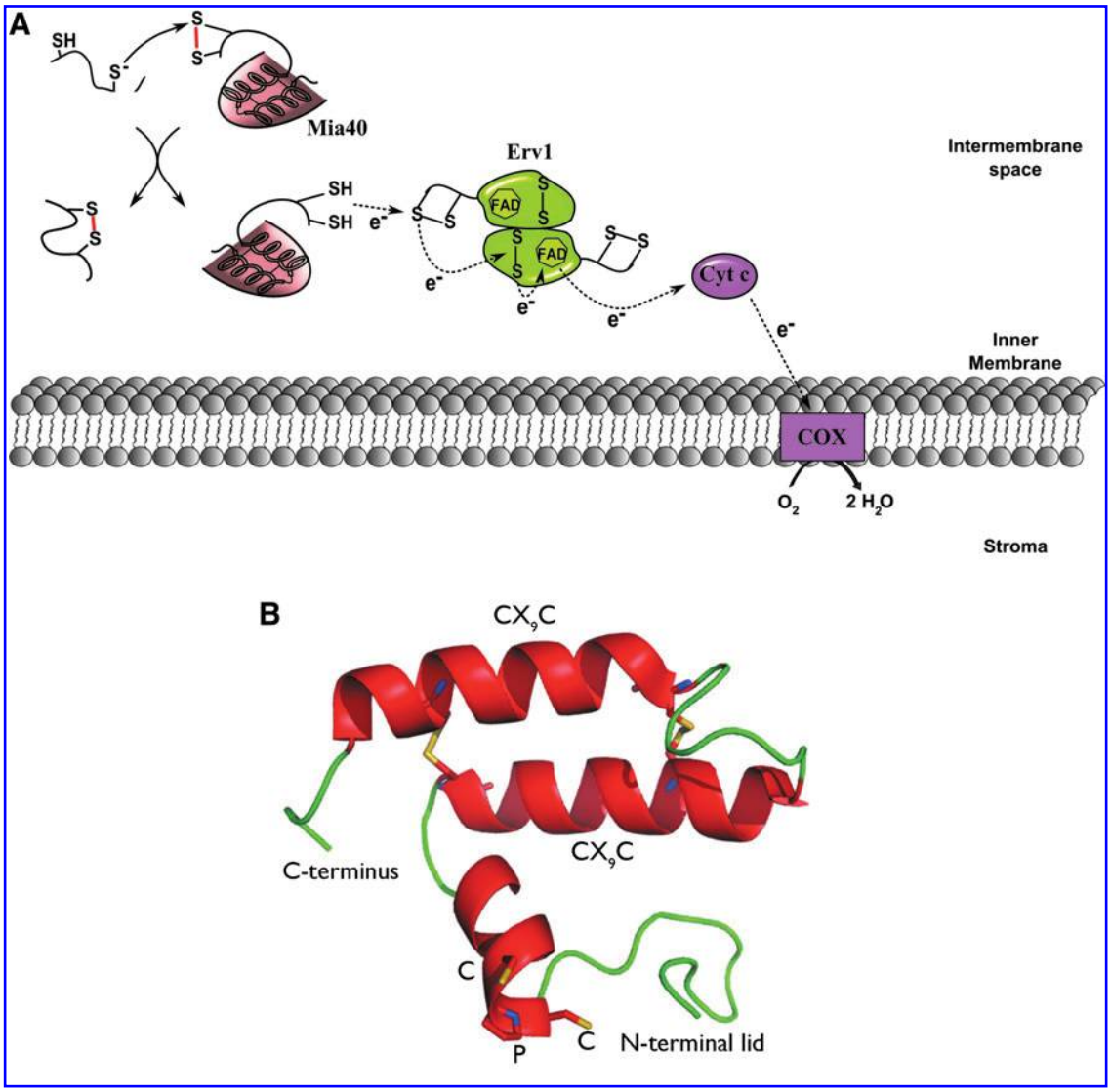

FIG. 5. Disulfide bond formation in the intermembrane space of mitochondria. (A) Proteins targeted to the intermembrane space of mitochondria have their disulfide bonds formed by Mia40. Mia40 is regenerated by the dimeric flavoenzyme Erv1 (protein essential for respiration and vegetative growth). e^- are then transferred successively to cytochrome c (Cyt c), cyclooxygenase (COX), and finally onto oxygen with the generation of a water molecule. The direction of electron flow is shown by the dotted arrows. **(B)** The solution structure of Mia40 (PDB entry code 2K3J) (10). The helix of the N-terminal lid and the helices composing the α-hairpin core are shown in red. Disulfides and the CPC residues are shown in stick presentation. The figure was generated using MacPyMol (Delano Scientific LLC 2006). (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

of Mia40, creating a hydrophobic cleft where the substrates likely bind (10).

