Pathways of disulfide bond formation in E. coli

2 AUTHORS, INCLUDING:

Joris Messens
Vlaams Instituut voor Biotechnologie, …
76 PUBLICATIONS   **2,275** CITATIONS

Review

Pathways of disulfide bond formation in *Escherichia coli*

Joris Messens [a,b], Jean-François Collet [c,*]

[a] *Laboratorium voor Ultrastructuur, Vrije Universiteit Brussel (VUB), Belgium*
[b] *Department of Molecular and Cellular Interactions, Vlaams interuniversitair Instituut voor Biotechnologie (VIB), Belgium*
[c] *Laboratory of Physiological Chemistry, Christian de Duve Institute of Cellular Pathology (ICP),*
Universite catholique de Louvain (UCL), BCHM-GRM 75-39, Av. Hippocrate, 1200 Brussels, Belgium

Abstract

Disulfide bond formation is required for the correct folding of many secreted proteins. Cells possess protein-folding catalysts to ensure that the correct pairs of cysteine residues are joined during the folding process. These enzymatic systems are located in the endoplasmic reticulum of eukaryotes or in the periplasm of Gram-negative bacteria. This review focuses on the pathways of disulfide bond formation and isomerization in bacteria, taking *Escherichia coli* as a model.
© 2005 Elsevier Ltd. All rights reserved.

Keywords: Disulfide; DsbA; DsbC; DsbD; Periplasm; *Escherichia coli*; Protein folding; Electrons

Contents

* Corresponding author. Tel.: +32 2 764 7562; fax: +32 2 764 7598.
  E-mail address: collet@bchm.ucl.ac.be (J.-F. Collet).

1. Introduction

Proteins face an obstacle race on their way to successful folding in the cell. The first hurdle is the crowding of the cytoplasm. When they emerge from the ribosome, nascent polypeptides expose hydrophobic surfaces and are prone to aggregation. To prevent this fatal process, cells possess a complex machinery of molecular chaperones that helps nascent proteins to fold. Another obstacle awaiting many secreted proteins is the formation of a disulfide bond between two cysteine residues.

Disulfide bonds are vital for the correct folding and tertiary structure stability of many secreted proteins. The formation of a disulfide bond between two cysteine residues stabilizes a protein structure, mainly by decreasing the conformational entropy of the denatured state. The stabilizing effect can be up to approximately 4 kcal/mol per disulfide formed (Clarke & Fersht, 1993; Pantoliano et al., 1987; Shaw & Bott, 1996). Failure to form proper disulfide bonds is likely to cause protein misfolding, leading to aggregation and degradation by proteases.

Disulfide bonds can form spontaneously in the presence of molecular oxygen. However, air oxidation is a rather slow process: in vitro, it can take several hours or even days of incubation to allow the formation of all the native disulfide bonds required for the correct folding of a protein. In contrast, disulfide bond formation in the cell is a much more rapid process and occurs within minutes or even seconds after synthesis. For instance, the refolding of RNAse A, a protein with four disulfide bonds, takes several hours in vitro but less than 2 min in vivo. The discrepancy between these in vivo and in vitro rates led to the discovery of the first catalyst of disulfide bond formation, protein disulfide isomerase (PDI). PDI is a eukaryotic protein present in the endoplasmic reticulum where it is part of a complex network of proteins involved in disulfide bond formation. The eukaryotic pathways of disulfide bond formation will not be discussed here (for a review, see Sevier & Kaiser, 2002). This review will focus on the pathways of disulfide bond formation and isomerization in *E. coli*. The pathways of

disulfide bond formation in other bacteria have recently been reviewed by Kadokura, Katzen, and Beckwith (2003).

2. The chemistry

A thiol–redox reaction between a molecule A containing two reduced cysteine residues and a molecule B containing one disulfide bond can be seen as the transfer of two electrons from A to B or as the transfer of a disulfide bond from B to A. The rate of the reaction depends on the accessibility of the reactive groups, on the difference of the redox potential between the redox partners and on the probability of the sulfur atoms to come within the distance required for thiol/disulfide exchange (Englander & Kallenbach, 1983).

3. The Dsb protein family

In *E. coli*, disulfide bonds are rare within cytoplasmic proteins. This may be because cysteine residues often play vital roles in the catalytic site of proteins and the formation of disulfide bonds where they are not normally found introduces three-dimensional constraints that may lead to protein misfolding and aggregation. To protect the cell against oxidizing reagents that could damage cytoplasmic proteins, *E. coli* possesses several overlapping pathways to keep these proteins reduced, including the thioredoxin–thioredoxin reductase system and the glutaredoxin–glutaredoxin reductase system (for a review, see Carmel-Harel & Storz, 2000). The glutathione buffer in the cytoplasm overall is relatively reducing. The glutathione concentration (about 5 mM) and the GSH/GSSG ratio, which ranges from 50/1 to 200/1, help to prevent disulfide bond formation in proteins (Hwang, Sinskey, & Lodish, 1992; Kosower & Kosower, 1978).

In contrast, proteins secreted into the periplasm are oxidized. The periplasm contains a family of proteins that catalyze disulfide bond formation in newly translocated proteins. These proteins belong to the "Dsb" family (Dsb stands for disulfide bond).
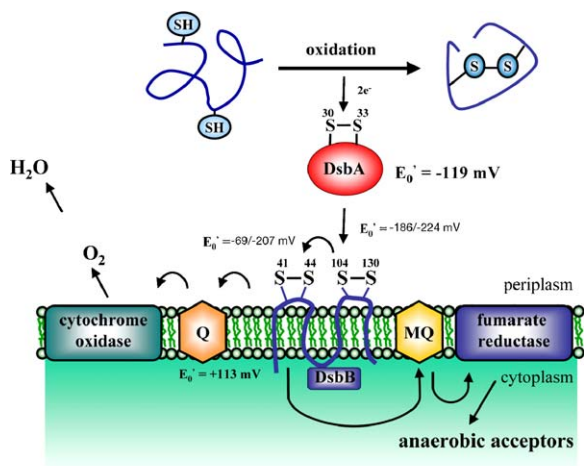
Fig. 1. The pathway of protein disulfide formation. The direction of electron flow is shown by black arrows. DsbA reacts with a newly translocated protein. Free thiols in this protein are oxidized to form a disulfide bond. After donating its disulfide bond to a target protein, DsbA is released in the reduced form. To start a new catalytic oxidation cycle, DsbA must be reoxidized. This reoxidation is accomplished by its partner enzyme DsbB, a quinone reductase. Then, electrons flow from DsbB to ubiquinones and then to terminal oxidases, such as cytochrome bd and bo oxidases. The terminal oxidases transfer the electrons to oxygen in reactions coupled to H^+ transfer and production of H_2O. Under anaerobic conditions, DsbB passes electrons from DsbA onto menaquinone (MQ), which is up-regulated upon oxygen depletion. Anaerobic oxidoreductases such as fumarate reductase serve to reoxidize menaquinone.

Most periplasmic Dsb proteins contain a thioredoxin fold and a catalytic CXXC motif. Depending on the function of the Dsb protein, the CXXC motif is kept in the reduced form (Dsb-$(SH)_2$; with a dithiol) or in the oxidized form (Dsb-S_2; with an intramolecular disulfide bond). The Dsb proteins are involved in two major pathways: an oxidation pathway (DsbA and DsbB) (Fig. 1) and an isomerization pathway (DsbC, DsbG, DsbD) (Fig. 4).

