Review

Disulfide bond isomerization in prokaryotes

Stefan Gleiter, James C.A. Bardwell *

Howard Hughes Medical Institute, University of Michigan, Ann Arbor, MI 48109-1048, USA
Department of Molecular Cellular and Developmental Biology, University of Michigan, Ann Arbor, MI 48109-1048, USA

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ABSTRACT

Proteins with multiple cysteine residues often require disulfide isomerization reactions before they attain their correct conformation. In prokaryotes this reaction is catalyzed mainly by DsbC, a protein that shares many similarities in structure and mechanism to the eukaryotic protein disulfide isomerase. This review discusses the current knowledge about disulfide isomerization in prokaryotes.

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1. Introduction

Disulfide bonds are important in the folding of secreted proteins. The formation of disulfides can conceptually be divided into two steps, the oxidation of cysteine residues to form disulfides, and the isomerization of incorrect disulfides to the correct disulfide bonded pattern. For proteins with more than two cysteines, the number of possible incorrect disulfide bonding patterns increases very rapidly with cysteine number, whereas the number of correct disulfide bonding patterns remains constant at one. Thus, disulfide isomerases play vital roles in the folding of proteins, particularly for proteins with multiple cysteine residues. In the early 1960s, Anfinsen and colleagues hypothesized the need for a protein to catalyze disulfide isomerization and discovered an endoplasmic reticulum protein, later called protein disulfide isomerase (PDI), involved in this process [1,2]. More recently, prokaryotes were shown to contain a related protein called DsbC that is involved in disulfide isomerization. Both PDI and DsbC act by thiol-disulfide exchange with secreted proteins, and function to catalyze protein folding [3]. This review focuses on disulfide isomerization in prokaryotes, particularly in *E. coli* since it is the best studied. The process of disulfide oxidation is only described as it is relevant to disulfide isomerization.

2. DsbA is the main oxidase in the periplasm of *E. coli*

Oxidative protein folding is catalyzed by the Dsb proteins present in the periplasmic space of prokaryotes [4]. DsbA serves as the direct donor of disulfides to secreted proteins, whereas DsbC serves as the isomerase of incorrect disulfide bonds. Strains that are mutant in the *dsbA* gene show a severe defect in disulfide bond formation.

DsbA acts by donating its disulfide to secreted proteins, thereby catalyzing their oxidative folding. DsbA oxidizes reduced polypeptides extremely rapidly, with rate constants of about 10^7 $M^{-1}s^{-1}$ at pH 7.5. This is about a million times faster than normal values for disulfide exchange reactions between proteins [43]. DsbA is also one of the most oxidizing thiol-disulfide oxidoreductases known, with a redox potential of −121 mV [5]. Given the very oxidizing redox potential of DsbA, it is not surprising that it can form incorrect disulfides. One reasonable model of DsbA function is that it oxidizes adjacent cysteines (consecutive disulfides) as they are secreted into the periplasmic space. Proteins secreted by the commonly used "sec" secretion apparatus are secreted from the N- to C-terminus. Thus, one simple model would be that DsbA may only be able to form correct disulfides if they all form between cysteines that are adjacent in the sequence [6]. Any other disulfide configuration (non-consecutive disulfide) would then require the disulfide isomerase DsbC. Thus, the paradigm that has been guiding the disulfide bond formation field for some time is that DsbA functions entirely as an oxidizing thiol-disulfide oxidoreductase, whereas DsbC functions as a disulfide isomerase. This is in contrast to the situation in the eukaryotic field, in which a single protein disulfide isomerase is thought to play roles in both isomerization and oxidation.

