ANTIOXIDANTS & REDOX SIGNALING
Volume 13, Number 8, 2010

DOI: 10.1089/ars.2010.3187

FORUM REVIEW ARTICLE

Mechanisms of Oxidative Protein Folding in the Bacterial Cell Envelope

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Abstract

Disulfide-bond formation is important for the correct folding of a great number of proteins that are exported to the cell envelope of bacteria. Bacterial cells have evolved elaborate systems to promote the joining of two cysteines to form a disulfide bond and to repair misoxidized proteins. In the past two decades, significant advances have occurred in our understanding of the enzyme systems (DsbA, DsbB, DsbC, DsbG, and DsbD) used by the gram-negative bacterium *Escherichia coli* to ensure that correct pairs of cysteines are joined during the process of protein folding. However, a number of fundamental questions about these processes remain, especially about how they occur inside the cell. In addition, recent recognition of the increasing diversity among bacteria in the disulfide bond–forming capacity and in the systems for introducing disulfide bonds into proteins is raising new questions. We review here the marked progress in this field and discuss important questions that remain for future studies. *Antioxid. Redox Signal.* 13, 1231–1246.

Introduction

Disulfide bonds contribute to the stability, activity, and folding of many secretory or extracytoplasmic domains of membrane proteins. Chemically, formation of a protein disulfide bond is an oxidation reaction, in which two electrons are removed from the protein. In contrast, breakage of a protein disulfide bond is a reduction reaction, in which two electrons are donated to the protein. *In vitro*, formation of protein disulfide bonds can occur spontaneously in the presence of an appropriate electron acceptor, although such a spontaneous process often requires hours or days of incubation. In contrast, formation of disulfide bonds in a folding protein *in vivo* is a much more rapid process, occurring within seconds or minutes after the synthesis of the proteins or even during their synthesis, thus necessitating enzyme catalysts for the process (10).

The enzymes that interact directly with folding proteins to introduce or break disulfide bonds are, in most cases, oxidoreductases belonging to the thioredoxin superfamily. The oxidoreductases of this superfamily often have an active site containing a CXXC motif (cysteines separated by two amino acids) embedded in a thioredoxin-fold that is observed in the three-dimensional structure of thioredoxin (6). In both prokaryotes and eukaryotes, the members of the thioredoxin superfamily exist in many cellular compartments and promote the oxidation or reduction of protein's thiols (97).

For the members of the thioredoxin superfamily to perform their functions, the cysteines of their active site must be maintained in the appropriate redox state. Increasingly, enzymes are being identified that are responsible for these maintenance functions. These enzymes, which usually do not belong to the thioredoxin superfamily, act on the direct catalysts of oxidative protein folding, providing them with oxidizing or reducing equivalents. These enzymes thereby connect the oxidative folding pathways to cellular metabolism (55, 97).

In *Escherichia coli*, approximately 300 proteins containing multiple cysteines are exported to the periplasm (27, 38). These proteins are potential substrates for disulfide bond–forming enzymes. Despite the large number of potential substrates, the components involved in disulfide-bond formation (Dsb proteins) are not essential for viability, although mutants exhibit a variety of defects (55). The nonessentiality of the components, the availability of strong genetic tools, the deep biologic knowledge of this organism available, and the ease with which the relevant proteins can be produced have greatly facilitated the analysis of disulfide-bond formation in this organism. In the past two decades, we have learned that a surprising variety of structures, mechanisms, and factors are required to carry out the process. However, the detailed mechanisms by which disulfide bonds are introduced into proteins by these systems, especially *in vivo*, are still unclear. Unless otherwise stated, we discuss the systems of *E. coli* that

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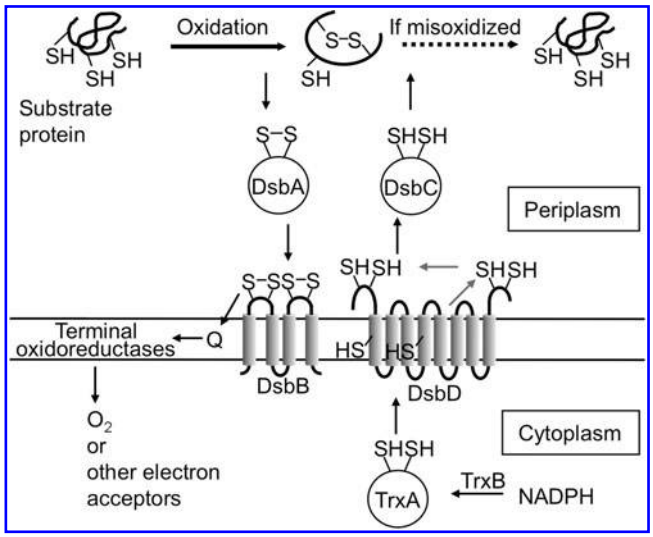

FIG. 1. Models for disulfide-bond formation in the periplasm of *E. coli*. The *solid black rightward arrow* indicates the oxidative folding reaction catalyzed by DsbA. In the instance in which the first folding reaction resulted in a misoxidized protein, DsbC may repair it by acting as a reductase (*dotted arrow*) or an isomerase (not shown in this figure) of the incorrect disulfide bond. The *thinner arrows* indicate the flow of reducing equivalents. Q, quinones (ubiquinone or menaquinone). For simplicity, DsbC, a dimeric molecule, is depicted as a monomer.

are responsible for introducing correct disulfide bonds into cell envelope proteins. We also describe some advances in our understanding of the disulfide bond–forming systems of other bacteria.

DsbA: A Disulfide Bond–Forming Enzyme

DsbA is a primary oxidant of many extracytoplasmic proteins (Fig. 1). In the absence of DsbA, many cell envelope proteins of *E. coli* acquire their disulfides much more slowly or fail to acquire them (10, 57). Thus, mutants that are missing DsbA show pleiotropic phenotypes, such as loss of motility and increased sensitivity to dithiothreitol and metal ions like Cd^{2+} and Hg^{2+} (55).

DsbA is a monomeric, periplasmic enzyme (21 kDa) having a redox active sequence, Cys30-Pro31-His32-Cys33, embedded in a thioredoxin fold (10, 74) (Fig. 2A). These two cysteines are kept oxidized (disulfide bonded) *in vivo* (60). DsbA rapidly oxidizes proteins secreted into the periplasm by donating its disulfide bonds to a pair of cysteines of the substrate. This process likely occurs through formation of an unstable mixed-disulfide complex between DsbA and its substrate (Fig. 3). Recently, such a DsbA-substrate complex, an apparent intermediate during oxidative protein folding, was detected and characterized *in vivo* (53).

The thioredoxin-fold, such as that found in DsbA, is described as an N-terminal $\beta\alpha\beta$ motif and a C-terminal $\beta\beta\alpha$ motif, connected by a loop of residues that incorporates an α-helix (75). After the N-terminal $\beta\alpha\beta$ motif, the thioredoxin-fold of DsbA has an insertion of a 65-residues α-helical domain that consists of four α-helices (Fig. 2A). It has been suggested that the α-helical domain protects the redox-active cysteines of DsbA from misreduction by DsbD (35) (for the description of DsbD, see later section).

Among the known enzymes that promote the exchange of thiols and disulfides, DsbA, with a standard redox potential of ~ -120 mV, is one of the strongest thiol oxidants (42). This enzyme can introduce disulfide bonds into its substrates quickly *in vivo* and *in vitro*. For comparison, thioredoxin has a much lower standard redox potential of -270 mV and *reduces* disulfide bonds in the cytoplasm (5).

The DsbA catalytic cysteine residues, Cys30 and Cys33, are located at the N-terminus of the first α-helix of the thioredoxin fold. Of the two cysteines, only Cys30 is surface exposed. The activity of DsbA as a strong oxidant derives in part from its biophysical properties. The Cys30 of DsbA has an unusually low pK_a value of $\sim$3.3 (the normal pK_a of cysteine is $\sim$9) (42, 81). Thus, Cys30 is in the thiolate anion state at physiologic pH. The structure of DsbA explains how the thiolate anion of Cys30 is stabilized (33). First, this residue is located at the N-terminus of the first helix of the thioredoxin-fold, allowing the partial positive charge from the dipole of the helix to stabilize the thiolate. Second, His32, which lies between Cys30 and Cys33, is hydrogen-bonded to Cys30 in the reduced but not in the oxidized DsbA, providing additional stabilization of the thiolate anion of Cys30 (Fig. 2B). In addition, the structure of reduced DsbA reveals a number of potential hydrogen bonds

