

Turning a disulfide isomerase into an oxidase: DsbC mutants that imitate DsbA

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There are two distinct pathways for disulfide formation in prokaryotes. The DsbA–DsbB pathway introduces disulfide bonds *de novo*, while the DsbC–DsbD pathway functions to isomerize disulfides. One of the key questions in disulfide biology is how the isomerase pathway is kept separate from the oxidase pathway *in vivo*. Cross-talk between these two systems would be mutually destructive. To force communication between these two systems we have selected *dsbC* mutants that complement a *dsbA* null mutation. In these mutants, DsbC is present as a monomer as compared with dimeric wild-type DsbC. Based on these findings we rationally designed DsbC mutants in the dimerization domain. All of these mutants are able to rescue the *dsbA* null phenotype. Rescue depends on the presence of DsbB, the native re-oxidant of DsbA, both *in vivo* and *in vitro*. Our results suggest that dimerization acts to protect DsbC's active sites from DsbB-mediated oxidation. These results explain how oxidative and reductive pathways can co-exist in the periplasm of *Escherichia coli*.

Keywords: disulfide bond/disulfide isomerase/disulfide oxidase/DsbC mutants/*Escherichia coli*

Introduction

The formation of disulfide bonds is one of the key steps during the folding of many secretory proteins; so important that proteins that lack their disulfides are often rapidly degraded. In bacteria, disulfide bond formation is catalyzed by the Dsb proteins (for reviews see Raina and Missiakas, 1997; Rietsch and Beckwith, 1998; Debarbieux and Beckwith, 1999). DsbA is a 21 kDa periplasmic protein with a CXXC motif in its active site. DsbA rapidly oxidizes cysteine residues in target proteins, thus leading to the formation of a disulfide bond. DsbA's extreme oxidizing power is responsible for the oxidizing nature of the periplasm as compared with the relatively reducing environment of the cell's cytosol (Wunderlich and Glockshuber, 1993). The inner membrane protein DsbB oxidizes DsbA efficiently (Bardwell *et al.*, 1993; Guilhot *et al.*, 1995). Thus, disulfide bonds flow from DsbB to

DsbA and then onto folding proteins. DsbB is re-oxidized directly by ubiquinone present in the inner membrane. Electrons flow from ubiquinone via terminal cytochrome oxidases onto oxygen. DsbB provides a link between disulfide bond formation and electron transport (Kobayashi *et al.*, 1997; Bader *et al.*, 1999, 2000).

A second independent pathway acts on wrongly formed disulfide bonds. DsbC and DsbG act to isomerize these incorrect disulfide bonds (Rietsch *et al.*, 1996; Darby *et al.*, 1998; Bessette *et al.*, 1999). Disulfide isomerization involves the nucleophilic attack of a wrong disulfide bond by the reduced catalyst forming a mixed disulfide bond between the enzyme and the target protein. The complex resolves upon formation of a disulfide bond in the target protein (Gilbert, 1997). In order to attack a disulfide bond and catalyze its rearrangement, the catalyst has to be kept in a reduced form. This is done by an inner membrane protein called DsbD, which uses the reducing power of thioredoxin to reduce DsbC (Missiakas *et al.*, 1995; Rietsch *et al.*, 1996, 1997).

A very important question concerning the DsbA–DsbB and DsbC–DsbD system is how these two pathways are kept separate. DsbA is found almost entirely in the oxidized form *in vivo*, while DsbC is entirely reduced (Joly and Swartz, 1997). This is in agreement with their divergent roles. Oxidases like DsbA need to be oxidized to be active, while isomerases need to be kept reduced. However, it begs the question of how the two systems avoid a futile cycle. Any cross-talk between the two pathways would make disulfide bond formation less efficient, as it would lead to the oxidation of the reductive pathway and vice versa. It has been shown that DsbA oxidizes DsbC very slowly *in vitro*, slow enough to essentially eliminate cross-talk *in vivo* (Darby *et al.*, 1998). Recently, we have found that the two pathways are also kinetically separated at the level of DsbB (Bader *et al.*, 2000). DsbB discriminates between DsbA and DsbC. The re-oxidation of DsbA by DsbB was found to be at least 500-fold faster than the re-oxidation of DsbC by DsbB, leading us to conclude that DsbB effectively oxidizes DsbA but not DsbC.

Here we provide evidence for the molecular basis of the separation of the oxidative and reductive pathways. We have isolated *dsbC* mutants that complement a *dsbA* null mutant, showing that they can actually replace *dsbA* *in vivo*. These mutants map to DsbC's dimerization interface and fail to form stable dimers. Based on this result we targeted the dimerization interface for mutagenesis, and in doing so constructed an additional four mutants that complement the *dsbA* null mutation. We also found that monomeric DsbC mutants were oxidized by DsbB *in vivo* and *in vitro*. Monomerization of DsbC allows it to switch allegiances and participate in the oxidative pathway.