4. The oxidation pathway

4.1. The catalyst of disulfide bond formation: DsbA

Until the early 1990s, disulfide bonds were thought to be formed in the periplasm spontaneously by molecular oxygen. However, the discovery of mutations in the *dsbA* gene revealed that disulfide bond formation is a catalyzed process (Bardwell, McGovern, & Beckwith, 1991; Kamitani, Akiyama, & Ito, 1992). Cells containing null mutations in the *dsbA* gene have a pleiotropic phenotype since the correct folding of many periplasmic proteins is affected. For instance, $dsbA^-$ are unable to fold FlgI, a component of the flagellar motor, which results in a loss of motility (Dailey & Berg, 1993). Moreover, $dsbA^-$ strains exhibit reduced levels of alkaline phosphatase, β-lactamase or the outer-membrane protein OmpA (Bardwell et al., 1991). All of these proteins normally contain disulfide bonds, in their absence the proteins have reduced stability. $DsbA^-$ mutants are also hypersensitive to dithiothreitol, benzylpenicillin and some metal cations like Hg^{2+} and Cd^{2+} (Missiakas, Georgopoulos, & Raina, 1993; Stafford, Humphreys, & Lund, 1999).

4.2. Properties of DsbA

The *dsbA* gene codes for a 21,000 Mr monomeric periplasmic protein containing a characteristic Cys30-Pro31-His32-Cys33 catalytic motif, embedded in a thioredoxin-like fold (Bardwell et al., 1991; Martin, Bardwell, & Kuriyan, 1993) (Fig. 2). In agreement with DsbA acting as a thiol-oxidase, the CXXC motif is found predominantly oxidized in vivo. The disulfide bond present in DsbA is very unstable and can be rapidly transferred to newly translocated proteins. DsbA is one of the strongest thiol oxidant known, with a standard redox potential of -119 mV (Zapun, Bardwell, & Creighton, 1993). The high redox potential of DsbA arises from the unusual low pK_a value of about 3.5 of Cys30 (Nelson & Creighton, 1994), the first cysteine of the CXXC motif. Cys30 is thus entirely in a thiolate anion state under physiological conditions. As explained in Section 4.3, the thiolate anion is stabilized by structural elements. The corollary of the stabilization of the reduced form of DsbA is the destabilization of its oxidized form which is 6.9 kcal/mol less stable than that of thioredoxin ($E'_o = -270$ mV). Thermodynamically, the reaction is therefore driven to the transfer of a disulfide from DsbA to a folding protein.

4.3. The structure of DsbA

The structure of DsbA has been solved in both the oxidized and reduced state and consists of a thioredoxin domain linked by a flexible hinge to an α-helical domain (Fig. 2). The orientation of the α-helical domain relative to the thioredoxin domain depends on the redox state of the CXXC motif.

The thioredoxin-fold of DsbA consists of a five-stranded β-sheet and three α-helices. The α-helical domain consists of four α-helices and is inserted within the thioredoxin-like fold.

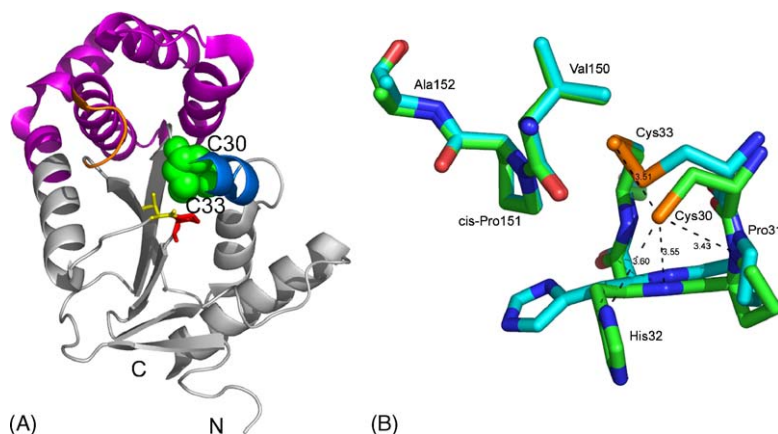The catalytic cysteine residues Cys30 and Cys33 are located at the N-terminus of the first α-helix of the

Fig. 2. Three dimensional structure of DsbA (PDB:1FVK (Martin et al., 1993)). The thioredoxin-domain is in grey and the helical domain in magenta. The N- and C-termini are labeled and the active site cysteines are shown in green spheres. The hinge that connects the two domains is coloured in orange and the *cis*-proline residue (Pro151) is shown in red sticks. Hinge-bending motions occur between the two domains (magenta and grey) and depend on the redox state of the CXXC motif. The oxidized form of DsbA is more flexible than the reduced form, which might facilitate the accommodation of various substrates. (B) Active site of oxidized (cyan, 1A2M (Guddat et al., 1998)) and reduced (green, 1A2L (Guddat et al., 1998)) DsbA in stick presentation. The hydrogen bonds within the characteristic CXXC motif of reduced DsbA that keep the cysteine 30 in thiolate form are indicated. This figure was generated by using PyMol version 0.98.

thioredoxin domain. The structure of reduced DsbA shows that the thiolate form of Cys30 is stabilized by the positive charge from the dipole of the first α-helix of the thioredoxin fold, the interaction with His32 in the Cys30-Pro32-His32-Cys33 active site and by several backbone NH hydrogen bonds of the CPHC motif (Guddat, Bardwell, & Martin, 1998; Schirra et al., 1998).

The CXXC motif is surrounded by a groove with an uncharged surface. A relatively deep hydrophobic pocket within the groove runs alongside the active site and can bind model peptides via hydrophobic interactions (Fig. 2) (Couprie, Vinci, Dugave, Qu, & Moutiez, 2000; Guddat, Bardwell, Zander, & Martin, 1997). On the other side of the active site, there is a conserved proline residue in a *cis* conformation. This *cis*-proline is conserved in most proteins with a thioredoxin fold. Mutation of the *cis*-proline dramatically affects the stability of the protein. Denaturation experiments showed a difference of more than 4.8 kcal/mol in ΔG between a Pro151Ala mutant and the wild type protein (Moutiez, Burova, Haertle, & Quemeneur, 1999). Interestingly, Kadokura, Tian, Zander, Bardwell, and Beckwith (2004) observed that the replacement of Pro151 by a threonine shifted the equilibrium of the oxidation reaction towards the formation of a mixed disulfide between DsbA and its substrates.

4.4. Substrates of DsbA

A bioinformatics search showed that there are more than 300 periplasmic proteins with at least two cysteine residues, suggesting that they are potential substrates for DsbA (Hiniker & Bardwell, 2004). The involvement of DsbA in the folding process of several of these proteins has been reported, suggesting that DsbA has a broad substrate specificity. Using a mutant of DsbA in which the *cis*-proline residue P151 is replaced by a threonine, Kadokura et al. were able to trap several DsbA-substrate complexes. For instance, they showed that DsbA is involved in the oxidative folding of LivK, a leucine-specific binding protein precursor with two cysteines, YodA a metal-binding lipocalin with two cysteines and RcsF, an exopolysaccharide regulator with six cysteines (Kadokura et al., 2004). Using two-dimensional gel electrophoresis to compare $dsbA^-$ and wild-type strains, Hiniker and Bardwell identified other DsbA substrates, such as ribonuclease I (four disulfides) and MepA, a periplasmic murein endopeptidase (three disulfides) (Hiniker & Bardwell, 2004). Finally, Leichert and Jakob (2004) developed a new differential thiol-trapping technique that allowed them to identify a number of additional DsbA substrates.

Interestingly, the oxidized form of DsbA is more flexible than the reduced form (Vinci, Couprie, Pucci, Quemeneur, & Moutiez, 2002). This might facilitate the accommodation of various substrates, while the more rigid nature of the reduced form only has to be recognized by DsbB for reoxidation (see Section 4.5) (Fig. 1). Also, in complex with a 14-mer peptide derived from DsbB, DsbA becomes more rigid as compared to the reduced form (Couprie et al., 2000), which might help the release of oxidized products.

