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Disulfide Bond Isomerization in Prokaryotes†

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A key step in protein folding is the formation of disulfide bonds between cysteine residues. Disulfide bonds frequently play an essential structural role within proteins, stabilizing their tertiary structures. These stabilizing forces are so important that simple reduction of disulfides can lead to protein unfolding. In organisms ranging from *Escherichia coli* to humans, disulfide bond formation is particularly important for the proper folding of secreted proteins, including bacterial virulence factors and mammalian secreted glycoproteins (*1*).

In 1961, Anfinsen and co-workers performed a classic experiment that demonstrated that oxidizing conditions are sufficient to correctly refold reduced and denatured RNaseA (*2*). This experiment was the first to show that a protein could fold into its correct tertiary structure with only the information provided in its amino acid sequence. Anfinsen and colleagues were also the first to note a potential caveat to their discovery. Although correct formation of RNase A's four disulfide bonds could occur spontaneously, there was an obvious discrepancy between the rate of refolding in vitro (hours−days) and in vivo (seconds−minutes), suggesting the presence of an in vivo catalyst for disulfide bond formation. Two years later Anfinsen and co-workers discovered the first of these catalysts, protein disulfide isomerase (PDI),[1] in the eukaryotic endoplasmic reticulum (*3*). In eukaryotes and prokaryotes, disulfide bond oxidation, reduction, and isomerization are catalyzed processes, facilitated by members of the thioredoxin superfamily.

The focus of this article is disulfide isomerases, proteins that catalyze the rearrangement of disulfide bonds. As the number of cysteine residues in a protein increases, the number of possible disulfide pairings increases rapidly and the need for a catalyst of disulfide isomerization also grows. Yet although they perform a vital cellular function, disulfide isomerases are less characterized than many thioredoxin-like proteins. This is largely due to the complexity of the eukaryotic enzyme protein disulfide isomerase (PDI), which has a multisubunit structure and numerous enzymatic functions. The recent discovery of prokaryotic disulfide isomerases allows the study of a less complicated system of disulfide isomerization in an organism with abundant genetic and biochemical tools. Despite large gains in knowledge, crucial questions remain unanswered. We begin with some fundamental information about disulfide bond formation that will allow in depth examination of the prokaryotic system of disulfide isomerization.

Subcellular Environment and Disulfide Bond Formation. Although disulfide bonds are essential stabilizing structures in many proteins, they are rarely found in the cytoplasm of

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[1] Abbreviations: PDI, protein disulfide isomerase; DTT, dithiolthreitol; BPTI, bovine pancreatic trypsin inhibitor; MGSA, melanocyte growth-stimulating activity; hGH, human growth hormone; anti-CD18, anti-CD18 antibody; IGF-1, interferon-like growth factor-1.

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prokaryotic or eukaryotic organisms. Instead, disulfide bonds are usually found in proteins destined for locations outside of the cytoplasm. In eukaryotes, the majority of disulfide bond-containing proteins are secreted, the bonds formed in the endoplasmic reticulum. In prokaryotes, these proteins are secreted or periplasmic, the disulfides formed within the periplasm.

There are several explanations for this observation. First, a number of cytoplasmic enzymes, such as ribonuclease reductase and methionine sulfoxide reductase, rely on a reduced cysteine residue in their active site (*4*). The presence of oxidized disulfides in the cytoplasm could lead to oxidation and inactivation of these enzymes. Second, a partially unfolded conformation is required for the translocation of many proteins across membranes (*5*). Cytoplasmic disulfide bond formation could hinder secretion of essential proteins. Finally, a number of prokaryotic virulence factors and toxins contain multiple disulfide bonds (*6*−*8*). One can imagine that a system preventing their premature activity within the cytoplasm would benefit the cell. Thus, disulfide bond formation rarely occurs in the cytoplasm, even in a protein that will eventually contain disulfide bonds. One exciting exception is work by Beckwith and others that manipulates cytoplasmic expression of redox-active proteins, allowing disulfide bond formation to occur in the cytoplasm (*9*, *10*).

To ensure that disulfide bonds formation only occurs in the specialized locations where it is productive, both eukaryotic and prokaryotic organisms have evolved impediments to disulfide bond formation in the cytoplasm. One important barrier is the extremely reducing environment of the cytoplasm, illustrated by the cytoplasmic ratio of reduced: oxidized glutathione of about 200:1 in prokaryotes (*11*). This environment, maintained by the thioredoxin/thioredoxin reductase, glutathione/glutathione reductase, and glutaredoxin/glutaredoxin reductase systems, helps ensure that cytoplasmic cysteine residues are kept in a reduced state (*12*). A second likely influence is that enzymes promoting disulfide bond formation are largely absent in the cytoplasm but abundant in the periplasm and endoplasmic reticulum.