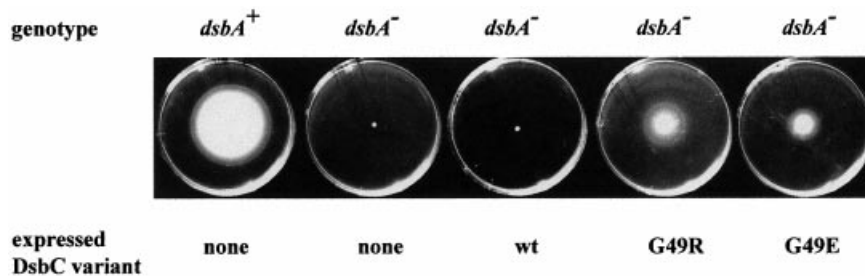


Fig. 1. Identification of two DsbC mutants that rescue the *dsbA* null phenotype. Colonies were stabbed onto M63 motility plates and grown for 24 h. A *dsbA* null mutation leads to a severe defect in disulfide bond formation causing the loss of motility (JCB817). This motility is not restored by the plasmid pMB69, which carries the wild-type *dsbC* gene. Two DsbC mutants that conferred motility were selected a number of times independently, following random mutagenesis of the *dsbC* gene. The two mutations were identified as *dsbC* G49R and G49E. The minimal medium contains 0.4% glycerol as carbon source and 0.2% arabinose to induce expression of DsbC under *ara* control. No rescue was observed in the absence of arabinose (data not shown).

Results

Identification of *dsbC* mutants that complement *dsbA*

In the periplasm of *Escherichia coli*, DsbA is the net donor of disulfide bonds while DsbC functions as a disulfide isomerase that corrects wrongly formed disulfide bonds (Bardwell *et al.*, 1991; Rietsch *et al.*, 1996). To avoid inactivation of the isomerization pathway by the oxidative pathway, the two must be kept isolated. To gain insight into what makes each pathway so specific for oxidation or isomerization, we decided to convert DsbC into a net donor of disulfide bonds; in effect, allowing DsbC to act like DsbA. *DsbA* null strains are completely non-motile, while *dsbA*⁺ strains are motile (Dailey and Berg, 1993). Based on this phenotype, we devised a powerful genetic selection allowing the identification of *dsbC* variants that could complement a *dsbA* null mutant. Since we did not know the basis of the separation of the two pathways, we decided to randomly mutate *dsbC* and select for mutants that could restore motility in a *dsbA* null mutant strain.

As described in Materials and methods, a plasmid library of random mutations in the *dsbC* gene (in which the expression of DsbC was under arabinose control) was created and transformed into a *dsbA* null strain. After incubation, 28 motile colonies were observed in which motility was dependent on the presence of arabinose. Figure 1 shows the motile phenotype. Plasmid DNA was prepared from these strains and used to re-transform a *dsbA* null mutant strain. After re-transformation, 26 of the 28 plasmids conferred motility to the previously immobile *dsbA* strain, confirming that, for these 26 plasmids, the motile phenotype was conferred by the plasmid. Although these mutants are not quite as motile as *dsbA*⁺ strains, they are strongly motile, whereas the starting *dsbA*⁻ strain is not at all motile. Sequencing of the 26 motile mutants revealed two different mutations, both of which altered the G49 residue in DsbC. A single base substitution from GGG to AGG was found in 17 clones. This resulted in a G49 to arginine substitution. In nine clones, a G49 to glutamic acid substitution was found. The complementation depends strictly on the presence of arabinose, which induces expression of DsbC from the pBAD33a derived plasmids (data not shown). Wild-type *dsbC*, present as a chromosomal copy or introduced on pMB69, is not able to

rescue the *dsbA* null phenotype under the conditions employed (Figure 1). To prove that these two mutations by themselves were sufficient to complement the *dsbA* null mutation, they were introduced into the *dsbC* gene by site-directed mutagenesis. After transformation into the *dsbA* null mutant strain JCB817, the mutant plasmids coding for DsbC G49R or G49E showed the same phenotype as the plasmids isolated by the selection. This confirms that the motile phenotype is due to the mutations G49R or G49E.

Missiakas *et al.* (1994) reported that massive overexpression of wild-type DsbC in rich media could rescue a *dsbA* null mutant. This rescue may be due to oxidized cystine in the media oxidizing DsbC, which is then able to oxidize substrate proteins. Indeed, the simple addition of cystine and other oxidants is sufficient to rescue some phenotypes of *dsbA* and *dsbB* null mutations, in the absence of DsbC overexpression (Bardwell *et al.*, 1991, 1993). To avoid the complicating effects of cystine and other small molecule oxidants present in rich media, we chose to perform our experiments in minimal media. Overproduction of wild-type DsbC, in strains grown in minimal media, resulted in no measurable motility. To investigate the level of DsbC overexpression from our promoter constructs, we prepared periplasmic extracts from the mutants and compared them to the starting strain. The level of expression of DsbC was unchanged in our mutants, showing that their ability to rescue *dsbA* null mutants was not due to enhanced DsbC expression (data not shown). Interestingly, SDS-PAGE revealed a 45 kDa protein that was present in all motile mutants and absent in both disulfide-negative strains (JCB817, JCB817 pMB69). The first 10 N-terminal amino acid residues of this band were sequenced and match exactly with the first 10 residues of *E. coli* alkaline phosphatase (RTPEMPVLEN). Alkaline phosphatase contains two disulfide bonds necessary for correct folding. If the disulfide bonds are not formed, the protein does not fold correctly and is degraded (Bardwell *et al.*, 1991). This result provides additional evidence that the DsbC variants G49R and G49E are efficiently forming disulfide bonds in the absence of DsbA, the native donor of disulfide bonds in the periplasm.