How is Mia40 reoxidized? After transfer of its disulfide to folding proteins, Mia40 is reoxidized by Erv1 (essential for respiration and vegetative growth), a soluble homodimeric flavoprotein present in the IMS (15, 98, 104, 113) (Fig. 5A). Noteworthy, Erv1, Mia40, and the substrate proteins seem to form a ternary complex *in vivo* (148).

Erv1 belongs to the conserved Erv/quiescin-sulfhydryl oxidase family of proteins whose members share a common flavin-binding domain found either alone or fused to a PDI-like domain (45, 152). Erv1 from fungi and animals is composed of two domains: a catalytic domain that contains a CXXC active site motif adjacent to the FAD cofactor and a tail segment. The tail segment located either N-terminally (*Saccharomyces cerevisiae*) or C-terminally (*Arabidopsis thaliana*) contains a second pair of cysteine residues arranged in a CXXC motif. These two cysteine residues have recently been shown to shuttle electrons from Mia40 to the CXXC motif in the catalytic domain of Erv1 (15).

The subunits of the Erv1 dimer cooperate in electron transfer: the electrons are shuttled from the tail segment of one subunit to the catalytic domain of the other subunit (15). Thus, electron transfer occurs intermolecularly and the tail segment of a first Erv1 protein functions as a flexible arm transferring electrons from Mia40 to the FAD domain of the second Erv1 subunit. This shuttling of electrons is similar to that described

for Ero1 or for other Erv proteins, such as Erv2 (56, 155). After reduction of the catalytic cysteines, electrons are transferred onto the FAD cofactor and then to Cyt c and the respiratory chain (31). Electrons are finally transferred to molecular oxygen to generate water molecules (16).

The complete mitochondrial disulfide bond formation pathway has recently been reconstituted *in vitro* using purified Mia40, Erv1, Cyt c, and Cox19, a Mia40 substrate (15). These experiments showed that Mia40 promotes the complete oxidation of Cox19 and revealed that reduced glutathione seems to play a role in the folding process by counteracting the formation of long-lived partially oxidized intermediates (15).

The substrates of Mia40 have CX_3C or CX_9C motifs. The substrates of Mia40 are small proteins [<20 kDa (41)] that contain either CX_3C or CX_9C motifs. Upon oxidation by Mia40, they form a helix-turn-helix hairpin fold in which the two helices are in antiparallel orientation and connected with two disulfide bonds (connecting cysteines C1-C4 and C2-C3, respectively) (1, 2). Mia40 substrates play essential roles in the IMS: twin CX_3C proteins are chaperones that assist proteins targeted to the inner membrane of mitochondria (142), whereas twin CX_9C proteins are mostly involved in the formation of Cyt c oxidase complex (18).

Protecting Single Cysteine Residues from Oxidation

Compartments in which disulfide bond formation occurs are significantly more oxidizing than the cytosol. This raises the question to know how cysteine residues that are not involved in disulfide bond formation are protected from oxidation in these compartments. Cysteine residues are indeed primary targets of ROS, such as H_2O_2 generated by the electron transport chain or, for instance, by Ero1 in the ER. Oxidation of the thiol (−SH) group of a cysteine by ROS leads to the formation of sulfenic acids (−SOH) (123) (Fig. 6). Sulfenic acids are highly reactive groups that tend to react rapidly either with other cysteine residues present in the vicinity to form a disulfide bond or to be further oxidized to sulfinic ($-SO_2H$) or sulfonic acids ($-SO_3H$) (123) (Fig. 6). The latter two oxidation states are considered to be irreversible (126). Protective mechanisms that prevent irreversible over-oxidation have been identified. For instance, sulfenic acids can react with glutathione (162), leading to glutathionylation. The glutathionylated cysteines can then be rescued by the reducing proteins of the glutaredoxin family (144). In another protection mechanism, the cysteine sulfenic acid reacts with the backbone amide nitrogen of the adjacent amino acid to form a sulfenamide (99, 134, 161), such as observed in phosphotyrosine phosphatases. However, enzymatic systems that reduce sulfenic acids and control the level of oxidation on sensitive thiolates are still