4.5. Maintaining DsbA oxidized: DsbB

After transfer of its disulfide-bond to a target protein, DsbA is released in the reduced form. To start a new catalytic oxidation cycle, DsbA must be reoxidized. This reoxidation is accomplished by its partner enzyme, DsbB (Bardwell et al., 1993). The fact that mutants lacking DsbB accumulate DsbA in the reduced state and exhibit the same phenotypes as *dsbA⁻* mutants was the first indication that both proteins are on the same pathway. This was confirmed in vitro when Bader, Muse, Ballou, Gassner, and Bardwell (1999) purified DsbB to homogeneity and showed that it can catalyze the re-oxidation of DsbA in the presence of molecular oxygen.

DsbB is a 20 kDa inner-membrane protein that is predicted to have four transmembrane helices and two periplasmic loops (Jander, Martin, & Beckwith, 1994). DsbB has four conserved cysteines, arranged in two pairs, that are required for activity. Both pairs are located in the periplasm on two different loops: Cys41 and Cys44 in the N-terminal loop and Cys104 and Cys130 in the C-terminal loop. The structure of DsbB is not known. However, DsbB contains no recognizable similarity to thioredoxin and seems to be the only Dsb protein without a thioredoxin fold. Recently, a number of similarities have been pointed out between DsbB and Ero1 and Erv2, two eukaryotic proteins that facilitate the formation of disulfide bonds (Sevier et al., 2005).

After the reoxidation of DsbA, DsbB needs to pass the electrons it gained from DsbA to an acceptor. Mutants defective in quinone and haem biosynthesis accumulate DsbB in a reduced state, so it was suggested that the electron transport chain recycles DsbB. Bader et al. (1999) confirmed this hypothesis by reconstituting the complete disulfide bond formation system in vitro. Using purified components, they showed that electrons flow from DsbB to ubiquinone then onto cytochromes oxidases *bd* and *bo* and finally to molecular oxygen. Under anaerobic conditions, electrons are transferred from DsbB to menaquinone then to fumarate or nitrate reductase (Fig. 1) (Bader et al., 1999; Takahashi, Inaba, & Ito, 2004).

DsbB is a unique enzyme that catalyzes the formation of a disulfide bond de novo from the reduction of a membrane-bound quinone. DsbB can transfer electrons between quinones and DsbA very efficiently: DsbA is extremely rapidly oxidized ($2.7 \times 10^6 \, M^{-1} \, s^{-1}$) by DsbB, while direct oxidation by ubiquinone bound to a cysteine-free DsbB mutant is 10^3 times slower (Grauschopf, Fritz, & Glockshuber, 2003). According to the current model, the first step is the oxidation of the CXXC motif of DsbA by the 104–130 disulfide of DsbB. Then, electrons flow from Cys104 and Cys130 to Cys41 and Cys44 (Fig. 1). Glockshuber and co-workers determined the redox potential of Cys41-Cys44 and Cys104-Cys130 to be −69 and −186 mV, respectively. The oxidation of the CXXC motif of DsbA ($E_o' = -119$ mV) by the 104–130 disulfide of DsbB is therefore an energetic uphill reaction ($\Delta G = +2.96$ kcal/mol) (Grauschopf et al., 2003), but the high redox potential of Cys41-Cys44, which lies between the redox potential of ubiquinone (+100 mV) and DsbA, provides a thermodynamical driving force allowing the oxidation of DsbA by the Cys104-Cys130 disulfide in DsbB. These results are disputed by a recent paper by Inaba et al. Using quinone-free DsbB prepared from cells unable to synthesize ubiquinone and menaquinone, they found that the redox potential of Cys41-Cys44 (−207 mV) and Cys104-Cys130 (−224 mV) are less oxidizing than DsbA (Inaba, Takahashi, & Ito, 2005). Similar values had been reported previously (Inaba & Ito, 2002; Regeimbal & Bardwell, 2002). The authors suggest that the interaction of DsbB with DsbA may affect the intrinsic redox properties of DsbB to facilitate the electron flow from DsbA to DsbB. Inaba and co-workers explain the discrepancies between their values and those reported by Grauschopf et al. by the fact that Grauschopf et al. prepared ubiquinone-Q8-depleted DsbB by washing the protein with detergents. According to Inaba et al. (but not to Grauschopf et al.), DsbB still contains ubiquinones after this procedure whereas no quinones can be present in a DsbB protein prepared from cells unable to synthesize them. The presence of residual quinones in the DsbB protein used by Grauschopf et al. might explain the very oxidizing redox potential they found for the Cys41-Cys44 cysteine pair.

A model has been proposed for the mechanism of DsbB where a quinhydrone-like complex plays a crucial role as a reaction intermediate (Regeimbal et al., 2003). A quinhydrone is a purple charge-transfer complex consisting of a hydroquinone and a quinone in a stacked conformation. According to this model, the DsbB mechanism would involve two quinone molecules. One of these quinones is very tightly bound to DsbB, whereas the other one is exchangeable. The resident quinone would be directly involved in disulfide bond formation and would undergo oxidation–reduction cycles: it would be reduced during the generation of a disulfide bond and then re-oxidized by an exchangeable quinone from the oxidized quinones pool.

Although the structure of DsbB is not known, residues important for the activity of DsbB have been identified. Residue Arg48 and residues 91–97 in the second periplasmic domain are important for quinone binding

(Kadokura, Bader, Tian, Bardwell, & Beckwith, 2000; Tan, Lu, & Bardwell, 2005; Xie, Yu, Bader, Bardwell, & Yu, 2002). Residues Q33 and Y46 are important for the interaction with DsbA and Leu43 and His91 seem to be important for the catalytic activity of DsbB (Tan et al., 2005).

4.6. Engineering of a new pathway

Recently, in collaboration with others, one of us (JFC) designed a new pathway for the formation of disulfide bonds in the periplasm (Masip et al., 2004). By imposing evolutionary pressure, we selected *E. coli* thioredoxin mutants that are able to catalyze disulfide bond formation in the periplasm independently of the action of DsbA and DsbB.

Thioredoxin is a small monomeric protein with a WCGPC active site sequence motif that reduces disulfide bonds formed in the cytoplasm. Thioredoxin was fused to the leader peptide of *E. coli* trimethyl N-oxide reductase (TorA) for export to the periplasm via the Tat-secretion pathway and the CXXC sequence motif of the catalytic site was randomized by PCR. The resulting library was expressed in a *dsbB⁻ dsbA⁻* strain and we screened for restoration of the bacterial motility (see Section 4.1). Mutants of thioredoxin that were able to restore disulfide bond formation in the periplasm were isolated. The hallmark of these mutants is the presence of a third cysteine in the catalytic site of the protein, replacing the CXXC motif by CX<u>C</u>C. The presence of this additional cysteine has a dramatic effect on thioredoxin, leading the protein to dimerize and to acquire a [2Fe–2S] iron–sulfur cluster. The first and the second cysteine of the CXCC motif are involved in cluster coordination (Collet, Peisach, Bardwell, & Xu, 2005; Masip et al., 2004). The most active of these mutants, TrxA(CACC), was able to catalyze disulfide bond formation both in vivo and in vitro.

Two models were proposed to explain the mechanism of disulfide bond formation by the TrxA(CACC) mutant. In the first one, the iron–sulfur cluster plays a direct role in disulfide bond formation (Fig. 3A). In the second model, the TrxA(CACC) dimer breaks down after export to the periplasm, releasing a redox active metal (iron) and a thiol–disulfide oxidoreductase (Fig. 3B); the released iron would oxidize TrxA(CACC) which in turn would oxidize substrate proteins.