DsbC G49R mutant is a monomer

We isolated two mutants that are able to replace the function of DsbA. We conclude that both DsbC G49R and

DsbC G49E are functionally more similar to DsbA than to wild-type DsbC. What distinguishes DsbC from DsbA? Although both DsbA and DsbC contain a thioredoxin fold, DsbC possesses an additional N-terminal domain, which causes DsbC to form a homodimer (Zapun *et al.*, 1995; McCarthy *et al.*, 2000). The N-terminal domain has also proven to be essential for DsbC's activity as an isomerase *in vitro* (Sun and Wang, 2000). G49 is located in the N-terminal dimerization domain of DsbC and is a very well conserved residue. In a sequence alignment of 15 different DsbC N-terminal domains, G49 is present in 14 out of 15 DsbC proteins. It is even found in the related disulfide isomerase DsbG (data not shown). This high degree of conservation suggests that G49 is a very important residue. In the recently solved crystal structure of DsbC, G49 is located between two consecutive β -strands (Figure 3A). These two strands are the core of the dimerization interface and form an extended β -sheet with the corresponding monomer. The high conservation of the small residue G49 led us to propose that the substitution of G49 with large charged residues, such as arginine or glutamate in the isolated mutants, will act to disrupt the dimer. We therefore predict that G49 is essential for the dimerization of DsbC. Indeed, we had previously constructed the same arginine substitution at exactly the same glycine in an effort to disrupt the dimerization by rational design (see below). This represents an astonishing congruence between the *in vivo* genetic selection approach and rational design.

In order to investigate the biochemical properties of the mutant protein, we overexpressed and purified DsbC G49R from strain BL21. The purified protein was analyzed by Sephadex-200 gel filtration. Figure 2 shows that DsbC G49R has a significantly altered retention time as compared with wild-type DsbC. A standard curve was generated with proteins of known molecular weight. The observed retention time for DsbC G49R corresponds to an apparent mol. wt of 31.6 kDa, while wild-type DsbC runs with an apparent mol. wt of 51.4 kDa. This indicates that DsbC G49R is monomeric. Surprisingly, the loss of functional dimerization leads to a different function. The mutant protein acts as an *in vivo* donor of disulfide bonds rather than an isomerase.

Rational design of dsbC mutants that complement a dsbA null mutation

Our results provide evidence that the dimerization of DsbC is essential for the separation of disulfide bond formation and isomerization. DsbC can be converted into a functional donor of disulfide bonds by two different *in vivo* selected mutations that disrupt its dimerization properties. We wanted to ascertain whether the disruption of the dimerization interface is what allows these mutants to function as oxidases. We reasoned that a rational design of mutants in the dimerization interface should reveal more DsbC variants that are able to rescue DsbA.

Substantial disruptions of the dimerization interfaces should lead to a disulfide-positive phenotype in a *dsbA* null mutant background. To design possible disrupting mutations, we focused on the interactions involving five residues in the interface. Figure 3 provides a view of the residues at the dimerization interface. The residues shown in Figure 3B and C are mostly hydrophobic and highly

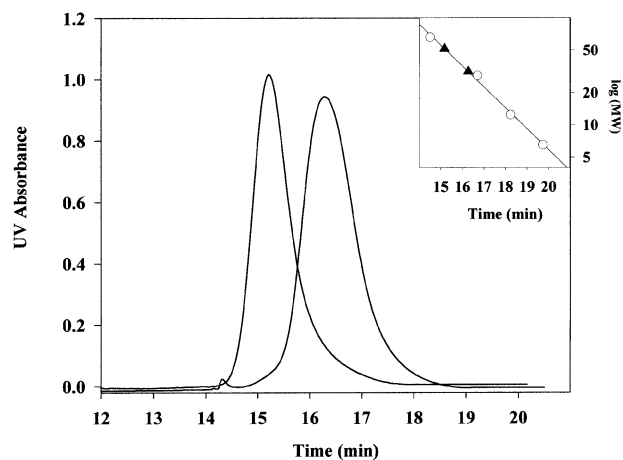


Fig. 2. DsbC G49R is a monomer. The DsbC mutant G49R was purified and analyzed by analytical gel filtration. DsbC G49R shows a clear difference in migration on a Sephadex 200 column as compared with wild-type DsbC. The inset shows a standard curve generated with proteins of known molecular weight (open circles): bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome *c* (12.4 kDa) and apoprotinin (6.5 kDa). The apparent molecular weights of wild-type DsbC and DsbC G49R (filled triangles) were determined according to this curve. An apparent mol. wt of 31.6 kDa was calculated for DsbC G49R. Wild-type DsbC runs with an apparent mol. wt of 51.4 kDa. This suggests that G49R is monomeric.

conserved. The surface area buried upon dimer formation is 1727 Å² or 15% of the total monomer surface (Figure 3D). Residues 8, 11, 12, 23–25, 27, 40, 43–55 and 59 of each molecule are buried in the dimer interface. There are hydrophobic interactions between isoleucine 47 from molecule 'a' of DsbC (abbreviated as I47a) and I47b, V54a and I46b, and M27a and M27b (data not shown). In addition, there is a potential salt bridge between H45a and D53b. To disrupt these interactions, residues I47, V54 or M27 were mutated to lysine. We reasoned that the charged lysine should be very unfavorable in the hydrophobic environment provided by the residues shown in Figure 3. Residue H45 was mutated to aspartate. This was hypothesized to lead to decreased affinity of the DsbC monomers for each other since this mutation destroys a vital salt bridge that links the two monomers in the crystal structure. The positions of these mutations are shown in Figure 3A. All the designed mutations, which we predicted would disrupt the dimer interface, are able to rescue the non-motile phenotype of the *dsbA* null mutant (data not shown).

