Molecular Microbiology (2002) **44**(1), 1–8

MicroReview

Oxidative protein folding in bacteria

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Summary

Ten years ago it was thought that disulphide bond formation in prokaryotes occurred spontaneously. Now two pathways involved in disulphide bond formation have been well characterized, the oxidative pathway, which is responsible for the formation of disulphides, and the isomerization pathway, which shuffles incorrectly formed disulphides. Disulphide bonds are donated directly to unfolded polypeptides by the DsbA protein; DsbA is reoxidized by DsbB. DsbB generates disulphides *de novo* from oxidized quinones. These quinones are reoxidized by the electron transport chain, showing that disulphide bond formation is actually driven by electron transport. Disulphide isomerization requires that incorrect disulphides be attacked using a reduced catalyst, followed by the redonation of the disulphide, allowing alternative disulphide pairing. Two isomerases exist in *Escherichia coli*, DsbC and DsbG. The membrane protein DsbD maintains these disulphide isomerases in their reduced and thereby active form. DsbD is kept reduced by cytosolic thioredoxin in an NADPH-dependent reaction.

Introduction

Proteins are synthesized on ribosomes as linear chains of amino acids. In order to be biologically active, they must fold into a unique three-dimensional structure. On the way to their active conformation, proteins are assisted by molecular chaperones that protect them from associating prematurely and forming insoluble aggregates. In addition, folding catalysts act to accelerate the rate-limiting steps in the folding pathway. One of these rate-limiting steps is the formation of correct disulphide bonds between cysteine residues in proteins.

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In eukaryotes, disulphide bond formation and isomerization is catalysed in the endoplasmic reticulum by protein disulphide isomerase (PDI) and related proteins. Although this eukaryotic system has been intensely studied for almost 40 years, many questions remain unanswered.

In 1991, it was discovered that disulphide bond formation in the prokaryotic periplasm is also a catalysed process (Bardwell *et al*., 1991). The availability of genetic tools in prokaryotes and the ease of handling the proteins involved has allowed rapid progress in understanding of the process of disulphide bond formation in the bacterial periplasm.

The oxidation pathway: from the discovery of DsbA to the reconstitution of the complete system

The first step: the discovery of DsbA

DsbA (for disulphide bond) was the first protein shown to be involved in disulphide bond formation in the bacterial periplasm. DsbA was simultaneously identified using two different genetic approaches. One approach involved the use of an artificially constructed disulphide indicator fusion protein (Bardwell *et al*., 1991). This protein had a portion of the inner membrane protein MalF fused to β-galactosidase. The MalF portion of this fusion directs the β-galactosidase to the inner membrane. In wild-type strains expressing this fusion protein, β-galactosidase is inactive because disulphide bonds are introduced in the portions of β-galactosidase protruding into the periplasm. When *dsbA* is mutated, disulphide bonds are not introduced and this restores β-galactosidase activity in the cytoplasm.

The second approach involved screening for mutations that affected the folding of alkaline phosphatase, a protein that contains two essential disulphides (Kamitani *et al*., 1992). Some of the mutants that showed reduced alkaline phosphatase activity by failing to form these disulphides were in the *dsbA* gene, again suggesting that it was involved in disulphide bond formation.

Cells containing null mutations in the *dsbA* gene have a pleiotropic phenotype as the correct folding of many periplasmic proteins is affected. They show reduced levels of proteins that contain disulphides such as alkaline phosphatase, β-lactamase or the outer membrane protein OmpA (Bardwell *et al*., 1991). *dsbA* mutants are hypersensitive to the reductant dithiothreitol, benzylpeni-

cillin and some metal ions, such as Hg^{2+} and Cd^{2+} (Missiakas *et al*., 1993; Stafford *et al*., 1999). It is also reported that they are unable to fold halocytochrome *c* (Metheringham *et al*., 1995) or the FlgI component of the flagellar motor, which results in an inability of *dsbA*$^-$ strains to swim (Dailey and Berg, 1993).

DsbA, the most oxidizing protein known

DsbA is a small periplasmic protein (21 kDa) which possesses two active site cysteine residues present in a CXXC motif. This motif is shared with several other oxidoreductases, including protein disulphide isomerase, thioredoxin and various other Dsb proteins (see below) (Raina and Missiakas, 1997). In order to be active, the two cysteines of DsbA need to be in the oxidized state. Oxidized DsbA is very unstable (Zapun *et al*., 1993) and reacts rapidly with unfolded proteins entering the periplasm. After the transient formation of a mixed disulphide between the substrate and the first cysteine of the CXXC motif (Cys-30 in DsbA), the disulphide bond is transferred onto the folding protein and reduced DsbA is released.