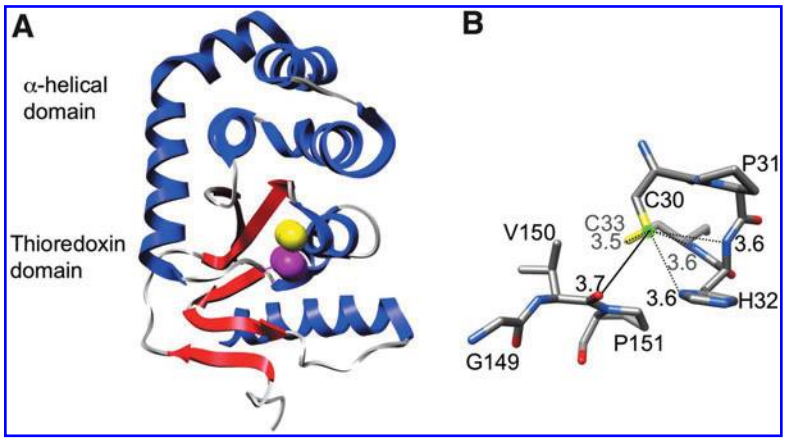

FIG. 2. The three-dimensional structure of DsbA. (**A**) The structure of the oxidized form of DsbA (PDB:1FVK) (74). DsbA has a thioredoxin-like fold with the insertion of an α-helical domain. The α-helices (blue), β-sheets (red), *cis*-Pro151 (magenta), and the sulfur atoms of the active-site cysteines (yellow) are indicated. Only the sulfur of Cys30 is surface-exposed. (**B**) The close-up view of the active site of the reduced form of DsbA (PDB: 1A2L) (33) in stick presentation. Possible hydrogen-bond interactions stabilizing the thiolate anion of Cys30 are indicated by *dotted lines*, with their distances in ångströms. A green ball represents the sulfur of Cys30. The distance between the sulfur of Cys30 and the main-chain oxygen of Val150 in the *cis*-Pro loop is also shown in ångströms with a *solid line*. Molecular graphics images were produced by using the UCSF Chimera (85). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

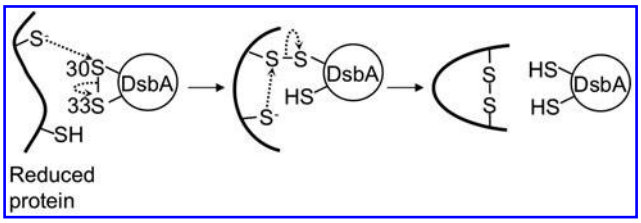

FIG. 3. Disulfide-bond formation by DsbA. Substrate oxidation by DsbA likely proceeds through two steps. First, a deprotonated cysteine of a substrate attacks the sulfur atom of Cys30 of the oxidized DsbA, leading to the formation of a disulfide-linked complex between DsbA and the substrate. In the next step, one of the remaining cysteines of the substrate is deprotonated and attacks the sulfur atom of the substrate cysteine that is disulfide bonded with Cys30 of DsbA. This reaction results in the formation of a disulfide bond in the substrate and reduction of DsbA.

that stabilize the thiolate anion of Cys30 (Fig. 2B). One of them is a hydrogen bond between the thiolate anion of Cys30 and the thiol of Cys33. The other two hydrogen-bond interactions are between the thiolate anion of Cys30 and the backbone amides of His32 and Cys33 (33). These electrostatic interactions, which are unique to the reduced form of this protein, cause the reduced form of this protein to be more stable than the oxidized form (109). In contrast, most proteins that are substrates of DsbA are stabilized by formation of disulfide bonds. This difference in the stabilities of oxidized and reduced forms provides a thermodynamic driving force for the transfer of disulfide bonds from DsbA to its substrates.

The *in vitro* redox properties of DsbA as well as other thioredoxin-fold proteins are strongly influenced by the amino acid sequence between the active-site cysteines (31, 87), as seen with His32 of DsbA (see earlier). However, the CXXC motif is not the only structural feature that greatly affects the function of DsbA. Most of the thioredoxin-superfamily members, including DsbA, have a highly conserved *cis*-proline residue in their structures (75) (Fig. 2A). The loop containing this residue is close in three-dimensional space to the CXXC motif but distant in sequence (Fig. 2B). Growing evidence suggests that the *cis*-proline loop plays important roles in the function of thioredoxin and its relatives.

First, biophysical studies of DsbA and thioredoxin indicate that the conserved *cis*-proline contributes to the stability and structure of these proteins (16). Second, a mutation that alters the Pro151 of DsbA, which corresponds to the highly conserved *cis*-proline in this enzyme, to threonine causes retardation of the resolution of intermediate complexes between DsbA and its substrates *in vivo*, suggesting that this residue is important structurally or functionally for substrate release (56). Third, in the crystal structure of a complex between DsbA and its model substrate peptide, a few residues preceding the *cis*-proline form an anti-parallel β-sheet with a part of the substrate, indicating that the *cis*-proline loop is important for substrate recognition (83). Finally, recent studies show that the residue just N-terminal to the *cis*-proline residue, Val150 in *E. coli* DsbA, strongly modifies the redox properties and substrate specificities of some thioredoxin-superfamily members (66, 89). Thus, in addition to the CXXC motif, the *cis*-proline loop is crucial in defining the properties of DsbA and other enzymes.

In *E. coli,* more than 300 potential substrates exist of DsbA (27, 38). This estimate is based on a bioinformatics analysis showing that that many cell envelope proteins occur with at least two cysteine residues. Some of these have been verified to be substrates of DsbA (38, 56, 67, 103). How is it possible for this enzyme to interact with so many different substrates? First, studies on the protease-accessible sites on the surface of DsbA revealed that the surface of the oxidized form is more flexible than that of the reduced form (104). Thus, the greater flexibility of the oxidized form may facilitate the accommodation of various substrates, and the more-rigid nature of the reduced form may expedite the release of the oxidized substrates. Second, in the crystal structures of a complex between DsbA and its polypeptide (see earlier), amino acids in the substrate polypeptide were hydrogen-bonded to the backbone atoms of the residues of the *cis*-proline loop in the anti-parallel β-sheet structure. The use of the backbone atoms, but not the side chains, for binding may allow the enzyme to interact with a greater variety of substrates (83).

Most structural disulfide bonds in the substrates of DsbA are buried and not solvent accessible in their final folded structures. Thus, DsbA must introduce disulfide bonds into its substrates before they are folded into their native structures. Conversely, DsbA avoids interaction with a subset of folded proteins, even if they contain cysteines that can be oxidized (*e.g.*, DsbC and DsbD; see later) (110). Consistent with its physiologic roles, DsbA appears preferentially to interact with unfolded proteins. For example, DsbA reacts about 10 to 25 times faster with unfolded proteins than it does with DTT (108). Further, in a complex between DsbA and unfolded RNase T1 (stably formed by mutating Cys33 of DsbA), the DsbA moiety is stabilized by 4.7 kJ/mol when it is linked with the unfolded RNase T1 (30). This strongly suggests that DsbA interacts noncovalently with its substrates. In addition, in the complex formed between DsbA and its model peptide (see earlier), the peptide was bound at the interface of the thioredoxin and α-helical domains of DsbA with multiple contacts (83). Such numerous interactions would not be available if the protein were already folded. These findings are consistent with the role of DsbA as a catalyst of disulfide-bond formation in folding proteins.

The surface structure of DsbA has a relatively deep hydrophobic groove running alongside the active site. Because of its hydrophobic nature, this groove has been postulated to be the binding site for the unfolded substrates (34). The recent crystal structure of a DsbA–DsbB complex shows that this groove constitutes a part of the binding site for DsbB (see later for DsbB) (44). However, no direct evidence indicates that this groove is actually used for the recognition of its unfolded substrates (83).

As in the case of other members of thioredoxin superfamily, thiol-disulfide exchange by DsbA likely proceeds by two steps (30, 53) (Fig. 3). In the first step, a deprotonated cysteine of a substrate attacks the sulfur atom of Cys30 in the Cys30–Cys33 disulfide bond. This reaction leads to the formation of an intermolecular disulfide bond between the cysteine of the substrate and Cys30 of DsbA and reduction of Cys33 of DsbA. In the next step, one of the remaining cysteines of the substrate is deprotonated and attacks the sulfur atom of the substrate cysteine that is disulfide bonded with Cys30 of DsbA. This reaction results in the formation of a stable disulfide bond in the substrate and in the reduction of both cysteines of DsbA.

How are these reactions facilitated on the enzyme? Studies on DsbL, a uropathogen-specific DsbA homologue, have provided some insights into this question. This protein is one of the most oxidizing thioredoxin superfamily members known to date (32, 105). By using molecular dynamics simulation, Narzi *et al.* (80) studied the active-site protonation characteristics of DsbL. Their characterization revealed that the buried active-site cysteine (Cys32) alternates between protonated and deprotonated states, which is achieved by proton shuffling between Cys32 and the neighboring Lys23. This proton shuffling avoids the negative-charge collision that would otherwise occur between the two active-site cysteines (Cys29 and Cys32) of DsbL, facilitating the smooth electron transfer from the substrate cysteines to the disulfide bond of DsbL. It is suggested that a residue with a similar function may also exist in other proteins sharing a CXXC active-site motif (80).

