Key Players Involved in Bacterial Disulfide-Bond Formation

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Protein Folding in the Cell

It has long been known that sufficient information for the correct folding of a protein is encoded in its amino acid sequence.[1] Although the information for the final fold of a protein is encoded in its primary sequence, other proteins have been implicated in the folding process in vivo.[2] Some of these proteins catalyze covalent interactions, such as disulfide-bond formation, required for the proper folding of such substrates. This review summarizes what is known about the catalysis of disulfide-bond formation in prokaryotes.

Disulfide-Bond Formation

Disulfide bonds form between the thiol groups of cysteine residues. Disulfide bonds are widely found in many exported proteins and contribute to the folding and stability of these proteins.[3–5] In contrast, disulfide bonds are very rare in cytosolic proteins. Occasionally they are formed transiently as part of the catalytic mechanism or are used to regulate enzymatic activity, but with rare exceptions, are not known to be used to stabilize cytosolic proteins. An overall description of the reactions that create (oxidize) or destroy (reduce) disulfide bonds can be represented by the following general redox scheme:

$$R{-}SH + R'{-}SH \rightleftharpoons R{-}S{-}S{-}R' + 2e^- + 2H^+ \quad (1)$$

The forward direction of Equation (1) is an oxidation reaction with respect to the thiols, and the reverse is a reduction reaction with respect to the disulfide. The oxidation of thiols requires an appropriate electron acceptor, and the reduction of a disulfide requires an electron donor. In vivo, the ultimate electron acceptor for thiol oxidation is usually O_2, except under anaerobic conditions where a suitable anaerobic electron acceptor such as fumarate is used. The ultimate source of electrons for disulfide reduction is usually NADPH.

In vivo, the oxidation, reduction, and isomerization of disulfides also involve multistep thiol–disulfide exchange reactions. Within these reactions, disulfides are neither created nor destroyed. Rather, they are transferred from one set of thiols to another [Eq. (2)].

$$R{-}S{-}S{-}R' + R''{-}SH \rightleftharpoons R{-}S{-}S{-}R'' + R'{-}SH \quad (2)$$

When R′ and R″ are on separate protein molecules, this reaction is an intermolecular disulfide exchange reaction. If they are on the same protein, this reaction is an intramolecular disulfide exchange, also known as disulfide isomerization. Both types of reactions occur in vivo. Disulfide isomerization reactions are especially important in proteins that contain multiple cysteine residues.

Mechanistically, the thiol–disulfide exchange occurs through the nucleophilic attack by a deprotonated incoming thiolate anion on one of the members of a disulfide; this displaces the other member as a thiolate anion. Thiols with lower pK_a values will serve as better nucleophiles (will be more reactive) because they will be deprotonated at physiological pH. Similarly, thiol–disulfide exchange will be enhanced if the leaving group can stabilize the negative charge of the thiolate anion. To occur to an appreciable extent, the exchange reaction should occur down a redox gradient in which electrons are donated from a molecule with a more negative to a more positive redox potential.

Disulfide-Bond Formation in vivo

In eukaryotes, an enzyme known as protein disulfide isomerase (PDI) is found in the endoplasmic reticulum (ER), and is responsible for catalyzing disulfide-bond formation as well as disulfide-bond rearrangements. In prokaryotes like *Escherichia coli*, enzymes such as thioredoxins (TrxA, TrxC) and glutaredoxins (GrxA, GrxB, GrxC) exist that serve as disulfide reductants in the cytoplasm. Herein, another group of proteins (DsbA, DsbB, DsbC, DsbD, DsbE, DsbG) involved in disulfide-bond formation or isomerization in prokaryotes will be reviewed.

DsbA, a Catalyst in Disulfide-Bond Formation

In vitro disulfide-bond formation is slow and susceptible to mistakes, while the in vivo process is fast and precise.[6,7] The search for agents that catalyze disulfide-bond formation has led to the discovery of DsbA by two separate groups.[8,9]

A genetic selection that made use of fusion of a cytoplasmic protein β-galactosidase and an inner-membrane protein MalF led to the identification of DsbA mutants.[8] Bardwell et al. showed that DsbA mutants have a defect in in vivo disulfide-bond formation in alkaline phosphatase, outer-membrane protein OmpA, or β-lactamase, and are resistant to M13 phage due to a defect in F pilus assembly.[8] They described the nonessential *dsbA* gene as coding for a 21 kDa periplasmic protein

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with a C30-P31-H32-C33 motif characteristic of the CXXC active sites of the thiol–disulfide oxidoreductase family, which includes protein disulfide isomerase (PDI), thioredoxin, and glutaredoxin.[10,11] They found that DsbA is able to reduce the disulfide bonds of insulin, an activity characteristic of disulfide oxidoreductases. The results of this work show that DsbA is required for disulfide-bond formation in the cell.