DsbA is the most oxidizing protein known. Its oxidation power originates from the structure of the CXXC motif and, more precisely, from an unusually low pK_a of the most N-terminal cysteine in this active site, Cys-30. This residue has the phenomenally low pK_a of ≈ 3, compared with normal pK_a vaules of cysteine residues of ≈ 9. Owing to this low pK_a value, Cys-30 is almost entirely in the thiolate anion state at physiological pH. The fact that this anion is stabilized drives the reaction towards the reduction of DsbA and the oxidation of the unfolded protein. The very oxidizing redox potential of DsbA (–120 mV) agrees with the oxidant role of the protein (Zapun *et al*., 1993). For comparison, thioredoxin, which acts as a reductant in the cytoplasm, has a standard redox potential of –270 mV.

The three-dimensional structure of DsbA (Martin *et al*., 1993) shows that DsbA consists of two domains linked by a flexible hinge. One domain has a thioredoxin-like fold, whereas the second domain is a compactly folded helical domain, inserted into the thioredoxin-like fold. A rotation motion occurs between the two domains upon binding of the substrate. Thioredoxin-like domains are present in DsbA, DsbC, DsbD and DsbG (see below), making the presence of a thioredoxin-like fold one of the hallmarks of the Dsb family. The only Dsb protein thought not to have a thioredoxin-like domain is DsbB.

DsbA contains a relatively deep hydrophobic groove running below the active site that is proposed to be involved in peptide binding (Guddat *et al*., 1997). Additional evidence that DsbA specifically interacts with unfolded proteins comes from the observations that conformational stability of DsbA is increased upon cross-linking to folded proteins. The detection by nuclear magnetic resonance (NMR) of intermolecular nuclear overhauser effects (NOEs) between DsbA and a cross-linked peptide indicates that DsbA binds peptides in a hydrophobic manner (Couprie *et al*., 2000).

In a paper entitled 'Does DsbA have chaperone-like activity' Zheng *et al*. (1997) raised the possibility that DsbA may function as a chaperone in addition to its function as an oxidoreductase. A chaperone activity for DsbA was previously suggested to be involved in pili assembly (Jacob-Dubuisson *et al*., 1994) and pullanase secretion (Sauvonnet and Pugsley, 1998). However, it was recently reported that the pullanase secretion defect is more likely due to a decreased disulphide bond formation activity in one of the secretion component (Pugsley *et al*., 2001).

Three-dimensional structure analysis and site-directed mutagenesis studies have helped us to understand why the active site Cys-30 of DsbA is so reactive. The abnormally low pK_a value of Cys-30 appears to be the result of electrostatic interactions that act to stabilize Cys-30 in a thiolate anion form (Guddat *et al*., 1997). The residues that are most important in this stabilization process are those that are located in between the active site $C_{30}PHC_{33}$ motif, particularly His-32. Crystal structure analysis revealed that this residue is hydrogen-bonded to Cys-30 in the reduced but not in the oxidized DsbA (Guddat *et al*., 1998). Mutations that alter His-32 greatly decrease the oxidizing power of DsbA and do so in a way that can be predicted directly from the way in which they alter the pK_a of Cys-30 (Grauschopf *et al*., 1995). Other residues nearby that are located near the active disulphide, such as Pro-31, also contribute to the oxidizing power of DsbA but are not as important.

How is DsbA reoxidized?

DsbA is reduced after the transfer of its disulphide bond to the target protein. In order for it to act catalytically, DsbA needs to be reoxidized. The protein responsible for DsbA's reoxidation is an inner membrane protein called DsbB.

Mutations in the DsbB gene were isolated by using the same MalF–β-galactosidase fusion approach that was used to discover DsbA (Bardwell *et al*., 1993). Dailey and Berg (1993) independently reported the identification of DsbB by the selection of mutants failing to assemble functional flagella, and Missiakas *et al*. (1993) also isolated mutants in *dsbB* and additional *dsb* genes by screening for dithiothreitol-hypersensitive mutants.

dsbB mutants exhibit the same pleiotropic phenotype as DsbA mutants. The fact that mutations in *dsbA* and *dsbB* produce similar defects in disulphide bond formation suggests that both proteins are on the same major oxidation pathway. This hypothesis was confirmed by the

observation that *dsbB*$^-$ strains accumulate DsbA in a reduced form, whereas DsbA is found oxidized in wild-type strains (Bardwell *et al*., 1993). The isolation of a DsbA–DsbB complex, connected via a mixed disulphide, suggested that DsbA and DsbB interact directly (Guilhot *et al*., 1995; Kishigami *et al*., 1995). We confirmed this by showing that DsbA is rapidly oxidized *in vitro* by a membrane preparation containing catalytic amounts of DsbB.