Good evidence indicates that the thiol-disulfide exchange reaction of thioredoxin-superfamily members, including DsbA, uses an S_N2 mechanism (20, 30, 53, 84, 106, 107). A reaction that adopts an S_N2 mechanism is predicted to be highly directional, proceeding through a transition state in which the three involved atoms form an ~180-degree angle (29, 107). For example, in the first step of DsbA-catalyzed protein oxidation reaction, the three involved atoms are the sulfur atom of the attacking cysteine of the substrate, and the two sulfur atoms (Cys30, and Cys33) of DsbA. Thus, the S_N2 reaction theory predicts that these three sulfur atoms must be linearly arranged at the transition state. Therefore, relative positions of these sulfur atoms must be important for efficient catalysis. Because an open space exists in front of Cys30 along the S-S axis of active-site cysteines of the oxidized DsbA, an attacking thiolate of a substrate will, at least, be able to access the sulfur atom of Cys30 in an S_N2 manner. To perform the reaction efficiently, DsbA must have evolved to bind its substrates in a manner that allows the attacking thiolate to be linearly placed at an ~180-degree angle from the Cys30–Cys33 disulfide of DsbA.

DsbB: A Membrane Protein That Maintains DsbA in the Oxidized Active State

After transfer of its disulfide bond to a target protein, DsbA must be reoxidized to repeat another catalytic cycle. This reoxidation is performed by a membrane protein DsbB (9). In wild-type cells, DsbA is maintained exclusively in the oxidized state. However, in *dsbB*-null mutants, DsbA fails to be reoxidized and remains in the reduced state. Thus, the *dsbB* mutants are strongly defective in disulfide-bond formation of many cell-envelope proteins. However, the extent of disulfide defect in the *dsbB* mutants is slightly less than that of the *dsbA* mutants, especially in rich medium containing a large amount of cystine or the oxidized form of glutathione, as DsbA can be oxidized, albeit much less efficiently, by these small-molecule oxidants (9).

DsbB restores the disulfide bond to DsbA by using the oxidizing power of the electron-transport chain (Fig. 1) (7, 63). Aerobically, ubiquinone (an aerobic quinone) receives electrons from DsbB. The reduced ubiquinone is then reoxidized by terminal oxidases, which pass electrons to O_2. Anaerobically, electrons are transferred from DsbB to menaquinone (an anaerobic quinone). The reduced menaquinone is then reoxidized by an anaerobic electron-transport system. The ability of DsbB to use both ubiquinone and menaquinone as electron acceptors allows the DsbA–DsbB system to work under both aerobic and anaerobic conditions (7).

DsbB (20 kDa) spans the membrane 4 times and has four essential cysteines. In the oxidized state of DsbB, the cysteines form disulfide bonds (Cys41–Cys44, and Cys104–Cys130). They are located in its first and second periplasmic loop, respectively, and are redox active (48, 62) (Fig. 4, stage 0). The *in vivo* and *in vitro* analysis of mutants in which these cysteines are altered has revealed the following picture for the function of each cysteine in oxidizing DsbA (54, 61). The Cys104–Cys130 pair undergoes an electron-transfer process with DsbA. This exchange is initiated by the attack of the reduced DsbA Cys30 on Cys104 in the Cys104–Cys130 disulfide bond of DsbB, resulting in the formation of an interprotein disulfide bond between Cys30(DsbA) and Cys104(DsbB) (54). Because the Cys41–Cys44 pair is required to maintain the Cys104–Cys130 pair in the oxidized state (61, 62), electrons are transferred from the Cys104–Cys130 pair to the Cys41–Cys44 pair. Further, because Cys41 and Cys130 can form an interloop disulfide linkage *in vivo* (54) and *in vitro* (45), the transfer of electrons from the second periplasmic loop to the first periplasmic loop is likely mediated by the attack of Cys130 on Cys41 in the Cys41–Cys44 disulfide bond (45, 54) (Fig. 4, stage 3).

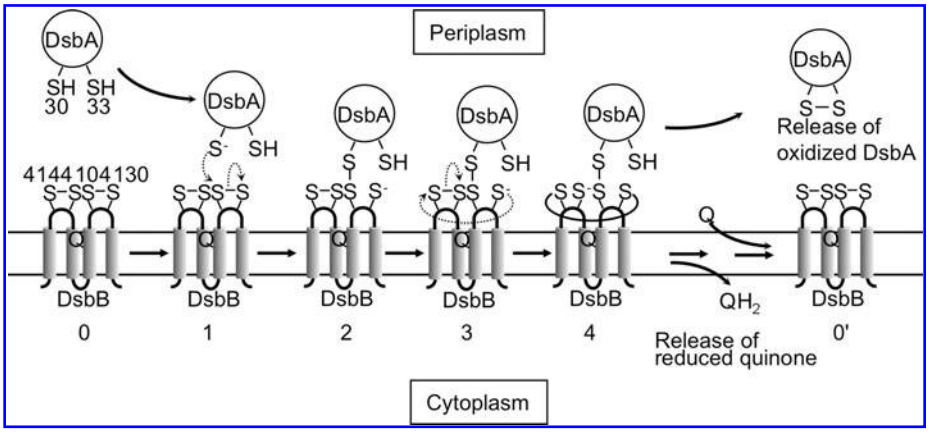

FIG. 4. A model for early steps in the reoxidation of DsbA by DsbB. DsbA is highly oxidizing. Thus, it tends to be reduced. The mechanism by which DsbB oxidizes such a highly oxidizing protein is a fundamental question. In this model, the interloop disulfide bond formed between Cys130 and Cys41 (stage 4) prevents the back reaction (stage 2 to stage 0) that would tend to occur because of the highly oxidizing nature of DsbA. Thus, DsbB coordinates the action of its four cysteines to oxidize DsbA. In this model, the DsbB–DsbA complex in stage 4 corresponds to the charge-transfer complex that is depicted in Fig. 5. In the alternative model, electron transfer from DsbA to quinone proceeds through a sequential set of two thiol-disulfide exchange reactions, and then reduction of quinones through a charge-transfer complex (not shown). Q indicates the position of ubiquinone bound to DsbB.

Finally, the Cys41–Cys44 pair is directly oxidized by quinones (62, 88). Thus, electrons from DsbA flow first to the Cys104–Cys130 pair, then to the Cys41–Cys44 pair, and from there to quinones in the respiratory chain.

The three-dimensional structures of DsbB show four transmembrane helices (TM1–TM4) arranged in a left-handed bundle (44, 73, 112). TM1 is bent slightly outside the bundle, creating a groove between TM1 and TM4 to accommodate ubiquinone (112). The redox-active ring structure of ubiquinone is located in close proximity to the Cys41–Cys44 disulfide and to Arg48 (73, 112). This arrangement of the ubiquinone ring is consistent with the model outlined earlier, in which quinones directly oxidize the Cys41–Cys44 pair of DsbB and also with the observation that Arg48 is important for the transfer of electrons from DsbB to quinone (52).

Then how is the Cys41–Cys44 pair of DsbB reoxidized by quinones? Quantum chemical simulation by Inaba *et al.* (46) suggested the formation of a charge-transfer complex between ubiquinone and DsbB. In this model, a thiolate anion of the reduced Cys44 transfers partial charge to the benzoquinone ring of ubiquinone (Fig. 5, stage 1). This charge-transfer complex formation may be followed by the nucleophilic attack on the ubiquinone by Cys44, resulting in the formation of a covalent adduct between ubiquinone and DsbB (Fig. 5, stage 2). Subsequently, Cys41 may attack Cys44 of DsbB (Fig. 5, stage 3), generating the Cys41–Cys44 disulfide bond and the reduced form of ubiquinone (Fig. 5, stage 4) (46).

The side chain of Arg48 likely stabilizes the charge-transfer complex by interaction with the Cys44 thiolate anion or the benzoquinone ring. The existence of a transient charge-transfer complex between the Cys44 thiolate and ubiquinone is supported by the observation that DsbB manifests a characteristic purple color on interaction with reduced DsbA (45). A similar absorbance in the visible region has been observed in a charge-transfer complex between a cysteine-thiolate and a flavin redox ring in glutathione reductase (58, 73). This charge-transfer model is consistent with the structures of DsbB, in which Cys44 is well positioned to form the charge-transfer complex (43, 44, 73, 112).

Paradoxically, the standard redox potential measurements indicate that the cysteine pairs of DsbB are significantly less oxidizing than the cysteine pair of DsbA, with redox potentials of −207 mV for Cys41–Cys44, and −224 mV for Cys104–Cys130 (47). The latter value indicates that transfer of reducing equivalent from DsbA to the Cys104–Cys130 pair of DsbB is energetically unfavorable.

Two models have been proposed that may explain how this energetic barrier is overcome (47, 54). In both models, the reaction is initiated by the nucleophilic attack of Cys30 of DsbA on the Cys104–Cys130 disulfide in DsbB, resulting in a Cys30(DsbA)–Cys104(DsbB) interprotein disulfide bond. The two models diverge after this step. In one model (Fig. 4), the reduced Cys130 immediately attacks Cys41 in the Cys41–Cys44 disulfide to form the Cys41–Cys130 disulfide bond (Fig. 4, stage 3). This interloop Cys41–Cys130 disulfide bond would function as a lock on Cys130, preventing it from attacking the disulfide bond between DsbB and DsbA (54). This attack would initiate the unwanted backreaction, yielding released reduced DsbA. This lock on Cys130 may be a necessary step because the redox potentials of the cysteine pairs involved would strongly favor this backreaction. After the formation of the interloop disulfide bond, the reduced Cys44 thiolate and ubiquinone would form a charge-transfer complex (46). This concerted model is supported by kinetic measurements by Tapley *et al*. (102), which showed that the rate of DsbA oxidation is the same as the rate of ubiquinone reduction. Such a concerted reaction may become possible because of the close proximity of all six involved cysteines in a model of DsbA–DsbB structure (112).