Substrates of DsbA

Although *dsbA* is nonessential for cell viability, disulfide-bond formation is crucial for the folding and stability of many secreted proteins. Protein-folding studies with DsbA have shown that DsbA stimulates disulfide-bond formation in in vitro-translated alkaline phosphatase as well as in denatured, reduced alkaline phosphatase and bovine RNaseA.[12,13] Reduced DsbA converts misfolded insulin-like growth factor I (IGF-I) to the correctly folded version, and oxidized DsbA stimulates refolding of reduced IGF-I.[14] Although DsbA has some isomerase activity, this activity might be physiologically insignificant. Comparison studies with bovine pancreatic trypsin inhibitor (BPTI) and α-lactalbumin as substrates show that its isomerase activity is much less than that of PDI.[15] Various groups have found that DsbA forms the disulfide bond in extracellular pullulanase,[16] type IV pilus,[17] heat-labile enterotoxin,[18] and heat-stable enterotoxins.[19,20] DsbA is required for formate-dependant nitrite reduction and cytochrome c synthesis,[21] and the secretion of ST_B, an extracellular heat-stable enterotoxin,[22] as well as folding of Cu,Zn superoxide dismutase.[23] Our laboratory has recently found ten substrates (DegP, PhoA, DppA, RNase I, FlgI, HisJ, LivJ, FliC, YggN, YbeJ) for DsbA by 2D gel analysis.[24] A number of the substrates we recovered coincide with the potential DsbA substrates found in collaboration with the Beckwith laboratory (OmpA, YodA, ZnuA, GltI, LivK, LivJ, DppA, OppA, OstA/Imp, YedD, RcsF, YbjP, YibQ, YcdO).[25]

The detection of these latter substrates involved the use of a mutation in DsbA that alters *cis*-Pro151. This mutant has the unique ability to stabilize disulfides. This process is thought to occur via formation of mixed disulfide complexes between DsbA and its substrates. However, these complexes are difficult to detect, probably because of their short-lived nature. Here we show that it is possible to detect such covalent intermediates in vivo by a mutation in DsbA that alters *cis*-Pro151. Further, this mutant allowed us to identify substrates of DsbA. Alteration of the *cis*-proline residue, highly conserved among thioredoxin superfamily members, might be useful for the detection of substrates and intermediate complexes in other systems.

DsbA Homologues

The Dsb system is widely distributed in many prokaryotic organisms. This highlights the importance of such enzymes in the cell. The organisms in which DsbA orthologs were first studied include *Vibrio cholerae*, *Haemophilus influenzae*, and *Erwinia chrysanthemi*. TcpG (*t*oxin *c*oregulated *p*ilus *G*) is a homologue of DsbA in *V. cholerae* found to be required for secretion of cholera toxin.[18,26,27] Por (*p*eriplasmic *o*xido*r*eductase), a functional homologue of DsbA in *H. influenzae*, is required for the assembly of cell envelope proteins involved in transformation.[28] DsbA of *E. chrysanthemi* is needed for full pectate lyase and cellulase activities,[29] while DsbA of *Shigella flexneri* facilitates release of invasions into the external medium.[30] Bdb, a secreted protein that can complement *E. coli* DsbA, is found in the gram-positive bacterium *Bacillus brevis*,[31] while another DsbA homologue is found in *Azotobacter vinelandii*.[32] Virulence proteins, Yops, in *Yersinia pestis*, need to be secreted by a secretion apparatus made of many proteins, one of which is YscC, and DsbA is required for disulfide-bond formation in YscC.[33] SrgA, a paralogue of DsbA in *Salmonella enterica*, is required for production of plasmid-encoded fimbriae.[34]

Properties of DsbA

To determine the basis for the oxidizing activity of DsbA, Yu et al. mutagenized the two active site cysteines of DsbA of *V. cholerae* and discovered that such mutations abolished assembly of the pentameric complex of enterotoxin, thus concluding that the cysteines are vital for the activity of DsbA.[35] The properties of these active site cysteines have been well studied in *E. coli*. The apparent pK_a of *E. coli* DsbA Cys30 is 3.5, a value that is extremely low as compared to approximately 9 for that of cysteines in unfolded peptides. The pK_a of Cys33, in contrast, is abnormally high (>10).[36] Because thiolate ions are active as the attacking species in thiol–disulfide exchange reactions, the pK_a of the cysteine is an important parameter for its reactivity. The pK_a of an amino acid characterizes the extent of ionization at any pH, hence affecting its reactivity. The very low pK_a of Cys30 in the active site of DsbA means that it is fully ionized over the entire physiological pH range and therefore is very reactive. This very low pK_a of Cys30 also allows DsbA to be involved in disulfide exchange reactions at acidic pH at rates not very different from that at neutral pH. This allows disulfide-bond formation to occur even when the bacterium is living in an acidic medium.[15,37,38]

Factors Contributing to the Different Redox Potentials of Members of the Thioredoxin Family

The active site cysteines in DsbA are separated by two amino acids, Pro31 and His32. Pro31 enhances the electrostatic interaction of the thiolate ion with the helix dipole,[39] while His32 electrostatically stabilizes the thiolate ion.[40,41] These two effects are important for fostering the low pK_a of Cys30, and making it so reactive. It was found that the fluorescence of the Trp76 residue of DsbA increases threefold upon reduction of the enzyme, and this characteristic was used to measure the redox potential of DsbA.[42,43] What contributes to the different redox potentials of members of the thioredoxin family? Why does DsbA have a redox potential of -122 mV,[38,42] which is so much more oxidizing than that of thioredoxin (-270 mV)[44] or PDI (-180 mV)?[45] It has been shown that changing the two residues between the active site cysteines in the active site

CXXC motif of thioredoxin-like proteins alters their redox potentials accordingly. DsbA has a CPHC, thioredoxin has a CGPC, and PDI has a CGHC active site. Substituting the two residues between the cysteines for each other moves the redox potential in the direction from which the residues are from, making a molecule more oxidizing when it has residues from a more oxidizing protein and vice versa.[46–51] The replacements do not, however, generate mutants with the exact same redox potentials as the wildtype proteins. The redox potentials are therefore not entirely determined by the amino acid residues in the active site. The two residues between the active site cysteines were mutated, and it was found that the pK_a of Cys30 increases with increased severity of mutation affecting the activity of DsbA, and that it varies in proportion to the oxidizing potential of the mutants. The oxidizing power of wildtype DsbA and its active-site mutants can be predicted just from considering the pK_as of the thiol groups involved.[52] In silico computation performed by Warwicker and Gane showing matches between the calculated pK_as for the DsbA mutants and the actual measured values, is added proof that researchers in this field understand the molecular dynamics determining the pK_a as well as the oxidizing power of DsbA.[41] The abnormally low pK_a of the active-site Cys30 residue in DsbA thus is a major determinant of its unusually oxidizing redox potential.