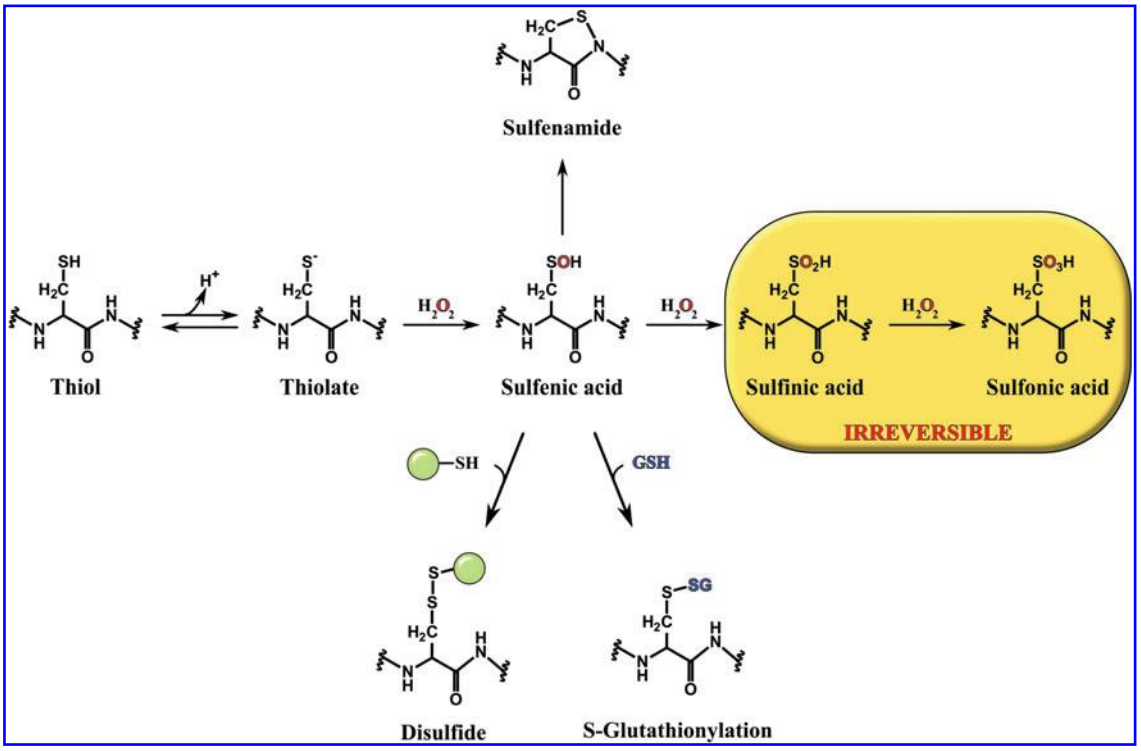

FIG. 6. Redox-active cysteines are sensitive to oxidation. Cysteine present in a thiolate form at physiological pH are more sensitive to reactive oxygen species. Exposure to hydrogen peroxide (H_2O_2) leads to the oxidation of the thiol group into the reversible sulfenic acid, whereas further exposure leads to irreversible cysteine oxidation states: sulfinic acid ($-SO_2H$) and sulfonic acid ($-SO_3H$). Sulfenic acids are protected from irreversible oxidation by different mechanisms: disulfide formation with another cysteine, mixed disulfide formation with glutathione (S-glutathionylation), or cyclization of the cysteine side chain by reaction with a backbone amide nitrogen to form a sulfenamide. Importantly, disulfide bond formation does not always proceed *via* a sulfenic acid intermediate, but can also result from the oxidation of two cysteine residues by oxidative protein folding catalysts (not shown). (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

