Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochimica et Biophysica Acta 1694 (2004) 111–119
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
http://www.elsevier.com/locate/bba

Review

Catalysis of disulfide bond formation and isomerization in the *Escherichia coli* periplasm

Hitoshi Nakamoto[1], James C.A. Bardwell*

Department of Molecular, Cellular and Developmental Biology, University of Michigan, 830, North University Dr., Ann Arbor, MI 48109-1048, USA

Received 2 December 2003; received in revised form 12 February 2004; accepted 16 February 2004
Available online 18 May 2004

Abstract

Disulfide bond formation is a catalyzed process in vivo. In prokaryotes, the oxidation of cysteine pairs is achieved by the transfer of disulfides from the highly oxidizing DsbA/DsbB catalytic machinery to substrate proteins. The oxidizing power utilized by this system comes from the membrane-embedded electron transport system, which utilizes molecular oxygen as a final oxidant. Proofreading of disulfide bond formation is performed by the DsbC/DsbD system, which has the ability to rearrange non-native disulfides to their native configuration. These disulfide isomerization reactions are sustained by a constant supply of reducing power provided by the cytoplasmic thioredoxin system, utilizing NADPH as the ultimate electron source.

Keywords: Disulfide bond; DsbA; DsbB; DsbC; DsbD; *Escherichia coli*

1. Introduction

Disulfide bond formation is crucial for the folding and stability of many secreted proteins. Failure to form proper disulfide bonds, or their slow formation in the cell, is likely to lead to protein aggregation and degradation by proteases. For example, alkaline phosphatase is a periplasmic protein that contains two disulfide bonds necessary for correct folding. If these disulfide bonds are not formed, the protein is almost completely degraded in vivo [1]. The role of disulfides in the in vitro folding of proteins has been studied in great detail for a number of eukaryotic model proteins, including ribonuclease A (RNaseA) and bovine pancreatic trypsin inhibitor (BPTI) [2]. Disulfides typically function to stabilize the tertiary structure of a protein. A protein whose cysteines are linked via disulfide bonds can take on many fewer conformations than a protein whose cysteines remain free. The decrease in chain entropy that accompanies disulfide bond formation is one of the key factors that help disulfide bonds disfavor the unfolded form of the protein.

Disulfide bonds can be formed spontaneously by molecular oxygen. For instance, under aerobic conditions, a thin layer of cystine is generated at the air–liquid interface when a cysteine solution is left exposed to air. However, this type of spontaneous, random air-oxidation reaction is very slow and cannot account for the rapid rates of disulfide bond formation needed by the cell. This discrepancy led Anfinsen to the discovery of the first catalyst for disulfide bond formation, the eukaryotic protein disulfide isomerase (PDI). PDI, together with Ero1, is part of the complex machinery responsible for the formation of disulfide bonds in the eukaryotic endoplasmic reticulum. The formation of disulfide bonds in the endoplasmic reticulum has been reviewed elsewhere and will not be discussed here [3]. Instead, this review will focus on the bacterial catalysts for disulfide bond formation and isomerization, using *Escherichia coli* as the model organism. In *E. coli*, disulfide bonds are introduced in the periplasm by the Dsb proteins ("Dsb" stands for *di*sulfide *b*ond formation). The Dsb proteins include DsbA and DsbB, which are involved in disulfide bond formation, and DsbC and

* Corresponding author. Tel.: +1-734-764-8028; fax: +1-734-647-0884.
E-mail address: jbardwel@umich.edu (J.C.A. Bardwell).
[1] Present address: Department of Biochemistry and Molecular Biology, Saitama University, Saitama 338-8570, Japan.

0167-4889/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2004.02.012

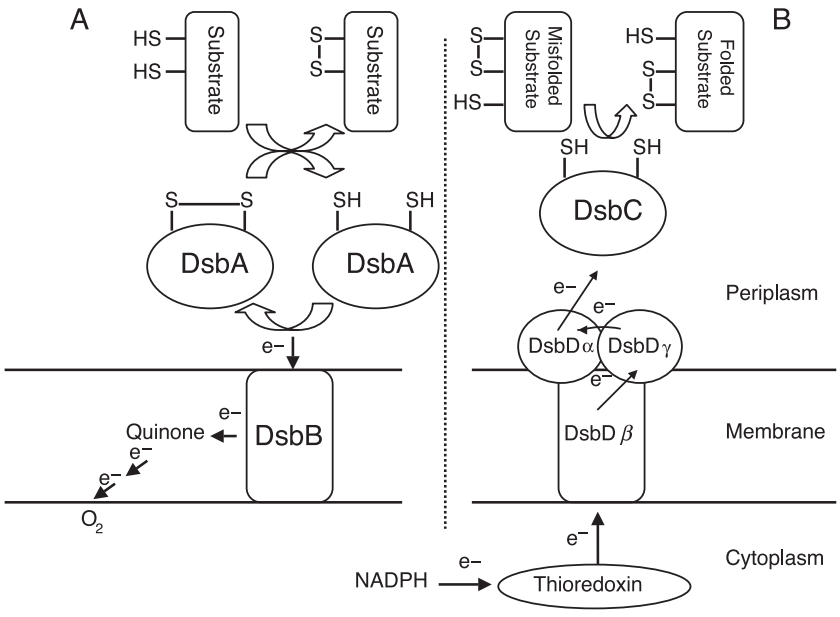

Fig. 1. The oxidation and isomerization pathways in the *E. coli* periplasm. (A) Disulfide bonds are introduced into newly secreted polypeptides by DsbA. DsbA is reoxidized by the inner membrane protein DsbB. In aerobic conditions, DsbB passes electrons to ubiquinone. Then electrons flow to molecular oxygen via the electron transport chain. (B) DsbA can form a non-native disulfide in proteins with more than two cysteine residues. The non-native disulfide is corrected by DsbC via disulfide shuffling. DsbC is kept reduced by the inner-membrane protein DsbD. DsbD receives electrons from the cytoplasmic thioredoxin system. Within DsbD, electrons flow from the β domain to the γ and α domains successively.