The highly oxidizing nature of DsbA can also be understood by considering the unstable nature of its disulfide bond. It was found that DsbA is destabilized by its disulfide bond, and that reactivity with reduced glutathione (GSH) is 1000 times greater than what is expected for a normal disulfide bond; this indicates that DsbA is a strong oxidant with a highly reactive disulfide bond well suited for driving the formation of disulfides.[38] The free energy of stabilization of oxidized and reduced DsbA for the chloride-induced folding/unfolding transition was calculated, and it was found that the reduced form is more stable.[53] However, rather than considering the oxidized form to be specifically destabilized, it is easier to think of it in terms of the reduced form as being stabilized. It has been shown that His32 stabilizes reduced Cys30; the negative charge on Cys30 could possibly be involved in stabilizing interactions with His32 and the partial positive charges of the helix dipole.[54] A study of mutant proteins in which His32 is altered has shown that such substitutions increase the stability of oxidized DsbA and that the increase in stability is probably due to electrostatic effects.[52,55] The negatively charged tyrosine substitution of His32 could, for example, interact with the partial positive charge of the helix dipole, or tyrosine could form stabilizing hydrophobic interactions with Phe36, a residue implicated in substrate binding, forming part of the proposed substrate binding groove.[55] It was suggested that the low pK_a of Cys30 that is dependent on the residues between the cysteines makes DsbA such an oxidizing catalyst.[52] Thus, the oxidizing power of DsbA can be understood in simple electrostatic terms.

Structure of DsbA

The structure of DsbA was solved to 2 Å resolution,[56] later refined to 1.7 Å resolution.[40] The structure of DsbA resembles that of thioredoxin even though the overall sequence homology between the two proteins is low.[56] One additional difference is that there are 76 extra residues in DsbA that comprise a helical domain inserted into the thioredoxin-like fold at position 63. This causes the active site of DsbA to be flanked by the thioredoxin (residues 1–62, 139–189) and helical domains (residues 63–138; Figure 1). Cys30 is exposed and on the surface of the molecule. Chemical modification of DsbA confirms that only one of the two cysteine residues (Cys30) is exposed and reactive, as only Cys30 can be modified in the absence of denaturants.[38] The buried Cys33 can only react with Cys30 and affect its reactivity.[36] Replacement of Cys30 and/or Cys33 with alanines indicates that Cys30 alone is sufficient to allow for a low level of catalysis of disulfide interchange reactions.[57] It therefore appears that a strongly nucleophilic thiol group, Cys30, and high reactivity of mixed disulfides between Cys30 and the substrate, are sufficient for disulfide interchange to occur.

In comparison to the detailed knowledge available about the structure and active site of DsbA, relatively little is known about how transcription of *dsbA* is controlled. A study by

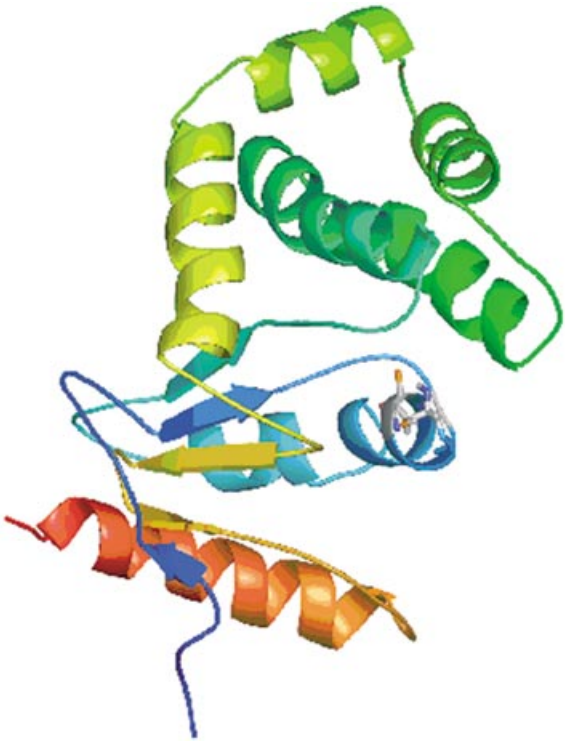


Figure 1. *DsbA has two domains flanking the active site.[60] The thioredoxin domain has a five-stranded β sheet and three α helices. The helical domain is made up of four α helices and is inserted into the thioredoxin domain. The active site, C30PHC33, is flanked by both domains. The active site cysteines, Cys30 and Cys33, are modeled in sticks (sulfur: orange, nitrogen: blue). The cysteine residues are located on the α1 helix.*

primer extension analysis indicated that the transcription of *dsbA* originates either from the P1 promoter, located in the distal portion of *yihE*, the gene preceding *dsbA*, or the P2 promoter, located upstream of *yihE*.[58] The Cpx signal transduction system, consisting of a sensor (CpxA) and a response regulator (CpxR),[59] controls *dsbA* transcription.[60,61] Stimulation of *dsbA* transcription by the Cpx system occurs at the P2 promoter, which is upstream of *yihE*, and not the P1 promoter, located in the distal portion of *yihE*.

