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Six conserved cysteines of the membrane protein DsbD are required for the transfer of electrons from the cytoplasm to the periplasm of *Escherichia coli*

Eric J.Stewart[1], Federico Katzen and Jon Beckwith[2]

Department of Microbiology and Molecular Genetics, Harvard Medical School, 200 Longwood Avenue, Boston, MA 02115, USA

[1]Present address: Institut Jacques Monod, 2 Place Jussieu, Tour 43, 75251 Paris, Cedex 5, France

[2]Corresponding author

The active-site cysteines of the *Escherichia coli* periplasmic protein disulfide bond isomerase (DsbC) are kept reduced by the cytoplasmic membrane protein, DsbD. DsbD, in turn, is reduced by cytoplasmic thioredoxin, indicating that DsbD transfers disulfide-reducing potential from the cytoplasm to the periplasm. To understand the mechanism of this unusual mode of electron transfer, we have undertaken a genetic analysis of DsbD. In the process, we discovered that the previously suggested start site for the DsbD protein is incorrect. Our results permit the formulation of a model of DsbD membrane topology. Also, we show that six cysteines of DsbD conserved among DsbD homologs are essential for the reduction of DsbC, DsbG and for a reductive pathway leading to *c*-type cytochrome assembly in the periplasm. Our findings suggest a testable model for the DsbD-dependent transfer of electrons across the membrane, involving a cascade of disulfide bond reduction steps.

Keywords: cytochrome/disulfide bond/electron transport/isomerization/membrane topology

Introduction

The formation of disulfide bonds in proteins often contributes to their proper folding into stable tertiary structures. In general, such bonds are found among those proteins that are exported out of the cytoplasm. For instance, in Gram-negative bacteria such as *Escherichia coli*, stable disulfide bonds have only been observed in proteins in the periplasm or inserted into one of the two cellular membranes (for a review see Rietsch and Beckwith, 1998).

We and others have identified a set of *E.coli* proteins (termed Dsb for disulfide bond) that are required for the formation of the appropriate disulfide bonds in proteins that are translocated across the cytoplasmic membrane. Most of these proteins contain a Cys-X_a-X_b-Cys active-site motif embedded in a domain termed a thioredoxin fold, which is superimposable over the structure of the prototype *E.coli* thioredoxin (Martin *et al.*, 1993; Frishman, 1996). The first of these to be identified, DsbA, catalyzes disulfide bond formation directly by transferring its own disulfide bond, present in a Cys-X_a-X_b-Cys motif, to substrate proteins (Bardwell *et al.*, 1991). The resultant reduced form of DsbA is re-oxidized by the inner membrane protein DsbB (Bardwell *et al.*, 1993; Dailey and Berg, 1993; Jander *et al.*, 1994; Guilhot *et al.*, 1995; Kishigami *et al.*, 1995; Bader *et al.*, 1998). DsbB, in turn, is re-oxidized by transferring electrons to components of the electron transport system in the cytoplasmic membrane (Kobayashi *et al.*, 1997; Zeng *et al.*, 1998; Bader *et al.*, 1999; Kobayashi and Ito, 1999).

It has long been recognized that proteins with more than one disulfide bond may, during their folding process, pass through intermediates with disulfide bonds that are not found in the final form of the protein. This observation suggested the existence of a protein disulfide bond isomerase (PDI) that would promote isomerization (shuffling) of disulfide bonds. While PDIs have been known in eukaryotic cells for some time (De Lorenzo *et al.*, 1966), it is only recently that a periplasmic protein with isomerase activity (DsbC) was found in bacteria (Missiakas *et al.*, 1994; Shevchik *et al.*, 1994; Rietsch *et al.*, 1996). DsbC contains a thioredoxin-like fold including an active-site Cys-X_a-X_b-Cys motif (Frishman, 1996). In order to isomerize disulfide bonds, the active-site cysteines of DsbC are maintained in the reduced state. This is necessary so that a free cysteine can make a nucleophilic attack on the disulfide bond in the substrate protein, resulting in a mixed disulfide between the isomerase and the substrate. The resolution of the mixed disulfide can take one of two paths. Either DsbC reduces the disulfide bond, leaving the substrate protein to be re-oxidized (perhaps correctly) by DsbA, or the mixed disulfide is resolved by interaction with another cysteine from the substrate protein, resulting in a changed arrangement of disulfide linkages in the substrate. When the former mechanism occurs, DsbC exits the reaction in the oxidized state (Darby *et al.*, 1998). Before DsbC can participate in further oxidation reactions, its active site must be reduced.