3. DsbB is a membrane protein that reoxidizes DsbA

The oxidation of a substrate protein by DsbA reduces DsbA. In order to be catalytic, DsbA must be reoxidized; this reoxidation is performed by the membrane protein DsbB. DsbB generates protein disulfides de novo, via the reduction of quinones [8,9]. DsbB is thus the

primary source of disulfides in prokaryotic organisms. Strains that have mutations in the *dsbB* gene showed accumulation of reduced DsbA in the periplasm and exhibited the same pleiotropic phenotypes as *dsbA* mutants. DsbB is reoxidized by components of the respiratory chain, which accept electrons from DsbB. Under aerobic conditions, DsbB transfers the electrons to oxidized ubiquinone. The reduced ubiquinone then becomes reoxidized by the terminal cytochrome oxidases, which finally transfer the electrons onto molecular oxygen [8,10]. Under anaerobic conditions, DsbB transfers electrons to menaquinone, which then passes them to the final electron acceptors fumarate or nitrate [8]. DsbB has four membrane-spanning segments and two pairs of essential cysteines, Cys^{41}-Cys^{44}, and Cys^{104}-Cys^{130}. One of each pair is located in each of the two periplasmic domains [11]. These cysteines are involved in a disulfide cascade reaction within DsbB that ends up transferring a disulfide to DsbA [9].

4. DsbC, a periplasmic isomerase

The role of the disulfide isomerase proteins DsbC and possibly DsbG is the isomerization of incorrect disulfides [3,12,13]. These members of the Dsb family were identified by two different genetic approaches [3,13]. DsbC and DsbG share 24% sequence identity with each other and about 10% sequence identity to thioredoxin [3,14].

DsbC can rearrange disulfides in vitro and in vivo. The addition of catalytic amounts of DsbC to fully reduced and denatured BPTI in vitro promotes the formation of correct disulfides in the presence of GSH/GSSG and leads to active BPTI [15]. In this case glutathione oxidizes BPTI and potentially incorrect disulfides are isomerized by DsbC. In an in vivo study BPTI was oxidized in the presence of DsbA, leading to mispaired disulfides, which were corrected in a DsbC-dependent manner. This is consistent with the results that we have obtained with RNaseA. DsbA can oxidize reduced RNaseA to the point that no free thiols are available, but this does not result in significant RNaseA activity; only upon addition of DsbC is RNaseA activity recovered [16]. We interpreted this to mean that the DsbA oxidizes the protein to completion but incorporates at least one incorrect disulfide, and DsbC then reshuffles the disulfides to the correct configuration. As shown in Fig. 1 there are several possible mechanisms by which DsbC may isomerizes incorrect disulfide bonds. DsbC attacks incorrect disulfides forming a mixed disulfide between the more N-terminal cysteine of DsbC and the protein substrate (step 1 in Fig. 1). This disulfide may be immediately transferred back to the protein substrate resulting in a different and in this case correct disulfide bond pattern (step 2 in Fig. 1). This mechanism is called the shuffling model for the purposes of this review. Alternatively the mixed disulfide may be resolved such

that DsbC is oxidized and the substrate is reduced (step 3 in Fig. 1) The reduced substrate may then be reoxidized by DsbA or DsbC (step 4 or 5 in Fig. 1). This mechanism is called the reduction/reoxidation model. This may result in a correct pattern of disulfides. Since correct disulfides are, in general, stable and buried they will not be attacked by reduced DsbC so steps 2 and 5 can be considered essentially irreversible. Incorrect disulfides in contrast are expected to be exposed and susceptible to attack and will undergo another round of isomerization. Steps 1, 3 and 4 can be considered to be reversible. It should be noted that often, particularly in the case where consecutive disulfides are correct, DsbA will correctly form disulfides in the first place, eliminating the need for isomerization.

Some evidence against the shuffling model and in favor of the reduction/reoxidation mechanism are the observations by Rietsch et al. [33]. They showed that DsbC mutants that lack the more C-terminal cysteine are strongly deficient in the expression of urokinase, implying that they are deficient in isomerization. They also showed that in a DsbA, DsbD double mutant, DsbC's active site cysteines are in a reduced state, while in a DsbD mutant DsbC is in the oxidized state. This seems to indicate that the oxidation of DsbC's active site cysteines is dependent on DsbA. Since DsbA and DsbC do not directly interact, the oxidation of DsbC likely takes place through DsbA oxidizing protein substrates which are then reduced by DsbC, favoring the reduction/isomerization model.

Compared to PDI, a well-studied eukaryotic isomerase, DsbC is not as catalytically active, with PDI being 10-fold faster in regard to the disulfide rearrangement in bovine pancreatic trypsin inhibitor (BPTI) [15]. Compared to DsbA, however, DsbC is much more active as an isomerase in the in vitro refolding of Ragi bifunctional inhibitor (RBI), a protein with five nonconsecutive disulfides. DsbC seems to be particularly efficient in rearranging buried, nonnative disulfides that have been formed by treatment of RBI with oxidants such as GSSG [17].