To better understand why the presence of a third cysteine in the catalytic site of thioredoxin causes such a dramatic modification of this protein, the structure of one of these thioredoxin variants, TrxA(CACA), was solved (Collet et al., 2005). TrxA(CACA) was preferred over TrxA(CACC) because its iron–sulfur cluster
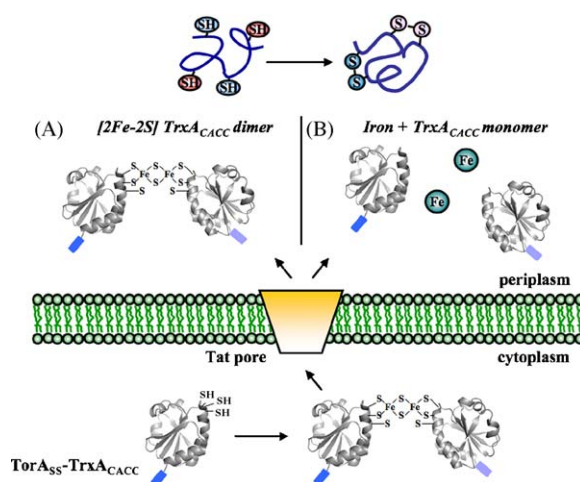

Fig. 3. A new pathway for the formation of protein disulfide bonds. The TrxCACC mutant with an [2Fe–2S] iron–sulfur cluster is exported to the periplasm where it can catalyze disulfide bond formation. In model A, the iron–sulfur cluster plays a direct role in disulfide bond formation. In model B, the TrxA(CACC) dimer breaks down after export to the periplasm: the released iron would oxidize TrxA(CACC) which in turn would oxidize substrate proteins.

is more stable. TrxA(CACA) crystallized as a dimer in which the iron–sulfur cluster is replaced by two inter-molecular disulfide bonds (PDB:1ZCP). The structure shows that both cysteine residues of the CACA motif are solvent-exposed, which contrast with wild-type thioredoxin where the second cysteine of the CXXC motif is buried. In addition, many inter-subunit contacts are observed at the dimer interface, suggesting that thioredoxin might form a weak dimer in vivo. On the basis of these observations, we proposed that the formation of a dimer by TrxA(CACA) brings Cys32 and Cys34 of one subunit in close proximity to Cys32 and Cys34 of a second one, allowing the coordination of an iron–sulfur cluster. In the case of wild-type thioredoxin, only one cysteine residue is exposed in the catalytic site, which would prevent the formation of an iron–sulfur cluster.

5. The isomerization pathway

DsbA is a powerful oxidant and quickly reacts with unfolded proteins as they enter the periplasm. However, DsbA is not a specific oxidant and can introduce non-native disulfide bonds into proteins with more than two cysteines. These incorrect disulfides have to be corrected to prevent protein misfolding and aggregation. To correct non-native disulfides, *E. coli* possesses a "disulfide isomerization system", which involves two protein disulfide isomerases, DsbC and DsbG, and a membrane protein, DsbD.

5.1. Identification of DsbC

The *dsbC* gene was isolated as a multicopy suppressor of a *dsbA* null mutation (Shevchik, Condemine, & Robert-Baudouy, 1994). Mutations in *dsbC* alter the ability of *E. coli* to grow on copper (Hiniker, Collet, & Bardwell, 2005) and affect the folding of proteins with multiple disulfide bonds, like alkaline phosphatase (two disulfides). The *dsbC* promoter is regulated by σ^E, which is induced upon accumulation of denatured proteins within the periplasm (Mecsas, Rouviere, Erickson, Donohue, & Gross, 1993; Missiakas, Betton, & Raina, 1996).

5.2. Properties of DsbC

DsbC is a dimeric protein with two 23.3 kDa subunits. Each subunit contains four conserved cysteine residues. Only two of them (Cys98 and Cys101), arranged in a CXXC motif, are essential for the oxidoreductase activity of the protein. The redox properties of DsbC are reminiscent to those of DsbA. The first cysteine residue of the CXXC motif, Cys98, has a low pK_a (Section 4.1) and is therefore in the thiolate form at neutral pH. The stabilization of the thiolate state of Cys98 by a hydrogen-bonding network within the CXXC motif and by a positively charged residue (Arg125) positioned near the active site contributes to make DsbC a rather oxidizing protein ($E_0' = -130$ mV) (Zapun, Missiakas, Raina, & Creighton, 1995).

Despite these similarities between the redox properties of DsbA and DsbC, DsbC is kept reduced in the periplasm, in contrast with DsbA, which is found oxidized. Keeping DsbC in its reduced form is required to allow it to function as an isomerase. According to current understanding, Cys98 performs a nucleophilic attack on a non-native disulfide. The reaction results in the formation of an unstable mixed disulfide complex between DsbC and the substrate. This mixed disulfide will be resolved either by attack of another cysteine of the misfolded protein, resulting in the formation of a more stable disulfide in the substrate and the release of reduced DsbC, or by the attack of the other cysteine of the CXXC motif, Cys101. In this latter case, DsbC is released in an oxidized state and will need to be reduced by DsbD (Fig. 4) (see below).

5.3. Structure of DsbC

McCarthy and co-workers solved the structure of DsbC. DsbC appears as a V-shaped homo-dimeric molecule (Fig. 5A). Each monomer forms one arm of
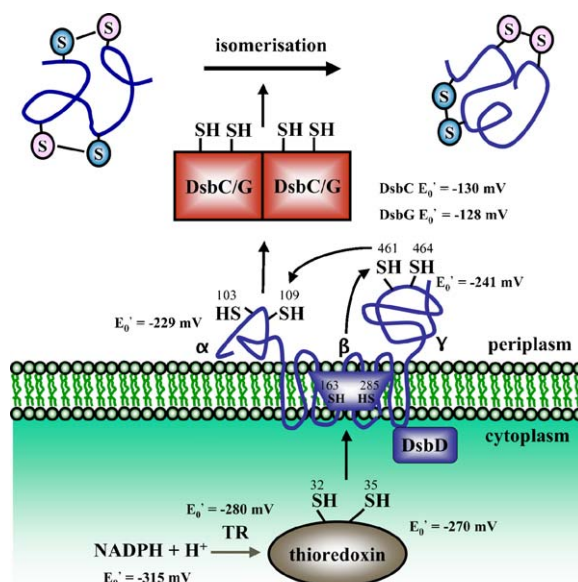

Fig. 4. The isomerization pathway. The direction of electron flow is indicated by the arrows. Disulfide-bond rearrangement is catalyzed by the thiol–disulfide oxidoreductases DsbC and DsbG, which are maintained in a reduced state by the membrane protein DsdD. DsbD is reduced by cytoplasmic thioredoxin, which is recycled by thioredoxin reductase (TR) in a NADPH-dependent manner. In DsbD, the electrons flow from the membranous (β domain) to the C-terminal domain (γ domain) and then to the N-terminal domain (α domain).

the V and consists of two domains: a C-terminal domain with a thioredoxin fold and a N-terminal dimerization domain (McCarthy et al., 2000).

The N-terminal domain (residues 1–61) from each monomer consists of a pair of six-stranded anti-parallel β-sheets each flanked on the concave side by a N-terminal helix. These sheets form the dimerization domain of the molecule. The structure shows that the two subunits are linked by the mutual exchange of two β-strands. The C-terminal domain has a typical thioredoxin fold and contains the Cys98-XX-Cys101 catalytic motif. The Sγ atom of the first cysteine residue of the CXXC motif Cys98 is partially solvent exposed, which is consistent with its role in the formation of mixed disulfide bonds with substrate proteins. The C-terminal domain is linked to the N-terminal domain by a hinged helix (Fig. 5A orange helix), which is sufficiently long to minimize the interactions between the two domains.