In the alternative model, electron transfer from DsbA to quinone proceeds through a sequential set of two thiol-disulfide exchange reactions and then reduction of quinones (47). Importantly, in this model, release of oxidized DsbA from the DsbA–DsbB complex occurs before the interloop electron transfer. In this model, the backreaction is prevented by relocation of Cys130 away from Cys104 in the DsbA–DsbB complex (44). This nonconcerted model is supported by *in vitro* analysis using quinone-free DsbB (47) (under steady-state conditions, DsbB has a bound quinone). The structures of DsbB are consistent with both of the two models (44, 112). These two models are still currently under debate.

DsbC: A Periplasmic Disulfide-Bond Isomerase

For a protein with two cysteines, only one possible disulfide bond can form within that protein. However, in a protein with three or more cysteines, two cysteines can be joined in a disulfide bond that does not appear in the native structure. Such nonnative disulfide bonds do occur in the process of disulfide-bond formation *in vivo*. In *E. coli*, a periplasmic protein DsbC repairs proteins with such incorrect disulfide bonds (12, 38, 90, 99, 110) (Figs. 1 and 6).

In *E. coli* mutants lacking DsbC, some disulfide-bonded proteins are reduced in activity or are degraded. These proteins include periplasmic endoribonuclease RNase I, penicillin-insensitive murine endopeptidase MepA, periplasmic acid

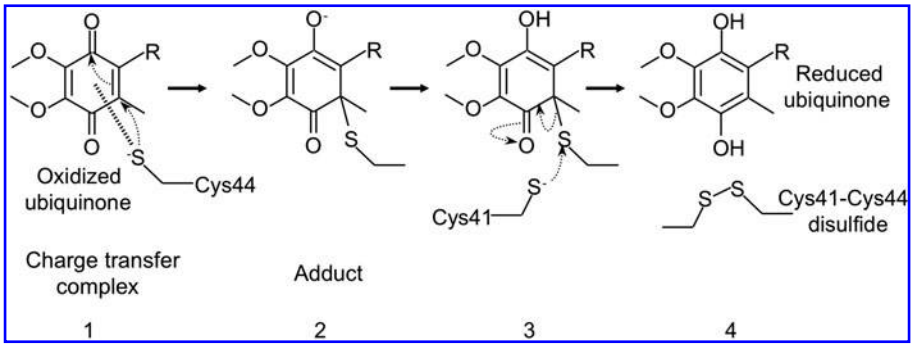

FIG. 5. A model for the oxidation of the N-terminal cysteine pair of DsbB by DsbB-bound ubiquinone. See text for the description of the model.

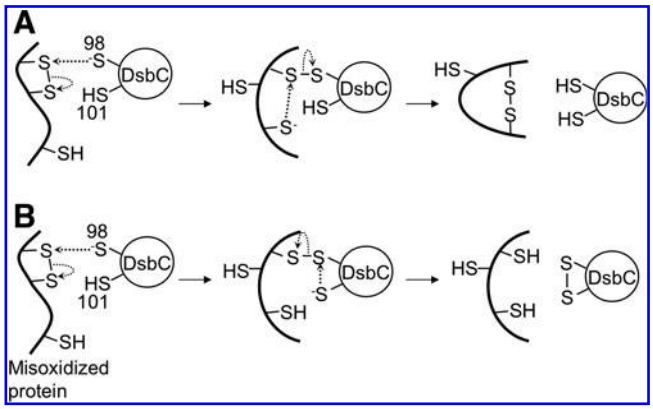

FIG. 6. Disulfide-bond isomerization (A) and reduction (B) by DsbC. Two mechanisms have been proposed for the repair of a misoxidized cysteine pair by DsbC. After the reduction of the substrate protein in sequence (**B**), DsbA can reoxidize the substrate, potentially generating the correct disulfide bond (not shown).

phosphatase AppA, and periplasmic endonuclease-1 End1 (12, 38, 78, 103). A common feature of these proteins is that they have or likely have, in their native structure, at least one nonconsecutive disulfide bond, a disulfide bond formed between two cysteines that do not appear in sequence in the protein. When a protein contains, in its native structure, one or more nonconsecutive disulfide bonds, it appears that the incorrect disulfide bonds are often made. By using AppA as a model substrate, Berkmen *et al.* (12) showed that DsbC is important for the folding of proteins with such nonconsecutive disulfide bonds. Another evident feature of *dsbC*-null mutants is that they show an increased sensitivity to copper. Because copper causes misoxidation of proteins in the presence of molecular oxygen, it has been proposed that the copper sensitivity of *dsbC* mutants is due to the inability of the mutants to repair the misoxidized proteins in the periplasm that have accumulated in the presence of copper (39).

DsbC is a homodimeric molecule with a V-shaped structure (Fig. 7A). Each monomer (23 kDa) of DsbC consists of a C-terminal thioredoxin domain with a CXXC active site and an N-terminal dimerization domain connected by a linker α-helix (77). Two cysteines (Cys98 and Cys101) in the active site from each monomer face the inside of the V-shape and are in the reduced state *in vivo* (49, 91). The dimeric structure of DsbC is crucial for its function: in contrast to DsbA, which is monomeric and oxidized by DsbB, the active site of DsbC is sterically protected from such unwanted oxidation by its dimeric state. A mutation in the dimer interface of DsbC, which results in the inability to form a dimer, allows DsbB to oxidize DsbC. The oxidized mutant DsbC can now act as an oxidant of exported proteins in the periplasm (8) (see later). DsbC has a standard redox potential of −130 mV (110), which is slightly lower than that of DsbA (−120 mV).

How can DsbC repair incorrect disulfide bonds of its substrates? Two models have been proposed (55, 91) (Fig. 6). In both cases, the reaction starts with the nucleophilic attack by Cys98 of DsbC on the incorrect disulfide bond. This reaction results in a mixed-disulfide bond between DsbC and its substrate, which can then be resolved in two ways. In the first model (Fig. 6A), the mixed disulfide is attacked by a third cysteine of the substrate, resolving the complex, allowing a more stable disulfide bond to form in the substrate and restoring a reduced DsbC. In this case, DsbC is acting as a true disulfide isomerase. In the second (Fig. 6B), the mixed disulfide is attacked by Cys101 of DsbC, generating a reduced substrate and an oxidized DsbC. The reduced substrate will then have a chance to be oxidized again by DsbA to give the correct disulfide bond. In the latter case, DsbC is acting as a reductase of disulfide bonds rather than as an isomerase. In both models, the active site of DsbC must be in the reduced state for the enzyme to carry out the first step in the repair of a nonnative disulfide bond.

Theoretically, nonnative disulfide bonds can be repaired by repeating the isomerization or reduction–oxidation reaction until the native disulfide connectivity is established in the substrate protein. Although distinguishing between these two possibilities for the repair of the nonnative disulfide bonds *in vivo* has remained elusive, a recent study with TrxP, a thioredoxin-fold protein from *Bacteroides fragilis*, showed that one of the mechanisms can work *in vivo*. TrxP has strong disulfide reductase activity but very weak isomerase activity. By expressing TrxP in the periplasm of *E. coli* cells lacking DsbC, Shouldice *et al.* (100) showed that the reduction–oxidation

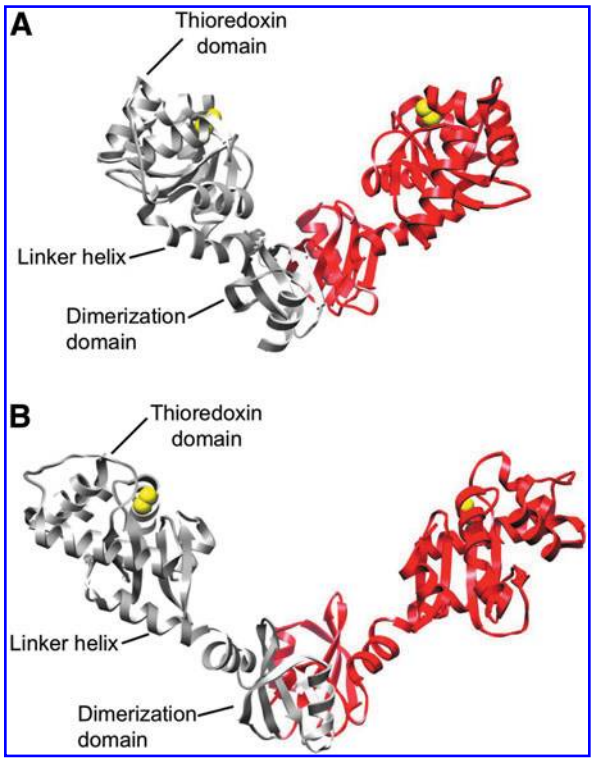

FIG. 7. The crystal structures of DsbC (PDB:1EEJ) (A) and DsbG (PDB:1V58) (B). DsbC and DsbG are V-shaped homodimers; each arm is a monomer containing an N-terminal dimerization domain and a C-terminal thioredoxin domain. The two domains are connected by a linker helix. The yellow spheres represent the sulfurs of active-site cysteines. The dimer form of DsbG was generated by applying crystallographic symmetry (85). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

mechanism can indeed operate *in vivo* to repair nonnative disulfide bonds.