In parallel to the oxidation pathway, where the cytoplasmic membrane protein DsbB is required to maintain DsbA in the oxidized state, in the isomerization pathway, the cytoplasmic membrane protein DsbD is necessary to maintain DsbC in the reduced state. However, in this case the source of electrons that DsbD uses to reduce disulfide bonds comes from the cytoplasmic thioredoxin pathway (Rietsch *et al.*, 1996, 1997). Thus, DsbD appears to transduce disulfide-reducing potential from the cytoplasm to the periplasm. DsbD is predicted to have multiple transmembrane domains, as well as a C-terminal thioredoxin-like domain with a Cys-X_a-X_b-Cys active site (Crooke and Cole, 1995; Fong *et al.*, 1995; Missiakas *et al.*, 1995).

DsbD is required for at least two additional reduction pathways in the cell envelope. First, DsbG, a periplasmic homolog of DsbC with unknown *in vivo* function, accumulates in the oxidized form in the absence of DsbD

(Andersen *et al.*, 1997; Bessette *et al.*, 1999). Secondly, DsbD is important for the assembly of the periplasmic *c*-type cytochromes. This assembly requires the formation of covalent bonds between heme groups and cysteine residues in the cytochrome polypeptide chain. For this reaction to take place, the cysteine residues must be maintained in the reduced state in the oxidizing environment of the periplasm. In the absence of DsbD, heme can not be bound to these cytochromes, probably because the cysteines are no longer reduced (Sambongi and Ferguson, 1994; Crooke and Cole, 1995). Fabianek *et al.* (1999) have proposed a reductive pathway involving intermediates between DsbD and the cysteines of the cytochromes. These intermediates are the membrane proteins CcmG (DsbE) and CcmH. CcmG also contains a thioredoxin-like domain located in the periplasm and may be a substrate for DsbD (Fabianek *et al.*, 1998).

In order to understand how the novel form of transmembrane electron transport facilitated by DsbD occurs, we have initiated an analysis of the structure and function of DsbD. Here we present evidence for the membrane topology of DsbD through the use of alkaline phosphatase (AP) fusions. During the course of this analysis it became clear that the previously published translational start site of DsbD was incorrect. Our analyses suggest that DsbD is much larger than previously proposed and that it includes an additional cysteine residue and transmembrane domain at the N-terminus of the protein. Translation of DsbD from the new translational start point predicts a protein of 565 amino acids containing eight cysteine residues. To study the function of DsbD, we mutated the eight cysteine residues to alanine and examined the phenotype of the mutants. The results of these two investigations allow us to speculate on the mechanism of transfer of disulfide-reducing potential across the cytoplasmic membrane.

Results

Determination of the topology of DsbD

We analyzed the membrane topology of DsbD by fusing the AP gene (*phoA*) missing its signal sequence to various positions within the *dsbD* gene (Manoil and Beckwith, 1986). The level of AP activity of these membrane protein fusions indicates whether the AP domain is in the cytoplasm (where AP has very low activity) or the periplasm (where AP has high activity), thus suggesting the topological localization of the domain of the membrane protein to which AP is fused (Manoil and Beckwith, 1986). To preserve the likely topology determinants of the protein, we placed the fusion joints at the predicted C-terminal end of each hydrophilic region as suggested by Boyd *et al.* (1993).

Initially, for these studies, we employed a plasmid that contained the portion of the *dsbD* gene which began with the previously reported start site for transcription (Missiakas *et al.*, 1995) and a *tac* promoter to drive transcription (data not shown). However, various manipulations of transcriptional and translational signals failed to yield a plasmid that could complement a chromosomal *dsbD* mutant. Furthermore, when we constructed the *dsbD–phoA* gene fusions on this plasmid they were expressed at extremely low levels. Finally, the results of the topological analysis using these fusions were