DsbB is a 21 kDa inner-membrane protein with four transmembrane segments. DsbB has two pairs of essential cysteine residues, one pair located in each of its two periplasmic domains. The first pair of cysteines is located in a CXXC arrangement, an arrangement reminiscent of the CXXC motif present in many thioredoxin-like proteins. The three-dimensional structure of DsbB has not yet been solved, but it is very unlikely that DsbB contains a domain with a thioredoxin-like fold. The first periplasmic domain is simply too small and, in addition, lacks the other features that are characteristic of thioredoxin-like proteins. The other pair of cysteines is in the second larger periplasmic domain.

The mechanism of how DsbB works is not yet fully understood. A preliminary mechanism has been proposed based on site-directed mutagenesis experiments in which the different cysteines of DsbB and DsbA were replaced in pair-wise combinations (Guilhot *et al*., 1995; Kishigami *et al*., 1995; Kishigami and Ito, 1996). Such experiments can allow for the accumulation of otherwise very unstable mixed disulphides, such as those that may form between DsbA and DsbB. These studies have provided us with a preliminary idea of the interactions that may occur *in vivo*. For instance, when Cys-33 of DsbA is replaced by a serine, a mixed disulphide between DsbB and DsbA accumulates because this complex cannot be resolved by Cys-33 (Kishigami *et al*., 1995). The observation that this complex is not formed when Cys-104 of DsbB is mutated suggests that Cys-104 of DsbB is required for the formation of the mixed disulphide (Guilhot *et al*., 1995; Kishigami and Ito, 1996). Indeed, a mixed disulphide between Cys-30 of DsbA and Cys-104 of DsbB has been observed (Guilhot *et al*., 1995; Kishigami and Ito, 1996). After the resolution of the mixed disulphide bond by the attack of DsbA's Cys-33, DsbA is released in the oxidized form while DsbB's cysteines 104 and 130 are reduced. These two residues are thought to be then regenerated by the two cysteines of the other periplasmic domain of DsbB, Cys-41 and Cys-44 (Kishigami and Ito, 1996).

This model is based on the assumption that the stable mixed disulphide bond that is observed between Cys-30 of DsbA and Cys-104 of DsbB is a normal intermediate in the reaction mechanism. However, reaction intermediates are generally highly unstable; the *more* stable the mixed disulphide is, the *less* likely it is that it represents a real intermediate. When studying disulphide exchange reactions within a multicysteine protein, removal of one cysteine does not usually stop the reaction in its tracks leading to accumulation of the intermediate normally present prior to the block. The unstable reaction intermediates have a tendency to rapidly rearrange to the most stable configurations. Thus, the exact direction of disulphide flow through DsbB needs to be confirmed by additional experiments.

Where do the electrons go?

DsbA is reoxidized by the membrane protein DsbB. This leads to the obvious question: what reoxidizes DsbB? The possibility that molecular oxygen and the electron transport chain could be involved in the reoxidation of DsbB was a hypothesis raised when DsbB was first isolated (Bardwell *et al*., 1993). Four years passed, until the first evidence in favour of this hypothesis was reported. In 1997, Kobayashi *et al* (1997) showed that mutants defective in haem or quinone biosynthesis accumulated DsbA and DsbB in a reduced form. In 1998, Bader *et al*. (1998) showed that the oxidation of DsbA by DsbB strongly depends on the presence of oxygen. One year later, the redox state of the four essential cysteines of DsbB was characterized (Kobayashi and Ito, 1999). In the presence of oxygen, the CXXC motif of DsbB is very difficult to reduce even at high dithiothreitol concentrations, but these cysteines become easy to reduce when the membranes are prepared from cells that do not contain quinone or haem (Kobayashi and Ito, 1999). Taken together, these results suggested the involvement of the respiratory chain in the reoxidation of DsbB and DsbA.

The *in vitro* reconstitution of the disulphide bond catalysis system using purified components verified the connection between electron transport and disulphide bond formation (Bader *et al*., 1999). DsbB transfers its electrons on to oxidized ubiquinone, which then donates them to cytochrome oxidases, which reduce oxygen (Fig. 1). Under anaerobic conditions, DsbB passes its electrons on to menaquinone and then on to fumarate reductase or nitrate reductase. Therefore, DsbB uses the oxidizing power of quinones to generate disulphides *de novo* (Fig. 2). This novel catalytic activity is apparently the major source of disulphide bonds *in vivo* (Bader *et al*., 2000). These results show where the oxidative power for disulphide formation originates and how one step in the catalysis of protein folding and cellular metabolism are linked.