In vivo DsbC has been shown to increase the yield of overall correctly disulfide bonded *E. coli* and eukaryotic proteins with non-consecutive disulfide bonds. For example, the formation of active murine urokinase containing 12 disulfides is decreased 100-fold in a *dsbC⁻* strain compared to the wild-type strain, which indicates that DsbC is required for the formation of the correct disulfide bonds [18]. Joly and Swartz investigated the expression of a variety of eukaryotic proteins in a *dsbC⁻* strain [19]. The influence of DsbC on the formation of active eukaryotic proteins strongly depends on the model protein used, and whether the disulfides are consecutive or nonconsecutive. For melanoma growth stimulating activity (MGSA) and insulin-like growth factor 1 (IGF-1), proteins that require nonconsecutive
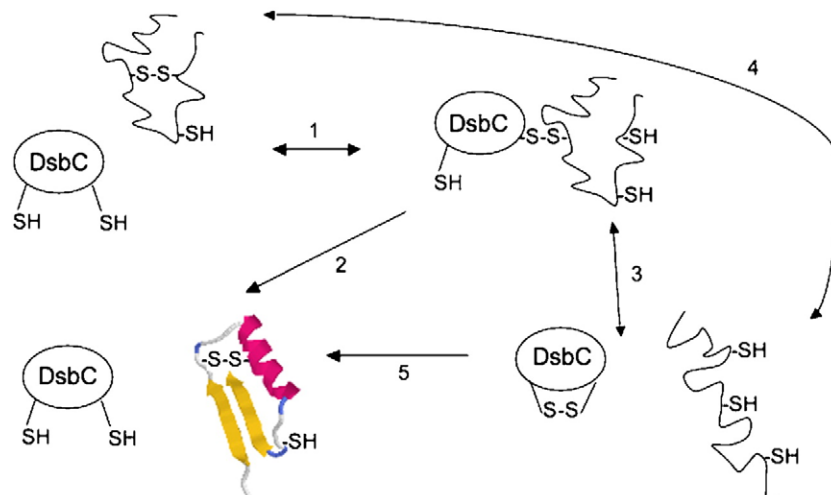

Fig. 1.

disulfides, the absence of DsbC reduced the amount of expressed protein. However, human growth hormone (hGH) and anti-CD18 antibody, proteins that contain consecutive disulfides, showed similar expression levels in a *dsbC*− and a wild-type strain [19]. The assembly of pertussis toxin, which requires 11 consecutive disulfides, requires DsbA but not DsbC [20]. Periplasmic proteins whose disulfide bonding pattern are known to be decreased in amount in *dsbC*− strains include RNaseI, and phytase, both of which contain a non-consecutive disulfide [21]. This ability of DsbC to specifically assist in the expression of proteins with nonconsecutive disulfides strongly suggests that DsbC acts as an isomerase.

Perhaps the strongest evidence for a specific requirement of DsbC in folding proteins with nonconsecutive disulfides comes from experiments conducted with phytase. Phytase is an *E. coli* periplasmic protein that contains three consecutive disulfide bonds and one nonconsecutive one. Its expression is DsbC-dependent, unless the nonconsecutive disulfide is removed by mutation, when it becomes DsbC-independent [6]. It has been proposed that DsbA acts immediately on proteins as they are translocated into the periplasm. Evidence for this also comes from a mutant in alkaline phosphatase (AP) in which the most amino-terminal cysteine was replaced with serine. Alkaline phosphatase normally contains two consecutive disulfides, one that links the first and second cysteines in the protein, and one that links the third and fourth. Upon removal of the first cysteine in AP, DsbA forms an incorrect disulfide. This disulfide is slowly resolved in a DsbC-dependent reaction. Interestingly, more active alkaline phosphatase results from expression of this mutant AP in a *dsbA* null strain than is formed in a *dsbA*+ strain. This implies that DsbA may be too oxidizing for the expression of proteins with nonpaired cysteines [7]. However, a recent report found that RNaseI is oxidized correctly by DsbA, even though it has a nonconsecutive disulfide [44].