The DsbA−*DsbB Pathway Generates de Novo Disulfides in Prokaryotes.* In the *E. coli* periplasm, disulfide bond formation and disulfide bond isomerization are catalyzed by two separate pathways (Figures 1 and 2). The DsbA−DsbB pathway oxidizes thiol groups to form disulfides de novo, while the DsbC−DsbD pathway isomerizes mismatched disulfides. The oxidative DsbA−DsbB pathway was discovered about 10 years ago, when it was shown that disulfide bond formation requires the presence of a previously unknown gene, thus named *dsbA* (*13*). The numerous phenotypes associated with *dsbA* or *dsbB* null mutants reflect the variety of proteins that require disulfide bonds for proper function. These include proteins required for flagella-driven motility, resistance to benzylpenicillin, and transformation with DNA (*14*, *15*). Virulence factors, such as enterotoxins I and II, often have multiple disulfides and *dsb* mutants can show attenuated virulence (*6*, *7*).

In the oxidative pathway, DsbA directly interacts with substrate proteins and oxidizes them. DsbA is a 21 kDa periplasmic protein. Like other members of the thioredoxin superfamily, including disulfide isomerases, DsbA contains the thiredoxin fold as well as two active site cysteines residues in a CXXC motif (*16*). Unlike disulfide isomerases,

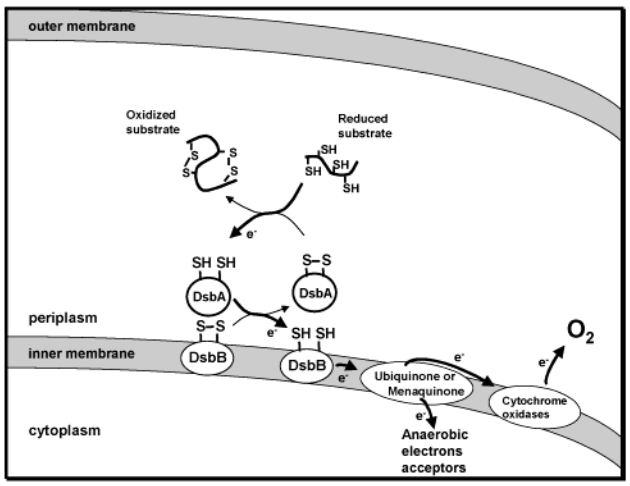

FIGURE 1: Disulfide bond formation is catalyzed by the periplasmic DsbA−DsbB pathway. DsbA interacts with a folding protein containing reduced cysteines, oxidizing them to form disulfide bonds. In this process, DsbA is reduced and must donate its electrons to the inner membrane protein DsbB in order to be reoxidized. Under aerobic conditions, DsbB donates electrons to ubiquinone, which passes them to cytochrome oxidases and finally to molecular oxygen. Under anaerobic conditions, DsbB donates its electrons to menaquinone, which donates them to anaerobic electron acceptors.

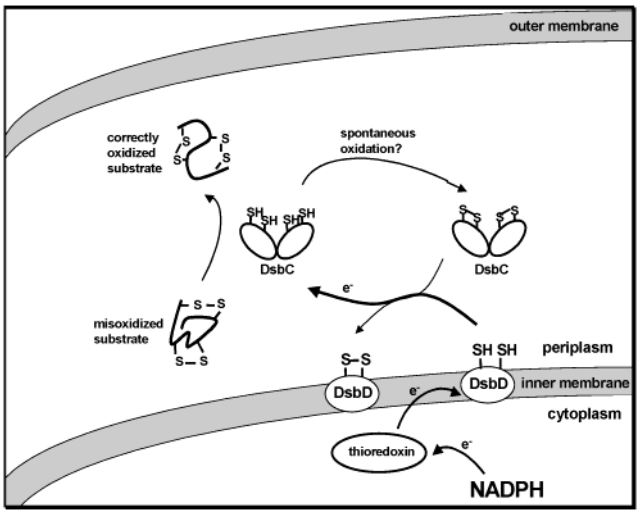

FIGURE 2: The DsbC−DsbD pathway catalyzes disulfide isomerization in the periplasm. DsbC interacts with substrate proteins that contain non-native disulfide bonds, allowing these disulfide bonds to rearrange to their native pairings. DsbC requires the action of DsbD in order to remain reduced in the highly oxidizing environment of the periplasm. The ultimate source of DsbD's reducing potential is cytoplasmic NADPH, which transfers electrons to thioredoxin, the cytoplasmic protein that directly reduces DsbD.