Titration experiments demonstrated that DsbB has only one quinone binding site, which binds quinones with a very high specificity (K_m of 2 μM and kcat/K_m ratio of 3×10^6 M^{-1} s^{-1}) (Bader *et al*., 2000). It has been suggested that the oxidation of DsbB by quinones takes place through the CXXC motif of the first periplasmic domain

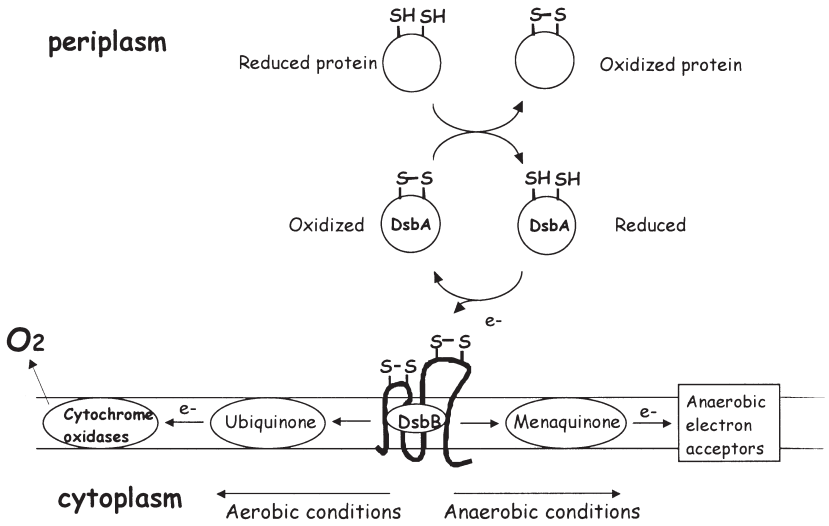

Fig. 1. Oxidation pathway in the *E. coli* periplasm. Reduced proteins are oxidized by the extremely oxidizing active site disulphide found at the active site of the periplasmic DsbA protein, a rapid and unidirectional process which helps to catalyse their folding. DsbA is reoxidized by the inner membrane protein DsbB. Under aerobic conditions, DsbB is reoxidized by molecular oxygen in a cytochrome oxidase- and ubiquinone-dependent reaction. Anaerobically alternative final electron acceptors are used in a menaquinone-dependent reaction.The direction of electron flow is shown by straight arrows. The direction of disulphide flow is shown by curved arrows.

(Kobayashi and Ito, 1999). It has been shown that a residue located close to the CXXC motif, Arg-48, seems to play an important role in the interaction between DsbB and quinones. Replacement of this amino acid by a histidine residue increases the DsbB's K_m for ubiquinone more than sevenfold (Kadokura *et al*., 2000). In addition, insertion or deletion in a segment located immediately after Cys-44 and ending at Arg-48 leads to the accumulation of an inactive DsbB in a reduced state (Kobayashi *et al*., 2001).

The isomerization pathway

The two prokaryotic disulphide isomerases: DsbC and DsbG

For any protein that contains more than two cysteines, the possibility exists that incorrect disulphide bonds will form. The fact that DsbA can form inappropriate disulphides in a protein was demonstrated *in vitro* in RNase A refolding experiments (Bader *et al*., 2000). DsbA, in the presence of DsbB and quinones, completely oxidizes RNase A, but the oxidized RNase A has no activity (Bader *et al*., 2000), unless glutathione redox buffers are added. The introduction of non-native disulphide bonds by DsbA is probably the reason that DsbA-oxidized RNase A is mainly misfolded. In the cell, it is vital to resolve quickly any incorrect disulphides introduced by DsbA to prevent accumulation of non-native proteins. The enzymes that are able to 'shuffle' disulphides around are called protein disulphide isomerases.

The first step in the discovery of the isomerization pathway in prokaryotes was the identification of DsbC in 1994. Two different groups independently isolated the gene; one group found DsbC by screening for dithiothreitol-sensitive mutations (Missiakas *et al*., 1994), whereas the other cloned genes that were able to complement DsbA. Like DsbA and DsbB mutants, DsbC mutants are defective in disulphide bond formation, but the defect in DsbC mutations is much milder (Rietsch *et al*., 1996). For instance, mutations in DsbC do not affect the cell motility or the formation of OmpA (Rietsch *et al*., 1996).

DsbC was first reported to catalyse disulphide bond formation (Missiakas *et al*., 1994). However, it is now thought that DsbC acts mainly as a disulphide isomerase *in vivo*. DsbC can efficiently catalyse disulphide bond isomerization in proteins that have multiple S–S bonds, such as bovine pancreatic trypsin inhibitor (Zapun *et al*., 1995) or RNase A (Bader *et al*., 2000). In addition, DsbC is required *in vivo* to ensure the folding of proteins with multiple disulphide bonds. The severity of the folding defect increases with the number of disulphide bonds formed in a protein. For instance, the production of urokinase, a protein with 12 disulphide bonds, is more than 100-fold reduced in DsbC mutants, whereas the production of alkaline phosphatase, which has only two disulphides, is only slightly affected (Rietsch *et al*., 1996). This is exactly what is expected for isomerase mutants, in which the number of possibly incorrect disulphides grows exponentially with the number of cysteines in a protein. Finally, it has been reported that DsbC is found in a reduced state *in vivo*. This is consistent with DsbC acting as an isomerase as the first step in the resolution on an incorrect disulphide is the attack of this disulphide by a reduced cysteine (Rietsch *et al*., 1997).