DsbD, which are involved in disulfide bond isomerization (Fig. 1). Since the discovery of DsbA in 1991 [1], disulfide bond formation and isomerization in the *E. coli* periplasm has become one of the best characterized experimental systems.

2. Oxidation of cysteine residues in the bacterial periplasm

2.1. DsbA catalyzes disulfide bond formation

DsbA, a 21-kDa soluble protein, is the immediate donor of disulfide bonds to proteins secreted into the *E. coli* periplasm [1]. In the absence of DsbA, periplasmic protein thiols accumulate in the reduced form. In *E. coli*, at least 300 proteins containing two or more cysteines are predicted to be secreted to the periplasm [4]. These proteins are therefore potential DsbA substrates. Thus, it is not surprising that *dsbA*$^-$ strains exhibit pleiotropic phenotypes [1]. For instance, *dsbA*$^-$ strains are hypersensitive to benzylpenicillin, dithiothreitol and metals [5,6]. They show reduced levels of several secreted proteins such as the outer-membrane protein OmpA and alkaline phosphatase [1]. They also show impaired folding of β-lactamase and the flagellar protein FlgI [1,7].

Like many other thiol-disulfide oxido-reductases, DsbA has a thioredoxin-like fold and a CXXC active site motif, consisting of Cys30 and Cys33. DsbA's active site cysteine residues are found oxidized in vivo, which agrees with the oxidase function of DsbA [8]. The protein acts as both an oxidant and a disulfide bond isomerase in vitro, depending on the redox state of the two cysteines in the protein; however, it is much more effective as an oxidase [9,10].

In general, disulfide bonds stabilize proteins. However, oxidized DsbA is less stable than reduced DsbA [11]. This instability of both the oxidized protein and a mixed disulfide with a target protein, which is probably an obligatory intermediate in thiol-disulfide exchange reactions, provides a thermodynamic driving force for the transfer of the disulfide bond from DsbA to a target protein (Fig. 1). The first step of this transfer reaction is the formation of an unstable mixed disulfide between DsbA and a target protein. Then, the mixed disulfide is attacked by another thiol group in the target protein. This results in the formation of a disulfide bond in the target protein and the reduction of Cys30 and Cys33 in DsbA.

Oxidized DsbA containing the Cys30–Cys33 disulfide bond is a potent oxidant, which is indicated by its high redox potential, −120 mV [11]. In comparison, thioredoxin, which functions as a cytoplasmic dithiol reductant, has a much more reducing redox potential (−270 mV). In fact, DsbA is the second most oxidizing protein known (see below). The low pK_a value of Cys30 of DsbA, the first cysteine of the CXXC motif, is critical for determining the extreme oxidizing power of DsbA [12]. Cys30 has a pK_a of ~3, which contrasts sharply with the normal pK_a of cysteine residues (~9). Thus, Cys30 is almost entirely in the thiolate anion state at physiological pH. Several studies have shown that this thiolate anion is stabilized by hydrogen bonds, electrostatic and helix

dipole interactions, the most important being an electrostatic interaction between His32 and Cys30 [13,14]. Stabilization of the thiolate anion is a key characteristic of DsbA, as it drives the reaction towards the reduction of DsbA and the transfer of a disulfide bond to a target protein. However, DsbC has a redox potential nearly as high as that of DsbA and yet it is not as potent an oxidant in vitro as DsbA [15]. Despite the thermodynamic similarities with DsbA as a reactant/product in its oxidoreduction reaction, DsbC is much more active than DsbA in catalyzing protein disulfide rearrangements. These facts indicate that a DsbA reaction proceeds differently from a DsbC reaction. As described below, DsbA and DsbC are distinguished in their tertiary and quaternary structures which are potentially involved in binding a target protein. The stability of a DsbA or DsbC–target protein complex may be one of the factors which influence the reaction pathway.

The crystal structure of DsbA has been solved, both in the oxidized and reduced states [13,16]. It shows that a 75-residue helical domain is embedded into the thioredoxin domain of DsbA. This domain covers the CXXC active site and contains several residues that form a hydrophobic patch. This patch, together with a groove running in the thioredoxin domain below the active site, is likely to interact in a hydrophobic manner with unfolded protein substrates [17]. Such noncovalent interactions have indeed been reported to take place [18,19]. It is not surprising that DsbA is able to bind proteins in a hydrophobic manner, as DsbA must interact with proteins while they are still unfolded or only partially folded in order to gain access to cysteine residues before they are buried upon folding. It is therefore reasonable to assume that DsbA specifically interacts noncovalently with unfolded substrate proteins during or immediately after their translocation into the periplasm. The association of DsbA with a target protein could also prevent the target protein from folding prior to disulfide bond formation, as once the protein is folded it would be very difficult for DsbA to gain access to the reduced cysteines. This association between DsbA and the target protein should be terminated immediately after intermolecular disulfide exchange, so that protein folding is not inhibited.

2.2. Reoxidation of DsbA by DsbB

In order to function as a catalyst in the periplasm, DsbA needs to be reoxidized. Genetic as well as biochemical evidence showed that the reoxidation of DsbA is ensured by the inner-membrane protein DsbB (Fig. 1) [5,20]. The fact that *dsbB*$^-$ mutants accumulate DsbA in a reduced state and have similar defects in disulfide bond formation as *dsbA*$^-$ strains suggested that both DsbA and DsbB lie on the same major oxidation pathway [20]. Moreover, the isolation of a mixed disulfide between DsbA and DsbB [21,22] indicated that DsbA and DsbB interact directly. This was confirmed in vitro when we showed that purified DsbB is able to catalytically oxidize DsbA in the presence of molecular oxygen [23].

DsbB is a 20-kDa protein predicted to have four transmembrane helices and two periplasmic loops [24]. Each periplasmic loop contains a pair of conserved cysteines; Cys41 and Cys44 in the N-terminal loop, and Cys104 and Cys130 in the C-terminal loop (Fig. 2). These cysteines can undergo oxidation–reduction cycles and are essential for the activity of DsbB. Removal of any one of these four cysteine residues causes the loss of DsbB's activity [22,24,25]. Like DsbA, DsbB contains a pair of cysteine residues (Cys41–Cys44) in a CXXC motif. Unlike DsbA, however, the region surrounding Cys41–Cys44 of DsbB is predicted to be too short to contain a thioredoxin-like fold.