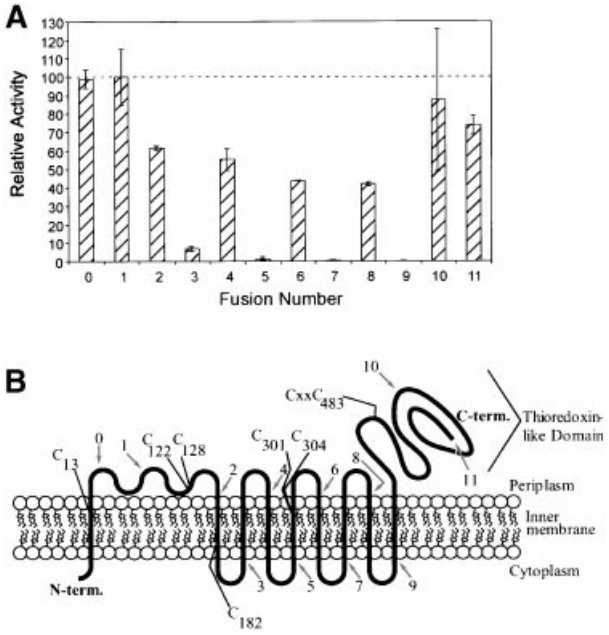

Fig. 1. (**A**) Specific activities of the DsbD–AP fusions and predicted topology of DsbD. Cultures were assayed for both their AP activity and their level of expression of the fusions. The AP activity was adjusted for the amount of fusion protein expressed and then normalized as a percentage of the highest activity fusion. This yielded the form of specific activity represented here. Error bars represent the standard deviation of duplicate cultures. (**B**) The numbered arrows in the model of DsbD (0–11) indicate the locations of the AP fusions. The exact fusion locations are after DsbD residues 29, 99, 160, 205, 243, 282, 320, 362, 388, 414, 523 and 563. Cysteine residues in DsbD are indicated by thin lines.

inconsistent with known characteristics of membrane protein topology. These findings led us to postulate that the published start site is incorrect. Therefore, we incorporated the fusions into a plasmid that included 722 bases of DNA upstream of the previously reported start site. These fusions were now expressed at high levels. This expression is not due to the *tac* promoter in the vector, as it is not responsive to induction of this promoter (data not shown). Our results (discussed further in the next section) strongly support a start site for transcription and translation considerably upstream from the previous proposal (Missiakas *et al.*, 1995). Both of these signals are included in the plasmid with the larger upstream *dsbD* fragment.

On this new plasmid, we generated 12 fusions of AP to DsbD. We assayed cells expressing these fusion proteins for AP activity. We also measured the rate of synthesis of each of the fusion proteins in order to ensure that the differences in activity were not due to differing levels of synthesis (San Millan *et al.*, 1989). Cells were pulse-labeled with radioactive methionine and the amount of radioactivity incorporated into each fusion quantitated after immunoprecipitation. Quantitation of the amount of OmpA expressed in the same period served as an internal control in each case. These data allowed us to calculate the specific activity of each fusion protein, i.e. the amount of AP activity per unit of AP protein (Figure 1A).

The fusions fall into two clear categories based on their specific activities: fusions 0, 1, 2, 4, 6, 8, 10 and 11 exhibited high specific activities, while fusions 3, 5, 7 and 9 exhibited low activities. These results suggest the

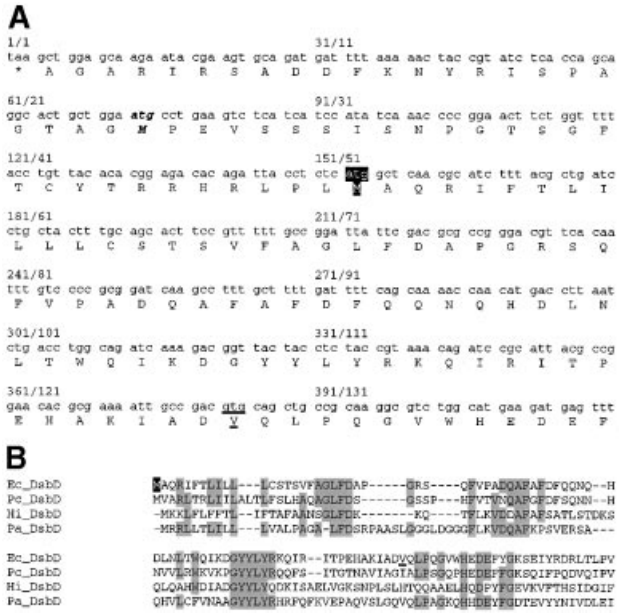

Fig. 2. The N-terminal region of DsbD. (**A**) The nucleotide and amino acid sequence of the N-terminus of *E.coli dsbD*. The three potential start codons in the *E.coli dsbD* gene are highlighted. The black codon is the most likely location of the initiation of translation. The previously proposed start codon is underlined, while the most upstream start codon (which is not necessary for functional DsbD) is shown in italics. (**B**) Comparison of the N-terminal regions of four DsbD proteins. The source organisms are: Ec, *E.coli*; Pc, *P.citrea*; Pa, *P.aeruginosa*; Hi, *H.influenzae*. The potential start codons are indicated as in (A). Conserved amino acids are shaded in gray.