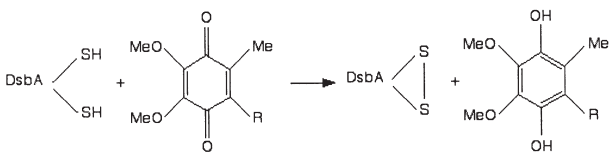

Fig. 2. DsbB is a quinone reductase that generates disulphides *de novo* using the oxidizing power of quinones.

DsbC is a dimer (Zapun *et al*., 1995) of two identical 23 kDa subunits. Each subunit has four cysteine residues. The Cys-98–Cys-101 pair is directly involved in DsbC's oxidoreductase activity (Zapun *et al*., 1995). The two other cysteines of DsbC form a stable disulphide bond, which probably plays a structural role (Zapun *et al*., 1995).

The mechanism that has been proposed for DsbC action can be summarized as followed: Cys-98 attacks an incorrect disulphide in a protein and a mixed disulphide is formed between DsbC and the misfolded protein. This mixed disulphide is then resolved either by attack of another cysteine of the misfolded protein, resulting in the formation of a more stable disulphide in the substrate and reduced DsbC, or by attack of Cys-101 of DsbC. In this case, DsbC becomes oxidized and needs to be reduced in order to be recycled (see below).

DsbC possesses a peptide-binding activity, which enhances the rate at which it interacts with folding proteins. Also, it has been reported that DsbC has a chaperone activity and can assist the refolding of lysozyme or glyceraldehyde-3-phosphate dehydrogenase (Chen *et al*., 1999).

The DsbC structure has recently been solved to a resolution of 1.9 Å (McCarthy *et al*., 2000). DsbC appears as a V-shaped protein, with each monomer forming one arm of the V. Each monomer is subdivided into two domains: an N-terminal dimerization domain and a C-terminal catalytic domain with a thioredoxin-like fold, similar to that of DsbA. The two active site CXXC motifs face each other in the interior of the V. The surface of this V-shaped cleft is composed mainly of hydrophobic and uncharged residues, suggesting that it might be involved in the non-covalent binding of substrate proteins.

That the N-terminal domains in DsbC are involved in the dimerization has been confirmed by limited proteolysis experiments. When the N-terminal domain is removed, the resulting DsbC fragment is no longer able to dimerize (Sun and Wang, 2000). These studies also demonstrated that the C-terminal fragment is entirely lacking in the isomerase activity, although it does contain the active CXXC motif and has some oxidoreductase activity. This suggested that dimerization is necessary for isomerase activity.

Another Dsb protein, DsbG, was identified by Andersen *et al*. (1997), who found that this gene, when present in multiple copies, could confer on DsbB mutants resistance to elevated concentration of dithiothreitol. DsbG is also a homodimer and shows 28% sequence identity and 56% sequence similarity to DsbC (Andersen *et al*., 1997). DsbG is a non-essential disulphide isomerase, like DsbC, but with presumably different substrate specificity (Bessette *et al*., 1999). Shao *et al*. (2000) showed that DsbG has a chaperone activity *in vitro*, which is more consistent with DsbG working as an isomerase.

DsbD, the electron import machine

In order to be functional as isomerases, DsbC and DsbG need to be kept reduced within the very oxidizing environment of the periplasm. The enzyme that keeps DsbC and DsbG reduced is an inner membrane protein called DsbD.

DsbD (also called DipZ or CutA2) was discovered independently by three groups working in different fields. This highlights DsbD's central and important role. DsbD mutants are hypersensitive to dithiothreitol (Missiakas *et al*., 1995) and Cu^{2+} (Fong *et al*., 1995) and are unable to synthesize mature *c*-type cytochromes (Crooke and Cole, 1995). A *dsbD* null mutation also affects the folding of proteins with multiple disulphides and leads to the accumulation of oxidized DsbC and DsbG (Missiakas *et al*., 1995; Andersen *et al*., 1997; Rietsch *et al*., 1997; Bessette *et al*., 1999). These results indicate that DsbD is required to keep DsbC and DsbG reduced.

In order to reduce DsbC and DsbG, the DsbD protein itself has to be reduced. The proteins required to facilitate DsbD reduction were elucidated by genetic studies. Rietsch and co-workers showed that mutations in either thioredoxin or thioredoxin reductase lead to the accumulation of oxidized DsbC (Rietsch *et al*., 1996; 1997). Thioredoxin is a cytoplasmic protein that reduces ribonucleotide reductase and helps to maintain cytoplasmic proteins in a reduced state. Thioredoxin itself is then reduced by thioredoxin reductase and NADPH. The similar phenotypes shared by thioredoxin, thioredoxin reductase and DsbD mutants indicate that these three enzymes are members of a single pathway (Fig. 3) that is involved in the transfer of reducing equivalents from the cytoplasm to DsbC or DsbG (Rietsch *et al*., 1996).