DsbA must have an oxidized active site in order to transfer disulfides to newly synthesized proteins (Figure 1). The reactivity of DsbA's active site is largely achieved by the CXXC motif's N-terminal cysteine, which has a remarkably low pK_a of 3 (the normal pK_a of cysteine residues is 9) (*17*). This extremely low pK_a causes the thiolate anion to predominate at physiologic pH. Since this thiolate anion is stabilized by an adjacent histidine only in reduced DsbA, reduced DsbA is much more stable than oxidized DsbA.

The physiologic result of this unusual pK_a is that DsbA is extremely unstable in oxidized form and is, in fact, the most oxidizing protein known (*17*, *18*). Oxidized DsbA reacts

quickly with newly synthesized substrate proteins to form mixed disulfides, then releases substrates in oxidized form. In this way, DsbA returns to its more stable, reduced state while creating de novo disulfide bonds in substrate proteins. DsbB's role in the DsbA−DsbB pathway is to reoxidize DsbA, allowing DsbA to regain activity.

DsbB is an inner membrane protein that contains two pairs of essential cysteines residues, one of which is located in a CXXC motif. Although the crystal structure has not been solved, DsbB's only predicted similarity to thioredoxin is this CXXC motif—it is very unlikely to be a member of the thioredoxin superfamily. When first discovered, null mutations in the *dsbB* gene showed a pleiotropic phenotype almost identical to that of *dsbA*$^-$ strains, suggesting that DsbA and DsbB are part of the same pathway (*14*). This was confirmed by demonstrating that reduced DsbA accumulates in a *dsbB* null strain, while oxidized DsbA is found in wild-type strains, indicating that DsbB is required for formation of catalytically active, oxidized DsbA (*19*).

DsbB becomes reduced after it has reoxidized DsbA. To function catalytically, DsbB must therefore be reoxidized. As soon as DsbB was discovered to be an inner membrane protein, Bardwell and co-workers proposed that the electron transport chain might be involved in reoxidizing DsbB (*19*). In support of this hypothesis, mutants defective in quinone or heme biosynthesis accumulate DsbA in reduced form (*20*). Bader and co-workers determined the precise pathway of oxidative protein folding through a series of in vitro experiments (*21*−*23*). DsbB was shown to require the presence of cytochrome oxidases for its activity. These cytochromes oxidases act as the terminal electron acceptor in the electron transport chain, transferring electrons from ubiquinone to molecular oxygen. Under aerobic conditions, electrons are transferred from DsbB to ubiquinone to cytochrome oxidases and finally to molecular oxygen (*21*−*23*). Under anaerobic conditions, DsbB is still reoxidized. Reoxidation under anaerobic conditions occurs by electron flow from DsbB to menaquinone to terminal acceptors such as fumerate reductase or nitrate reductase (*21*−*23*).

The DsbC−DsbD Pathway Isomerizes Disulfide Bonds in E. coli. The DsbA−DsbB pathway catalyzes formation of de novo disulfide bonds between two cysteines residues. However, as the number of cysteines residues in a protein increases, the number of possible disulfide pairings grows very rapidly. This raises the question, how does the cell catalyze formation of the correct cysteines pairings? It is hypothesized that DsbA rapidly introduces disulfide bonds into partially unfolded proteins as they are being translocated into the periplasm, suggesting that DsbA may introduce disulfides between sequential cysteines residues, even if these particular disulfide pairings do not exist in the native protein. In the case of a protein with multiple disulfides, the DsbA−DsbB system does not always introduce the correct disulfides (*23*). In an in vitro folding experiment using RNase A, Bader and co-workers demonstrated that, although DsbA can catalyze the complete oxidation of RNase A, this RNase A is catalytically inactive (*23*). Refolded RNase A inactivity is most likely caused by introduction of non-native disulfides by DsbA causing the misfolding of RNase A. When this experiment is done in the presence of glutathione redox buffers, RNase A is catalytically active. Glutathione is a nonphysiologic reoxidant of DsbA that is not present in the *E. coli* periplasm. What allows proteins with multiple disulfides to fold correctly in vivo?