To confirm and to quantify the extent of the rescue, we performed alkaline phosphatase assays. Alkaline phosphatase is a periplasmic protein that requires two disulfide bonds for activity. The amount of alkaline phosphatase activity in a cell is often used as a measure of the extent of disulfide defect. We observed nearly full restoration of alkaline phosphatase activity in all of our mutants that were designed to disrupt the dimerization interface (Figure 4A). The simplest explanation for these results is that dimerization acts to shield DsbC from the re-oxidant DsbB. This explanation makes two predictions: (i) that the dimerization domain should be dispensable for the observed complementation; and (ii) that complementation should be DsbB dependent. To test the first prediction, we

constructed a DsbC variant in which the entire dimerization domain had been deleted by site-specific mutagenesis. This variant, expressing only the thioredoxin domain of DsbC, is able to complement the *dsbA* null phenotype for both motility and alkaline phosphatase activity (Figure 4A).

Complementation depends on the presence of DsbB

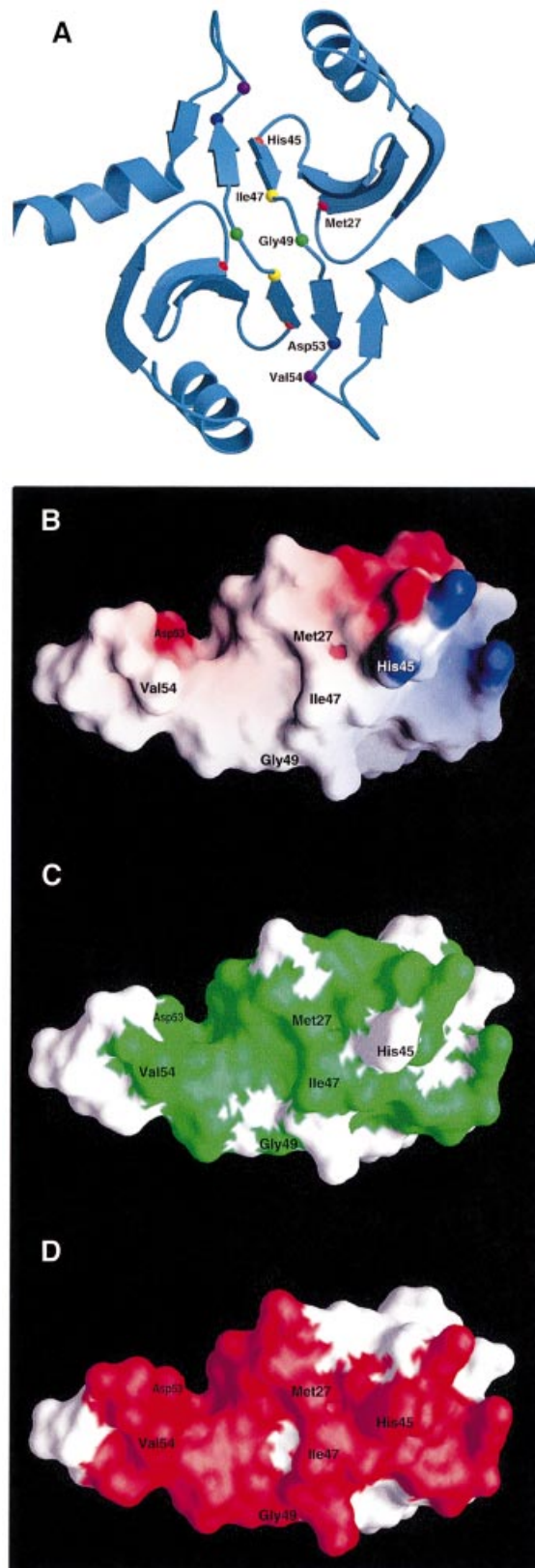
We have isolated and rationally designed mutants in the disulfide isomerase DsbC that act like the oxidase DsbA. We speculated that for these mutants to be efficient disulfide oxidases, they have to be re-oxidized by DsbB. We therefore tested whether the mutant DsbC proteins were still able to complement a disulfide-negative phenotype in a *dsbA dsbB* null strain. Figure 4B shows that none of the mutants can complement *dsbA* deficiency in the absence of *dsbB*. This strongly suggests that DsbB directly or indirectly is responsible for the re-oxidation of DsbC proteins containing any of the mutations G49E, G49R, H45D, I47K, V54K or M27K in the dimerization domain. We also performed AMS trapping experiments, which showed that the DsbC mutants were partially oxidized *in vivo* in a *dsbA*⁻ background and that this oxidation was *dsbB* dependent (data not shown). It appears that we have been able to get DsbC to switch allegiance from being part of the isomerization pathway to becoming part of the DsbB-dependent oxidative pathway.

DsbD depletion is not sufficient for full complementation by wild-type DsbC

We considered the possibility that our DsbC mutant proteins could also be impaired in their recognition by DsbD, the *in vivo* reductant of DsbC. The observed phenotypes could therefore be in part due to a loss of interaction between mutant DsbC proteins and DsbD. If the entire rescue were due to lack of interaction with DsbD, we would not expect the rescue to be DsbB