The N-terminal dimerization domains form an extended cleft in the DsbC structure. This cleft is predominantly composed of hydrophobic residues that are likely to be involved in peptide binding. Depending on the redox state of the CXXC motif, the cleft switches between an open and a closed conformation (Haebel,
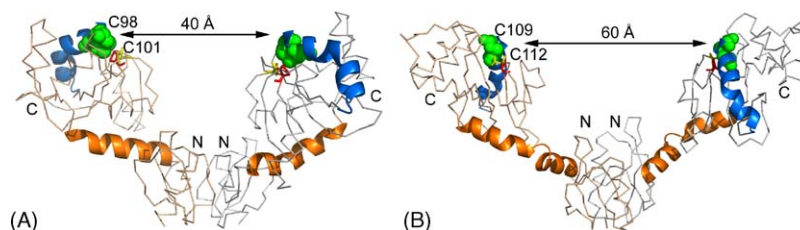
Fig. 5. The three-dimensional structures of DsbC (PDB:1EEJ (McCarthy et al., 2000)) and DsbG (PDB:1V58 (Heras et al., 2004)—the biological dimer was generated by applying crystallographic symmetry). Each DsbC (A) and DsbG (B) monomer (respectively, grey and wheat) consists of a N-terminal dimerization domain (N), a linker helix (orange), and a C-terminal catalytic domain (C) that has a thioredoxin-fold. The active site disulfide is shown as green spheres. In DsbC, *cis*-Pro183 (red sticks) and Thr182 (yellow sticks) and in DsbG, *cis*-Pro201 (red stick) and Thr200 (yellow stick) are shown. These figures were generated by using PyMol version 0.98.

Goldstone, Katzen, Beckwith, & Metcalf, 2002). This flexibility should help DsbC to accommodate a variety of unfolded structures by adapting to the size and the shape of the binding partner.

5.4. Substrates of DsbC

DsbC is able to catalyze the isomerization of non-native disulfide bonds both in vitro and in vivo. In vivo, DsbC can assist the folding of eukaryotic recombinant proteins expressed in *E. coli* such as RNase A, bovine pancreatic trypsin inhibitor (BPTI), and urokinase (Rietsch, Bessette, Georgiou, & Beckwith, 1997; Sun & Wang, 2000; Zapun et al., 1995). So far, only three physiological substrates of DsbC have been identified, RNase I, MepA (Hiniker and Bardwell, 2004) and AppA (Berkmen, Boyd, & Beckwith, 2005). Studies performed on the latter show that DsbC is particularly important for the folding of proteins with non-consecutive disulfide bonds (Berkmen et al., 2005).

In addition to the isomerase activity, DsbC has chaperone activity. In vitro, DsbC can assist the refolding of denatured proteins like lysozyme or glyceraldehyde-3-phosphate dehydrogenase (Chen et al., 1999). The active site residues are not required for this chaperone activity (Liu & Wang, 2001).

5.5. Importance of the dimerization domain

The dimerization of DsbC seems to be crucial for both the isomerization and chaperone activity of DsbC as truncated forms of the protein lacking the N-terminal dimerization domain are inactive as isomerase and chaperone (Sun & Wang, 2000). The involvement of the dimerization domain in the isomerization and chaperone activity of DsbC has been confirmed recently. Fusion of the N-terminal domain of DsbC to other oxidoreductases, like DsbA and thioredoxin, two proteins that do not exhibit isomerization activity, causes dimerization and confers disulfide-bond rearrangement and chaperone activity, although to a lower extent compared to native DsbC (Segatori, Paukstelis, Gilbert, & Georgiou, 2004; Zhao, Peng, Hao, Zeng, & Wang, 2003). The formation of a V-shaped dimer with a hydrophobic cleft by these hybrid proteins is likely to be at the origin of these new properties.

Another important role of the dimerization domain of DsbC is to prevent the oxidation of the protein by DsbB. If DsbB were able to oxidize the active site cysteines of DsbC, the protein would no longer be able to act as a disulfide isomerase. It has been reported that DsbB is unable to efficiently oxidize DsbC and that large kinetic barriers separate DsbC from the oxidation pathway (Bader, Xie, Yu, & Bardwell, 2000; Joly & Swartz, 1997; Rietsch et al., 1997). The dimerization of DsbC seems to be the molecular basis for this discrimination as truncated versions of DsbC lacking the dimerization domain can be oxidized by DsbB both in vivo and in vitro and can substitute for DsbA (Bader et al., 2001). Recent findings by Segatori et al. (2004) suggest that additional structural features are also involved in the kinetic isolation of DsbC from DsbB.

5.6. Properties of DsbG

A second protein disulfide isomerase, DsbG, about four-fold less abundant than DsbC, is present in the periplasm. The *dsbG* gene was identified as a suppressor of DTT sensitivity of mutants lacking DsbB (Andersen, Matthey-Dupraz, Missiakas, & Raina, 1997). DsbG is a 25.7 kDa protein that forms a dimer and shares 24% amino acid identity with DsbC. DsbG has two conserved cysteine residues, Cys109 and Cys112, which are present in a CXXC motif (Bessette, Cotto, Gilbert, & Georgiou, 1999) (Fig. 5B). DsbG has a redox potential similar to this of DsbC (values range between −126 mV (Bessette

et al., 1999) and −129 mV (van Straaten, Missiakas, Raina, & Darby, 1998)).

5.7. Substrates of DsbG

Like DsbC, DsbG is able to correct non-native disulfide bonds in eukaryotic proteins, both in vitro and in vivo. However, in contrast with DsbC, DsbG does not catalyze the oxidative refolding of RNAse A in vitro (Bessette et al., 1999) and is not critical for the in vivo activity of RNAse I (Hiniker & Bardwell, 2004). The in vivo substrates of DsbG are still unknown and it seems likely that DsbG has a different and perhaps more limited substrate specificity than DsbC. Like DsbC, DsbG has some chaperone activity and can assist the folding of luciferase and citrate synthase (Shao, Bader, Jakob, & Bardwell, 2000).

5.8. Structure of DsbG

The structure of DsbG has been solved recently. Interestingly, the structure reveals a mixture of reduced and oxidized forms, which suggests that DsbG's disulfide is unstable (Heras, Edeling, Schirra, Raina, & Martin, 2004). The protein appears as a V-shaped protein with two domains. The N-terminal dimerization domain has a cystatin-like fold, a fold found in cysteine protease inhibitors (Bode et al., 1988). A helical linker (orange helix in Fig. 5B) connects the N-terminal domain to the thioredoxin domain. The CXXC catalytic motif is located at the N-terminus of the first helix (blue helix in Fig. 5B) of the thioredoxin fold.

The overall structure of DsbG resembles DsbC but there are a few significant differences. First, DsbG's dimensions are larger and the linker helix connecting the two domains (orange helix in Fig. 5) is 2.5 turns longer. This alters the position of the catalytic domain relative to the dimerization domain (Heras et al., 2004). Second, the V-shaped cleft is lined with several acidic residues that form negatively charged patches. A third difference is a charged groove formed by residues in a loop connecting two β strands of the N-terminal domain. This loop is longer in DsbG and the extra residues are charged. The insertion is conserved in DsbG homologues, which indicates that it is important for the function of the protein.

These structural differences may provide some clues about DsbG substrates. The size and surface charge of DsbG suggest that its substrates are larger and have less hydrophobic surfaces than the substrates of DsbC, which might indicate that DsbG preferentially catalyze disulfide bond isomerization in folded or partially folded proteins.

Interestingly, there is a conserved threonine residue immediately preceding the *cis*-proline (Fig. 5). This residue is also conserved in DsbC, but not in DsbA and thioredoxin. It has been suggested that this residue favors the reduced form of DsbC and DsbG by interacting with the first cysteine of the CXXC motif.

5.9. Keeping DsbC and DsbG reduced: DsbD

To be active as protein disulfide isomerases, DsbC and DsbG have to be kept reduced in the oxidizing environment of the periplasm. The protein that keeps DsbC and DsbG reduced is the inner-membrane protein called DsbD. Mutants lacking DsbD are hypersensitive to DTT (Missiakas, Schwager, & Raina, 1995), are unable to synthesize mature c-type cytochromes and cannot grow on copper (Fong, Camakaris, & Lee, 1995). The fact that a *dsbD* null mutation leads to the accumulation of DsbC and DsbG in an oxidized state indicates that DsbD is required to keep DsbC and DsbG reduced.