2.3. Reoxidation of DsbB

The fact that heme- or quinone-depleted cells accumulate reduced DsbA and DsbB suggested that the oxidizing power for protein disulfide bond formation in *E. coli* is provided by the respiratory chain [26]. We confirmed this hypothesis by reconstituting the complete disulfide bond formation system in vitro [23,27]. Using purified components, we showed that quinones could act as direct recipients of electrons from DsbB. Thus, DsbB has the novel ability to generate a disulfide in DsbA by reducing quinones. This quinone reductase activity is the primary source of disulfides in *E. coli*. Reduced quinones are then reoxidized by the terminal oxidases cytochrome *bd* and *bo*, which finally transfer electrons to oxygen (Fig. 1). Under anaerobic conditions, DsbB switches to utilizing menaquinone as its immediate electron acceptor. These results show that disulfide bond formation is

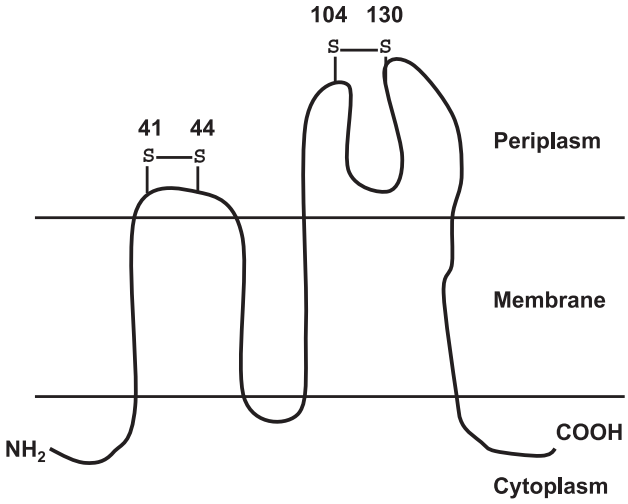

Fig. 2. Membrane topology of DsbB. DsbB is predicted to contain four transmembrane stretches and two periplasmic loops, each of which contains a pair of cysteines.

driven by the electron transport system and explain how disulfides can be formed under both aerobic and anaerobic conditions.

2.4. A model for the mechanism of action of DsbB

On the basis of in vivo results, Kishigami and Ito [25] proposed a model for the mechanism of action of DsbB. According to this model, the solvent-exposed thiolate anion of Cys30 of DsbA attacks the Cys104/Cys130 disulfide bond of DsbB and a mixed disulfide complex is formed. This mixed disulfide is then attacked by Cys33 of DsbA, which results in the transfer of the disulfide bond from DsbB to DsbA and the reduction of Cys104 and Cys130. This was suggested by the detection of a mixed disulfide between Cys104 of DsbB and Cys30 of a DsbA mutant lacking Cys33 [21,22,25]. The reduced Cys104 and Cys130 residues are then reoxidized intramolecularly by the Cys41–Cys44 disulfide bond. Finally, cysteine residues Cys41 and Cys44 are reoxidized by quinone reduction.

Results obtained by us and others do not entirely agree with this model [28–30]. However, Grauschopf et al. [31] recently reported strong evidence in favor of this mechanism. They showed that Cys104–Cys130 can directly transfer a disulfide bond to DsbA and is then reoxidized by the Cys41–Cys44 disulfide bond. They also determined the redox potential of Cys41–Cys44 and Cys104–Cys130 disulfide bonds of DsbB to be −69 and −186 mV, respectively. It should be noted that these redox potentials were measured in the absence of bound quinone. This makes the Cys41–Cys44 disulfide in DsbB the most oxidizing disulfide bond described in a protein to date. The Cys104–Cys130 disulfide, with a redox potential of −186 mV, is less oxidizing than DsbA, which would appear to make it not suitable to be the direct reoxidant of DsbA. However, the very strongly oxidizing potential of the Cys41–Cys44 disulfide drives the overall reaction to completion.

2.5. The novel quinone reductase activity of DsbB

DsbB is a unique enzyme that has the novel ability to use the oxidizing power of quinones to generate disulfides de novo [32]. The interaction of DsbB with quinones has been partially characterized. We previously showed that DsbB has at least one quinone-binding site, and that DsbB has a K_m of 2 μM for ubiquinone [32]. DsbB mutants in the residue Arg48 in the first periplasmic loop show a major in vivo defect in their ability to catalyze disulfide formation [33]. Purified DsbB R48H exhibits an apparent K_m for ubiquinone seven times greater than that of the wild type DsbB [33]. These results suggest that Arg48 plays an important role in quinone binding. Xie et al. [34] showed that a photoactivatable ubiquinone analog can cross-link to a region of the second periplasmic loop of DsbB spanning residues 91 to 97. This suggests that this region might also be involved in the interaction of DsbB with quinones. Finally, we showed that the Cys41–Cys44 pair in DsbB is oxidized directly by ubiquinone [29].

We recently reported that purified DsbB has a dramatic purple color [35]. Our evidence suggests that this is due to the presence of a bound quinhydrone, a charge-transfer complex consisting of a hydroquinone and a quinone in a stacked conformation. We propose that the quinone reductase activity of DsbB involves two quinone molecules that can be trapped on DsbB as a quinhydrone. One of these quinones is bound tightly 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 reoxidized by an exchangeable quinone derived from the oxidized quinones pool. It is during this quinone to quinone reoxidation that a quinhydrone-like complex is likely to form. Inaba et al. [64] have also very recently observed that DsbB has a strong reddish color, but they attribute this to a single quinone bound to DsbB in an unusual electronic state, not to a quinhydrone.