Fig. 3. Rational design of mutants in the dimerization domain of DsbC. (A) The crystal structure of DsbC is shown with the mutated residues represented as small spheres. The monomers interact via an N-terminal dimerization interface that consists of two very short consecutive β -strands from each monomer. Two extended β -sheets are formed, which consist of two strands from one monomer and four strands from the other. Two DsbC mutants that were found to complement a *dsbA* null mutant were mapped to G49 (drawn as a green sphere), which lies in between the interacting β -strands. These mutations replace G49 with either arginine or glutamate. An additional five residues, which were predicted to provide crucial interactions in the dimerization interface and were subject to mutagenesis, are also shown as small spheres. (B) Surface potential of DsbC's dimerization interface. Only one monomer is shown. Values range from +7kT (blue) to 0 (white) to -7kT (red). M27, V54 and I47 were mutated to lysines. H45 forms a salt bridge with Asp53 on the other monomer (compare with Figure 3A), and was mutated to an aspartate. (C) Same as (B), except that the green color highlights conserved residues. Conserved residues were mapped according to the sequence alignment of 15 DsbC molecules. A BLAST search was performed with the entire DsbC protein sequence at the NCBI database of finished and unfinished bacterial genomes (http://www.ncbi.nlm.nih.gov/Microb_blast/unfinishedgenome.html). Fifteen microbial sequences were aligned with CLUSTAL W (Thompson *et al.*, 1994). Identical and similar residues that are found in at least 60% of the aligned sequences are shaded in green. (D) Molecular surface of the dimerization domain showing residues involved in dimer formation. Note that most of the residues involved in dimerization are conserved.

dependent. In addition, wild-type DsbC should be able to confer a motile phenotype if DsbD is removed by deletion. However, we did not observe restoration of alkaline phosphatase activity and motility for wild-type DsbC in a *dsbA dsbD* null strain (Table 1). We conclude that the



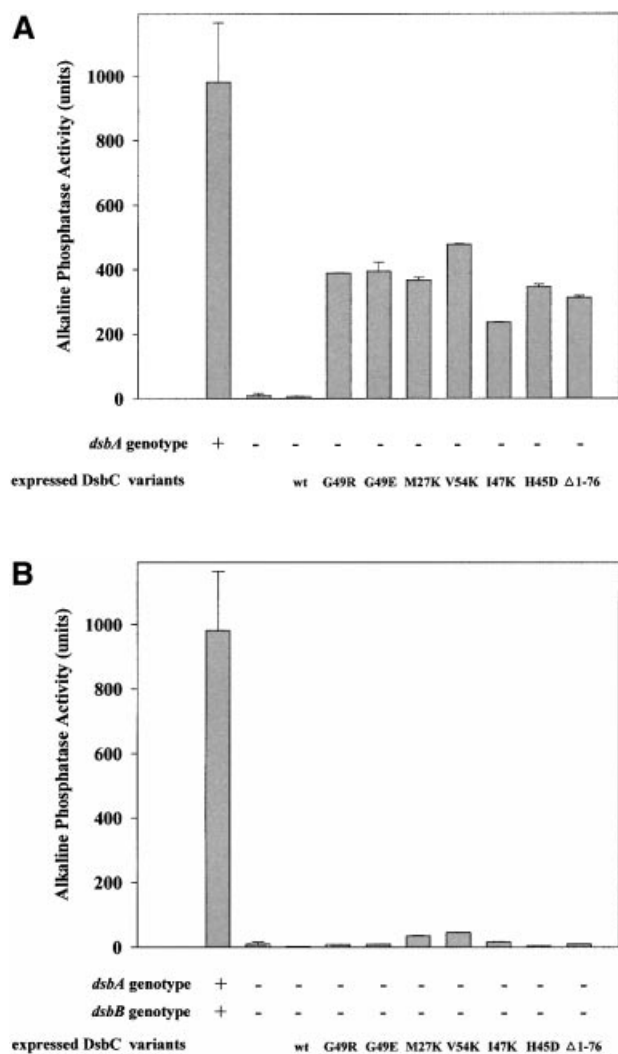


Fig. 4. (A) All mutant DsbCs promote folding of alkaline phosphatase *in vivo*. Plasmids encoding DsbC or DsbC variants, respectively, were transformed into JCB817 (*dsbA*⁻). The strains were grown in M63 media supplemented with 0.4% glycerol as carbon source and 0.2% arabinose to induce expression from the pBAD33 derived plasmids. The activity of alkaline phosphatase was determined by a standard assay described in Materials and methods. JCB816 (wild type) served as a positive control. The different plasmids are listed in Table II. (B) Rescue of the *dsbA* null phenotype strongly depends on the presence of DsbB. Conditions were essentially as in (A), except that JCB818 (*dsbA*⁻, *dsbB*⁻) served as a strain background.

ability of our DsbC mutants to rescue the *dsbA* null phenotype can not solely be due to the loss of DsbC–DsbD interaction. In an effort to determine whether this loss of interaction may contribute in some small way to the observed phenotype, we measured the extent of rescue of these mutants in a *dsbA*⁻ *dsbD*⁻ strain and compared it with the extent of rescue in a *dsbA*⁻ *dsbD*⁺ strain. The mutant DsbC plasmids allowed the expression of similar levels of alkaline phosphatase as a percentage of wild type independent of whether the strain background was *dsbA*⁻ or *dsbA*⁻ *dsbD*⁻ (compare Figure 4A with Table I). The small amount of increased expression in the *dsbD*⁻ strain may be due to the generally more oxidizing environment present in this background. If loss of DsbC–DsbD

interaction is playing a role in DsbC acting as an oxidase, it is only playing a small one. In contrast, DsbC is unable to serve as an oxidase in a *dsbB*⁻ background (Figure 4B). The most important new feature of our mutant DsbC proteins is thus their ability to serve as substrates for DsbB rather than their inability to be reduced by DsbD.