topological model presented in Figure 1B. According to this model, the N-terminus of DsbD is in the cytoplasm and the C-terminal thioredoxin-like domain is in the periplasm. The model also predicts a second, large (~145 amino acids), periplasmic domain near the N-terminus. This domain contains three cysteine residues. According to this proposed structure, the cysteine residues at positions 301 and 304 are located near the periplasmic face of the membrane, while cysteine 182 would be the only cysteine near the cytoplasmic side.

Evidence for a start site for initiation of translation upstream of that previously published

The original proposal for a start site for DsbD translation placed the GUG initiation codon at a position corresponding to codon 126 of an open reading frame (ORF) (Missiakas *et al.*, 1995). We find that fusion 0, which is 49 codons upstream of the supposed GUG start codon, expresses a level of AP protein and activity as high or higher than that of the fusions following the original proposed start site (Figures 1 and 2). As these fusions are expressed from an endogenous promoter upstream of the gene and are all in the same reading frame, translation that begins upstream of the location of fusion 0 (in a wild-type *dsbD* transcript) must continue through, translating the rest of the gene as one polypeptide.

In the reading frame that we conclude contains the *dsbD* gene, there are two potential AUG start codons (Figure 2A). The sizes of fusions 1 and 2, as estimated by SDS–PAGE, are consistent with the predicted mol. wts of 60–62 and 66–68 kDa that would result from translation initiating from these upstream AUG codons. The apparent

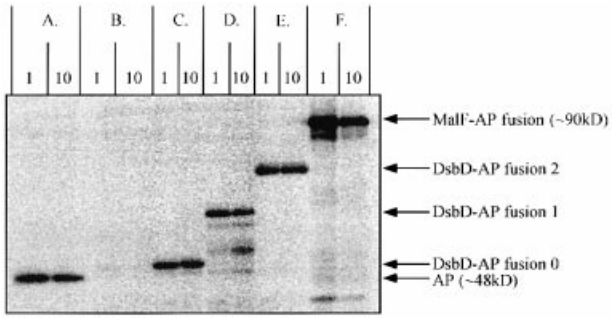

Fig. 3. Immunoprecipitation of the first three fusions. Radiolabeled samples were immunoprecipitated with anti-AP antibodies and then separated on a 12% SDS–polyacrylamide gel. The samples are as follows: (A) wild-type AP; (B) a strain expressing no AP or fusion protein; (C) DsbD–AP fusion 0; (D) DsbD–AP fusion 1; (E) DsbD–AP fusion 2; and (F) MalF–AP fusion control. A number 1 above a lane indicates that the sample was taken after 1 min of labeling, while a 10 indicates that the sample was taken after 1 min of labeling and 10 min of chase with cold methionine. The extrapolated sizes of fusions 0, 1 and 2 are ~51, 62 and 75 kDa, respectively.

sizes are not consistent with the predicted masses of 52 and 58 kDa that would result from translation beginning at the previously proposed GUG start codon (Figure 3). In addition, alignment of the predicted N-terminal protein sequence of the *E.coli* DsbD sequence with the three most closely related homologs (from *Haemophilus influenzae*, *Pseudomonas aeruginosa* and *Pantoea citrea*) shows amino acid homology in the region between the second AUG codon (at base 73 of the ORF) and the putative GUG start site (Figure 2B). Finally, the *dsbD* gene was cloned into a vector such that expression of the protein initiates at the AUG codon at base 73 under the control of the arabinose promoter. The protein thus expressed is fully functional (see the following section; Figures 4 and 5). We propose that this codon, 228 bases upstream of the GUG codon, is the actual translation start site. Expression from this AUG would result in a DsbD 76 amino acids longer than previously proposed. This predicted DsbD molecule contains an additional transmembrane domain and an additional cysteine residue. The numbering of the residues of the DsbD protein in this paper is therefore relative to the methionine encoded by this AUG codon.