DsbB, a Recycler of Functional Oxidized DsbA

DsbA needs to be reoxidized to donate its disulfide moiety to proteins requiring them for folding. How is DsbA reoxidized to its disulfide form? In vitro, spontaneous air oxidation of DsbA occurs on the hour or day time scale, suggesting that this is far too slow to be the in vivo reaction. DsbB, a 20 kDa cytoplasmic membrane protein that is required for the reoxidation of DsbA has been identified by several groups independently.[62–64] DsbB mutants accumulate DsbA in a reduced form and are hypersensitive to dithiothreitol (DTT).[64,62] The folding of periplasmic phosphatases Agp and AppA requires DsbA.[65] A Tn*10* mutant that lacks *agp*, *appA*, and *phoA* expression displayed normal *dsbA* expression, and growth of the mutant in the presence of 5 mM oxidized glutathione (GSSG) restored *appA* expression.[66] The mutation, located at 26.2 min in *E. coli*, is required for reoxidizing DsbA, and we now know this factor to be DsbB.

DsbB has six cysteine residues, four of which (Cys41, Cys44, Cys104, Cys130) are essential for its function.[67] The topology of DsbB has been determined by the use of alkaline phosphatase fusions to DsbB. In this approach, alkaline phosphatase is much more enzymatically active when fused to a periplasmic region and less active when fused to a cytoplasmic domain of a membrane protein. Results indicate that DsbB is an integral membrane protein containing two periplasmic domains and four transmembrane segments (Figure 2).[67] Its two pairs of essential cysteine residues Cys41–Cys44 and Cys104–Cys130 are located in the first and second periplasmic domains of DsbB, respectively.

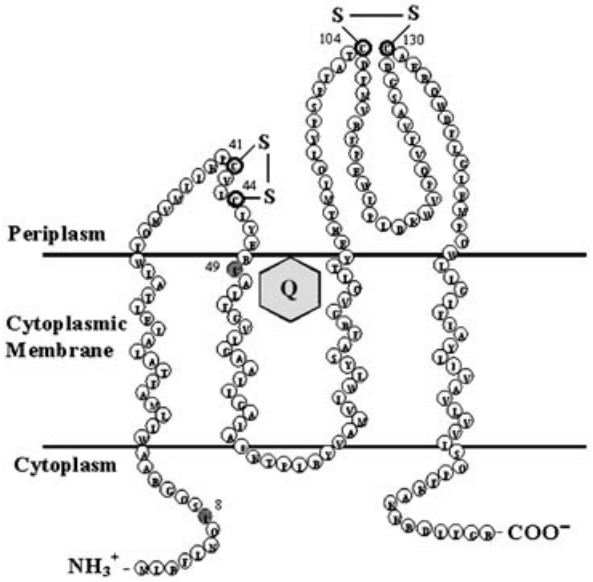
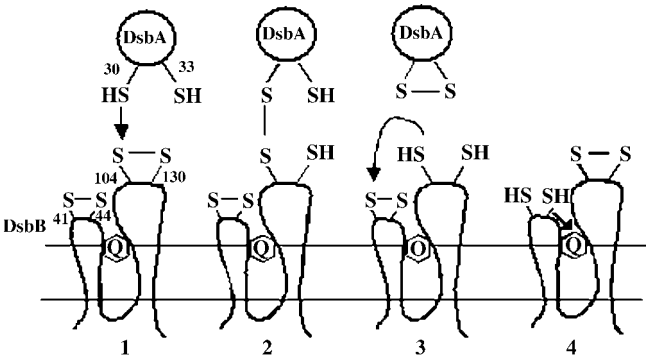


Figure 2. *Predicted membrane topology of DsbB.[73] Essential cysteines (Cys41, Cys44, Cys104, Cys130) are in bold circles while nonessential cysteines (Cys8, Cys49) are in shaded circles. Q = quinone.*



Scheme 1. *Reoxidation of DsbA by DsbB. Reduced DsbA is reoxidized by the Cys104–Cys130 disulfide of DsbB, thereby regenerating catalytically active DsbA via a DsbA Cys30–DsbB Cys104 mixed disulfide (1, 2). Reduced Cys104–Cys130 of DsbB is reoxidized by the flow of electrons to Cys41–Cys44 of DsbB (3). Reduced Cys41–Cys44 of DsbB is reoxidized by reducing a quinone (4), which leaves fully oxidized DsbB ready for another round of DsbA reoxidation (1). Q, quinone.*