3. Disulfide reshuffling

3.1. The two periplasmic protein disulfide isomerases: DsbC and DsbG

In any protein with more than two cysteines, there is the potential for incorrect disulfide bond formation. For example, a protein with four pairs of cysteines has less than a 1% chance of attaining the correct four disulfides by random oxidation. For a protein to fold correctly, any incorrect disulfides that have been formed will need to be broken and reformed correctly. DsbA is a very strong oxidant and oxidizes thiol compounds nonspecifically and extremely rapidly. Thus, it has the potential of introducing non-native disulfides into proteins with multiple cysteines [36]. If disulfide bonds are formed immediately following translocation to the periplasm, incorrect disulfides will be formed unless each pair of cysteines that is destined to form a native disulfide bond is located consecutively in the primary structure of the protein. Incorrect disulfide bonds formed by DsbA may trap proteins in nonnative conformations. In vivo, DsbA is present in the oxidized state [8] and has a very low ability to reduce incorrect disulfides. Thus, it cannot provide proofreading activity for improperly formed disulfides. Incorrect disulfides will need to be reshuffled and corrected before the protein can attain its proper conformation.

Disulfide reshuffling involves an intramolecular thiol/disulfide exchange reaction; i.e., the nucleophilic attack of an incorrect disulfide bond by a thiolate anion. In the periplasm, this reaction is catalyzed by the disulfide bond

isomerase DsbC [36,37]. There is another periplasmic homolog to DsbC, that is, DsbG [38]. Its cellular function is not yet known. It may function as a disulfide bond isomerase since overproduction of DsbG could restore the ability of *dsbC* mutants to express multidisulfide-containing BPTI [38]. However, unlike DsbC, DsbG could neither catalyze insulin reduction in vitro or oxidative protein refolding of RNase efficiently, indicating functional differences between DsbC and DsbG.

DsbC and DsbG are both homodimeric proteins ($2 \times \sim 25$ kDa) with a CXXC active site motif [15,37,38]. They share 30% sequence identity and 49% sequence similarity. DsbG is expressed at approximately 25% the level of DsbC. In contrast to DsbA, the two active-site cysteine residues of DsbC and DsbG are kept reduced in the periplasm [38,39]. This allows the N-terminal thiolate group of the CXXC motif of DsbC or DsbG to attack the incorrect disulfide bond of a target protein, forming a mixed disulfide bond between them. The mixed disulfide bond is then attacked by a thiolate group in the target protein, resulting in a new disulfide bond in the target protein and reformation of a thiolate group in the catalyst (Fig. 3). Alternatively, the disulfide isomerase could simply remove the incorrect disulfide, becoming oxidized in the process.

3.2. DsbC is a V-shaped dimer

McCarthy et al. [4] reported the 1.9-Å resolution crystal structure of DsbC. DsbC forms an overall V-shaped structure where each arm of the V is a DsbC monomer consisting of two separate domains, an N-terminal domain and a C-terminal catalytic domain. The N-terminal domains from each monomer join to form the dimer interface at the base of the V. The C-terminal catalytic domain has a thioredoxin-like fold. The sulfur of the first cysteine of the active site CXXC motif (Cys98) is partially solvent exposed, consistent with its expected function of forming a mixed disulfide bond with a substrate protein. A second disulfide bond is present in DsbC, between Cys141 and Cys163. This disulfide is also partially solvent exposed, but is thought to play a more structural role. This structural disulfide bond is not as highly conserved among DsbCs from different bacterial species as the catalytic disulfide bond. *E. coli* DsbG lacks the second disulfide bond.

3.3. DsbC and DsbG have chaperone-like activity

As native disulfide bonds are generally buried in the structure of folded proteins [40] and often in hydrophobic environments, the reduction of such a disulfide bond must be preceded by a local or global unfolding step that exposes the bond to a reductant. Non-native disulfides could be buried or exposed. The ability of DsbC and DsbG to isomerize disulfide bonds suggests that they might interact with hydrophobic regions of folding, but not with fully folded polypeptides. These properties are reminiscent of those of molecular chaperones. Not surprisingly, DsbC and DsbG appear to possess peptide-binding and molecular chaperone activities, in that they assist the in vitro refolding of model substrates such as lysozyme, glyceraldehyde-3-phosphate dehydrogenase, citrate synthase and luciferase [41,42]. The chaperone activity of DsbG is independent of its redox state [42].

Unlike DsbA, DsbC does not have a helical domain that is large enough to form a cap over the active CXXC site, and the

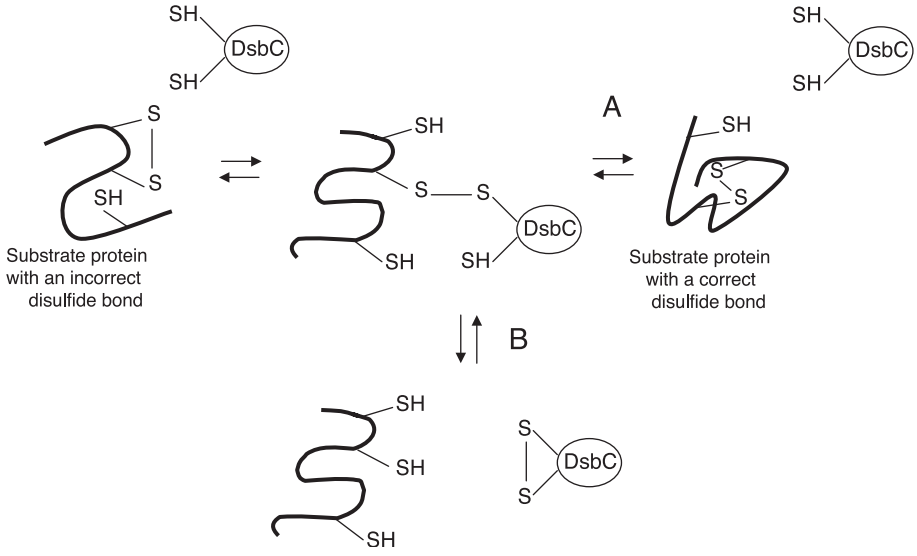

Fig. 3. The isomerization of a wrongly formed disulfide by DsbC. Reduced DsbC attacks an incorrect disulfide bond of a substrate protein and an intermolecular disulfide is formed. Then, the intermolecular disulfide is exchanged for the correct intramolecular disulfide in the substrate protein, releasing DsbC in a reduced state (A). Alternatively, the intermolecular disulfide between the substrate protein and DsbC is exchanged for an intramolecular disulfide within DsbC, releasing the substrate protein in a reduced state and DsbC in an oxidized state (B). This is then followed by an oxidation reaction of the substrate protein catalyzed by DsbA and a reduction of DsbC by DsbD (not shown).