The initial step in answering this question occurred with the discovery of DsbC in 1994. Two groups separately isolated the *dsbC* gene through distinct genetic approaches. Using a screen that had previously yielded the *dsbA* and *dsbB* genes, Missiakas and co-workers identified *dsbC* by screening for *E. coli* mutants that are hypersensitive to the reductant dithiolthreitol (DTT) (*24*). This method yielded mutations in *dsbC*, as well as two other dsb genes, *dsbD* and *dsbG* (DsbD and DsbG will be discussed in more detail later). A second approach screened for *Erwinia chrysanthemi* genes that complemented the *E. coli dsbA* gene when expressed on multicopy plasmids (*25*). This group discovered both the *E. chrysanthemi dsbA* gene and a second gene, *dsbC*, that when overexpressed could complement all the phenotypic changes associated with a *dsbA* null mutation (*25*).

These two early papers immediately identified DsbC as a periplasmic protein with thiol-disulfide oxidoreductase activity both in vivo and in vitro (*24*, *25*). Although DsbC is most similar to DsbG, it is also clearly a member of the thioredoxin family whose members include DsbA, PDI, and thioredoxin. However, the precise role of DsbC was debated: although the possibility that DsbC might, like PDI, function as an isomerase, was immediately recognized, Missiakas and colleagues also proposed that DsbC acts as a second oxidase in the *E. coli* periplasm.

DsbC Can Function as a Disulfide Isomerase in Vitro and in Vivo. DsbC can rearrange incorrectly formed disulfides both in vitro and in vivo. In vitro, catalytic amounts of purified DsbC facilitate the refolding of fully reduced bovine pancreatic trypsin inhibitor (BPTI) and allow formation of native BPTI (*26*). BPTI is an especially good substrate for in vitro folding experiments: it contains 3 nonconsecutive disulfide bonds and its in vitro folding pathway is very well characterized. In the presence of an oxidant (either oxidized glutathione or DsbA), DsbC increases the rate of formation of native BPTI without increasing the rate of disappearance of the fully reduced BPTI (*26*). This occurs because DsbC causes disulfide rearrangements in the stable misfolded intermediates of BPTI, allowing the native disulfide pairings to occur. Interestingly, DsbC cannot catalyze rearrangements of these quasi-native intermediates as well as eukaryotic PDI does: spontaneous rearrangement has a half time of 2 h, in the presence of DsbC this half time drops to 10 min, and in the presence of PDI, to 1 min (*26*).

In vivo studies of DsbC's isomerase activity have relied mainly on expression of eukaryotic proteins with multiple disulfide bonds. In fact, the only known in vivo substrate of DsbC is a periplasmic protein with two disulfides, alkaline phosphatase (*27*). However, the folding yield of native alkaline phosphatase decreases by only 15% in a *dsbC* null strain (*27*). More strikingly, when the eukaryotic protein mouse urokinase (containing 12 disulfide bonds) is expressed in a *dsbC* null strain, its activity shows a 100-fold reduction compared to activity in a wild-type strain(*27*). Furthermore, certain eukaryotic proteins with multiple disulfides rely heavily on the presence of DsbC while others do not. While the yield of human growth hormone (hGH) and the anti CD-18 antibody (anti CD-18) is nearly identical in wild-type and *dsbC*$^-$ strains, melanocyte growth-stimulating activity (MGSA) and interferon-like growth factor-1 (IGF-1) show marked

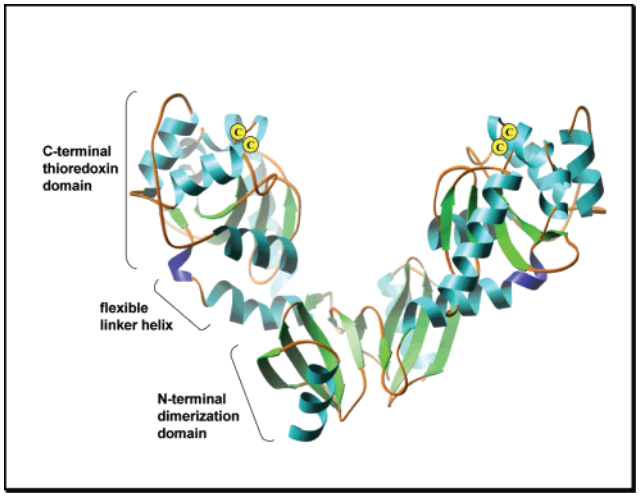

FIGURE 3: The recent crystal structure of DsbC shows that it is a dimer consisting of two DsbC monomers. Each monomer is composed of a C-terminal thioredoxin domain containing the CXXC active site, a flexible linker helix that allows DsbC to adjust to interact with other proteins, and an N-terminal dimerization domain.

reduction in the *dsbC*$^-$ strain (*28*). This difference may be explained by the disulfide pairings in the native structure: while hGH and anti CD-18 both contain disulfides between consecutive cysteine residues, both IGF-a and MGSA require disulfides to form between nonconsecutive cysteines residues.