DsbB re-oxidizes monomeric DsbC *in vitro*

The DsbC H45D mutant was purified, its active site disulfide was reduced and its oligomerization state was analyzed by gel filtration using a Sephadex-200 column. DsbC H45D runs with a retention time of 16.90 min as compared with 15.17 min for wild-type DsbC. This corresponds to a change in apparent mol. wt from 51.4 to 37.4 kDa, suggesting that the H45D mutant is monomeric. The change is not due to degradation since the protein runs as a single band on SDS–PAGE of the same molecular weight as wild-type DsbC (data not shown). This result agrees with the prediction from the *in vivo* data for this mutant (Figure 4) and further supports our model. To obtain direct evidence for interaction between a mutant DsbC protein and DsbB, an enzymatic assay for the quinone reductase activity of DsbB was employed. DsbB provides the oxidative power for the periplasm by reducing ubiquinone, thus linking disulfide bond formation and electron transport (Bader *et al.*, 1999, 2000). We mixed the reduced H45D or G49R mutants with catalytic quantities of DsbB and oxidized ubiquinone. We monitored both the reduction of ubiquinone and the oxidation of DsbC by trapping with the sulfhydryl reagent AMS. No reaction occurred when quinone alone was added. However, when catalytic quantities of DsbB were added, DsbC H45D and G49R were oxidized (Figure 5, insert). The oxidation of DsbC was accompanied by the reduction of ubiquinone, which could be quantified by following the change in absorption at 275 nm. Figure 5 shows that DsbB efficiently oxidizes both the DsbC H45D and G49R mutant proteins *in vitro*, but can not effectively oxidize wild-type DsbC. This suggests that monomerizing DsbC forces it to become a specific substrate for DsbB. The finding that DsbB oxidizes monomeric DsbC *in vitro* agrees well with the *in vivo* observation of *dsbB* dependency.

Discussion

Two distinct pathways assist the proper formation of disulfide bonds within folding proteins in the periplasm (Bardwell *et al.*, 1993; Rietsch *et al.*, 1996). The DsbA–DsbB system drives the net introduction of disulfide bonds by providing a link to the oxidative power of the electron transport chain. DsbA is a strong oxidant that has the potential of introducing wrong disulfide bonds not normally found in the native structure of proteins (Rietsch *et al.*, 1996; Sone *et al.*, 1997). Efficient folding of proteins containing multiple disulfide bonds requires an enzyme that shuffles incorrectly formed disulfide bonds toward the ones found in the native state of the protein. DsbC is a disulfide isomerase that is thought to correct wrongly formed disulfide bonds by rearrangement towards the native ones. DsbC is part of the reductive pathway that is linked, via DsbD, to the thioredoxin system of the cytosol (Rietsch *et al.*, 1997).

Table I. Effect of *dsbD* depletion on motility and alkaline phosphatase activity

Strain	Relevant genotype	DsbC variant expressed	Motility	Alkaline phosphatase activity (% of wt)
MC1000	wt	–	+++	100
FED126	<i>dsbD</i> ⁻	–	+++	109
MB102	<i>dsbA</i> ⁻ <i>dsbD</i> ⁻	–	–	10
MB102 pMB69	<i>dsbA</i> ⁻ <i>dsbD</i> ⁻	wt	–	16
MB102 pMB89	<i>dsbA</i> ⁻ <i>dsbD</i> ⁻	G49R	++	76
MB102 pMB90	<i>dsbA</i> ⁻ <i>dsbD</i> ⁻	G49E	++	88
MB102 pMB91	<i>dsbA</i> ⁻ <i>dsbD</i> ⁻	M27K	++	82
MB102 pMB92	<i>dsbA</i> ⁻ <i>dsbD</i> ⁻	I47K	++	74
MB102 pMB93	<i>dsbA</i> ⁻ <i>dsbD</i> ⁻	V54K	++	73
MB102 pMB94	<i>dsbA</i> ⁻ <i>dsbD</i> ⁻	H45D	++	75
MB102 pMB96	<i>dsbA</i> ⁻ <i>dsbD</i> ⁻	Δ1–76	++	44

Although we have learned much about the flow of electrons through the two pathways, little was known about how they are able to co-exist. This work now shows that wild-type DsbC can be converted into a net donor of disulfide bonds *in vivo* by a single mutation at residue G49. Both DsbC G49E and G49R are able to rescue the *dsbA* null phenotype. These mutants are able to restore motility as well as alkaline phosphatase activity *in vivo*. Both alkaline phosphatase and the P ring of the flagellar motor need to be oxidized to be active (Bardwell *et al.*, 1991; Dailey and Berg, 1993). Restoration of these two activities indicates that the mutant DsbC proteins are capable of restoring oxidative power to the periplasm. Substitution of G49 for arginine disrupts the ability of DsbC to form a stable dimer. This loss of function is accompanied by the gain of a new function: monomeric DsbC acts as a donor of disulfide bonds *in vivo*. The finding that loss of functional dimerization allows DsbC to act as an oxidase led us to believe that other mutations disrupting the dimerization interface may cause the same effect. The resulting mutants M27K, H45D, I47K, V54K, and one that removes the entire dimerization domain, complement a *dsbA* null mutant for motility and alkaline phosphatase.

The finding that different *dsbC* mutants were able to promote the formation of disulfide bonds raised the question of how these mutant proteins are re-oxidized. Mutant DsbC proteins do not rescue in the absence of a functional DsbB *in vivo*. This finding suggests that the mutant DsbC proteins are re-oxidized *in vivo* by DsbB, while wild-type DsbC is not. We tested this observation *in vitro* and found that monomeric DsbC is re-oxidized enzymatically by DsbB, whereas wild-type DsbC is not. We conclude that the failure to form a functional dimer turns DsbC into a substrate for DsbB. Hence, monomeric DsbC becomes part of the oxidative pathway. These results demonstrate how the delicate balance between the oxidative and reductive pathway is controlled by the dimerization of DsbC. It explains how DsbA and DsbC exhibit rather different redox activities in the same cellular compartment without interfering with each other.