hydrophobic cleft proposed to bind a substrate peptide to DsbA is not present in DsbC. Instead, the inside of the V-like structure of dimeric DsbC is covered with uncharged residues, forming a large central cleft [4]. The active sites of the N-terminal thioredoxin-like domain from each DsbC monomer face inwards across the cleft. Substrate peptides may bind noncovalently to the cleft, allowing the reduced active site Cys98 thiol to attack a substrate disulfide bond forming a covalently bound mixed DsbC–substrate intermediate. A mixed disulfide bond between DsbC and a model peptide composed of residue 4–31 of BPTI is 40- to 100-fold more stable than the corresponding complex between the model peptide and DsbA [43]. The higher stability of a mixed disulfide complex between peptide and DsbC is likely to result from enhanced peptide binding by DsbC's larger cleft, which may allow the peptide to search various conformations for the correct disulfide bond to form. Burial of hydrophobic residues upon protein folding that accompanies correct disulfide bond formation may destabilize the DsbC–substrate protein complex, allowing the correctly folded protein to dissociate from DsbC.

3.4. Dimerization of DsbC is important for isomerase and chaperone activity

The dimeric nature of DsbC is essential for its isomerase function and the thioredoxin domain alone is inactive as an isomerase [44]. Monomeric DsbC also lacks molecular chaperone activity in vitro [44]. These results could indicate that the broad hydrophobic cleft plays an essential role in the association of DsbC with a target protein and its disulfide reshuffling/protein folding activities. It could also be that the presence of two active sites in the DsbC dimer enhances its activity. Evidence in favor of the latter hypothesis came from experiments performed with heterodimers of DsbC where one of the two active sites is inactivated by carboxymethylation [44]. The structural and functional properties of DsbC are reminiscent of those of eukaryotic PDI [45]. PDI is thought to have two roles, the oxidation of disulfides and the isomerization of incorrect disulfides [46]. PDI, a resident of the endoplasmic reticulum, is composed of five separate domains [47]. Four of these domains (except c), a, b, b′, and a′, all belong to the thioredoxin family. These individual domains lack the full isomerase activity exhibited by the complete PDI molecule [48].

3.5. Reduction of DsbC and DsbG by DsbD

DsbC and DsbG have to be kept reduced in the periplasm in order to stay active as isomerases. The reduction of DsbC and DsbG is carried out by the inner-membrane protein DsbD [38,49]. DsbD in turn is kept reduced by the cytoplasmic protein thioredoxin (Fig. 1) [36,39].

DsbD is a 59-kDa protein which consists of three domains: an N-terminal periplasmic domain (α), a central domain with eight transmembrane segments (β) and a C-terminal periplasmic domain (γ). Each domain of DsbD possesses a conserved pair of cysteine residues, which has been shown by site-directed mutagenesis experiments to be required for its disulfide transport activity [50,51].

3.6. A model for the transfer of disulfide bonds across the membrane

Determining the mechanism of action of DsbD is of interest because DsbD and its homologues are the only proteins capable of transporting disulfide bonds across the membrane. It has been postulated that electron flow within DsbD occurs via a succession of disulfide exchange reactions, where disulfides are transferred from DsbC to the α domain, then on to the γ domain, and finally on to the β domain (Fig. 1). The β domain is then reduced by thioredoxin and thioredoxin reductase in an NADPH-dependent reaction [52].

Most of the steps that have been proposed by this model are well characterized. The identification of a mixed-disulfide between thioredoxin and the β domain argues in favor of a direct interaction between thioredoxin and the β domain [52]. The periplasmic steps of the DsbD reaction are even more characterized. Using purified γ and α domains, we showed in vitro that electrons flow from the γ domain to the α domain and then to DsbC or DsbG [53]. We determined the redox potential of the γ and α domains to be −241 and −229 mV, respectively, which indicates that the electron flow is thermodynamically driven. In vitro, we showed that the α domain can efficiently reduce DsbC and DsbG with a K_m of 5 μM. The structure of the γ and α domains have been determined [54,55]. The γ domain has a thioredoxin-like fold whereas the α domain is a part of the immunoglobulin superfamily. The structure of a complex between the α domain and DsbC has also been solved [56]. The α domain binds the central cleft of the V-shaped DsbC dimer. This enables the complex to adopt a closed conformation, allowing exclusive interactions between the two DsbC catalytic domains and the α domain.

3.7. The β domain: DsbD's black box

The membranous part of the DsbD reaction is still obscure. It is clear that the β domain is required to transfer electrons from thioredoxin to the γ domain as removal of the β domain leads to the accumulation of oxidized γ and α domains. We also purified the β domain and showed that it can transfer electrons from thioredoxin to the γ domain in vitro [53].

However, *how* electrons are transferred from the β domain to the γ domain is still unclear. Is it by pure disulfide exchange? Is a cofactor, for instance quinone, required like in DsbB ? So far, there is no evidence that the cysteine residues of the β domain interact directly with the cysteine residues of the γ domain and attempts to isolate mixed disulfides between the two domains were unsuccessful. Moreover, both cysteine residues of the β domain seem

to be on the cytoplasmic side of the membrane [57]. If such is the case, how can they reduce the cysteines of the γ domain, which are in the periplasm? The important question of how electrons are transferred across the membrane is still unanswered and further work is required.

3.8. The oxidation and isomerization systems are kept separated in the periplasm

As we described above, the formation of correct disulfide bonds in a target protein involves two different pathways: (1) disulfide bond formation by DsbA and the reoxidation of DsbA by DsbB and the respiratory system, and (2) disulfide reshuffling or isomerization by DsbC and re-reduction of DsbC by DsbD and the cytosolic thioredoxin system. DsbA is found almost entirely in the oxidized state in vivo, while DsbC is entirely reduced [58]. This indicates that cross-talk between these two systems is avoided in the *E. coli* periplasm.