Analysis of the DsbD cysteine mutants

In order to study the role of specific amino acid residues in DsbD function, we began by determining the role of the cysteines. We chose to alter the cysteines for two reasons. First, in the pathways of disulfide bond formation and isomerization, the various protein components involved in the transfer of electrons achieve this transfer mainly through cysteine residues via transient disulfide bonds. For example, the four cysteines of DsbB appear to be the most important amino acids in the functioning of this protein and to be directly involved in receiving electrons from DsbA (Jander *et al.*, 1994; Guilhot *et al.*, 1995; Kishigami *et al.*, 1995; Kishigami and Ito, 1996). DsbD, in all likelihood, receives its electrons via the Cys-X_a-X_b-Cys active site of thioredoxin and ultimately transfers those electrons to the analogous active sites of proteins such as DsbC and DsbG. It seemed possible then that the cysteines of DsbD might be involved in a similar mechanism for transferring electrons. Secondly, six of the

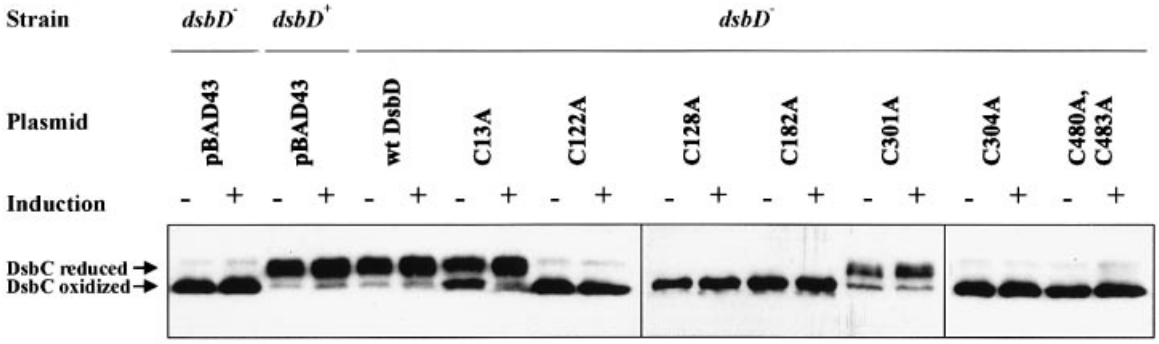

Fig. 4. Western blot showing the *in vivo* redox state of DsbC. The redox state of DsbC was trapped by acid precipitation and AMS alkylation. The third and fourth lanes represent wild-type controls expressing DsbD from the chromosome (strain RI89). All other lanes use a strain with a null allele of *dsbD* on the chromosome (RI242). The cysteine variants of DsbD were expressed from pBAD43. DsbC that is reduced becomes alkylated by AMS and displays a higher molecular weight than oxidized DsbC. Cells were grown without induction (–) or induced in the presence of 0.1% arabinose plus 0.1% glucose (+).

eight cysteine residues in DsbD are conserved in all of the known homologs (*E.coli*, *H.influenzae*, *P.aeruginosa*, *Neisseria gonorrhoeae*, *P.citrea*, *Salmonella typhimurium* and *Chlamydia trachomatis*; data not shown).

Therefore, we mutated the *dsbD* gene so as to generate a set of *dsbD* versions carrying mutations of sites corresponding to each of the eight cysteine residues. For the thioredoxin domain of DsbD, both cysteine codons were altered in a single construct. We cloned the mutated *dsbD* genes into a plasmid under the control of the BAD promoter of the arabinose operon with an optimized Shine–Dalgarno sequence. As seen below, the expression of wild-type DsbD from this promoter in the absence of the inducer, arabinose, was still sufficient to complement a mutant missing the *dsbD* gene. The *dsbD* mutations were chosen so as to change cysteines to alanines in all cases. We substituted alanines rather than serines because the latter can contribute to monothiol reactions such as isomerization and mixed-disulfide reduction, probably through hydrogen bonding to an active cysteine (Jeng *et al.*, 1995). To determine whether the cysteine residues are necessary for the function of DsbD, the mutant proteins were analyzed for their ability to function in the reduction of DsbC and DsbG, and in the assembly of *c*-type cytochromes. In addition, expression levels of the proteins were determined by Western blot analysis (see the next section).