It has been reported that thioredoxin or protein disulfide isomerase (PDI), when exported to the periplasm, is able to rescue a *dsbA* null phenotype (Jonda *et al.*, 1999; Debarbieux and Beckwith, 2000). The observed rescue depends on the presence of the *dsbB* gene, suggesting that thioredoxin and PDI are re-oxidized by DsbB. According

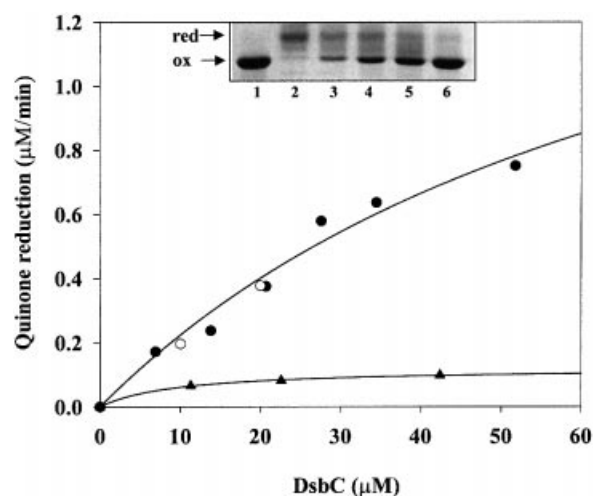


Fig. 5. DsbB re-oxidizes monomeric DsbC *in vitro*. DsbB's ability to re-oxidize monomeric DsbC (H45D, filled circles; G49R, open circles) was measured by its ubiquinone reductase activity. The assay buffer consists of 100 mM sodium phosphate pH 6.0, 0.1% dodecyl-maltoside, 20 µM ubiquinone and 1 µM DsbB. The concentration of reduced DsbC H45D was steadily increased up to 50 µM. Activity of DsbB was plotted as quinone reductase activity. Reduced wild-type DsbC served as a control (filled triangles), and a much lower ubiquinone reductase activity was observed. This suggests that monomeric DsbC mutants are a superior substrate to wild-type DsbC. Insert: to visualize the redox state of DsbC after incubation with DsbB and ubiquinone. Lane 1, purified oxidized DsbC; lanes 2–6, samples taken after 0.5, 1, 2, 3 and 4 h and precipitated with 5% TCA. Free thiols were modified with AMS essentially as described before (Kobayashi *et al.*, 1997).

to our results, DsbB also re-oxidizes the thioredoxin fold of monomeric DsbC. Thus, DsbB seems to be rather promiscuous in recognizing the thioredoxin folds of DsbA, monomeric DsbC, thioredoxin and PDI. However, this promiscuity does not interfere with disulfide bond isomerization in wild-type cells because the thioredoxin fold of wild-type DsbC is not oxidized by DsbB. It is very likely that DsbC evolved from thioredoxin and that evolutionary pressure forced DsbC to find a mechanism whereby its active site is protected from DsbB. Apparently, this was achieved by dimerization of the thioredoxin domains of DsbC, thus allowing DsbC to function as an isomerase despite the presence of DsbB.

DsbC is kept in a reduced state by DsbD. In order to achieve this, DsbD must distinguish between DsbC and

DsbA since any cross-talk between DsbD and DsbA would lead to inactivation of the oxidative pathway. This is very similar to the problem of how the cell avoids inactivation of the isomerization pathway due to DsbB. On the other hand, DsbD contains a thioredoxin fold, which has to be protected from DsbB-mediated oxidation. Once again, it is quite surprising how the thioredoxin folds of the various players of these systems, despite their structural similarity, seem to be protected from destructive cycles of oxidation and reduction. Very little is known about the actual mechanisms of how the isomerization pathway operates. It is therefore possible that additional barriers exist between the two pathways, which have not been identified yet.

Materials and methods

Bacterial strains and plasmid constructs

The strains and plasmids used in this study are shown in Table II. Mutations were introduced into the *dsbC* gene using the Stratagene QuikChange kit. The forward primers used are listed in Table III. A DsbC variant (pMB96) lacking the first 76 amino acids of the native protein was constructed following the Stratagene Exsite Protocol with some modifications. In brief, whole plasmid pMB69 was amplified by PCR using the phosphorylated primers listed in Table III. The linear PCR product was ligated *in vitro*, *DpnI* digested and transformed into XL1-blue. Transformants were screened by the loss of a *BsmI* site. All variants were confirmed by sequence analysis of the entire *dsbC* gene.

Selection for DsbC mutants that complement a *dsbA* null phenotype

Random mutagenesis of the *dsbC* gene was carried out in the Stratagene XL-1 Red Epicurean Coli mutator strain (Stratagene, CA). Plasmid

Table II. Strains and plasmids

	Genotype
Strain	
MC1000	<i>araD139, Δ[ara-leu]7679, galU, galK, Δ[lac]174, rpsL, thi-1</i>
JCB816	MC1000 <i>phoR</i>
JCB817	MC1000 <i>phoR dsbA</i> ⁻
JCB818	MC1000 <i>phoR dsbA</i> ⁻ , <i>dsbB</i> ⁻
BL21 (DE3)	F ⁻ <i>ompT hsdSB (r_B⁻ m_B⁻) gal [dcm] [lon]</i>
Plasmid	
pMB69	pBAD33a with <i>dsbC</i>
pMB89	pBAD33a with <i>dsbC</i> G49R
pMB90	pBAD33a with <i>dsbC</i> G49E
pMB91	pBAD33a with <i>dsbC</i> M27K
pMB92	pBAD33a with <i>dsbC</i> I47K
pMB93	pBAD33a with <i>dsbC</i> V54K
pMB94	pBAD33a with <i>dsbC</i> H45D
pMB96	pBAD33a with <i>dsbC</i> Δ1–76
pMB66	pET22 with <i>dsbC</i> G49R
pMB78	pET28a with <i>dsbC</i> H45D