Why does DsbA or DsbB not oxidize DsbC or DsbD and why does DsbC or DsbD not reduce DsbA or DsbB? DsbA and DsbB have more oxidizing redox potentials than either DsbC or DsbD. Thus, the cross-talk should be thermodynamically driven. Compounding this problem is the observation that DsbB seems to be rather promiscuous in recognizing the thioredoxin folds of different proteins such as DsbA, monomeric DsbC, thioredoxin and PDI.

Biochemical and genetic evidence suggests that DsbD cannot reduce DsbA. The α domain transfers electrons to DsbC, but not to DsbA in vitro [53]. Biochemical evidence suggests that DsbA and DsbB are unable to oxidize DsbC in vivo. DsbA oxidizes DsbC very slowly in vitro, slow enough to essentially eliminate any cross-talk in vivo [15]. DsbB reoxidizes the thioredoxin fold of dimeric DsbC at least 500-fold more slowly than does DsbA [32]. Bader et al. [59] concluded that the two pathways are also partitioned by the dimerization of DsbC. Various mutations in the dimerization interface of DsbC, which prevented dimer formation, allowed the DsbC monomers to become oxidized by DsbB, both in vivo and in vitro, and these DsbC monomers can complement a *dsbA* null mutation. The simplest explanation for these results is that dimerization acts to shield DsbC from the reoxidant DsbB.

4. The practical implications of the DsbA–DsbC pathway for protein expression

The periplasm of *E. coli* is a useful compartment for the expression of proteins with multiple disulfide bonds. It is an oxidative environment containing disulfide bond formation machinery. Just like in the folding of endogenous proteins with disulfide bonds in *E. coli* periplasm as described above, the Dsb proteins play essential roles in the folding of foreign proteins [60]. Overexpression of Dsb proteins has been employed to increase the yield and the stability of the overexpressed recombinant proteins with multiple disulfide bonds [61–63]. However, when expressed in the periplasmic space of *E. coli*, eukaryotic proteins often fail to form the proper disulfide linkages, though they are oxidized to completion. This is a major problem in the commercial expression of pharmacologically active eukaryotic proteins. Almost all *E. coli* proteins contain two or fewer disulfides, and mismatches are not likely to occur. In contrast, many heterologous proteins contain multiple disulfides with complex disulfide bonding patterns; expression in the *E. coli* periplasm often results in ‘‘scrambled’’ proteins as the incorrect thiol groups become linked, thus resulting in a non-native inactive protein. The strong oxidizing power of the disulfide catalyst DsbA and the disulfide isomerase DsbC drives the extremely oxidizing nature of periplasm. These proteins are much more oxidizing than the eukaryotic analogue, PDI. It may be necessary to down-regulate the oxidizing power of these proteins, or enhance the isomerization power of DsbC in order to obtain optimal expression of eukaryotic proteins in the periplasm of *E. coli*.

5. Concluding remarks

Disulfide bond formation in the periplasm is not the result of a spontaneous oxidation reaction. It is a catalyzed process in which each Dsb protein plays a distinct role. Since the discovery of DsbA in 1991, much work has been done and most of the important steps involved in the formation of native disulfide bonds have been unraveled. DsbA and DsbB are responsible for de novo disulfide bond formation while DsbC and DsbD rescue proteins that have non-native disulfides. DsbA is the direct donor of disulfide bonds and is maintained in an oxidized state by the novel quinone reductase activity of DsbB. In this way, the oxidizing power of the DsbA–DsbB pathway is sustained by the electron transport system. Proteins that contain non-native disulfides are freed from this folding trap by the disulfide isomerase DsbC. To function properly, DsbC is maintained in a reduced state by DsbD. DsbD taps into the reducing power of the thioredoxin system in the cytosol and delivers reducing equivalents to DsbC.

However, some very intriguing problems remain unsolved. We still do not know exactly how DsbB can generate disulfide bonds from quinone reduction, a reaction which appears to be the source of the vast majority of disulfides in *E. coli*. DsbD acts to transport disulfides across the membrane; how it does so is almost entirely unclear. Further characterization of the β domain of DsbD will help to understand how DsbD solves this unique problem. Both DsbB and DsbD can be purified in large amounts, so further biochemical as well as structural progress is likely. Another interesting question that remains unanswered is, what is the function of DsbG? If it functions as a disulfide bond isomerase, then why does *E. coli* have two homologous

protein disulfide bond isomerases, DsbC and DsbG? Do DsbC and DsbG have different substrate specificities?

Acknowledgements

We thank Jean-Francois Collet for many suggestions and help in completing this review. We are grateful to James Regeimbal and Annie Hiniker for reading the manuscript.