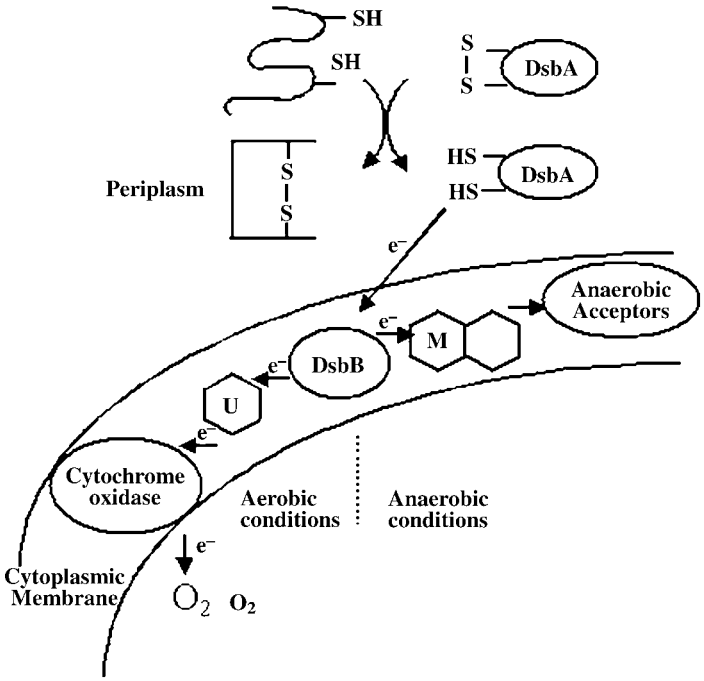
DsbB Reoxidizes DsbA

There is evidence that DsbA is oxidized by DsbB by initially forming a mixed disulfide between Cys30 of DsbA and Cys104 of DsbB. Cys33 of DsbA attacks this disulfide to form Cys33–S–S–Cys30 while leaving Cys104 and Cys130 of DsbB reduced (Scheme 1). This regenerates the active oxidized form of DsbA.[68,69] A C33S mutant of DsbA leads to accumulation of mixed-disulfide DsbB Cys104–DsbA Cys30 complexes, while a DsbB C104S mutant fails to form such complexes, thus leading to the proposal that DsbB Cys104–Cys130 directly reoxidizes reduced DsbA.[68,69,70] The oxidation of DsbA by Cys104–Cys130 of DsbB is then followed by an intramolecular disulfide transfer from Cys41–Cys44.[70] Accumulation of reduced Cys104–Cys130 in a DsbB mutant where Cys41 and Cys44 have been replaced with serines suggests that Cys41–Cys44 directly oxidizes reduced Cys104–Cys130.[71] The inability of C104S,C130S and C104S DsbB mutants but the ability of C41S,C44S and C130S DsbB mutants to form DsbA–DsbB complexes imply that C104 is required for the formation of mixed disulfides.[71] A very recent study described the redox potential of Cys41–Cys44 of DsbB to be −69 mV, and that of Cys104–Cys130 to be −186 mV.[72] It was found that the less oxidizing Cys104–Cys130 disulfide bond is still able to exchange disulfides with DsbA that has a redox potential of −122 mV. A model was thus put forth whereby Cys104–Cys130 oxidizes reduced DsbA, and the electrons get passed onto Cys41–Cys44, which then gets reoxidized by ubiquinone, a far more oxidizing molecule with a redox potential of ~100 mV.[73]

DsbB is Reoxidized by Quinones

How is DsbB maintained in its oxidized state? Cells deprived of protoheme, ubiquinone, and menaquinone accumulate DsbA in its reduced state and DsbB in its DsbA–DsbB complex state.[74] The Cys41–Cys44 disulfide of DsbB is resistant to reduction by DTT, but only in the presence of quinones or heme.[71] This suggests that the respiratory chain plays a role in keeping DsbB catalytically active, and that oxygen might be the ultimate electron acceptor. Our laboratory showed that DsbB-containing membranes reoxidize DsbA in an oxygen-dependant manner.[75]

DsbB possesses a unique enzymatic activity: it uses the oxidizing power of quinones to generate disulfides.[76] DsbB has two substrates, DsbA and quinones. Experiments conducted in our laboratory showed that components of the electron transport chain reoxidize DsbB and serve as the immediate electron acceptors of DsbB (Scheme 2).[77] DsbB transfers its electrons to quinones present in the cytoplasmic membrane. Thus, ubiquinone is the major immediate donor of oxidizing power to DsbB.[76] Ubiquinone is in turn reoxidized by terminal oxidases such as cytochrome bd and bo oxidases that finally transfer electrons to oxygen. Under anaerobic conditions, *E. coli* switches its immediate electron acceptor from ubiquinone to menaquinone, which is upregulated upon oxygen depletion.



Scheme 2. *DsbA–DsbB pathway.[83] Electrons (e^-) flow from newly synthesized peptides in the periplasm to DsbA upon disulfide-bond formation, which allows the formation of disulfide bonds for proper folding of the peptides. Reduced DsbA is reoxidized by membrane-bound DsbB. Under aerobic conditions, DsbB passes electrons to ubiquinone (U), and these electrons are ultimately transferred to oxygen (O_2) by cytochrome oxidases. Under anaerobic conditions, DsbB passes its electrons to menaquinone (M), which is then reoxidized by anaerobic oxidases. The end result is reoxidized DsbB that is ready to undergo another round of reoxidizing DsbA.*

Instead of cytochrome oxidases, anaerobic oxidases such as fumarate reductase serve to reoxidize menaquinone.[77]

Mutants in a highly conserved residue, Arg48 of DsbB, are only partially active.[78] These mutants are defective in β-lactamase oxidation under anaerobic but not under aerobic conditions, and display a sevenfold increase in K_m for ubiquinone while retaining a wildtype K_m for DsbA. These results suggest that Arg48 of DsbB is directly or indirectly important for its interaction with quinones. An interaction between DsbB and quinone was later shown by the ability of DsbB to reduce quinones and that purified DsbB is bound to ubiquinone-40.[76]

The spectral properties of wildtype DsbB (purple) and DsbB cysteine mutants (yellow) are very similar to those of quinhydrone charge-transfer complexes and oxidized quinone, respectively.[79] Our laboratory proposed a model for the involvement of quinones in the function of DsbB. DsbB is initially bound to a reduced quinone. A second, transiently bound reduced quinone is replaced by an oxidized quinone from the cellular quinone pool. The bound reduced quinone transfers electrons to the oxidized transiently bound quinone, forming a purple charge-transfer quinhydrone complex. When DsbB reoxidizes DsbA, electrons are passed from the C-terminal dithiol to the N-terminal cysteine residues, and subsequently to the bound quinone. Inactive cysteine mutants of DsbB are locked in the oxidized quinone state, therefore having their characteristic yellow color, while active wildtype DsbB is able to transfer disulfides and form the quinhydrone charge-transfer complex, therefore bestowing a characteristic purple color upon the protein.