DsbD has a molecular mass of 59 kDa, making it the largest protein in the Dsb family. DsbD is synthesized as a precursor with a cleavable signal peptide (Chung *et al*., 2000; Gordon *et al*., 2000). Topological studies have revealed that DsbD has three distinct domains (Chung *et al*., 2000; Gordon *et al*., 2000): an N-terminal periplasmic domain (α-domain), followed by a hydrophobic core with eight transmembrane segments (β-domain) and, finally, a second periplasmic portion (γ-domain), which is predicted to have a thioredoxin-like fold.

Each domain of DsbD has two conserved cysteine residues. From site-directed mutagenesis experiments it seems that these cysteine residues are important for the mechanism of DsbD action, but whether or not they are essential remains controversial. Single cysteine-to-alanine mutants accumulate oxidized DsbC and DsbG and are unable to produce active urokinase, similar to DsbD null mutants (Stewart *et al*., 1999; Chung *et al*., 2000; Gordon *et al*., 2000). However, copper sensitivity and cytochrome biosynthesis are affected only when two

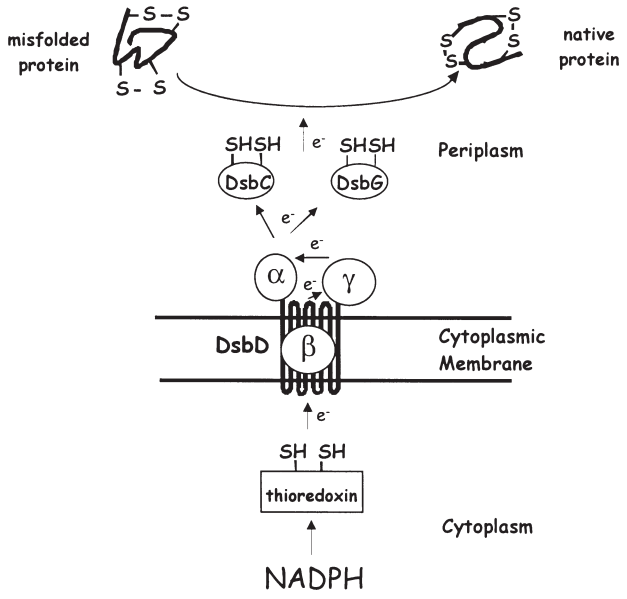

Fig. 3. Isomerization pathway in the *E. coli* periplasm. DsbC and DsbG are disulphide isomerases. In order for them to be able to attack misformed disulphides they need to be in the reduced form. They are kept this way by the membrane protein DsbD. The proposed direction of electron flow within the DsbD domains α, β and γ is shown by straight arrows and that of disulphide flow is shown by curved arrows. DsbD is kept reduced by the reducing power of thioredoxin, which is reduced by thioredoxin reductase (not shown) and then by NADPH.

or more cysteines are replaced simultaneously; individual replacements are reported to have no effect (Gordon *et al*., 2000).

A model for the mechanism of DsbD action has recently been proposed which involves the successive transfer of electrons from thioredoxin to the β-domain of DsbD and then successively on to the γ- the α-domains and finally on to DsbC. This model is based upon the observation of disulphide cross-links between DsbC and the α-domain (Katzen and Beckwith, 2000; Krupp *et al*., 2001) and preferential accumulation of some domains in a reduced form if the domain downstream is absent from the expression system (Katzen and Beckwith, 2000). In addition, purified α-domain was recently shown to be able to reduce DsbC *in vitro*. It is proposed that this succession of disulphide exchange reactions involves major conformational change of DsbD.

It should be noted that this DsbD mechanism, like the DsbB mechanism, depends on the assumption that the mixed disulphide intermediates observed correspond to real reaction intermediates. As for the oxidation system, it will be helpful to reconstitute the isomerization system *in vitro* to study this mechanism in detail.

CcdA, a stripped down version of the DsbD protein that lacks the α- and γ-domains and contains only the β-domain, has been identified in at least 35 prokaryotic species, including both Gram-positive and Gram-negative organisms. Similar to DsbD mutants, CcdA mutants are defective in *c*-type cytochrome biosynthesis (Deshmukh *et al*., 2000). CcdA is a ~26 kDa integral membrane protein with six transmembrane segments and two conserved cysteines residues (Deshmukh *et al*., 2000).These cysteines, which align with the conserved cysteines of the β-domain, are required for CcdA activity in *c*-type cytochrome biosynthesis (Deshmukh *et al*., 2000).