DsbD consists of 546 amino acids and has three different domains: N-terminal domain (α), a transmembrane domain (β) and a C-terminal domain (γ) (Fig. 4). Two of these domains (α and γ) are in the periplasm, whereas the third one, β, has eight transmembrane segments. Each domain contains one pair of invariant cysteine residues that are essential for activity (Stewart, Katzen, & Beckwith, 1999).

The function of DsbD is to give electrons to the periplasmic protein disulfide isomerases. In vivo and in vitro studies have shown that DsbD itself receives electrons from cytoplasmic thioredoxin. Thioredoxin is kept reduced in the cytoplasm by thioredoxin reductase which uses NADPH. DsbC/G are thus kept reduced by DsbD at the expense of NADPH oxidation in the cytoplasm (Rietsch et al., 1997). The electrons coming from the cytoplasm are also distributed by DsbD to CcmG (also called DsbE), a membrane-tethered thioredoxin-like protein essential for cytochrome c maturation (Stirnimann et al., 2005; Thony-Meyer, 2002). The reactions between DsbD and its periplasmic substrates are fast whereas a large kinetic barrier prevents DsbD to be oxidized by DsbA (Rozhkova et al., 2004).

The mechanism by which DsbD transfers electrons across the membrane is still unclear. In vivo and in vitro experiments suggest that electrons are transferred via a succession of disulfide bond exchange reactions between the three domains.

According to the current model, oxidizing equivalents are successively transferred from oxidized DsbC
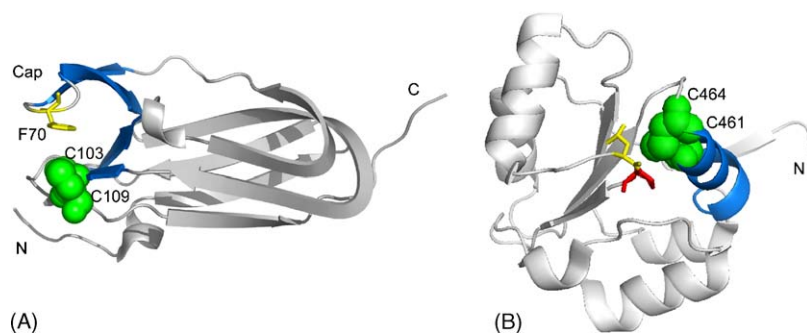
Fig. 6. (A) The structure of the α domain of the transmembrane electron transporter DsbD (PDB:1JZD—chain C (Haebel et al., 2002)). The catalytic subdomain (marine blue) containing the active site is inserted at the antigen-binding site of the immunoglobulin fold. The active site cysteines Cys103 and Cys109, represented by the green spheres, form a disulfide bond. The active site is shielded by a cap formed by two β strands connected by a loop containing Phe70 (yellow sticks). (B) The structure of the γ domain of the transmembrane electron transporter DsbD (PDB:1UC7—chain A (Kim et al., 2003)). The active site cysteines are oxidized and drawn as green spheres. These figures were generated by using PyMol version 0.98.

or DsbG to Cys103 and Cys109 of the α domain ($E'_o = -229\,mV$), then to Cys461 and Cys464 of the γ domain ($E'_o = -241\,mV$) via a mixed disulfide between Cys461 and Cys109 (Rozhkova et al., 2004), then to Cys163 and Cys285 in the transmembrane β domain and finally to thioredoxin in the cytoplasm (Fig. 4). The reactions taking place in the cytoplasm and in the periplasm have been well characterized (Collet, Riemer, Bader, & Bardwell, 2002; Katzen & Beckwith, 2000; Rozhkova et al., 2004), but we still do not know how the cysteine residues of the β domain can interact successively with thioredoxin on one side of the membrane and the γ domain on the other.

5.10. Structure of DsbD

So far, only the structures of both periplasmic domains have been solved. The γ domain of DsbD (Kim, Kim, Jeong, Son, & Ryu, 2003) has a typical thioredoxin fold containing a CXXC motif and an extended N-terminal stretch (Fig. 6B). Crystallized α domain DsbD assumes an immunoglobulin-like fold that encompasses two active site cysteines, C103 and C109, forming a disulfide bond between two beta-strands (Fig. 6A) (Goulding et al., 2002). This disulfide is shielded from the environment and capped by a phenylalanine (Phe70).

Recently, the structure of a trapped reaction intermediate between the α and γ domains of DsbD has been solved (Rozhkova et al., 2004), as well as the structures of mixed-disulfide complexes between the α domain and either DsbC (Haebel et al., 2002) or CcmG (Stirnimann et al., 2005). Structural comparisons show that a few residues form contacts that are specific to the protein partners. However, the majority of the residues of the

α domain involved in the binding of either the γ domain, DsbC, or CcmG are common (Stirnimann et al., 2005). The α domain must therefore be able to undergo large displacements to interact successively with the γ domain and the periplasmic oxido-reductases.

6. Conclusions and future perspectives

The discovery of DsbA, the first protein folding catalyst identified in prokaryotes, paved the way to the unravelling of most of the steps involved in disulfide bond formation and isomerization in the *E. coli* periplasm. In less than 15 years, five other Dsb proteins have been discovered and characterized. The kinetic parameters of most of the interactions have been determined and the structures of all the soluble components of the pathways have been reported.

However, some very interesting problems remain unsolved. Only two substrates have been found for DsbC and we do not know whether this protein interact with other substrates. The function of DsbG is still unclear, as no in vivo substrates have been found for this protein. More work is also required to better understand how the two membrane proteins of the Dsb family, DsbB and DsbD, perform their reactions. This is particularly true for DsbD as the mechanism used by this protein to transport electrons across the membrane is almost entirely unclear. Does DsbD use a cofactor to shuttle electrons across the membrane? Are there major conformational changes taking place upon oxidation/reduction of the catalytic cysteines of the β domain? A better biochemical characterization of both DsbB and DsbD is required, but the major breakthrough is likely to come from the determination of the three-dimensional structure of these proteins.

Acknowledgements

We would like to thank Jim Bardwell, Emile Van Schaftingen and Lode Wyns for helpful criticism of this manuscript. JFC is Chercheur Qualifié of the FNRS. JM is a project leader of the VIB.

References

Andersen, C. L., Matthey-Dupraz, A., Missiakas, D., & Raina, S. (1997). A new *Escherichia coli* gene, *dsbG*, encodes a periplasmic protein involved in disulphide bond formation, required for recycling DsbA/DsbB and DsbC redox proteins. *Mol. Microbiol.*, *26*, 121–132.

Bader, M., Muse, W., Ballou, D. P., Gassner, C., & Bardwell, J. C. (1999). Oxidative protein folding is driven by the electron transport system. *Cell*, *98*, 217–227.

Bader, M. W., Hiniker, A., Regeimbal, J., Goldstone, D., Haebel, P. W., Riemer, J., et al. (2001). Turning a disulfide isomerase into an oxidase: DsbC mutants that imitate DsbA. *Embo J.*, *20*, 1555–1562.

Bader, M. W., Xie, T., Yu, C. A., & Bardwell, J. C. (2000). Disulfide bonds are generated by quinone reduction. *J. Biol. Chem.*, *275*, 26082–26088.

Bardwell, J. C., Lee, J. O., Jander, G., Martin, N., Belin, D., & Beckwith, J. (1993). A pathway for disulfide bond formation in vivo. *Proc. Natl. Acad. Sci. U.S.A.*, *90*, 1038–1042.

Bardwell, J. C., McGovern, K., & Beckwith, J. (1991). Identification of a protein required for disulfide bond formation in vivo. *Cell*, *67*, 581–589.

Berkmen, M., Boyd, D., & Beckwith, J. (2005). The nonconsecutive disulfide bond of *Escherichia coli* phytase (AppA) renders it dependent on the protein-disulfide isomerase DsbC. *J. Biol. Chem.*, *280*, 11387–11394.