The abundance of a number of proteins, including MepA, End1, YebF, and Ivy, is decreased in the absence of DsbC, suggesting that these *E. coli* proteins are in vivo substrates for DsbC [22]. A larger number of proteins are decreased in amount in *dsbA*− strains, implying that DsbA, as the presumptive oxidase, is more important for the folding of proteins than is DsbC, the presumptive isomerase. Darby et al. measured the rates at which a 28-mer model peptide reacts with DsbC and compared it with the rates at which DsbA reacts with the same peptide. To function as an isomerase, DsbC needs to attack incorrect disulfide bonds. The initial rate at which DsbC reacts with the model peptide's disulfide is about 6×10^3 more rapid than the same reaction of the oxidized peptide with reduced DsbA. For isomerization to occur, a free thiol needs to attack this mixed disulfide, resulting in a different disulfide. This reaction occurs 26- to 43-fold more rapidly with DsbC than it does with DsbA. These kinetic differences help explain the greater isomerase activity of DsbC than DsbA [23].

The crystal structure of oxidized DsbC [24] reveals two 23 kDa monomers of DsbC forming a V shaped homodimer [24]. Each monomer of DsbC consists of two domains: a C-terminal thioredoxin-like domain containing the active site Cys[98]-Gly-Tyr-Cys[102] motif, a structural disulfide, and an N-terminal dimerization domain connected via a flexible linker helix. Mutations that remove the active site cysteines, as expected, show that they are required for the thiol-disulfide exchange activities of DsbC, but not the chaperone activity. Mutations that remove the structural disulfide increase the sensitivity of DsbC to denaturants and decrease its folding rate [25]. Dimerization of DsbC acts to form a large cleft built of uncharged and hydrophobic residues, which may be the binding site for substrate proteins of DsbC. The active site cysteines of each monomer are oriented facing each other. The thioredoxin-like domains are separated from the dimerization domain by a linker alpha helix. Relative movement of the active sites and the presumptive peptide binding cleft at the junction of the arms of the V may be important in DsbC's isomerase or chaperone activities. It has been suggested that the cleft may act to both bind and

partially shield incompletely folded proteins while the protein explores various conformations and disulfide bonding patterns [24]. DsbC reacts with peptides up to a million-fold faster than it reacts with glutathione, and the mixed disulfide formed between the peptide and DsbC is 10,000 times more stable than glutathione-DsbC mixed disulfides. Noncovalent binding interactions that occur between the peptide and DsbC likely contribute to these very rapid kinetics of substrate utilization and the increased stability of the mixed disulfide [23]. In reduced DsbC, the catalytic domains are shifted and twisted up to 13 Å around an alpha helical hinge located between the catalytic and dimerization domains. This apparent flexibility of DsbC may play a role in accommodating substrates in the cleft between the catalytic domains.

The function of DsbC as isomerase and chaperone is strictly dependent on it's dimerization [26]. A screen to find mutants of *dsbC* that complement a *dsbA* deficiency revealed that mutations in the dimerization domain of DsbC lead to monomeric DsbC that can act as an oxidase and, in addition, can interact with DsbB to become reoxidized [27]. The dimerization of DsbC therefore, is a structural safeguard against the interaction with DsbB, protecting the chaperone and isomerase functions of DsbC. A heterodimer that has had one of the CXXC active sites inactivated by carboxymethylation has no isomerase activity [26]. This suggests strongly that both active sites of the dimer are required for isomerization.

Deletion mutations in the alpha helical linker domain that connects the dimerization domain to the catalytic domain allow DsbC to be oxidized by DsbB and also decrease DsbC's isomerization activity [29,30]. In wild-type DsbC, the active site cysteines are facing each other; these deletion mutations probably function by rotating the catalytic domains with respect to each other. The ability of the mutants to oxidize proteins was inversely related to their ability to isomerize incorrect disulfide bonds. A number of orientations (including an inward pointing orientation) of the active sites appear to be associated with high isomerase activity; other orientations leave DsbC accessible to DsbB, and DsbC thus acquires oxidase activity. All of the alpha helical linker truncations tested by Segatori et al. are capable of serving as both an oxidant and an isomerase [29,30]. This mimics the situation present in eukaryotes, in which protein disulfide isomerase is thought to serve both roles.