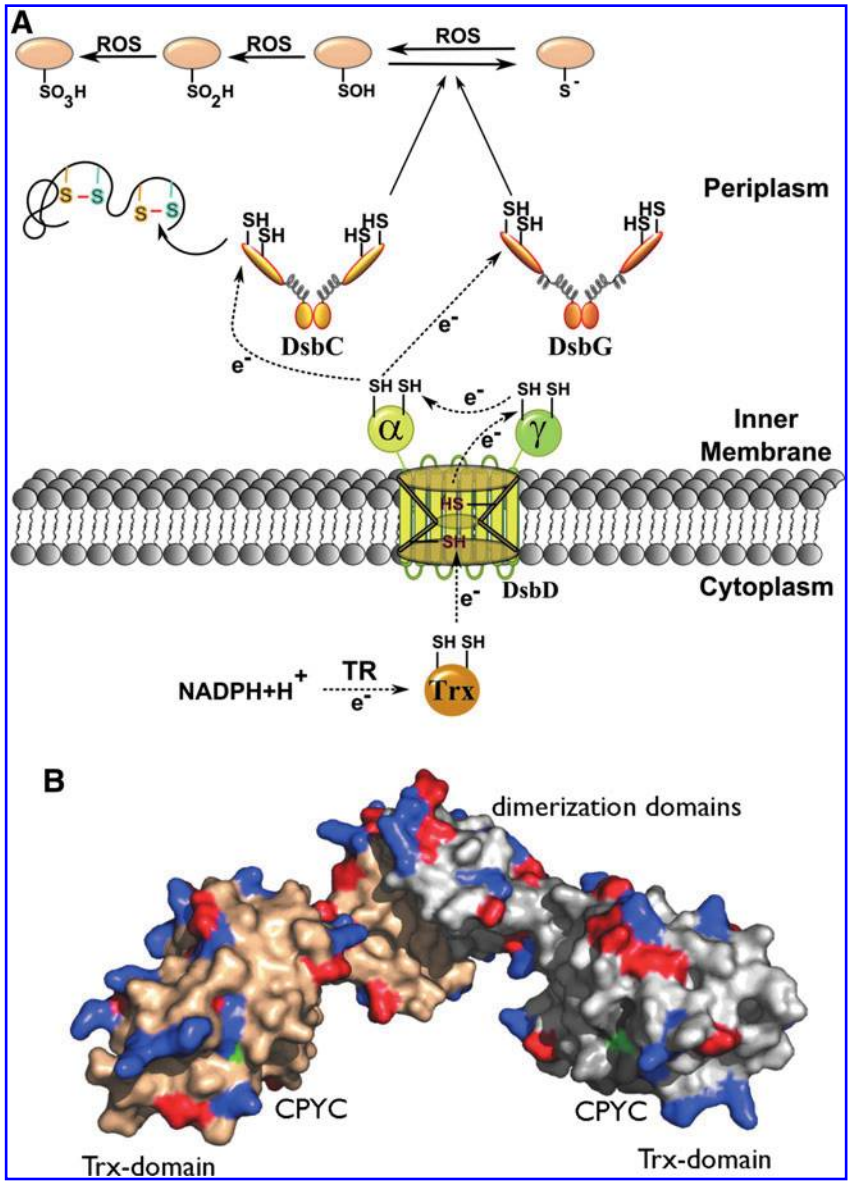

FIG. 7. Rescue of sulfenylated cysteine residues in the periplasm. (A) Proteins with single cysteine residues are easy prey for reactive oxygen species (ROS) present in the periplasm. They form sulfenic acids (–SOH) that are susceptible to irreversible oxidation to sulfinic ($-SO_2H$) and sulfonic acids ($-SO_3H$). DsbG functions as a reductase that rescues sulfenylated cysteine residues. DsbC, which resembles DsbG, seems to serve as a back up for DsbG. Both DsbC and DsbG are kept reduced by DsbD. Thus, e^- originating from the cytoplasmic pool of nicotinamide adenine dinucleotide phosphate (NADPH) protect sensitive cysteine residues in the periplasm. The direction of electron flow is shown by the dotted arrows. **(B)** Surface representation of dimeric DsbG (PDB endtry code 1V58—the biological dimer was generated by applying crystallographic symmetry) (62). Monomers are colored wheat and gray. The active site cysteines are in green. All lysine and arginine residues are in blue, and all aspartate and glutamate residues are in red. This figure was generated using MacPyMol (Delano Scientific LLC 2006). (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

poorly characterized, and so far, no general sulfinic or sulfonic acid reductases have been discovered.