In addition to its isomerase activity, DsbC has been shown to have chaperone and peptide-binding activity. A chaperone can interact nonspecifically with hydrophobic regions of a peptide to prevent the formation of aggregates by that peptide and assist in its proper folding. Work by Chen and colleagues demonstrated that DsbC is able to assist in the refolding of lysozyme and glyceraldehyde-3-phosphate dehydrogenase (*29*). These activities are believed to be in keeping with DsbC's ability to refold misfolded substrates. To rearrange a misfolded substrate, DsbC needs to be able to bind substrate during the disulfide rearrangement process. In particular, it has been proposed that DsbC must bind substrate until it has tried out various disulfide pairings and reached the most stable, native, conformation (*30*). In support of this theory, it has been shown that DsbC forms a disulfide with a model peptide, residues 4−31 of BPTI, that is 100-fold more stable than the DsbA-BPTI 4−31 mixed disulfide (*31*).

DsbC's Structure Is Consistent with Its Activities. The crystal structure of DsbC has recently been solved to a resolution of 1.9 A (*32*). It shows DsbC to be a 2 × 23 kDa V-shaped homodimer with each monomer forming one arm of the V (*32*). Each DsbC monomer consists of two separate domains—an N-terminal dimerization domain joined by a linker helix to a thioredoxin-like C-terminal domain that contains the CXXC active site (Figure 3). The N-terminal dimerization domains interact by noncovalent bonds between corresponding β-sheets of each monomer to form the dimer interface. The dimerization domains also form an extended hydrophobic cleft large enough (40 × 40 × 25 A) to bind a small peptides. The presence of this cleft may explain DsbC's ability to bind peptide. Limited proteolysis experiments support the importance of DsbC's hydrophobic cleft in its peptide-binding activities: when the dimerization domain (amino acids 1−65) is removed by cleavage with trypsin, DsbC becomes inactive as a chaperone. Furthermore, this monomeric DsbC has little activity as an isomerase in vitro, despite the fact that the remaining thioredoxin-like domain retains both a similar pK_a of the active site thiol and shows only small differences in overall conformation compared to wild-type DsbC (*33*).

In DsbC's wild-type dimeric state, the CXXC active site of each monomer appears to function independently (*26*). The postulated reaction mechanism of DsbC will thus be discussed for one active site in the dimer. DsbC performs a nucleophilic attack of the N-terminal cysteine (Cys98) of the CXXC active site onto the substrate protein, causing formation of a mixed disulfide between DsbC and substrate. Attack of a third substrate thiol group onto the mixed disulfide allows resolution of the DsbC-substrate mixed disulfide and causes rearrangement of substrate disulfides to a more stable conformation. Some support for this mechanism comes from the fact that DsbC is found almost entirely in reduced form in wild-type cells, which is necessary if DsbC must perform the initial nucleophilic attack on substrate protein.

DsbG Is a Second Putative Disulfide Isomerase. A screen for DTT-sensitive mutants that also cause an increase in σ^E-dependent periplasmic heat shock response led to the discovery of a second putative disulfide isomerase, DsbG (*34*). Additionally, DsbG confers resistance to high concentrations of DTT in a *dsbB*$^-$ strain if expressed from a multicopy plasmid (*34*). DsbG was also identified through its sequence similarity to DsbC (*35*). DsbG has 49% homology and 30% identity to DsbC, which immediately suggests that DsbG likely has a similar function to DsbC and is probably a second disulfide isomerase (*35*).

Much less research has been performed on the newly discovered DsbG than on its possible counterpart, DsbC. In fact, there is still some controversy over the particular oxidoreductase function of DsbG in vivo. While initial work suggested that DsbG functions as a disulfide oxidase (*34*), more recent research provides evidence that DsbG is a second disulfide isomerase (*35*). In vitro, DsbG has been shown to have both isomerase and chaperone activity (*35*, *36*). As a chaperone, DsbG was shown to prevent in vitro thermal aggregation of both citrate synthase and luciferase (*36*). DsbG's chaperone activity is independent of its disulfide redox state and is not affected in a cysteine-less DsbG mutant (*36*).