It should be noted that other possible mechanisms exist for the isomerization of disulfide bonds. For example, oxidized DsbC generated after the reduction of a misoxidized substrate (Fig. 6B) may, in turn, act to introduce a disulfide bond into the reduced substrate. It may also be possible that a free cysteine on a misoxidized protein may attack a nonnative disulfide bond on the protein to isomerize, by itself, the disulfide bond (internal rearrangement). However, currently no experiments have tested these mechanisms *in vivo*.

In addition to the isomerase or reductase activity, DsbC has chaperone activity that allows this protein to bind unfolded proteins. For this activity, the active-site cysteine residues are not required (70). The chaperone activity of DsbC may allow this enzyme to capture its misoxidized (thus likely unfolded) substrates efficiently.

The recognition site in DsbC for unfolded structures likely exists in the cleft formed by the inside of the V-shape (Fig. 7A). First, this cleft is large enough (40 Å×40 Å×25 Å) to bind small or unstructured/unfolded proteins. Second, the inner surface is covered with uncharged and hydrophobic residues, a feature often seen in domains that bind misfolded or unfolded proteins (77). Third, fusion of the N-terminal dimerization domain of DsbC to other oxidoreductases, like DsbA, that do not exhibit chaperone activity, confers both chaperone and disulfide isomerase activities (see later) (96, 111). Finally, disruption of the dimerization domain, by a mutation in the dimer interface, results in the loss of both chaperone and disulfide isomerase activities (8). Thus, the dimeric nature of the bottom of the cleft is necessary for the chaperone activity.

Because of the dimerization, DsbC has two catalytic domains, raising the question of whether both catalytic sites are required for its activity. To answer this question, Arredondo *et al*. (4) engineered a DsbC variant, in which the DsbC sequence is tandemly duplicated on a single polypeptide. Surprisingly, a sufficiently long linker sequence placed between the two monomer sequences allowed the polypeptide to fold into an enzyme that displayed essentially identical catalytic and biochemical properties to the authentic dimeric DsbC. Intriguingly, because two monomer sequences are on the same polypeptide, it is possible to design heterodimer-like DsbC variants that have mutations only on the one of the two monomer sequences. With this system, the authors showed that, even if DsbC is missing one of the two catalytic domains, the protein still exhibits the isomerase activity. However, such a mutant was oxidized by DsbB and acted also as an oxidant *in vivo*. Thus, the possession of two catalytic domains is not essential for the activity but is required to protect the active site from misoxidation by DsbB.

DsbG: A DsbC Homologue That Is Able to Protect Single Cysteines from Hyperoxidation

DsbG is a periplasmic thiol-oxidoreductase that is homologous to DsbC (2, 14). DsbG shares 24% amino acid sequence identity with DsbC. The size of the monomer is 26 kDa. Like DsbC, DsbG is dimeric and assumes a V-shaped structure with its N-terminal dimerization domain and its C-terminal thioredoxin domain connected by a linker helix (36). Two cysteines (Cys109 and Cys112) in its active-site CXXC motif are maintained in the reduced state *in vivo* by DsbD (14). DsbG has a redox potential of −126 mV, which is similar to that of DsbC (14).

Although the overall structure of DsbG resembles that of DsbC (Fig. 7A and B), significant differences exist. Most strikingly, the size of the cleft formed by the V-shaped structure in DsbG is almost twice the size of that of DsbC. Additionally, DsbG has several acidic residues lining the cleft, which form a negatively charged surface that is absent in DsbC. These properties of DsbG led Heras *et al*. (36) to propose that DsbG can interact with proteins that are larger and have less hydrophobic surfaces than do the substrates of DsbC.

Then what is the role of DsbG *in vivo*? DsbG exhibits molecular chaperone and protein disulfide isomerase activity *in vitro* (14, 98), although its protein disulfide isomerase activity was poor as compared with that of DsbC (14, 40). Consistently, overexpression of DsbG suppressed some of the defects of *dsbC*-null mutations (12, 14). Yet, the *in vivo* function of DsbG had remained unknown because of the inability to define its physiologic substrates (38, 103).

Recently, Depuydt *et al*. (23) identified the substrates of DsbG, results that led to the discovery of an unexpected function for both DsbG and DsbC. This group found that DsbG preferentially interacts with three periplasmic proteins. All of them belong to the same family of L,D-transpeptidases, which are responsible for cross-linking the major outer-membrane lipoprotein to the peptidoglycan of *E. coli* (71). Importantly, they possess a sole cysteine, essential for activity. By using dyes that specifically detect sulfenic acid (Cys-S-OH), Depuydt *et al.* (23) showed that such a cysteine can be oxidized to a sulfenic acid in the periplasm and that both DsbG and DsbC can protect the cysteine from sulfenylation. The level of sulfenylation was highest in cells lacking both DsbG and DsbC. However, even the cells lacking DsbG alone, but not those lacking DsbC alone, showed increased sulfenylation of sole cysteines over the wild-type cells. Thus, DsbG appears to be more proficient than DsbC in protecting free cysteines in proteins from sulfenylation.

As discussed earlier, the cleft formed by the V-shape of DsbG is much larger and more hydrophilic than that of DsbC (36). Because the preferred substrates of DsbG are folded proteins with an oxidized single cysteine, these unique features of DsbG may allow this enzyme to bind its substrates more efficiently than DsbC.

DsbD: A Membrane Protein That Maintains Both DsbC and DsbG in the Reduced Active State

Both DsbC and DsbG must be maintained in the reduced state to be active. This task is performed by a membrane protein, DsbD (14, 79, 91). In *dsbD* mutants, both DsbC and DsbG fail to be reduced and, thus, cannot act as isomerases or reductases of misoxidized proteins. As a result of reducing DsbC or DsbG, DsbD becomes oxidized. The oxidized DsbD is, in turn, reduced by cytoplasmic thioredoxin (TrxA), which itself is reduced by thioredoxin reductase (TrxB). Thus, reducing power to maintain DsbC and DsbG in their reduced state originated from NADPH (91).

DsbD (59 kDa) has three domains: an N-terminal periplasmic domain (DsbDα) with an immunoglobulin-like fold (35), a hydrophobic core domain (DsbDβ) with eight transmembrane segments (18), and a C-terminal periplasmic domain (DsbDγ) with a thioredoxin-like fold (94). Each domain

has a pair of cysteines that are essential for the protein's activity (59).

How does DsbD catalyze the transmembrane electron transfer from TrxA in the cytoplasm to DsbC in the periplasm? Katzen and Beckwith (59) reconstituted DsbD activity by expressing three domains of DsbD separately *in vivo*. With this system, they revealed that this electron-transfer reaction is carried out through a sequential reduction and oxidation of the three structural domains: DsbDα directly reduces oxidized DsbC; oxidized DsbDα, thus generated, is then reduced by DsbDγ; the resulting oxidized DsbDγ is reduced by DsbDβ; and finally, oxidized DsbDβ is reduced by thioredoxin in the cytoplasm. Subsequent *in vitro* results are consistent with this cascade model (19, 35, 94).

The analyses described revealed that electrons from TrxA in the cytoplasm are passed through DsbD to the oxidized DsbC or DsbG in the periplasm in the following order: TrxA, DsbDβ, DsbDγ, DsbDα, to DsbC/DsbG. The standard redox potentials of their active-site cysteine pairs are −270 mV(TrxA), −246 mV (DsbDβ), −241 mV (DsbDγ), −229 mV (DsbDα), −130 mV (DsbC), and −126 mV(DsbG) (19, 93). Thus, electron-transfer reactions between every two active-site cysteine pairs along the DsbD-mediated electron flow are thermodynamically favorable (19, 93). This is in contrast to the oxidation of DsbA by the second periplasmic domain of DsbB (see earlier).

Two cysteines of DsbDβ receive electrons from TrxA in the cytoplasm and transfer them to DsbDγ in the periplasm (59). How is it possible for the two cysteines of DsbDβ to perform the reactions on both sides of the membrane? Recent studies by Cho *et al.* (17, 18) suggest that DsbDβ exhibits hourglass-like openings on both sides of the membrane, having its two active-site cysteines at the constriction site. This physical arrangement appears to allow two cysteines in DsbDβ to interact with the cysteines of thioredoxin on the cytoplasmic side and with the cysteines of DsbDγ on the periplasmic side of the membrane, facilitating the electron-transfer reaction across the membrane.

DsbD represents a unique type of two-electron transporter. Still unclear is the mechanism that would allow the passage of electrons across the membrane without perturbing the function of the membrane as a permeation barrier. Determination of the three-dimensional structure of DsbDβ may be necessary to arrive at an answer to this question.