A new link between oxidative protein folding and cysteine rescue

Recently, we found that DsbG, an enzyme present in the periplasm of *E. coli*, controls the level of sulfenylation in this compartment (35) (Fig. 7). DsbG is homologous to the PDI DsbC. DsbG, like DsbC, is a V-shaped dimeric protein whose catalytic CPYC motif is kept reduced by DsbD (62). We showed that DsbG protects periplasmic proteins containing a single cysteine residue from oxidation to sulfenic acid. Moreover, our results suggest that DsbC functions as a backup for DsbG. Thus, the electron flux originating from the cytoplasmic pool of nicotinamide adenine dinucleotide phosphate and transferred to the periplasm by DsbD provides the reducing equivalents required for both the correction of incorrect disulfides and the rescue of sulfenylated cysteines. It is tempting to speculate that other proteins from the Trx superfamily, such as, for instance, some of the PDI family members, play similar roles in controlling the global sulfenic acid content of eukaryotic cellular compartments.

Conclusion

Since the discovery of PDI, the field of thiol-based redox biology has expanded to both prokaryotes and eukaryotes and to several sub-cellular compartments. One of the most exciting breakthrough that has been obtained over the past 50 years is the discovery that oxidative protein folding mechanisms are connected to the physiological electron flow pathways.

Ironically, whereas disulfide bond formation has first been uncovered in the eukaryotic ER, it is probably the compartment where it is still the less characterized. Here, mechanisms are more complicated, several redox players are involved, and regulation is of prime importance. Intriguing fundamental questions remain unsolved regarding the oxidative protein folding in the ER. For instance, we still do not know

the *in vivo* function of several of the oxidoreductases from the PDI family present in the ER. Moreover, it has been reported that most of those oxidoreductases are maintained in a reduced state in this compartment (20). However, it is not clear whether the ER contains a reductive pathway that specifically maintains these proteins in a reduced state. Reduced glutathione is likely to be a source of reducing equivalents in the ER (21). However, we do not know how glutathione is recycled: is GSSG converted back to GSH within the ER or is it exported from this compartment to regulate the redox environment? Another challenging problem will be to understand how the oxidative protein folding process is coordinated with protein translocation and with ER quality control mechanisms.

Remarkably, some of the questions that are still unsolved in the ER are also unanswered in bacteria. We do not know how the periplasmic protein folding catalysts that are involved in disulfide bond formation coordinate their action with the various chaperones that ensure the correct folding of secreted proteins. How glutathione is secreted and how this redox peptide is involved in the global redox homeostasis of the periplasm is also not known. It is likely that a better understanding of those problems in bacteria will prove very useful in gaining some insights into the similar mechanisms that occur in the ER.

Finally, it has been considered for a long time that cysteine residues in secreted proteins form disulfide bonds and the fate of sensitive single cysteine residues in extracytoplasmic compartment has been overlooked. By identifying *E. coli* DsbG as a protein responsible for rescuing sensitive cysteine residues from oxidation in the periplasm, we unraveled a new reducing system that is connected to the pathways of oxidative protein folding. It is likely that similar systems exist in eukaryotes, as the rescue of single cysteines in oxidizing compartments is probably critical to maintain the activity of enzymes with redox-sensitive cysteines.

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Address correspondence to:
Prof. Jean-Francois Collet
de Duve Institute
Université catholique de Louvain
75-39 Ave. Hippocrate
1200 Brussels
Belgium

E-mail: jfcollet@uclouvain.be

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Abbreviations Used

Bdb = bacillus disulfide bond protein
COX = cyclooxygenase
Cyt c = cytochrome c
Dsb = disulfide bond protein
e^- = electron
ER = endoplasmic reticulum
Erv1 = protein essential for respiration and vegetative growth
FAD = flavin adenine dinucleotide
GSH = reduced glutathione
GSSG = oxidized glutathione
H_2O_2 = hydrogen peroxide
IMS = intermembrane space of mitochondria
MQ = menaquinone
NADPH = nicotinamide adenine dinucleotide phosphate
PDB = protein database
PDI = protein disulfide isomerase
PhoA = alkaline phosphatase
Q = ubiquinone
ROS = reactive oxygen species
TR = thioredoxin reductase
Trx = thioredoxin
VKOR = vitamin K epoxide reductase

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