As mentioned above, DsbG was originally discovered by Andersen and co-workers because it conferred resistance to high levels of DTT when overexpressed as well as causing DTT sensitivity when mutated (*34*). These authors also asserted that *dsbG* is an essential gene since a *dsbG* null linked to Tet^R could not be crossed onto the chromosome in the absence of low molecular weight oxidants (*34*). However, these authors also found that a *dsbG* null linked to Kan^R could be crossed onto the chromosome without additional oxidants (*34*). They hypothesized that *dsbG* is essential and that the ability of the *dsbG*::Ω Kan to be transduced onto the chromosome was the result of suppressor mutations.

In contrast to the work of Andersen and colleagues, later work by Georgiou and co-workers suggests that DsbG is most likely a disulfide isomerase (*35*). This research demonstrated that *dsbG* null mutations can be crossed onto the chromosome with normal frequency under a variety of conditions and into a variety of strains (*35*). Furthermore,

the transduction frequency of a Δ*dsbG* allele into a wild-type background was equivalent in strains containing multiple copies of *dsbG* present on plasmid and those lacking plasmid *dsbG* (*35*). These authors also found that DsbG catalyzes the isomerization of certain substrates in vitro. Interestingly, DsbG has varying efficacy in catalyzing disulfide rearrangement of two different eukaryotic substrates, BPTI and urokinase (*35*). Although overexpression of DsbG allowed near wild-type yields of BPTI in a *dsbC*$^-$ strain, overexpression of DsbG produces only about 15% yield of wild-type urokinase in a *dsbC*$^-$ strain. This 15% yield is an improvement over the *dsbC*$^-$ strain in which no active urokinase is produced (*35*).

The in vivo function of DsbG remains unknown—no in vivo substrates of the protein have been discovered—however, the most convincing evidence supports the hypothesis that DsbG, like DsbC, is a disulfide isomerase. First, DsbG is found in reduced form in vivo, consistent with an isomerase rather than oxidase role. Second, DsbG has been shown to complement a *dsbC*$^-$ strain in the refolding of certain eukaryotic proteins. Finally, and perhaps most convincing, DsbG has a high degree of sequence homology to DsbC, suggesting that they have similar in vivo functions. Since no in vivo substrates requiring DsbC or DsbG for proper folding of the majority of substrate have been discovered and since most periplasmic proteins do not contain multiple disulfide bonds, it remains unclear why *E. coli* has two putative disulfide isomerases in the periplasm.

DsbD Reduces DsbC and DsbG. As illustrated above, both DsbC and DsbG require their CXXC active site to be reduced in order to act as isomerases. In vivo data show that both DsbC and DsbG are indeed maintained in reduced form despite the fact that they are located in the strongly oxidizing environment of the bacterial periplasm (*37*). DsbC and DsbG are kept reduced by the action of the inner membrane protein DsbD.

Evidence supporting DsbD's essential role in the isomerization pathway comes from genetic work examining the effects of a *dsbD* null mutant on periplasmic protein folding (*27*). In the *dsbD* null mutant, DsbC and DsbG accumulate in oxidized form (*27*). Like *dsbC*$^-$ strains, *dsbD*$^-$ strains accumulate a misoxidized form of alkaline phosphatase. Furthermore, the effect of the double *dsbC*$^-$ *dsbD*$^-$ mutant on alkaline phosphatase expression is not cumulative, suggesting that DsbC and DsbD are part of the same pathway (*27*).

In order for DsbD to reduce DsbC and DsbG, DsbD itself must be reduced (Figure 2). Like DsbB, DsbD is located in the inner membrane where it could potentially interact with periplasmic, inner membrane, or cytoplasmic components. The same genetic work that illustrated the role of DsbD in transferring reducing equivalents to DsbC and DsbG also resulted in the isolation of mutants in the cytoplasmic thioredoxin system as components of the pathway that allows DsbC and DsbG to remain reduced in the highly oxidizing periplasmic environment (*27*). The current model for maintenance of DsbC and DsbG in reduced form involves passage of electrons from the reducing environment of the cytoplasm to the oxidizing periplasm. In this model, thioredoxin, which is kept in reduced form by thioredoxin reductase and NADPH, passes electrons to DsbD. DsbD's position in the inner membrane allows it to receive electrons from cytoplasmic thioredoxin and transfer them to the periplasmic proteins DsbC and DsbG.