The *ccdA* gene is adjacent to an open reading frame (ORF) encoding a thioredoxin-like protein in at least seven bacteria, including *Streptomyces*, *Corynbacterium* and *Mycobacterium*. This suggests that evolution may have acted to combine two modular proteins, CcdA and a thioredoxin like protein, into one functional unit, DsbD. However, a protein corresponding to the α-domain is not found in bacterial genomes containing the ccdA gene, even though this domain is essential for the DsbD activity. In addition, some organisms, such as *Rhodobacter capsulatus* and *Haemophilus influenzae*, contain both CcdA and DsbD, suggesting that these two proteins are not simply redundant.

How are the oxidative and reductive pathways kept separate?

DsbA needs to be kept oxidized in order to function as a disulphide donor, and DsbC needs to be kept reduced for it to work as a disulphide isomerase. The coexistence of these two systems, which have opposite goals, within the same periplasmic compartment raises the interesting question of how the two systems are kept separate. If barriers that prevent cross-talk between the two systems did not exist there would be a futile cycle in which DsbB would oxidize DsbC while DsbD reduced DsbC. How does DsbB discriminate between DsbA and DsbC?

DsbB reoxidizes DsbC at least 500-fold more slowly than does DsbA, suggesting that DsbB can distinguish between these two proteins (Bader *et al*., 2000). The molecular basis of this discrimination became clear when we selected for DsbC mutants that complement a *dsbA* null mutation (Bader *et al*., 2001). Analysis of these mutants revealed that all the mutations were in the dimerization interface of DsbC and produced monomeric proteins. The ability of these monomeric mutants to rescue a *dsbA*$^{-}$ phenotype depended on the presence of an active DsbB. These results indicated that the DsbC active site cysteines are protected from DsbB oxidation by dimerization.

Conclusions and future directions

A decade ago it was generally accepted that disulphide bond formation in the bacterial periplasm is a sponta-

neous process. With the identification of five Dsb proteins involved in two different pathways, we now have a detailed understanding of how this process is catalysed.

However, several important problems remain, including the relative substrate specificity of the isomerases DsbG and DsbC and the mechanism of DsbB and DsbD action. For instance, how reducing equivalents cross the cytoplasmic membrane remains to be elucidated.

The solution to these problems will not only increase our understanding of the system, but will also be very helpful in other fields, e.g. biotechnology, with the production of pharmacologically important proteins that have multiple disulphides.

Moreover, more insight into the different pathways involving the Dsb proteins will also be very useful in studying disulphide bond formation in the much more complex eukaryotic endoplasmic reticulum.

Acknowledgements

We would like to thank Ursula Jakob for helpful criticism of this manuscript. This work was supported by an NIH grant to J.C.A.B. J.F.C. is Charge de Recherches of the Belgian Fonds National de la Recherche Scientifique. J.C.A.B. is a Pew Scholar.

References

Andersen, C.L., Matthey-Dupraz, A., Missiakas, D., and 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.W., Hiniker, A., Regeimbal, J., Goldstone, D., Haebel, P.W., Riemer, J., Metcalf, P., and Bardwell, J.C. (2001) Turning a disulfide isomerase into an oxidase: DsbC mutants that imitate DsbA. *EMBO J* **20:** 1555–1562.

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

Bader, M., Muse, W., Zander, T., and Bardwell, J. (1998) Reconstitution of a protein disulfide catalytic system. *J Biol Chem* **273:** 10302–10307.

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

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

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

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

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

Chung, J., Chen, T., and Missiakas, D. (2000) Transfer of electrons across the cytoplasmic membrane by DsbD, a membrane protein involved in thiol-disulphide exchange and protein folding in the bacterial periplasm. *Mol Microbiol* **35:** 1099–1109.

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

Crooke, H., and Cole, J. (1995) The biogenesis of *c*-type cytochromes in *Escherichia coli* requires a membrane-bound protein, DipZ, with a protein disulphide isomerase-like domain. *Mol Microbiol* **15:** 1139–1150.

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

Deshmukh, M., Brasseur, G., and Daldal, F. (2000) Novel *Rhodobacter capsulatus* genes required for the biogenesis of various c-type cytochromes. *Mol Microbiol* **35:** 123–138.

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

Gordon, E.H., Page, M.D., Willis, A.C., and Ferguson, S.J. (2000) *Escherichia coli* DipZ: anatomy of a transmembrane protein disulphide reductase in which three pairs of cysteine residues, one in each of three domains, contribute differentially to function. *Mol Microbiol* **35:** 1360–1374.

Grauschopf, U., Winther, J.R., Korber, P., Zander, T., Dallinger, P., and Bardwell, J.C. (1995) Why is DsbA such an oxidizing disulfide catalyst? *Cell* **83:** 947–955.