Factors Influencing DsbA-mediated Oxidative Protein Folding

Despite considerable knowledge of the structure and biochemical properties of the enzymes involved in disulfide bond formation, we know very little about how each disulfide bond forms in a protein that is translocated into the bacterial periplasm *in vivo*. When during the process of translation, membrane translocation, and folding does disulfide bond formation take place? How important are the properties of the cysteines themselves in determining which cysteines are covalently joined? Here we discuss factors that may affect the process of oxidative protein folding.

Vectorality of protein export and oxidative folding in the periplasm

For the protein substrates of DsbA to cross the membrane through the Sec channel, the polypeptides must be maintained in the cytoplasm in an unfolded state (41, 69). As the proteins pass through the Sec channel, the N-terminus of the protein appears in the periplasm first (51, 82). These mechanistic features of protein export appear to affect the mechanisms of oxidative protein folding. Both indirect and direct evidence support a model in which disulfide bonds are often formed sequentially between two cysteines of a protein as they appear in the periplasm.

If disulfide bond formation does proceed vectorially, it would cause a problem for those proteins that have nonconsecutive disulfide bonds in their native structure. Such a mechanism would lead to the formation of incorrect disulfide bonds in these proteins, necessitating the action of an isomerase. As described earlier, the disulfide isomerase DsbC is required for the efficient folding of such proteins (12, 38), consistent with the vectorial model.

This model also predicts that the formation of a disulfide bond between a correct pair of cysteines would be compromised if there were an unpaired cysteine before or between cysteines that are meant to be joined in the native structure. Interestingly, bioinformatics analysis by Dutton *et al.* (27) revealed that, for those organisms that make disulfide bonds, the majority of bacterial cell envelope proteins appear to have evolved to avoid an unpaired cysteine, apparently to prevent the formation of incorrect disulfide bonds (see later), consistent with the vectorial model.

More-direct evidence for this model came from a recent study of oxidative folding of PhoA, a periplasmic protein of *E. coli* having two disulfide bonds (53). This protein crosses the membrane by a mixed co-translational/posttranslational mechanism in which some of the protein is exported during its synthesis on the ribosome, and some is exported after completion of its synthesis (51). During cotranslational folding of PhoA, the formation of the N-terminal disulfide bond took place before that of the C-terminal disulfide bond, consistent with the order in which each cysteine pair appears in the periplasm. In addition, steps of electron transfer leading to the formation of the C-terminal disulfide bond began by Cys286 attacking DsbA when the PhoA peptide elongated to ~43 kDa in length, thus forming a mixed-disulfide complex between DsbA and the nascent chain of PhoA (Fig. 8). Further elongation of the peptide allowed Cys336 to attack the mixed disulfide, resulting in the resolution of the complex and formation of a Cys286–Cys336 disulfide in the nascent chain, completing the process. Thus, two cysteines that form a disulfide bond also acted sequentially in the order in which they appear in the periplasm (53), further supporting the vectorial model.

These results indicate that the vectorality in the appearance of nascent polypeptide from the Sec machinery can be an important factor affecting oxidative protein folding in the periplasm. However, recent evidence indicates the existence of nonvectorial folding and other factors that may affect the oxidative protein folding.

Partially correct folding of RNase I even in the absence of DsbC

RNase I of *E. coli* has four disulfide bonds, one of which is formed between a nonconsecutive cysteine pair. Messens *et al.* (78) studied the DsbA-catalyzed formation of disulfide bonds in this protein *in vivo* and *in vitro* in the presence or absence of

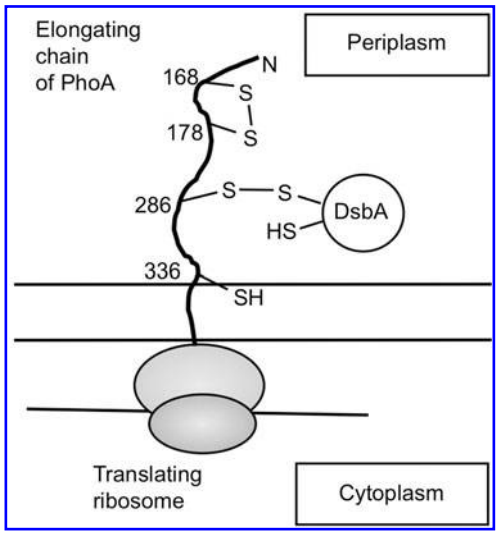

FIG. 8. A model for the disulfide-linked DsbA-substrate complex that forms during the cotranslational folding of PhoA. PhoA has two pairs of disulfide bonds. During cotranslational folding of PhoA, the PhoA Cys286 attacked DsbA to form a disulfide-linked complex (this model) between DsbA and the elongating chain of PhoA. This reaction took place when the PhoA polypeptide was elongated to ~43 kDa in length. Further elongation of the polypeptide allowed Cys336 to attack the mixed-disulfide bond, completing the formation of the C-terminal disulfide bond of PhoA (not shown) (53).

DsbC. They found that DsbA activity alone is capable of yielding some of the protein containing its correct disulfide bonds. This finding suggests that a factor, other than vectorial export, must be influencing the interaction between DsbA and its substrate, leading to the fairly correct oxidative folding of this protein despite the protein's nonconsecutive disulfide bond. One possible explanation is that the speed with which DsbA acts on its emerging substrates is slow enough so that some fraction of the polypeptide chains achieve a sufficient degree of folding to make it possible for DsbA to join the correct cysteines.

Disulfide-bond formation guided by the structural changes of a protein

Elastase of *Pseudomonas aeruginosa* is synthesized as a preproenzyme. The mature protein has two disulfide bonds. Interestingly, disulfide-bond formation in this protein takes place nonvectorially by three steps (15). In the first step, DsbA introduces the C-terminal disulfide bond into the proenzyme. This step is required for the autocatalytic processing of its propeptide. In the final step, DsbA introduces the N-terminal disulfide bond before translocation of the mature protein across the outer membrane. Thus, it appears that disulfide-bond formation in this protein by DsbA *in vivo* is an ordered process, guided by structural changes of the protein.

The activity of the ribosome can affect the extracytoplasmic protein folding

Analysis of the oxidative folding process of PhoA during cotranslational or posttranslational folding revealed an unexpected effect of the ribosome in the folding process of a protein in the bacterial periplasm (53) (Fig. 9).

As we described earlier, during the cotranslational formation of the Cys286–Cys336 disulfide bond of PhoA, Cys286 was the cysteine used to form the mixed-disulfide complex with DsbA. However, surprisingly, during posttranslational folding of PhoA, both Cys286 and Cys336 were used to form the disulfide-linked complexes between DsbA and PhoA (53), suggesting that the process of disulfide-bond formation during posttranslational folding may be different from that of cotranslational folding. Further, in a PhoA variant lacking its second cysteine, Cys178, the first cysteine, Cys168, is unpaired because it does not have its native partner for the formation of the Cys168–Cys178 disulfide bond. When PhoA was exported cotranslationally by using an SRP-dependent signal sequence, the folding of PhoA was more sensitive to the presence of the unpaired cysteine than was the same protein exported both cotranslationally and posttranslationally with its authentic signal sequence. These results suggest that oxidative folding of PhoA occurs more vectorially during its cotranslational folding than that of the same protein during posttranslational folding (53).

It has been estimated that the rate of posttranslational export is more than 10 times greater than that of cotranslational export (86). Thus, when the polypeptide is crossing the membrane in a cotranslational manner, it may be the slowness of the appearance of the polypeptide in the periplasm that results in polypeptide folding more vectorially from the N-terminus to the C-terminus in this experiment. In contrast, the more rapid posttranslational export may cause the protein folding to outcompete the early action of DsbA on a substrate even more significantly.

Increasing evidence suggests that the ribosome plays an important role in the folding of proteins in the cytoplasm (65). This finding on the unexpected link between the activity of ribosome and the extracytoplasmic protein folding may expand our understanding regarding the role of the ribosome in protein folding.

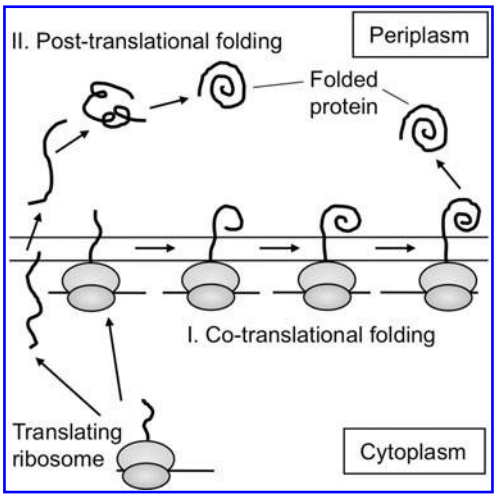

FIG. 9. Cotranslational and posttranslational oxidative folding of PhoA. Results (53) suggest that the mode of translocation (cotranslational versus posttranslational) can affect the folding process of a protein in the periplasm.

Is the oxidative-folding process regulated by the reactivity of cysteines?