References

[1] J.C. Bardwell, K. McGovern, J. Beckwith, Identification of a protein required for disulfide bond formation in vivo, Cell 67 (1991) 581–589.
[2] W.J. Wedemeyer, E. Welker, M. Narayan, H.A. Scheraga, Disulfide bonds and protein folding, Biochemistry 39 (2000) 4207–4216.
[3] C.S. Sevier, C.A. Kaiser, Formation and transfer of disulphide bonds in living cells, Nat. Rev., Mol. Cell Biol. 3 (2002) 836–847.
[4] A.A. McCarthy, P.W. Haebel, A. Torronen, V. Rybin, E.N. Baker, P. Metcalf, Crystal structure of the protein disulfide bond isomerase, DsbC, from *Escherichia coli*, Nat. Struct. Biol. 7 (2000) 196–199.
[5] D. Missiakas, C. Georgopoulos, S. Raina, 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 (1993) 7084–7088.
[6] S.J. Stafford, D.P. Humphreys, P.A. Lund, 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 (1999) 179–184.
[7] F.E. Dailey, H.C. Berg, Mutants in disulfide bond formation that disrupt flagellar assembly in *Escherichia coli*, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 1043–1047.
[8] S. Kishigami, Y. Akiyama, K. Ito, Redox states of DsbA in the periplasm of *Escherichia coli*, FEBS Lett. 364 (1995) 55–58.
[9] Y. Akiyama, S. Kamitani, N. Kusukawa, K. Ito, In vitro catalysis of oxidative folding of disulfide-bonded proteins by the *Escherichia coli* dsbA (ppfA) gene product, J. Biol. Chem. 267 (1992) 22440–22445.
[10] J.C. Joly, J.R. Swartz, Protein folding activities of *Escherichia coli* protein disulfide isomerase, Biochemistry 33 (1994) 4231–4236.
[11] A. Zapun, J.C. Bardwell, T.E. Creighton, The reactive and destabilizing disulfide bond of DsbA, a protein required for protein disulfide bond formation in vivo, Biochemistry 32 (1993) 5083–5092.
[12] U. Grauschopf, J.R. Winther, P. Korber, T. Zander, P. Dallinger, J.C. Bardwell, Why is DsbA such an oxidizing disulfide catalyst? Cell 83 (1995) 947–955.
[13] J.L. Martin, J.C. Bardwell, J. Kuriyan, Crystal structure of the DsbA protein required for disulphide bond formation in vivo, Nature 365 (1993) 464–468.
[14] L.W. Guddat, J.C. Bardwell, R. Glockshuber, M. Huber-Wunderlich, T. Zander, J.L. Martin, Structural analysis of three His32 mutants of DsbA: support for an electrostatic role of His32 in DsbA stability, Protein Sci. 6 (1997) 1893–1900.
[15] A. Zapun, D. Missiakas, S. Raina, T.E. Creighton, Structural and functional characterization of DsbC, a protein involved in disulfide bond formation in *Escherichia coli*, Biochemsitry 34 (1995) 5075–5089.
[16] L.W. Guddat, J.C. Bardwell, J.L. Martin, Crystal structures of reduced and oxidized DsbA: investigation of domain motion and thiolate stabilization, Structure 6 (1998) 757–767.
[17] L.W. Guddat, J.C. Bardwell, T. Zander, J.L. Martin, The uncharged surface features surrounding the active site of *Escherichia coli* DsbA are conserved and are implicated in peptide binding, Protein Sci. 6 (1997) 1148–1156.
[18] J. Couprie, F. Vinci, C. Dugave, E. Quemeneur, M. Moutiez, Investigation of the DsbA mechanism through the synthesis and analysis of an irreversible enzyme–ligand complex, Biochemistry 39 (2000) 6732–6742.
[19] C. Frech, M. Wunderlich, R. Glockshuber, F.X. Schmid, Preferential binding of an unfolded protein to DsbA, EMBO J. 15 (1996) 392–398.
[20] J.C. Bardwell, J.O. Lee, G. Jander, N. Martin, D. Belin, J. Beckwith, A pathway for disulfide bond formation in vivo, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 1038–1042.
[21] S. Kishigami, E. Kanaya, M. Kikuchi, K. Ito, DsbA–DsbB interaction through their active site cysteines. Evidence from an odd cysteine mutant of DsbA, J. Biol. Chem. 270 (1995) 17072–17074.
[22] C. Guilhot, G. Jander, N.L. Martin, J. Beckwith, Evidence that the pathway of disulfide bond formation in *Escherichia coli* involves interactions between the cysteines of DsbB and DsbA, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 9895–9899.
[23] M. Bader, W. Muse, T. Zander, J. Bardwell, Reconstitution of a protein disulfide catalytic system, J. Biol. Chem. 273 (1998) 10302–10307.
[24] G. Jander, N.L. Martin, J. Beckwith, Two cysteines in each periplasmic domain of the membrane protein DsbB are required for its function in protein disulfide bond formation, EMBO J. 13 (1994) 5121–5127.
[25] S. Kishigami, K. Ito, Roles of cysteine residues of DsbB in its activity to reoxidize DsbA, the protein disulphide bond catalyst of *Escherichia coli*, Genes Cells 1 (1996) 201–208.
[26] T. Kobayashi, S. Kishigami, M. Sone, H. Inokuchi, T. Mogi, K. Ito, 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. U. S. A. 94 (1997) 11857–11862.
[27] M. Bader, W. Muse, D.P. Ballou, C. Gassner, J.C. Bardwell, Oxidative protein folding is driven by the electron transport system, Cell 98 (1999) 217–227.
[28] K. Inaba, K. Ito, Paradoxical redox properties of DsbB and DsbA in the protein disulfide—introducing reaction cascade, EMBO J. 21 (2002) 2646–2654.
[29] J. Regeimbal, J.C. Bardwell, DsbB catalyzes disulfide bond formation de novo, J. Biol. Chem. 277 (2002) 32706–32713.
[30] H. Kadokura, J. Beckwith, Four cysteines of the membrane protein DsbB act in concert to oxidize its substrate DsbA, EMBO J. 21 (2002) 2354–2363.
[31] U. Grauschopf, A. Fritz, R. Glockshuber, Mechanism of the electron transfer catalyst DsbB from *Escherichia coli*, EMBO J. 22 (2003) 3503–3513.
[32] M.W. Bader, T. Xie, C.A. Yu, J.C. Bardwell, Disulfide bonds are generated by quinone reduction, J. Biol. Chem. 275 (2000) 26082–26088.
[33] H. Kadokura, M. Bader, H. Tian, J.C. Bardwell, J. Beckwith, 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 (2000) 10884–10889.
[34] T. Xie, L. Yu, M.W. Bader, J.C. Bardwell, C.A. Yu, Identification of the ubiquinone-binding domain in the disulfide catalyst disulfide bond protein B, J. Biol. Chem. 277 (2002) 1649–1652.
[35] J. Regeimbal, S. Gleiter, B.L. Trumpower, C.A. Yu, M. Diwakar, D.P. Ballou, J.C. Bardwell, Disulfide bond formation involves a quinhydrone-type charge-transfer complex, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 13779–13784.
[36] A. Rietsch, D. Belin, N. Martin, J. Beckwith, An in vivo pathway for disulfide bond isomerization in *Escherichia coli*, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 13048–13053.
[37] D. Missiakas, C. Georgopoulos, S. Raina, The *Escherichia coli* dsbC (xprA) gene encodes a periplasmic protein involved in disulfide bond formation, EMBO J. 13 (1994) 2013–2020.
[38] P.H. Bessette, J.J. Cotto, H.F. Gilbert, G. Georgiou, In vivo and in vitro function of the *Escherichia coli* periplasmic cysteine oxidoreductase DsbG, J. Biol. Chem. 274 (1999) 7784–7792.
[39] A. Rietsch, P. Bessette, G. Georgiou, J. Beckwith, Reduction of the periplasmic disulfide bond isomerase, DsbC, occurs by passage of electrons from cytoplasmic thioredoxin, J. Bacteriol. 179 (1997) 6602–6608.