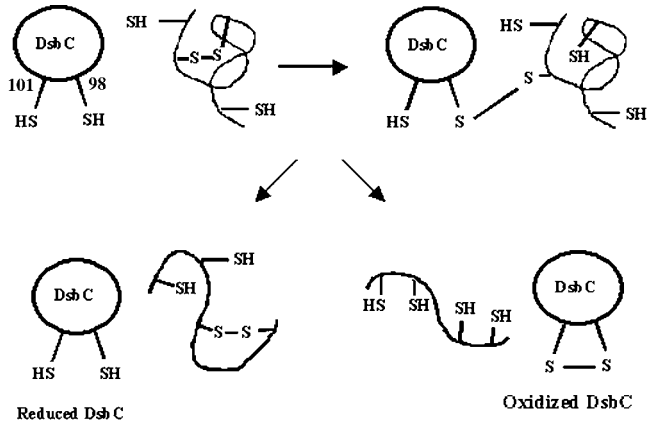
In contrast to DsbA and DsbB, thioredoxin is a disulfide reductase found in the cytoplasm. We have engineered a pathway for the formation of disulfide bonds. By imposing evolutionary pressure, we identified mutations that changed thioredoxin, which is a monomeric disulfide reductase, into a [2Fe-2S]-bridged dimer capable of catalyzing O2-dependent sulfhydryl oxidation in vitro. Expression of the mutant protein in *E. coli* with oxidizing cytoplasm and secretion through the Tat pathway restored disulfide-bond formation in strains that lacked the complete periplasmic oxidative machinery (DsbA and DsbB). The evolution of [2Fe-2S] thioredoxin illustrates how mutations within an existing scaffold can add a cofactor and markedly change protein function. Such mutants require both an iron–sulfur cluster and the CXXC motif for oxidative protein folding. It appears that changing the structure of thioredoxin and allowing it to incorporate an iron–sulfur cluster, has therefore converted a reductase into an oxidase.

Like *dsb*A, little is known about the regulation of *dsbB* expression. Two promoters of *dsbB* were found by primer extension analysis.[64] *dsbB* is transcribed predominantly from the P1 promoter, located 237 nucleotides upstream of the start codon of *dsbB*, under normal conditions. Diminished transcription from P1, but activated transcription from P2, located 40 nucleotides upstream of *dsbB*, was observed in strains lacking *rpoH*, *katF*, or *lrp*.[64] Heat-shock proteins are synthesized in increased amounts when an organism is exposed to high temperatures. Many of these heat-shock proteins are chaperones or proteases, and aid in the refolding of proteins or elimination

of irreversibly damaged proteins.[80] The synthesis of heat-shock proteins is activated by the transcriptional regulator σ^{32}, a subunit of RNA polymerase, encoded by *rpoH*.[81] Stationary phase increases the resistance of an organism to harmful conditions by altering cellular composition and metabolism.[82] The regulator of stationary phase transcription is σ^{S}, which is encoded by *katF*.[83] The leucine-responsive regulatory protein (Lrp) controls Ntr (nitrogen-regulated response) gene expression. The Ntr response is due to the limited nitrogen supply, which consequently affects the expression of approximately 100 genes.[84] Missiakas et al. thus postulate that the P2 promoter is required for *dsbB* transcription under stress conditions as null mutations in regulators of the heat-shock response, the stationary phase general response, and amino acid starvation cause increased transcription of *dsbB* from such a promoter.[64]

DsbC, a Disulfide Isomerase

Proteins with more than two cysteine residues have the potential to form incorrect disulfide bonds. DsbC, a soluble periplasmic protein, isomerizes incorrectly paired cysteines.[85,86,87] Cys98 of DsbC forms a mixed disulfide with an incorrectly paired cysteine (Scheme 3). This mixed disulfide is then resolved by either attacking another cysteine in the misfolded protein, giving rise to reduced DsbC and correctly paired cysteines, or attacking Cys101 of DsbC, leading to oxidized DsbC and reduced cysteines in the polypeptide. The reduced cysteines are now ready to be oxidized again, hopefully forming the correct disulfide bonds the second time around. RNase I, which has one nonconsecutive disulfide moiety, has recently been shown by our laboratory both in vivo and in vitro to be a substrate for DsbC; this suggests that DsbC acts on substrates with nonconsecutive disulfides.[24]



Scheme 3. *DsbC isomerizes incorrectly formed disulfide bonds. The isomerase, DsbC, forms a mixed disulfide with the substrate containing the aberrant disulfide bond, which allows the formation of the correct disulfide bond.[5]*

The structure of DsbC has been solved, showing DsbC to be a V-shaped homodimer consisting of two thioredoxin-like folds (Figure 3).[88] DsbC has two active site cysteines (C98GYC101) in each monomer responsible for its activity.[89] The active sites from each monomer face inwards toward a cleft thought to be the substrate binding site.[88] Dimerization of the two mature 23.3 kDa monomers is required for the isomerase activity of DsbC,[90] and protects the active sites from oxidation by DsbB.[91] In our laboratory, a plasmid library of *dsbC* clones was randomly mutagenized and transformed into a *dsbA* null strain.[91] Mutants that could restore motility to a *dsbA* null strain were selected. The monomeric DsbC G49R mutant, as well as a DsbC variant consisting of just the thioredoxin domain, are both able to complement a *dsbA* null for motility and alkaline phosphatase activity. Such complementation is dependent on the presence of DsbB. Monomeric DsbC acts as a substrate for oxidation by DsbB. The dimerization of DsbC appears, therefore, to enable the separation of the isomerization and oxidative pathways by preventing DsbB from oxidizing DsbC.