Table III. Primers used in this study

pMB89	5'-AAACATATCATTTCAGAGGCCTAT GTATGACGTTAGTGGCACGGC
pMB90	5'-AAACATATCATTTCAGAGGCCCATGTATGACGTTAGTGGCACGGC
pMB91	5'-CGCCTGTAGCTGGCAAGAAGACAGTGTAACTAACACGCG
pMB92	5'-GATGATGGTAAACATATCAAGCAGGGCCCAATGTATGACGTTAG
pMB93	5'-GGGGCCAATGTATGACAAGAGTGGTACCGCTCCGGTCAATGTCACC
pMB94/pMB78	5'-CATCACCGATGATGGTAAAGATATCATTTCAGGGGCC
pMB96	P-GAGATGATCGTTTATAAAGCGCCG
	P-AGCCTGAGCAAAGCCTGAAAACG
pMB66	5'-AAACATATCATTTCAGAGGCCTATGTATGACGTTAGTGGCACGGC

pMB69 containing wild-type *dsbC* was transformed into XL-1 Red. pMB69 is derived from pBAD33a (Guzman *et al.*, 1995), and was obtained from G.Gergioui (University of Texas). This strain lacks three key enzymes for DNA repair, which leads to a 5000-fold increase in the mutation rate. DNA extracted from ~1500 individual colonies was pooled and electroporated into the *dsbA* null mutant JCB817. Mutant colonies that conferred a *dsbA*⁺ phenotype were selected by their ability to swim through 0.3% agar minimal media motility plates. Transformants from each library were pooled and serially diluted in 150 mM NaCl. Aliquots of 100 μl from these dilutions were added to 4 ml of cooling motility medium containing 0.05% arabinose and poured on top of a portion of pre-poured motility plates. Plates were incubated at 37°C until motile puffs arose that were moving into the portion of the motility plates that had not been overlaid with the mutagenized library (~48 h). Motile puffs were streaked out onto chloramphenicol plates. Plasmids from these colonies were extracted and re-transformed into JCB817. For all of these strains, motility depended upon supplementation of the media with arabinose, suggesting that expression of DsbC was necessary for their motility. Stabbing a single colony onto minimal plates containing 0.3% agar assessed the motility of these colonies. The minimal media contained 0.4% glycerol, 0.2% arabinose, 0.2% ammonium sulfate, 1 mM magnesium sulfate, 0.1% casamino acids, 2 μg/ml biotin, 2 μg/ml nicotinamide, 0.2 μg/ml riboflavin and 2 μg/ml thiamin.

Expression and purification of proteins

DsbB was purified as described previously (Bader *et al.*, 1999). The DsbC variant G49E was purified by anion exchange chromatography as has been described previously for wild-type DsbC (Missiakas *et al.*, 1994). His-tagged wild-type DsbC and the variant H45D were purified over HiTrap Chelating columns (Pharmacia) charged with nickel according to the manufacturer's manual. DsbA and DsbC were reduced with 10 mM dithiothreitol (DTT); excess DTT was removed by gel filtration on PD10 columns (Pharmacia). All proteins were >95% pure after purification.

Biochemical assays

In vivo alkaline phosphatase activity was measured using cells prepared from 25 ml overnight cultures grown in the same minimal media as described above. The OD₆₀₀ for each culture was measured and a 1 ml aliquot of each overnight culture (1:10) was trapped with 0.1 M iodoacetamide for 20 min at 0°C. The cells were then washed with ice-cold 67 mM MOPS, 83 mM sodium chloride, 13 mM ammonium chloride pH 7.3, supplemented with 10 mM magnesium chloride and 10 mM iodoacetamide. The washed cells were used in the assay described previously (San Millan *et al.*, 1989; Boyd *et al.*, 1993). The substrate used was Sigma 104 (Sigma Diagnostics, St Louis, MO).

DsbB activity was determined by measuring its quinone reductase activity at 275 nm. In brief, 20 μM decyl-ubiquinone was incubated with reduced DsbC H45D in 100 mM sodium phosphate pH 6.0, 0.1% dodecyl-maltoside. The reaction was started by the addition of DsbB to a final concentration of 1 μM. As a control, quinone reduction was determined in the presence of wild-type DsbC. To visualize the redox state of DsbC after incubation with DsbB and ubiquinone, samples were taken and precipitated with 5% trichloroacetic acid (TCA). Free thiols were modified with 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS) essentially as described before (Kobayashi *et al.*, 1997).

Analytical gel filtration of DsbC variants was performed on Sephadex 200 (Pharmacia) in 10 mM HEPES pH 7.5 and 300 mM NaCl. Wild-type DsbC served as a control. The presence of the His tag did not affect the retention time on this column as compared with the protein without a His tag. The Sigma low molecular weight gel filtration standard was used to generate a standard curve in order to obtain apparent molecular weights for DsbC variants.

Structure analysis

Residues buried in the dimer interface area were calculated using CNS (Brunger *et al.*, 1998). Program O (Jones *et al.*, 1991) was used to visualize the structure and to determine atom distances. Molecular surface representations were generated by GRASP (Nicholls *et al.*, 1991). Coordinates for DsbC are available at the Protein Data Bank (accession code 1eej).

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