Further evidence that the presence of two active sites is important in facilitating isomerase action is the observation that the addition of a dimerization domain onto monomeric thioredoxin-like proteins generates a protein with significant isomerase activity in vivo and in vitro. Dimerization of DsbA, thioredoxin, or monomeric PDI domains by the fusion of these proteins to the dimerization domain of DsbC results in a protein that is capable of supporting the formation of active vtPA in the periplasm and RNaseA in vitro, indicating that these artificial dimeric proteins have acquired disulfide isomerization activity [29,30]. Formation of a mixed disulfide between these dimeric disulfide isomerases and their substrates will greatly increase the local concentration of the active sites relative to the substrate. In contrast, monomeric disulfide oxidoreductases like thioredoxin and DsbA are very inefficient disulfide isomerases. This higher isomerase activity of DsbC compared to DsbA is proposed to be due to a number of factors in addition to the increased effective concentration of active sites upon formation of mixed disulfides; these factors include a higher peptide affinity and a slower and less random oxidation of substrates by DsbC.

It appears that the equilibrium redox potential alone does not determine whether a protein primarily functions in the cell as an oxidase or an isomerase, since DsbC and DsbA both have very similar and strongly oxidizing redox potentials, and both react very rapidly with folding proteins and peptides [15]. The ability of mutants in DsbA to catalyze the folding of multidisulfide proteins does not correlate with their redox potential [31]. To function as an isomerase, DsbC is kept in the reduced form in vivo by DsbD [18,19,28,32,33]. In contrast,

DsbA functions as an oxidase in part because it is kept in the oxidized state by DsbB. Large kinetic barriers separate the oxidation pathway from the isomerization pathway. Nonphysiological interactions between DsbC-DsbB, DsbA-DsbD, and DsbC-DsbA are three to seven orders of magnitude slower than the physiological interactions [34].

DsbC mutants are copper sensitive. Copper is a redox metal, which catalyzes disulfide bond formation and appears to introduce incorrect disulfides more frequently than DsbA does. Thus, the copper sensitivity of *dsbC⁻* strains may arise from the inability of the cell to rearrange copper-catalyzed incorrect disulfide bonds. These results suggest that the primary role of DsbC may be to rearrange incorrect disulfide bonds that are formed during certain forms of oxidative stress [35]. The structure of DsbC in complex with a fragment of DsbD revealed that the large cleft can adopt two conformations: an open and a closed conformation [36]. This flexibility presumably allows DsbC to adopt the cleft for different substrates. The interface between the DsbC-DsbD alpha subunit involves only residues from the thioredoxin domain of DsbC. The contact residues are highly conserved, which helps explain how a variety of DsbC homologues isolated from gamma proteobacteria are capable of complementing *E. coli* DsbC.

In addition to functioning as an isomerase, DsbC also has chaperone activity and can assist the refolding of single chain Fv fragments [37], lysozyme, and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). For the latter two proteins, this chaperone activity is independent of the presence of DsbC's active site cysteines [25]. The dual mode of action of DsbC is probably the key to its functionality. As a chaperone, DsbC recognizes misfolded proteins, then the isomerase function resolves incorrectly formed disulfides. DsbC's ability to bind to peptides probably contributes to its chaperone activity. A mixed disulfide between a peptide derived from BPTI and DsbC is 100-fold more stable than between the peptide and DsbA [23].