We determined the thiol–disulfide redox state of DsbC in each of the *dsbD* cysteine mutants by trapping the protein with trichloroacetic acid (TCA) followed by alkylation of the free cysteines with 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS) (Joly and Swartz, 1997; Kobayashi *et al.*, 1997). AMS only alkylates free cysteines, not those engaged in disulfide bonds. The mass added by reaction of AMS (~0.5 kDa per AMS molecule) with each reduced cysteine is sufficient to significantly retard the mobility of the protein on an SDS–polyacrylamide gel. Thus, the reduced form of DsbC will display a higher molecular weight than the oxidized form.

Almost all of the DsbC produced from the control strains (expressing wild-type DsbD from the chromosome or from the plasmid pEJS92) had its active-site cysteines in the reduced state (Figure 4). Even without induction of the arabinose promoter responsible for the expression of DsbD from pEJS92, DsbC was as reduced as in the strain expressing DsbD from the chromosome. The phenotypes of the mutant strains fell into two distinct categories. Those expressing DsbD-C122A, C128A, C182A, C304A and C480/483A accumulate fully oxidized DsbC whether induced with arabinose or not. Therefore, these cysteines are essential to complete the thiol–disulfide redox pathway from the cytoplasm to DsbC. On the other hand, DsbD-C13A and C301A, like wild-type DsbD, show predominantly reduced DsbC, demonstrating that these cysteines are not required for this process. For the C13A mutant, in contrast to C301A, induction with arabinose is necessary to achieve a wild-type level of DsbC reduction, presumably because of the lower steady-state level of the DsbD-C13A protein (see below). Without induction of DsbD-C13A, DsbC is partially reduced.

In addition to measuring the redox state of DsbC, we also assessed the functionality of the mutant forms of DsbD in its two other known reactions. First, we showed that the reduction of DsbG, the periplasmic isomerase that is a homolog of DsbC, is affected by the same cysteine mutants as DsbC (data not shown). Secondly, we determined whether the *c*-type cytochromes were able to bind heme *in vivo*, an assay for the presence of reduced cysteines in the cytochromes. The *c*-type cytochromes are ordinarily only expressed under anaerobic growth conditions. To stimulate cytochrome *c* maturation, the *ccm* gene region together with the genes for the *c*-type cytochromes NapB and NapC were overexpressed from the plasmid pEC66. This system allowed the analysis of cytochrome *c* maturation in a background of various *dsbD* cysteine mutants during aerobic conditions.

The heme group in cytochromes can be visualized on SDS–polyacrylamide gels by staining the gel with 3,3′,5,5′-tetramethylbenzidine (see Materials and methods). In a background where the wild-type DsbD is synthesized, we were able to detect only three heme stainable bands (Figure 5). Since pEC66 is absolutely required for the presence of these bands (not shown), we presume that they represent the holocytochromes NapB and NapC, and the hemoprotein CcmE encoded by the plasmid (Fabianek *et al.*, 1999). In contrast, in a *dsbD* background, the formation of the holocytochrome NapB is completely abolished. In the same background, biosynthesis of the holocytochrome NapC, although not abolished, is strongly reduced. This residual assembly activity might be due to

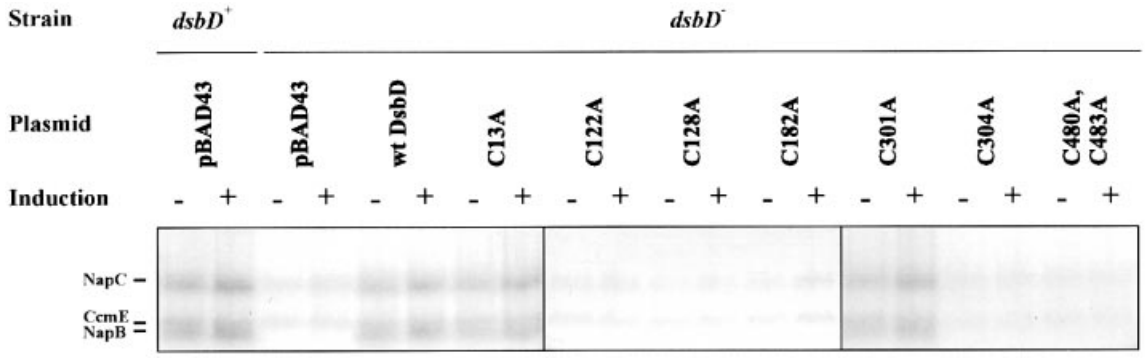

Fig. 5. Heme-stained SDS–PAGE of total proteins from strains carrying different DsbD cysteine mutants. MC1000 cells (*dsbD*$^+$) or FED126 cells (*dsbD*$^-$) carrying the vector (pBAD43), or plasmids encoding either DsbD (wt DsbD) or the DsbD cysteine mutants were grown as indicated in Materials and methods without induction (–) or induced in the presence of 0.1% arabinose plus 0.1% glucose (+). All the strains harbor plasmid pEC66. CcmE binds heme through a histidine residue and therefore is not dependent on DsbD.

a DsbD-independent reductive pathway. Since the heme chaperone CcmE was shown to bind heme by way of a covalent bond to a histidine residue (Schulz *et al.*, 1998), it is not surprising that in the absence of DsbD the biosynthesis of this hemoprotein is largely unaffected.