[40] J.M. Thornton, Disulphide bridges in globular proteins, J. Mol. Biol. 151 (1981) 261–287.
[41] J. Chen, J.L. Song, S. Zhang, Y. Wang, D.F. Cui, C.C. Wang, Chaperone activity of DsbC, J. Biol. Chem. 274 (1999) 19601–19605.
[42] F. Shao, M.W. Bader, U. Jakob, J.C. Bardwell, DsbG, a protein disulfide isomerase with chaperone activity, J. Biol. Chem. 275 (2000) 13349–13352.
[43] N.J. Darby, S. Raina, T.E. Creighton, Contributions of substrate binding to the catalytic activity of DsbC, Biochemistry 37 (1998) 783–791.
[44] X.X. Sun, C.C. Wang, The N-terminal sequence (residues 1–65) is essential for dimerization, activities, and peptide binding of *Escherichia coli* DsbC, J. Biol. Chem. 275 (2000) 22743–22749.
[45] K.J. Woycechowsky, R.T. Raines, Native disulfide bond formation in proteins, Curr. Opin. Chem. Biol. 4 (2000) 533–539.
[46] R. Noiva, Protein disulfide isomerase: the multifunctional redox chaperone of the endoplasmic reticulum, Semin. Cell Dev. Biol. 10 (1999) 481–493.
[47] D.M. Ferrari, H.D. Soling, The protein disulphide-isomerase family: unravelling a string of folds, Biochem. J. 339 (1999) 1–10.
[48] N.J. Darby, T.E. Creighton, Functional properties of the individual thioredoxin-like domains of protein disulfide isomerase, Biochemistry 34 (1995) 11725–11735.
[49] D. Missiakas, F. Schwager, S. Raina, Identification and characterization of a new disulfide isomerase-like protein (DsbD) in *Escherichia coli*, EMBO J. 14 (1995) 3415–3424.
[50] E.J. Stewart, F. Katzen, J. Beckwith, 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 (1999) 5963–5971.
[51] J. Chung, T. Chen, D. Missiakas, 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 (2000) 1099–1109.
[52] F. Katzen, J. Beckwith, Transmembrane electron transfer by the membrane protein DsbD occurs via a disulfide bond cascade, Cell 103 (2000) 769–779.
[53] J.F. Collet, J. Riemer, M.W. Bader, J.C. Bardwell, Reconstitution of a disulfide isomerization system, J. Biol. Chem. 277 (2002) 26886–26892.
[54] J.H. Kim, S.J. Kim, D.G. Jeong, J.H. Son, S.E. Ryu, Crystal structure of DsbDgamma reveals the mechanism of redox potential shift and substrate specificity(1), FEBS Lett. 543 (2003) 164–169.
[55] C.W. Goulding, M.R. Sawaya, A. Parseghian, V. Lim, D. Eisenberg, D. Missiakas, Thiol-disulfide exchange in an immunoglobulin-like fold: structure of the N-terminal domain of DsbD, Biochemistry 41 (2002) 6920–6927.
[56] P.W. Haebel, D. Goldstone, F. Katzen, J. Beckwith, P. Metcalf, The disulfide bond isomerase DsbC is activated by an immunoglobulin-fold thiol oxidoreductase: crystal structure of the DsbC–DsbDalpha complex, EMBO J. 21 (2002) 4774–4784.
[57] F. Katzen, J. Beckwith, Role and location of the unusual redox-active cysteines in the hydrophobic domain of the transmembrane electron transporter DsbD, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 10471–10476.
[58] J.C. Joly, J.R. Swartz, In vitro and in vivo redox states of the *Escherichia coli* periplasmic oxidoreductases DsbA and DsbC, Biochemistry 36 (1997) 10067–10072.
[59] M.W. Bader, A. Hiniker, J. Regeimbal, D. Goldstone, P.W. Haebel, J. Riemer, P. Metcalf, J.C. Bardwell, Turning a disulfide isomerase into an oxidase: DsbC mutants that imitate DsbA, EMBO J. 20 (2001) 1555–1562.
[60] M. Ostermeier, G. Georgiou, The folding of bovine pancreatic trypsin inhibitor in the *Escherichia coli* periplasm, J. Biol. Chem. 269 (1994) 21072–21077.
[61] J.C. Joly, W.S. Leung, J.R. Swartz, Overexpression of *Escherichia coli* oxidoreductases increases recombinant insulin-like growth factor-I accumulation, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 2773–2777.
[62] Y. Kurokawa, H. Yanagi, T. Yura, Overexpression of protein disulfide isomerase DsbC stabilizes multi-disulfide-bonded recombinant protein produced and transported to periplasm in *Escherichia coli*, Appl. Environ. Microbiol. 66 (2000) 3960–3965.
[63] Z. Zhang, Z.-H. Li, F. Wang, M. Fang, C.-C. Yin, Z.-Y. Zhou, Q. Lin, H.-L. Huang, Overexpression of DsbC and DsbG markedly improves soluble and functional expression of single-chain Fv antibodies in *Escherichia coli*, Protein Expr. Purif. 26 (2002) 218–228.
[64] K. Inaba, Y.-H. Takahashi, N. Fujieda, K. Kano, H. Miyoshi, K. Ito, DsbB elicits a red-shift of bound ubiquinone during the catalysis of DsbA oxidation, J. Biol. Chem. 279 (2004) 6761–6768.