Guddat, L.W., Bardwell, J.C., Zander, T., and 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.

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

Guilhot, C., Jander, G., Martin, N.L., and Beckwith, J. (1995) Evidence that the pathway of disulfide bond formation in *Escherichia coli* involves interactions between the cysteines of DsbB and DsbA. *Proc Natl Acad Sci USA* **92:** 9895–9899.

Jacob-Dubuisson, F., Pinkner, J., Xu, Z., Striker, R., Padmanhaban, A., and Hultgren, S.J. (1994) PapD chaperone function in pilus biogenesis depends on oxidant and chaperone-like activities of DsbA. *Proc Natl Acad Sci USA* **91:** 11552–11556.

Kadokura, H., Bader, M., Tian, H., Bardwell, J.C., and 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 USA* **97:** 10884–10889.

Kamitani, S., Akiyama, Y., and 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., and Beckwith, J. (2000) Transmembrane electron transfer by the membrane protein DsbD occurs via a disulfide bond cascade. *Cell* **103:** 769–779.

Kishigami, S., and Ito, K. (1996) Roles of cysteine residues of DsbB in its activity to reoxidize DsbA, the protein disulphide bond catalyst of *Escherichia coli*. *Genes Cells* **1:** 201–208.

Kishigami, S., Kanaya, E., Kikuchi, M., and Ito, K. (1995) DsbA–DsbB interaction through their active site cysteines. Evidence from an odd cysteine mutant of DsbA. *J Biol Chem* **270:** 17072–17074.

Kobayashi, T., and Ito, K. (1999) Respiratory chain strongly oxidizes the CXXC motif of DsbB in the *Escherichia coli* disulfide bond formation pathway. *Embo J* **18:** 1192–1198.

Kobayashi, T., Kishigami, S., Sone, M., Inokuchi, H., Mogi, T., and Ito, K. (1997) Respiratory chain is required to maintain oxidized states of the DsbA-DsbB disulfide bond formation system in aerobically growing *Escherichia coli* cells. *Proc Natl Acad Sci USA* **94:** 11857–11862.

Kobayashi, T., Takahashi, Y., and Ito, K. (2001) Identification of a segment of DsbB essential for its respiration- coupled oxidation. *Mol Microbiol* **39:** 158–165.

Krupp, R., Chan, C., and Missiakas, D. (2001) DsbD-catalyzed transport of electrons across the membrane of *Escherichia coli*. *J Biol Chem* **276:** 3696–3701.

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

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

Metheringham, R., Griffiths, L., Crooke, H., Forsythe, S., and Cole, J. (1995) An essential role for DsbA in cytochrome c synthesis and formate- dependent nitrite reduction by *Escherichia coli* K-12. *Arch Microbiol* **164:** 301–307.

Missiakas, D., Georgopoulos, C., and 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 USA* **90:** 7084–7088.

Missiakas, D., Georgopoulos, C., and Raina, S. (1994) The *Escherichia coli dsbC* (*xprA*) gene encodes a periplasmic protein involved in disulfide bond formation. *EMBO J* **13:** 2013–2020.

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

Pugsley, A.P., Bayan, N., and Sauvonnet, N. (2001) Disulfide bond formation in secreton component PulK provides a possible explanation for the role of DsbA in pullulanase secretion. *J Bacteriol* **183:** 1312–1319.

Raina, S., and Missiakas, D. (1997) Making and breaking disulfide bonds. *Annu Rev Microbiol* **51:** 179–202.

Rietsch, A., Belin, D., Martin, N., and Beckwith, J. (1996) An *in vivo* pathway for disulfide bond isomerization in *Escherichia coli*. *Proc Natl Acad Sci USA* **93:** 13048–13053.

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

Sauvonnet, N., and Pugsley, A.P. (1998) The requirement for DsbA in pullulanase secretion is independent of disulphide bond formation in the enzyme. *Mol Microbiol* **27:** 661–667.

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

Stafford, S.J., Humphreys, D.P., and Lund, P.A. (1999) Mutations in *dsbA* and *dsbB*, but not *dsbC*, lead to an enhanced sensitivity of *Escherichia coli* to Hg^{2+} and Cd^{2+}. *FEMS Microbiol Lett* **174:** 179–184.

Stewart, E.J., Katzen, F., and 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.

Sun, X.X., and 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.

Zapun, A., Bardwell, J.C., and 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., and Creighton, T.E. (1995) Structural and functional characterization of DsbC, a protein involved in disulfide bond formation in *Escherichia coli*. *Biochemistry* **34:** 5075–5089.

Zheng, W.D., Quan, H., Song, J.L., Yang, S.L., and Wang, C.C. (1997) Does DsbA have chaperone-like activity? *Arch Biochem Biophys* **337:** 326–331.