Bessette, P. H., Cotto, J. J., Gilbert, H. F., & Georgiou, G. (1999). In vivo and in vitro function of the *Escherichia coli* periplasmic cysteine oxidoreductase DsbG. *J. Biol. Chem.*, *274*, 7784–7792.

Bode, W., Engh, R., Musil, D., Thiele, U., Huber, R., Karshikov, A., et al. (1988). The 2.0 Å X-ray crystal structure of chicken egg white cystatin and its possible mode of interaction with cysteine proteinases. *Embo J.*, *7*, 2593–2599.

Carmel-Harel, O., & Storz, G. (2000). Roles of the glutathione- and thioredoxin-dependent reduction systems in the *Escherichia coli* and *Saccharomyces cerevisiae* responses to oxidative stress. *Annu. Rev. Microbiol.*, *54*, 439–461.

Chen, J., Song, J. L., Zhang, S., Wang, Y., Cui, D. F., & Wang, C. C. (1999). Chaperone activity of DsbC. *J. Biol. Chem.*, *274*, 19601–19605.

Clarke, J., & Fersht, A. R. (1993). Engineered disulfide bonds as probes of the folding pathway of barnase: Increasing the stability of proteins against the rate of denaturation. *Biochemistry*, *32*, 4322–4329.

Collet, J. F., Peisach, D., Bardwell, J. C., & Xu, Z. (2005). The crystal structure of TrxA(CACA): Insights into the formation of a [2Fe–2S] iron–sulfur cluster in an *Escherichia coli* thioredoxin mutant. *Protein Sci.*, *14*, 1863–1869.

Collet, J. F., Riemer, J., Bader, M. W., & Bardwell, J. C. (2002). Reconstitution of a disulfide isomerization system. *J. Biol. Chem.*, *277*, 26886–26892.

Couprie, J., Vinci, F., Dugave, C., Qu, E., & Moutiez, M. (2000). Investigation of the DsbA mechanism through the synthesis and analysis of an irreversible enzyme–ligand complex. *Biochemistry*, *39*, 6732–6742.

Dailey, F. E., & Berg, H. C. (1993). Mutants in disulfide bond formation that disrupt flagellar assembly in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.*, *90*, 1043–1047.

Englander, S. W., & Kallenbach, N. R. (1983). Hydrogen exchange and structural dynamics of proteins and nucleic acids. *Quart. Rev. Biophys.*, *16*, 521–655.

Fong, S. T., Camakaris, J., & Lee, B. T. (1995). Molecular genetics of a chromosomal locus involved in copper tolerance in *Escherichia coli* K-12. *Mol. Microbiol.*, *15*, 1127–1137.

Goulding, C. W., Sawaya, M. R., Parseghian, A., Lim, V., Eisenberg, D., & Missiakas, D. (2002). Thiol–disulfide exchange in an immunoglobulin-like fold: Structure of the N-terminal domain of DsbD. *Biochemistry*, *41*, 6920–6927.

Grauschopf, U., Fritz, A., & Glockshuber, R. (2003). Mechanism of the electron transfer catalyst DsbB from *Escherichia coli*. *Embo J.*, *22*, 3503–3513.

Guddat, L. W., Bardwell, J. C., & Martin, J. L. (1998). Crystal structures of reduced and oxidized DsbA: Investigation of domain motion and thiolate stabilization. *Structure*, *6*, 757–767.

Guddat, L. W., Bardwell, J. C., Zander, T., & Martin, J. L. (1997). The uncharged surface features surrounding the active site of *Escherichia coli* DsbA are conserved and are implicated in peptide binding. *Protein Sci.*, *6*, 1148–1156.

Haebel, P. W., Goldstone, D., Katzen, F., Beckwith, J., & Metcalf, P. (2002). The disulfide bond isomerase DsbC is activated by an immunoglobulin-fold thiol oxidoreductase: Crystal structure of the DsbC–DsbDalpha complex. *Embo J.*, *21*, 4774–4784.

Heras, B., Edeling, M. A., Schirra, H. J., Raina, S., & Martin, J. L. (2004). Crystal structures of the DsbG disulfide isomerase reveal an unstable disulfide. *Proc. Natl. Acad. Sci. U.S.A.*, *101*, 8876–8881.

Hiniker, A., & Bardwell, J. C. (2004). In vivo substrate specificity of periplasmic disulfide oxidoreductases. *J. Biol. Chem.*, *279*, 12967–12973.

Hiniker, A., Collet, J. F., & Bardwell, J. C. (2005). Copper stress causes an in vivo requirement for the *Escherichia coli* disulfide isomerase DsbC. *J. Biol. Chem.*, *280*, 33785–33791.

Hwang, C., Sinskey, A. J., & Lodish, H. F. (1992). Oxidized redox state of glutathione in the endoplasmic reticulum. *Science*, *257*, 1496–1502.

Inaba, K., & Ito, K. (2002). Paradoxical redox properties of DsbB and DsbA in the protein disulfide-introducing reaction cascade. *Embo J.*, *21*, 2646–2654.

Inaba, K., Takahashi, Y. H., & Ito, K. (2005). Reactivities of quinone-free DsbB from *Escherichia coli*. *J. Biol. Chem.*, *280*, 33035–33044.

Jander, G., Martin, N. L., & Beckwith, J. (1994). Two cysteines in each periplasmic domain of the membrane protein DsbB are required for its function in protein disulfide bond formation. *Embo J.*, *13*, 5121–5127.

Joly, J. C., & Swartz, J. R. (1997). In vitro and in vivo redox states of the *Escherichia coli* periplasmic oxidoreductases DsbA and DsbC. *Biochemistry*, *36*, 10067–10072.

Kadokura, H., Bader, M., Tian, H., Bardwell, J. C., & Beckwith, J. (2000). Roles of a conserved arginine residue of DsbB in linking protein disulfide-bond-formation pathway to the respiratory chain of *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.*, *97*, 10884–10889.

Kadokura, H., Katzen, F., & Beckwith, J. (2003). Protein disulfide bond formation in prokaryotes. *Annu. Rev. Biochem.*, *72*, 111–135.

Kadokura, H., Tian, H., Zander, T., Bardwell, J. C., & Beckwith, J. (2004). Snapshots of DsbA in action: Detection of proteins in the process of oxidative folding. *Science*, *303*, 534–537.

Kamitani, S., Akiyama, Y., & Ito, K. (1992). Identification and characterization of an *Escherichia coli* gene required for the formation of correctly folded alkaline phosphatase, a periplasmic enzyme. *Embo J.*, *11*, 57–62.

Katzen, F., & Beckwith, J. (2000). Transmembrane electron transfer by the membrane protein DsbD occurs via a disulfide bond cascade. *Cell*, *103*, 769–779.

Kim, J. H., Kim, S. J., Jeong, D. G., Son, J. H., & Ryu, S. E. (2003). Crystal structure of DsbDgamma reveals the mechanism of redox potential shift and substrate specificity(1). *FEBS Lett.*, *543*, 164–169.

Kosower, N. S., & Kosower, E. M. (1978). The glutathione status of cells. *Int. Rev. Cytol.*, *54*, 109–160.

Leichert, L. I., & Jakob, U. (2004). Protein thiol modifications visualized in vivo. *PLoS Biol.*, *2*, e333.

Liu, X., & Wang, C. C. (2001). Disulfide-dependent folding and export of *Escherichia coli* DsbC. *J. Biol. Chem.*, *276*, 1146–1151.

Martin, J. L., Bardwell, J. C., & Kuriyan, J. (1993). Crystal structure of the DsbA protein required for disulphide bond formation in vivo. *Nature*, *365*, 464–468.

Masip, L., Pan, J. L., Haldar, S., Penner-Hahn, J. E., DeLisa, M. P., Georgiou, G., et al. (2004). An engineered pathway for the formation of protein disulfide bonds. *Science*, *303*, 1185–1189.