How is DsbD able to transfer electrons from the cytoplasm to the periplasm? DsbD has a molecular mass of 59kDa and is the largest protein in the Dsb family. It is made up of three domains: an N-terminal periplasmic domain with an immunoglobulin-like fold (α-domain), a hydrophobic transmembrane domain consisting of eight transmembrane segments (β-domain), and a C-terminal thioredoxin-like domain, also located in the periplasm (γ-domain) (*38*). Each domain has a pair of conserved cysteine residues that participate in consecutive disulfide exchange reactions in order to transfer electrons from the cytosol to the periplasm. Recent work involving in vivo thiol trapping and an in vitro reconstition of the disulfide isomerization system shows that the transfer of electons occurs sequentially (*39*,*40*). Using an in vitro system composed of purified domains of DsbD, it was demonstrated that electrons are transferred from thioredoxin to DsbD's β-domain and then to the periplasmic γ-domain. DsbD's γ-domain passes electrons to the α-domain, which can then reduce DsbC or DsbG. Additionally, this pathway of electron flow is thermodynamically driven, as illustrated by the increasingly oxidizing redox potentials of the pathway components (*40*). This work confirms the model for electron transport proposed as a result of an elegant series of in vivo thiol trapping experiments (*39*). The DsbD α domain has recently been crystallized in complex with DsbC, demonstrating that the α domain bind the central cleft of the DsbC dimer, which allows the passage of electrons specifically to DsbC (*41*).

Finally, it should be noted that DsbD has a number of in vivo functions aside from its role in maintaining the periplasmic isomerization system. A telling illustration of the diversity of DsbD's roles is the fact that DsbD was discovered by three groups working in very different fields (*42*, *43*). Aside from its role in reducing DsbC and DsbG, DsbD plays an essential role in cytochrome C biosynthesis and is also important in copper resistance (*42*, *43*). Mutants lacking the ability to synthesize all five c-type cytochromes have been mapped to *dsbD* (then called *dipZ*); these mutations can be complemented by the addition of specific thiol-containing compounds to the growth medium (*41*, *44*). It is known that DsbD reduces CcmG; however, the precise mechanism by which heme is attached to c-type apocytochromes by CcmF-H remains unclear (*45*). Some species of bacteria, such as *Bacillus subtilis* and *Rhodobacter capsulatis*, contain CcdA rather than DsbD. CcdA is homologous to DsbD but contains only a domain similar to DsbD's β domain and lacks both of DsbD's periplasmic domains (α and γ) (*46*). Like DsbD, CcdA is required for cytochrome *c* biogenesis; however, unlike DsbD, CcdA seems to be uninvolved in disulfide isomerization (*46*, *47*). A recent investigation into the relationship between DsbD and CcdA suggests that fusion between *ccdA* and other redox-active domains (that became the α and γ domains) allowed DsbD to broaden its substrate specificity to include DsbC and DsbG (*47*).

Structural and Functional Comparison of DsbC and PDI. Protein disulfide isomerase (PDI) is a key catalyst of disulfide bond isomerization in eukaryotes. The importance of PDI is revealed both by its abundance and by the fact that, in some organisms, it is an essential protein. PDI is a highly conserved

protein and is present in a wide range of tissues (*48*). It is found predominantly in the lumen of the endoplasmic reticulum (ER) and can reach near millimolar concentrations in the ER of some tissues (*49*). PDI is essential in *S. cerevesiae*, and some evidence suggests that it is PDI's disulfide isomerase function that is necessary for survival, though this is very much debated (*50*−*52*). PDI has different redox roles under different redox conditions—it has been shown to oxidize, reduce, and isomerize disulfide bonds in vitro as well as to have chaperone activity (*53*). Here we focus on the disulfide isomerase function of PDI as a possible source of information about the prokaryotic disulfide isomerization system.

Eukaryotic PDI is both structurally and functionally more complex than DsbC or DsbG. Like DsbC, PDI is a member of the thioredoxin superfamily because it contains at least one thioredoxin-like domain. While the DsbC monomer contains one thioredoxin fold per monomer, the PDI monomer has a multisubunit structure consisting of 4 thiredoxin-like domains as well as a C-terminal acidic tail (*54*). From N to C terminus of PDI, the order of subunits is a-b-b′-a′, with a linker region between b′ and a′ and a C-terminal extension following the a′ domain.

NMR work on the recombinant a domain has demonstrated that the a domain is indeed a stable domain with a thioredoxin fold (*54*). Since a and a′ are highly homologous to each other as well as to thioredoxin, it is not surprising that preliminary NMR work on a′ indicates that it too contains a thioredoxin fold (*55*). Like thioredoxin, both a and a′ contain a redox active CXXC motif. The b and b′ domains have significant sequence similarity to each other but not to a, a′, or thioredoxin. Although both b and b′ contain a thioredoxin fold neither contains the CXXC active site (*54*). PDI's C-terminal acidic domain, or c domain, contains amino acids 465 to the C terminus. The c domain is postulated to contain a low-affinity, high-capacity Ca^{2+} binding site (*56*). Importantly, the c domain contains a KDEL sequence that localizes soluble PDI to the endoplasmic reticulum (*57*). This domain is entirely absent in DsbC, and DsbC has no known affinity for Ca^{2+} (*32*).