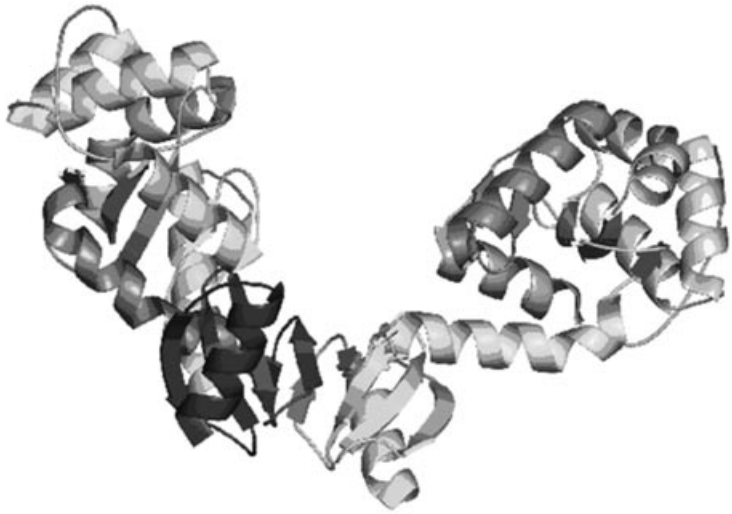
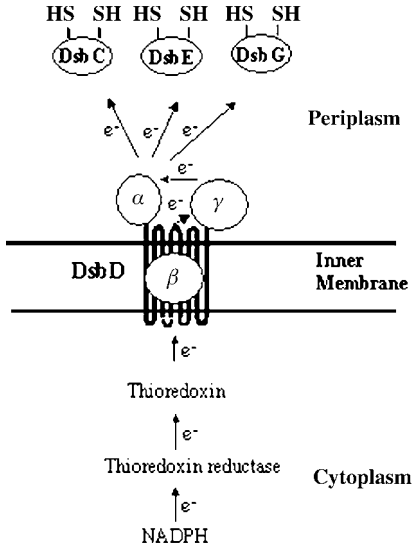


Figure 3. *DsbC is a homodimer.[94] Dimerization of the two monomeric units containing the thioredoxin-like folds protects the active sites of DsbC from oxidation by DsbB.*

The promoter for *dsbC* is regulated by σ^{E}.[92] The σ^{E} regulon in *E. coli* is induced when there is an imbalance in outer membrane protein synthesis[93] or when translocated polypeptides are misfolded.[94] DsbC has been shown to exhibit chaperone activity that promotes reactivation and suppresses aggregation of denatured D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH).[95] Using simple unfolded peptides with one or two cysteine residues, Darby et al. observed that the DsbC-peptide mixed-disulfide complex is more stable than a disulfide bond in an unfolded protein, and more stable than a DsbA-peptide mixed-disulfide complex. This suggests that DsbC interacts with peptides more strongly than DsbA does and might help to explain the superior isomerase activity of DsbC.[96]

DsbD, a Recycler of Reduced DsbC, DsbE, and DsbG

The *dsbD* gene was identified in screens for mutants deficient in cytochrome c synthesis,[97] disulfide-bond formation in periplasmic proteins,[98] or copper resistance.[99] DsbC and DsbG are disulfide isomerases. DsbE (CcmG) keeps the cysteine residues that coordinate the heme groups of cytochromes reduced. Thus, DsbC, DsbE, and DsbG all need to be kept reduced to be active. This is accomplished by DsbD, a 59 kDa integral inner-membrane protein, by transferring electrons from cytoplasmic NADPH (Scheme 4).[100–104] The electrons flow from NADPH to thioredoxin reductase, and subsequently to thioredoxin.[105,106]



Scheme 4. *Pathway of electron flow through DsbD.[116] DsbD has a hydrophilic N terminus, eight transmembrane helices, and a large hydrophilic C terminus. Electrons (e^-) flow from NADPH to thioredoxin reductase, from thioredoxin reductase to thioredoxin, and subsequently to DsbD. DsbD is therefore able to keep DsbC, DsbE, and DsbG reduced.*

The electrons are then passed from thioredoxin to DsbD.[85] This is in keeping with the overall function of thioredoxin, which is to maintain cytoplasmic proteins in the reduced form.[107–109] DsbD has three functionally separable domains: α and γ in the periplasm, and β in the membrane.[103,110] Each domain has a pair of reactive cysteine residues, and electrons flow from β to γ ($E^{0'} = -241$ mV) to α ($E^{0'} = -229$ mV; Scheme 4).[102–104,111,110] The six conserved cysteine residues, one pair in each periplasmic domain and the third pair in the transmembrane segment, have been shown by mutagenesis experiments to be required for DsbD to maintain DsbC in its reduced state.[101,102,112] The γ domain is a thioredoxin-like domain that acts as a middleman between the two other domains, specifically recognizing the α domain, and not interacting with DsbC or DsbG.[110] The recent structure of the γ domain solved to 1.9 Å resolution implicates the hydrophobic residue, Val462, in the C461XXC464 motif of the γ domain to be important in recognizing the α domain.[113]