5. DsbG, a second disulfide isomerase?

Although multigene families are less common in prokaryotes than they are in eukaryotes, they do occasionally occur, the disulfide isomerase family is one example. In general individual members of multigene families are assumed to have different substrate specificities, patterns of expression, or localizations. In some cases overlapping substrate specificity and expression patterns may lead to redundancy. A second putative isomerase in the periplasm of *E. coli*, DsbG, was discovered using a DTT based screen and because of its 24% sequence identity to DsbC [12]. Originally DsbG was described as a disulfide oxidase [12], but it is now thought that DsbG acts as a second isomerase in the periplasm of *E. coli* [14]. In vitro, DsbG has chaperone and isomerase activity [14,38]. Overexpression of DsbG, like DsbC overexpression, enhances the proper expression of single chain Fv antibody fragments, and the effect of DsbC and DsbG appears to be additive. However, DsbG does not catalyze insulin reduction, a standard thiol-disulfide redox reaction, and fails to catalyze the oxidative refolding of RNaseA [14]. The structures of DsbC and DsbG closely resemble each other [39]. However, DsbG is significantly larger because the helix that links the dimerization and catalytic domains is about 2.5 turns longer in DsbG. This acts to substantially increase the dimensions of the substrate binding cleft. In addition, DsbG possesses some conserved acidic residues in the generally hydrophobic substrate binding cleft that are not present in DsbC. These differences have led to the hypothesis that DsbC and DsbG vary in substrate specificity. DsbC is proposed to interact with unfolded hydrophobic polypeptide chains, whereas DsbG is proposed to interact with larger, at least partially folded substrates with charged surfaces [39]. Although a number of *E. coli* substrates are known for DsbC, none has yet been detected for DsbG. DsbG is normally expressed at lower levels than is DsbC. Since no protein that is abundant under normal laboratory growth conditions is present in dramatically reduced

concentrations in DsbG null mutants it seems likely that DsbG is acting on proteins that are not normally abundant. In vivo, over-expression of DsbG in *dsbC⁻* strains allows the production of comparable levels of BPTI as in *dsbC⁺ E. coli* strains, suggesting that DsbG also functions in vivo as an isomerase [14]. Mutations in DsbG have been isolated that allow it to complement the copper sensitivity of *dsbC* null strains [40].

That simple overexpression of the isomerases DsbC and DsbG assists in the folding of a number of eukaryotic proteins indicating that the periplasm of *E. coli* can be altered to improve the folding of eukaryotic disulfide containing proteins. Most peptides and proteins with therapeutic applications contain disulfides. The proper formation of these disulfides is one of the rate limiting steps in the commercial production of these proteins. Because the purification of the correctly disulfide bonded form of the protein as the active pharmaceutical ingredient turns out to be very complicated when multiple possibilities of disulfide bonding are possible, these therapeutic proteins are very expensive. Further optimization of the periplasm for thiol-disulfide exchange is clearly of great interest for the commercial production of disulfide containing proteins. It has been observed that DsbC variants that contain alterations in the dipeptide sequence between the active site cysteine residues increase the yield of mouse urokinase up to five-fold [31]. Further, optimization of the periplasm for thiol-disulfide exchange is clearly of great interest for the commercial production of disulfide containing proteins. Selection for chromosomal mutations that enhance the folding of tissue plasminogen activator yielded mutants that up-regulate DsbC due to mutations that result in reduced function of RNaseE. This suggests a critical role of RnaseE in regulating DsbC expression [41].

6. Comparison between the prokaryotic disulfide isomerases DsbC and DsbG, and eukaryotic PDI

The recently solved crystal structure of eukaryotic PDI reveals a number of striking similarities to DsbC and DsbG, including domain arrangement, active site location, and surface features [42]. Interestingly, these structural similarities are in large part due to convergent evolution. Nevertheless, the overall effect is very similar. Both prokaryotic and eukaryotic disulfide isomerases have a V or U shape, with two active site CXXC motifs embedded in a thioredoxin-like fold that face each other across the U. The eukaryotic PDI achieves this by covalently linking four thioredoxin domains, with the N- and C-terminal domains possessing the active site CXXC motifs, which face each other across the interior of the U; the two internal domains are catalytically inactive. In contrast, DsbC and DsbG utilize a thioredoxin unrelated domain to dimerize two monomers, each of which possesses a single CXXC active site containing a thioredoxin-like domain. It appears that two thioredoxin-like domains in close spatial proximity on opposite sides of a large cleft are required for disulfide isomerase activity. Individual catalytic domains of PDI, though redox active, are essentially inactive as isomerases, and heterodimers of DsbC in which one active CXXC motif is carboxymethylated are also inactive as an isomerase in vivo [26]. The two active sites of PDI presumably act in a concerted fashion on its substrates.

Acknowledgements

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