PDI is functionally more complicated than DsbC, as illustrated by its ability to catalyze disulfide oxidation, reduction, and isomerization (*58*). To elucidate the role of each PDI domain in PDI's overall function, redox assays using combinations of these domains have been performed (*57*, *58*). Because the isolated a and a′ domains have a thioredoxin fold and also contain the CXXC active site, it is not surprising that simple oxidation and reduction catalysis requires only the presence of either the a or a′ domain. Isomerizations that do not require large changes in conformation require the b′ domain in combination with either a or a′. Isomerization of complex substrates, particularly those in which substantial conformational rearrangements must occur, requires all four of the thioredoxin-like domains, both the two with CXXC active sites and the two without. For complex isomerizations to occur, the substrate protein must remain in contact with PDI while various disulfide pairings are scanned for their stability. Consistent with this requirement and also with the need for the b′ subunit in complex isomerizations, it has been shown that the b′ domain is the principle peptide binding site of misfolded proteins (*61*). Like PDI, DsbC contains an area suitable for peptide binding—the N terminal dimerization domains of DsbC form a central cleft that has recently been shown to alter in response to binding of another protein (*41*). Because both PDI and DsbC likely bind substrates that are partially unfolded, it is not surprising that both have been shown to have chaperone activity (*29*, *62*).

Finally, a large number of homologues to PDI exist in the ER. In yeast, the essential *PDI1* gene has four nonessential homologues: *MPD1*, *MPD2*, *EUG1*, and *EPS1* (*50*). All contain at least one thioredoxin fold but vary in the number of thioredoxin folds present and the active site sequence. Overexpression of any of these homologues can rescue lethality of a *PDI1* deletion, though rescue often depends on enodogenous levels of other homologues (*50*, *63*). The ability of these nonessential homologues to restore viability to a *PDI1* mutant demonstrates that there is some functional overlap of these genes. If this case is extrapolated to the prokaryotic system, it suggests that both DsbC and DsbG may be prokaryotic disulfide isomerases, perhaps with different substrate specificity.

CONCLUSIONS AND FUTURE DIRECTIONS

In prokaryotes and eukaryotes, disulfide bond isomerization is a catalyzed process; in prokaryotes, periplasmic isomerases DsbC and DsbG are believed to catalyze disulfide rearrangements, while in eukaryotes, PDI and its homologues are responsible for disulfide isomerization. Overexpression of DsbC or PDI has been shown to dramatically improve yields of multidisulfide eukaryotic proteins expressed in *E. coli* (*64*−*66*). Not surprisingly, optimization of eukaryotic protein folding in *E. coli* is an area of intense research, as this will likely have significant biotechnological impact. Disulfide isomerization is often the rate-limiting step in protein folding reactions: understanding isomerase function is of vital importance to optimizing protein folding of many eukaryotic proteins. One essential sources of information about the prokaryotic isomerization system is the in vivo substrates of DsbC and DsbG; however, with the exception of alkaline phosphatase, these remain undiscovered. The in vivo substrates of DsbC and DsbG will provide information about the substrate specificity of these two proteins as well as confirming their in vivo roles as disulfide isomerases.

The presence of two disulfide isomerases in *E. coli* is particularly surprising when one looks at the number of prokaryotic periplasmic or membrane proteins that might need an isomerase. Unlike eukaryotic proteins, prokaryotic proteins rarely contain more than two disulfide bonds. Additionally, these disulfides are generally consecutive, suggesting that they can be correctly formed by DsbA. If one uses the eukaryotic system as a model to understand the prokaryotic system, it seems plausible that DsbG, like some of the newer members of the PDI family, is a second disulfide isomerase with different substrate specificity from DsbC. This is suggested by the ability of DsbG to refold only some of the eukaryotic proteins that DsbC rearranges. However, the analogy between the eukaryotic and prokaryotic systems should only be taken so far: the seemingly small number of prokaryotic proteins that may require a disulfide isomerase reminds us that prokaryotic disulfide isomerization system is a discrete entity from the eukaryotic system and may function quite differently from that of eukaryotes. To

further define the in vivo roles of DsbC and DsbG, their physiologic substrates must be discovered.

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