McCarthy, A. A., Haebel, P. W., Torronen, A., Rybin, V., Baker, E. N., & Metcalf, P. (2000). Crystal structure of the protein disulfide bond isomerase, DsbC, from *Escherichia coli*. *Nat. Struct. Biol.*, *7*, 196–199.

Mecsas, J., Rouviere, P. E., Erickson, J. W., Donohue, T. J., & Gross, C. A. (1993). The activity of sigma E, an *Escherichia coli* heat-inducible sigma-factor, is modulated by expression of outer membrane proteins. *Genes Dev.*, *7*, 2618–2628.

Missiakas, D., Betton, J. M., & Raina, S. (1996). New components of protein folding in extracytoplasmic compartments of *Escherichia coli* SurA FkpA and Skp/OmpH. *Mol. Microbiol.*, *21*, 871–884.

Missiakas, D., Georgopoulos, C., & Raina, S. (1993). Identification and characterization of the *Escherichia coli* gene dsbB, whose product is involved in the formation of disulfide bonds in vivo. *Proc. Natl. Acad. Sci. U.S.A.*, *90*, 7084–7088.

Missiakas, D., Schwager, F., & Raina, S. (1995). Identification and characterization of a new disulfide isomerase-like protein (DsbD) in *Escherichia coli*. *Embo J.*, *14*, 3415–3424.

Moutiez, M., Burova, T. V., Haertle, T., & Quemeneur, E. (1999). On the non-respect of the thermodynamic cycle by DsbA variants. *Protein Sci.*, *8*, 106–112.

Nelson, J. W., & Creighton, T. E. (1994). Reactivity and ionization of the active site cysteine residues of DsbA, a protein required for disulfide bond formation in vivo. *Biochemistry*, *33*, 5974–5983.

Pantoliano, M. W., Ladner, R. C., Bryan, P. N., Rollence, M. L., Wood, J. F., & Poulos, T. L. (1987). Protein engineering of subtilisin BPN′: Enhanced stabilization through the introduction of two cysteines to form a disulfide bond. *Biochemistry*, *26*, 2077–2082.

Regeimbal, J., & Bardwell, J. C. (2002). DsbB catalyzes disulfide bond formation de novo. *J. Biol. Chem.*, *277*, 32706–32713.

Regeimbal, J., Gleiter, S., Trumpower, B. L., Yu, C. A., Diwakar, M., Ballou, D. P., et al. (2003). Disulfide bond formation involves a quinhydrone-type charge-transfer complex. *Proc. Natl. Acad. Sci. U.S.A.*, *100*, 13779–13784.

Rietsch, A., Bessette, P., Georgiou, G., & Beckwith, J. (1997). Reduction of the periplasmic disulfide bond isomerase, DsbC, occurs by passage of electrons from cytoplasmic thioredoxin. *J. Bacteriol.*, *179*, 6602–6608.

Rozhkova, A., Stirnimann, C. U., Frei, P., Grauschopf, U., Brunisholz, R., Grutter, M. G., et al. (2004). Structural basis and kinetics of inter- and intramolecular disulfide exchange in the redox catalyst DsbD. *Embo J.*, *23*, 1709–1719.

Schirra, H. J., Renner, C., Czisch, M., Huber-Wunderlich, M., Holak, T. A., & Glockshuber, R. (1998). Structure of reduced DsbA from *Escherichia coli* in solution. *Biochemistry*, *37*, 6263–6276.

Segatori, L., Paukstelis, P. J., Gilbert, H. F., & Georgiou, G. (2004). Engineered DsbC chimeras catalyze both protein oxidation and disulfide-bond isomerization in *Escherichia coli*: Reconciling two competing pathways. *Proc. Natl. Acad. Sci. U.S.A.*, *101*, 10018–10023.

Sevier, C. S., Kadokura, H., Tam, V. C., Beckwith, J., Fass, D., & Kaiser, C. A. (2005). The prokaryotic enzyme DsbB may share key structural features with eukaryotic disulfide bond forming oxidoreductases. *Protein Sci.*, *14*, 1630–1642.

Sevier, C. S., & Kaiser, C. A. (2002). Formation and transfer of disulphide bonds in living cells. *Nat. Rev. Mol. Cell Biol.*, *3*, 836–847.

Shao, F., Bader, M. W., Jakob, U., & Bardwell, J. C. (2000). DsbG, a protein disulfide isomerase with chaperone activity. *J. Biol. Chem.*, *275*, 13349–13352.

Shaw, A., & Bott, R. (1996). Engineering enzymes for stability. *Curr. Opin. Struct. Biol.*, *6*, 546–550.

Shevchik, V. E., Condemine, G., & Robert-Baudouy, J. (1994). Characterization of DsbC, a periplasmic protein of *Erwinia chrysanthemi* and *Escherichia coli* with disulfide isomerase activity. *Embo J.*, *13*, 2007–2012.

Stafford, S. J., Humphreys, D. P., & Lund, P. A. (1999). Mutations in *dsbA* and *dsbB*, but not *dsbC*, lead to an enhanced sensitivity of *Escherichia coli* to Hg2+ and Cd2+. *FEMS Microbiol. Lett.*, *174*, 179–184.

Stewart, E. J., Katzen, F., & Beckwith, J. (1999). Six conserved cysteines of the membrane protein DsbD are required for the transfer of electrons from the cytoplasm to the periplasm of *Escherichia coli*. *Embo J.*, *18*, 5963–5971.

Stirnimann, C. U., Rozhkova, A., Grauschopf, U., Grutter, M. G., Glockshuber, R., & Capitani, G. (2005). Structural basis and kinetics of DsbD-dependent cytochrome c maturation. *Structure (Cambridge)*, *13*, 985–993.

Sun, X. X., & Wang, C. C. (2000). The N-terminal sequence (residues 1–65) is essential for dimerization, activities, and peptide binding of *Escherichia coli* DsbC. *J. Biol. Chem.*, *275*, 22743–22749.

Takahashi, Y. H., Inaba, K., & Ito, K. (2004). Characterization of the menaquinone-dependent disulfide bond formation pathway of *Escherichia coli*. *J. Biol. Chem.*, *279*, 47057–47065.

Tan, J., Lu, Y., & Bardwell, J. C. (2005). Mutational analysis of the disulfide catalysts DsbA and DsbB. *J. Bacteriol.*, *187*, 1504–1510.

Thony-Meyer, L. (2002). Cytochrome c maturation: A complex pathway for a simple task? *Biochem. Soc. Trans.*, *30*, 633–638.

van Straaten, M., Missiakas, D., Raina, S., & Darby, N. J. (1998). The functional properties of DsbG, a thiol–disulfide oxidoreductase from the periplasm of *Escherichia coli*. *FEBS Lett.*, *428*, 255–258.

Vinci, F., Couprie, J., Pucci, P., Quemeneur, E., & Moutiez, M. (2002). Description of the topographical changes associated to the different stages of the DsbA catalytic cycle. *Protein Sci.*, *11*, 1600–1612.

Xie, T., Yu, L., Bader, M. W., Bardwell, J. C., & Yu, C. A. (2002). Identification of the ubiquinone-binding domain in the disulfide catalyst disulfide bond protein B. *J. Biol. Chem.*, *277*, 1649–1652.

Zapun, A., Bardwell, J. C., & Creighton, T. E. (1993). The reactive and destabilizing disulfide bond of DsbA, a protein required for protein disulfide bond formation in vivo. *Biochemistry*, *32*, 5083–5092.

Zapun, A., Missiakas, D., Raina, S., & Creighton, T. E. (1995). Structural and functional characterization of DsbC, a protein involved in disulfide bond formation in *Escherichia coli*. *Biochemistry*, *34*, 5075–5089.

Zhao, Z., Peng, Y., Hao, S. F., Zeng, Z. H., & Wang, C. C. (2003). Dimerization by domain hybridization bestows chaperone and isomerase activities. *J. Biol. Chem.*, *278*, 43292–43298.