As described earlier, during cotranslational formation of the C-terminal disulfide bond of PhoA, Cys286 attacked DsbA to initiate the reaction. The resulting DsbA–PhoA complex was resolved by Cys336, completing the reaction (53). This finding indicates that two cysteines of a folding protein can play different roles in the formation of their disulfide bond in a reaction catalyzed by a thioredoxin-superfamily member.

Two factors could be important in determining which cysteine will attack DsbA during disulfide-bond formation. First, the cysteine on a substrate must be sterically available to DsbA to initiate the process. Second, as the deprotonation of its sulfhydryl group is essential for a cysteine to attack a disulfide bond (29, 106), the reactivity of a substrate cysteine may also be affected by its pK_a. During the cotranslational formation of the C-terminal disulfide bond of PhoA, the availability of the cysteine (the order of appearance in the periplasm) most likely determined which cysteine attacked DsbA (53).

Can difference in the reactivity of cysteines, if it exists during oxidative folding, regulate this process *in vivo*? Before addressing this question, we should consider whether DsbA is able to choose a proper pair of cysteines from among others to form a correct disulfide bond. DsbA often makes mistakes. This protein can introduce disulfide bonds into proteins that do not have disulfide bonds in their native state. For example, when a cytoplasmic enzyme, β-galactosidase, is artificially exported to the periplasm, the cysteines of this protein, normally in the reduced state, are joined by DsbA to form disulfide bonds, resulting in the misfolding of this protein (10). In addition, as we have seen in the section for DsbC, DsbA tends to introduce incorrect disulfide bonds into proteins that have one or more nonconsecutive disulfide bonds in their native structure (12). These observations have led to a view that DsbA is an indiscriminating enzyme that does not distinguish among the cysteines it joins in forming disulfide bonds (100). However, as we saw previously, some evidence suggests that this is not necessarily the case. For example, a significant proportion of RNase I is oxidized by DsbA to give the correct disulfide bonds, even in the absence of DsbC *in vivo* and *in vitro*, although a nonconsecutive disulfide bond exists in this protein (78). Further, disulfide-bond formation in elastase of *Pseudomonas aeruginosa* by DsbA of this organism is a regulated process occurring in a manner guided by structural changes of this protein (15). These findings suggest that DsbA may, at least in some cases, be able to discriminate between a proper pair of cysteines and others to form a correct disulfide bond.

At least two ways exist to imagine that DsbA is able to discriminate in the recognition of substrate cysteines. First, the substrate-binding site of DsbA may have evolved to recognize a specific set of substrate cysteines (*e.g.*, by their ionic state or the local conformation in their vicinity). In this regard, the residue preceding the conservative *cis*-proline of DsbA was recently suggested to be important for the redox properties and substrate specificities of this enzyme (89). Second, some substrates of DsbA may have evolved to allow the reactivity of each of their cysteines toward DsbA to be regulated during oxidative folding, as discussed earlier. Although the substrate cysteine's reactivity could be potentially important in understanding the mechanisms of oxidative protein folding, our knowledge on this aspect is greatly limited because of the difficulty in detection and characterization of the intermediate complexes.

Diversity among Bacteria in Disulfide Bond–Forming Capacity and Mechanism

Searches for enzymes involved in pathways leading to disulfide-bond formation in bacteria other than *E. coli* have largely been based on seeking homologues of the DsbA and DsbB proteins. However, given the vast range of environments in which bacteria live, one might expect to find variability in seeking answers to the following questions: (a) Do all bacteria make proteins with structural disulfide bonds? (b) Do those bacteria that make disulfide bonds all use the DsbA and DsbB enzymes? and (c) Is it the case for all bacteria that proteins with disulfide bonds are absent from their cytoplasms and are found only in proteins translocated through the cytoplasmic membrane?

In recent years, a number of approaches have been used to answers these questions. These studies reveal diversity in the capacity and mechanism for disulfide-bond formation among bacterial species. Bioinformatic approaches for analyzing genome sequences have yielded predictions for whether and how an organism carries out disulfide-bond formation. Biochemical and genetic studies of individual organisms have revealed unexpected variants on the *E. coli* system. Finally, the novel mechanisms for achieving disulfide-bond formation in bacteria in laboratory-based evolution experiments have provided plausible scenarios for what may be found in bacterial species that have yet to be examined in detail.

Disulfide-bond formation in the cytoplasm

Until recently, it has generally been assumed that structural disulfide bonds are not present in cytoplasmic proteins. Two lines of evidence have challenged that assumption. First, *E. coli* strains have been mutationally altered in their reductive pathways, such that disulfide bonds are formed in certain proteins with high efficiency (13, 24). The proteins that did acquire disulfide bonds in these strains were those that were ordinarily exported from the cell but, in these studies, were expressed in the cytoplasm by deleting their signal sequences. Second, combining a computational approach with structural characterization of the proteins of a collection of microbial hyperthermophiles, Yeates and co-workers (72) discovered that some of these organisms, particularly the Archaea, do contain cytoplasmic proteins with disulfide bonds (11, 72). This unexpected finding was explained as being due to the evolutionary adaptation of these organisms to the harsh environments in which they live, requiring the added stability conferred on their proteins by disulfide bonds.

Proteins in cells or compartments in which disulfide bonds are made tend to have even numbers of cysteines

The computational approach used by Yeates' group assumed that cellular compartments that had many disulfide-bonded proteins would be enriched for proteins with even numbers of cysteines (11, 72). This assumption is reasonable, given the properties of a protein's cysteines in environments that are oxidizing or in which an enzyme catalyzing disulfide-

bond formation is present, or both. The assumption comes, in part, from the knowledge that the prototypical disulfide bond–forming enzyme, DsbA, is relatively indiscriminate in forming disulfide bonds. That is, DsbA often catalyzes formation of the wrong disulfide bonds and even catalyzes disulfide-bond formation in proteins that do not ordinarily have such bonds (1, 10). Incorrect disulfide-bond formation can be a particular problem for proteins that contain an odd number of cysteines, in which the process may involve sequential joining of cysteines as they appear in the periplasm during the translocation process (12, 53). A second relevant property of cysteine is that it is the amino acid most sensitive to oxidation, often being converted to sulfenic acid and higher oxidation states in the presence of such oxidants as hydrogen peroxide. As a result, proteins with odd numbers of cysteines, even if some of those cysteines were joined in disulfide bonds, would still have a free cysteine that could have oxidative damage or be joined in a disulfide bond with a cysteine in another protein. Considering these problems, a preference in evolution may have been to avoid odd numbers of cysteines in those proteins destined for export to compartments that are oxidative or where disulfide bonds are made or both. With hyperthermophilic Archaea, Yeates *et al.* (72) translated all of the open reading frames of these organisms into protein sequences and found that their entire proteome showed a strong preference for proteins containing even numbers of cysteines. This finding was in contrast to the same analysis applied to the entire *E. coli* proteome, in which no marked preference for even numbers of cysteines was found. Because the bulk of the cell's proteins are cytoplasmic, the overall preference for even numbers of cysteines in the hyperthermophiles suggested that disulfide-bonded proteins were made within the cell's cytoplasm. This presumption was confirmed by the finding of cytoplasmic disulfide-bonded proteins in these organisms. These results are in contrast to the findings with *E. coli* in which structural disulfide bonds in proteins are made only in the periplasm.

An extended computational approach to diversity

The even/odd computational approach pioneered by Yeates and co-workers (72) has been modified by us to extend it beyond the analysis of the entire proteome of the bacterial cell to an analysis that distinguishes between cytoplasmic and exported proteins. The modification requires separate calculations of the overall amount of cysteine present in proteins in the cytoplasm and of cysteine present in proteins of extracellular compartments and secreted proteins. Distinguishing between the locations of proteins required bioinformatic means of predicting from sequences which proteins are exported and which domains of membrane proteins extend outside the cytoplasmic membrane. In addition, the analysis included a search for homologues of the DsbA and DsbB proteins in the sequenced genomes. This approach has been applied to 374 sequenced bacterial genomes. The data obtained from this combined approach leads to the following predictions (27):