DsbE, a Disulfide Bond Reductase

Missiakas and Raina[114] identified genes in addition to *dsbA*, *dsbB*, *dsbC*, and *dsbD* that affect the sensitivity of *E. coli* to DTT. One of these genes, *dsbE*, is thought to play a role in the reduction of cytochrome c.[115] *DsbE* is identical to *ccmG*, which encodes for an inner-membrane protein involved in cytochrome biosynthesis in *E. coli* and other bacteria such as *Rhodobacter capsulatus*, *Rhizobium leguminosarum*, *Bradyrhizobium japonicum*, and *Paracoccus denitrificans*.[115–119] Maturation of cytochrome c requires the reduction of two cysteine residues, which is catalyzed by DsbE.[115,120–122] DsbE/CcmG contains a thioredoxin-like CXXC motif, with a standard redox potential of −175[123] or −217 mV,[119] as determined by the respective groups.

DsbG, a Paralogue of DsbC

The *dsbG* gene was identified as a suppressor of DTT sensitivity in a *dsbB*$^-$ background.[124] DsbG is a 25.7 kDa periplasmic protein containing the conserved thioredoxin-like active site CXXC motif (CPTC). DsbG is maintained in its reduced form by DsbD.[125] Overproduction of DsbG suppresses folding defects in a *dsbC*$^-$ strain, but DsbG seems to possess a narrower substrate specificity than DsbC in the folding of recombinant proteins.[125,126] DsbG is found at a quarter of the level of DsbC in the cell.[125] DsbG shows homology to DsbC, is dimeric, and is maintained in its reduced state by DsbD, just like DsbC.[100] DsbG is unable to reduce insulin, refold RNase, or form disulfides in alkaline phosphatase. This provides evidence that DsbG is involved in disulfide bond isomerization like DsbC, and not disulfide-bond formation like DsbA.[125] In our laboratory, we have shown that DsbG acts like a chaperone in the correct folding of substrates such as luciferase and citrate synthase, just like DsbC.[127]

Implications for Recombinant Protein Expression

Many commercially important proteins are secreted and to be functional, their cysteine residues must be oxidized to disulfides. The periplasm of *E. coli* is of interest for the heterologous expression of recombinant proteins as it provides a more oxidizing environment than the cytoplasm.[128] However, when these heterologous proteins are successfully overproduced in *E. coli*, they often accumulate in inclusion bodies.[129–131] Overproduction of DsbA in *E. coli* increases the recovery of recombinant proteins requiring disulfide bonds.[132,133] By coexpressing *dsbA* in *E. coli*, groups have successfully expressed α-amylase/trypsin inhibitor,[132] functional T-cell receptors,[134] insulin-like growth factor-I (IGF-I),[135] and human leptin.[136] The production of proteins with many disulfides is complicated, yet active human tissue-type plasminogen activator (17 disulfides) has been successfully produced by co-overproducing DsbA and DsbC.[137] Other groups have fused heterologous proteins to DsbA. Bovine enterokinase was expressed periplasmically as a fusion to DsbA.[138] IGF-I, IGFBP-3, 3C proteinase, sTGF-β-RII,

and GFP when fused to a mutant form of DsbA were not only soluble, but active.[139] Human proinsulin fused to the C terminus of DsbA was expressed in higher yields.[140]

Enhancing disulfide isomerization has been useful in the expression of heterologous proteins. Overproducing DsbC prevents the non-native disulfide-bond formation in retinol-binding protein; this enhances the yield of native protein.[141] Addition of GSSG and overproduction of DsbA does not increase the yield of α-amylase/trypsin inhibitor containing five disulfide bonds, whereas the addition of GSH together with the overproduction of DsbA increases the yield. This suggests that reducing aberrant disulfide bonds during oxidative folding is important.[132] Indeed, the co-overproduction of DsbA and DsbC increases efficiency of α-amylase/trypsin inhibitor folding.[142] The human nerve growth factor in *E. coli* has successfully been overexpressed by simultaneously overproducing the isomerase system, DsbC, and DsbD.[143] Moreover, DsbC or DsbG fusions to single-chain Fv antibodies have been used recently for the functional expression of such genetically engineered antibodies.[144]

Developing a clear understanding of the Dsb system will allow us to create strains in which copious amounts of recombinant proteins are made. Because many proteins that are needed in large amounts for therapeutics and diagnosis have disulfide bonds, this could lead to a more economical means of producing proteins for human therapeutics in bacteria, which is advantageous due to cost and simplicity.

Summary

In *E. coli*, the DsbA–DsbB pathway is responsible for net disulfide-bond formation. Under physiological conditions, DsbA—a small periplasmic protein with a highly unstable disulfide bond—directly oxidizes dithiols to disulfides, a process necessary for folding proteins. This leads to the reduction of the active site C30XXC33 motif of DsbA. To maintain its catalytic activity, DsbA is rapidly reoxidized by the integral membrane protein DsbB. DsbB thus serves as the protein that links disulfide-bond formation to the electron-transport chain. DsbA has no proofreading ability, and the formation of aberrant disulfide bonds needs to be corrected by the disulfide isomerases DsbC, DsbE, and DsbG, which are in turn kept in their catalytically active reduced forms by the membrane protein DsbD.

Keywords: disulfide bonds · isomerization · protein folding · recombinant proteins

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