1. Many bacteria incorporate disulfide bonds into a sizable fraction of their exported proteins, by using the same DsbA/DsbB system as does *E. coli*. These organisms include most of the α-, β-, and γ-proteobacteria. This prediction is consistent with structural studies on proteins from many of these organisms.
2. Certain other classes of bacteria, including many Actinobacteria, Cyanobacteria, and aerobic δ-proteobacteria, also make disulfide bonds in many of their proteins by using DsbA, but instead of DsbB, they use a homologue of vertebrate vitamin K epoxide reductase (VKOR) to reoxidize DsbA (27, 101). Vertebrate VKOR catalyzes reduction of vitamin K, a step in the set of enzymatic reactions that leads to blood coagulation (68, 92). Studies so far indicate that the mechanism of action of bacterial VKOR is quite similar to that of DsbB, that structural similarities exist between the two proteins, but that bacterial VKOR evolved separately and is not a homologue of DsbB. Remarkably, the VKOR of *Mycobacterium tuberculosis*, when expressed in *E. coli*, can substitute for DsbB and is inhibited by warfarin, a blood thinner that is used to prevent blood clotting in humans (26).
3. Many bacteria do contain homologues of DsbB and DsbA, but the cysteine-counting analysis predicts that they either do not make disulfide bonds in proteins at all or make them only in a small fraction of their exported proteins. In these cases, evidence suggests that some of these organisms may use relatively specialized DsbA homologues to introduce disulfide bonds into a very limited number of proteins. For example, genes encoding a DsbA and a DsbB were found in an operon with a gene for an arylsulfate sulfotransferase in a number of bacterial species, including *Geobacter metallireducens* and a uropathogenic *E. coli* (27, 32). (Some of these bacteria, including the latter organism, also contain genes encoding prototypical DsbA and DsbB proteins.) Evidence suggested that these DsbAs do not have the broad specificity of the prototypical DsbA and may have been designed to catalyze disulfide bond formation efficiently in the arylsulfate sulfotransferase. In another example, the gram-positive firmicute *Bacillus subtilis* encodes two DsbA/DsbB-like pairs (the Bdb proteins), for each of which only one known disulfide-bonded substrate has been found (64).
4. Many bacteria appear to have neither DsbA nor DsbB (nor VKOR) and from cysteine-counting are predicted not to make disulfide bonds. Many of these are obligate anaerobes, such as *Bacteroides fragilis,* or grow obligately only intracellularly (*e.g.*, Rickettsia) (27). These organisms, because the extracellular space available to them or their periplasm represents a strongly reducing environment, may always maintain their cysteines in the reduced state. This may be an active process promoted by extracytoplasmic reductants or simply due to the absence of any oxidative catalysts of disulfide-bond formation. Evidence supporting the proposal that these organisms do not allow disulfide-bond formation comes from the finding that cysteines in proteins that are ordinarily disulfide bonded in their native host, *E. coli*, when expressed in *Bacteroides fragilis*, remain in the reduced state. Nevertheless, other organisms such as these may not encounter oxygen, yet can use electron acceptors such as nitrate or fumarate to permit disulfide-bond formation.
5. Some organisms exist for which the results of the various bioinformatic analyses may appear inconsistent. For example, organisms such as *Staphylococcus aureus*

have a DsbA but no apparent DsbB, nor do they have a VKOR. In this case, based on the structure and the unusual stability of the oxidized form of this DsbA, Glockshuber and co-workers (37) proposed that the SaDsbA is more effective than the prototypical DsbA at using small-molecule oxidants as a replacement for DsbB.

Bioinformatic analysis in a number of laboratories has revealed added variability in the biology of the Dsb proteins. Although the DsbA of gram-negative bacteria is usually a soluble protein localized to the periplasmic space, gram-positive bacteria express DsbA homologues that are membrane bound either as lipoproteins or by transmembrane-spanning segments (64). This membrane attachment may be necessitated by the absence of the outer-membrane barrier found in gram-negative bacteria. Finally, some bacteria contain several homologues of Dsb proteins. For example, *Neisseria meningitides* expresses three DsbA homologues that may play different roles in the interaction of the bacteria with the host, again suggesting the possibility of differing specificities of the homologues (66).

Laboratory-generated evolution of alternative disulfide bond–forming systems in bacteria

After the discovery that the DsbA and DsbB proteins were responsible for disulfide bond formation in *E. coli* in the early 1990s, much of the research in this area has focused on the mechanisms of action of these two enzymes and on their presence in many other bacteria. However, as described in the previous section, work published in recent years has revealed a more complex and diverse picture of mechanisms of disulfide-bond formation in bacteria. These findings raise the possibility that there exist even more diverse mechanisms for disulfide-bond formation than have been detected. One approach to asking what other pathways for disulfide-bond formation might exist in bacteria is to evolve new pathways in the laboratory. Bioinformatic approaches can then be used to ask whether any evidence for such laboratory-evolved pathways can be detected among the array of bacteria species.

A number of studies have reported instances in which the requirement for DsbA for disulfide-bond formation has been bypassed. For example, when signal sequences are attached to cytoplasmic thioredoxin, it is exported to the periplasm of *E. coli*, where it is oxidized by DsbB (21, 22, 50). Even though thioredoxin has a very low redox potential and is a powerful reductant, it still catalyzed a measurable degree of disulfide-bond formation. This oxidizing activity was greatly enhanced by altering the two amino acids between the active-site cysteines of thioredoxin, so as to increase its redox potential to favor oxidation.

In another example, the disulfide isomerase DsbC, a thioredoxin family member, which ordinarily does not serve as an oxidant, can be converted by mutation or genetic engineering to an effective catalyst of disulfide-bond formation. Ordinarily, the homodimeric DsbC cannot act as an oxidant, as its active sites are protected from oxidation by DsbB, and it is efficiently reduced by DsbD. However, mutations of *dsbC* that interfere with dimerization cause DsbC monomers to accumulate, which can then be oxidized by DsbB, allowing the protein to catalyze disulfide-bond formation (see earlier) (8). Furthermore, deletions of amino acid residues from the DsbC linker helix that cause the DsbC active site to be more exposed also allow it to be oxidized by DsbB (95). Conversely, fusions of DsbA to dimerization domains result in a dimeric DsbA that now acts like DsbC as a disulfide isomerase (3, 96, 111). These manipulations hardly point to a potential new mechanism of disulfide-bond formation but, rather, may simply be recapitulating the evolution of thioredoxin family members, such as DsbA and DsbC.

However, two novel pathways for disulfide-bond formation have been generated in the laboratory. In the first case, mutagenesis and forced export of thioredoxin yielded a version of the protein in which the CXXC motif has been replaced with a CCXC motif (76). This version of thioredoxin formed a dimeric complex in which a [2Fe-2S] bridge linked the monomers via the cysteines. This dimeric complex was itself an effective catalyst of disulfide-bond formation in the absence of DsbB and DsbA and appeared to depend simply on oxygen. A second novel pathway involved the forced export of a glutaredoxin (glutaredoxin 3) of *E. coli* to the periplasm. In this case, disulfide-bond formation was also achieved without a requirement for DsbB. Rather, the ability of periplasmic glutaredoxin 3 to oxidize substrate proteins in the periplasm depended on the presence of the glutathione biosynthetic pathway in the cytoplasm (28). These results were interpreted to mean that the periplasmic glutaredoxin was able to promote disulfide-bond formation by using oxidized glutathione exported by cells. Although no evidence exists that either of these novel pathways is used normally by any bacterial species, their discovery should alert us to the possibility that they may be found as either the primary or a secondary pathway for disulfide-bond formation in some organisms.

Conclusion and Perspectives

In the past two decades, we have learned that a surprising variety of structures, mechanisms, and factors are involved in carrying out the process of disulfide-bond formation. However, fundamental questions remain about the molecular mechanism by which each component accomplishes its task. For example, DsbA introduces disulfide bonds into proteins in the periplasm. The proteins presumably begin to fold when a portion of the polypeptide appears in the periplasm. We do not know at what point of protein folding each disulfide bond is introduced into the protein by DsbA. Knowing how substrate cysteines attack DsbA to initiate the process will certainly increase our understanding of oxidative protein folding (53). In addition to DsbA, DsbC, and DsbG, *E. coli* possess a number of proteins that may affect protein folding in the periplasm. It will be important to determine the coordination between the disulfide catalysts and other factors such periplasmic molecular chaperones, peptidyl-prolyl *cis-trans* isomerases, export machinery proteins, and the ribosome (25, 53). Important features of the mechanisms by which the membrane components DsbB and DsbD carry out electron-transfer processes also remain largely unknown. How the C-terminal cysteine pair of DsbB oxidizes DsbA is still under debate. DsbB has a bound quinone, which is apparently reduced after oxidation of DsbA by DsbB. However, it is unclear at this point how the oxidized quinone is restored on DsbB. The mechanism by which the DsbD β domain promotes transmembrane electron transfer also remains unclear. Furthermore, many questions remain about the newly discovered system for repair of hyperoxidized single cysteines by DsbG

and DsbC (23). How do these enzymes recognize and reduce the modified cysteines on folded proteins? Recent bioinformatic analysis reveals that some bacteria evolved multiple or totally different enzyme systems to form disulfide bonds (27). For many bacteria that are the causative agents of human disease, disulfide-bonded proteins, toxins, adherence factors, and others are essential for pathogenesis. Thus, the disulfide-bond–formation systems may serve as therapeutic targets, making it important to understand the mechanisms involved in disulfide-bond formation. Answering all of these questions will require a variety of genetic, biochemical, structural, and bioinformatic approaches.

Acknowledgments

We thank Fredric Åslund and Seung-Hyun Cho for discussions, Jean-François Collet for communicating his group's results before publication, and Rachel Dutton for comments on the manuscript. J.B. is an American Cancer Society Professor. This work was supported by NIH grant GM41883 (to J.B.) and by an international research fellowship from the Global COE program in NAIST from MEXT of Japan and Grant-in-Aid for Scientific Research C (21580092) (to H.K.).

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Date of first submission to ARS Central, March 13, 2010; date of acceptance, April 1, 2010.

Abbreviations Used

DTT = dithiothreitol
SRP = signal-recognition particle
TrxA = thioredoxin
TrxB = thioredoxin reductase
VKOR = vertebrate vitamin K epoxide reductase

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