With the cytochrome *c* pathway, as with DsbG reduction, the phenotypes of the cysteine residues fell into the same two groups seen in the analysis of DsbC: those that are dispensable for the reductive activity (13 and 301) and those that are essential for this process (122, 128, 182, 304 and the pair at 480/483) (Figure 5). These results indicate the use of a common mechanism for these different targets of reduction.

The expression level of the DsbD cysteine mutants

For each of the cysteine mutants used in the analyses above, the steady-state level of DsbD was determined by Western blot analysis with anti-DsbD antibody. When induced with 0.1% arabinose plus 0.1% glucose, the expression level of the various DsbD proteins from the plasmid (with one exception) is ~10-fold higher than that seen from the wild-type chromosomal *dsbD* gene. Without induction the protein levels are ~5-fold lower than that from the chromosome. The plasmid encoding DsbD C13A proved to be the exception to these results. The induced level of protein from this plasmid is ~2-fold higher than that of the chromosomal level, and the uninduced level is nearly undetectable (data not shown). Whether this lower level of protein is due to reduced expression or decreased stability was not determined.

Discussion

The DsbD protein performs an unusual function in the *E.coli* cytoplasmic membrane. It receives electrons from cytoplasmic components and transduces them across the membrane. To analyze the mechanism of this novel mode of electron transfer it is essential to understand the organization of this protein in the membrane and to define the amino acid residues essential for its function. Here we report results providing a topological model for DsbD and the identification of six cysteine residues that are essential for its activity. Together, these results allow the proposal of testable models for the mechanism of electron transfer by DsbD.

The proposed topology of DsbD includes nine transmembrane segments, five periplasmic domains and four cytoplasmic domains. The subcellular location of the cysteine residues in this structure is of particular interest, since DsbD is involved in a pathway that transfers electrons from thiol–disulfide redox proteins in the cytoplasm to thiol–disulfide redox proteins in the periplasm (e.g. DsbC). These proteins, all members of the thioredoxin superfamily, contain Cys-X_a-X_b-Cys sequences as part of their active sites. Of the eight cysteine residues in DsbD, six are conserved in the DsbD homologs found in other bacterial species. According to our topological model, these six cysteines are positioned as follows: Cys122 and Cys128 are located in the first periplasmic domain; Cys182 in the second transmembrane segment, perhaps close to the cytoplasmic face of the membrane; Cys304 in transmembrane segment 5 or near it in periplasmic domain 3; and Cys480 and Cys483, representing the active site of the periplasmic thioredoxin-like domain (Figure 1B). These positional assignments must be considered tentative, as topological analysis of this sort (as well as any method other than direct structural techniques) can not give a precise picture of, for example, the exact extent of a transmembrane segment.

The most striking finding of this report is the demonstration that all six conserved cysteines are essential for the functioning of DsbD. Mutants lacking any one of them are fully defective in all the known functions of this protein. It is often difficult to obtain strong mutants of a protein in residues other than those that are part of the active site. For example, we and others have extensively sought mutations inactivating DsbA and DsbB, but found that the only residues that appear to be fully essential are the residues involved in the transfer of disulfide bonds; two cysteines in DsbA and four cysteines in DsbB (Jander *et al.*, 1994; H.-P.Tian, G.Jander, L.Debarbieux, H.Kadokura and J.Beckwith, unpublished results). In light of this, our findings with DsbD suggest a central role for these six essential cysteines in the transmembrane electron transport process that this protein appears to facilitate.

The results with the DsbD proteins with altered cysteines make it highly tempting to postulate a model for the movement of electrons that involves a cascade of reactions utilizing disulfide bonds. Assuming that the topological structure in Figure 1B is a close representation of the actual structure, we suggest the following as a working model for this process (Figure 6). We propose that newly

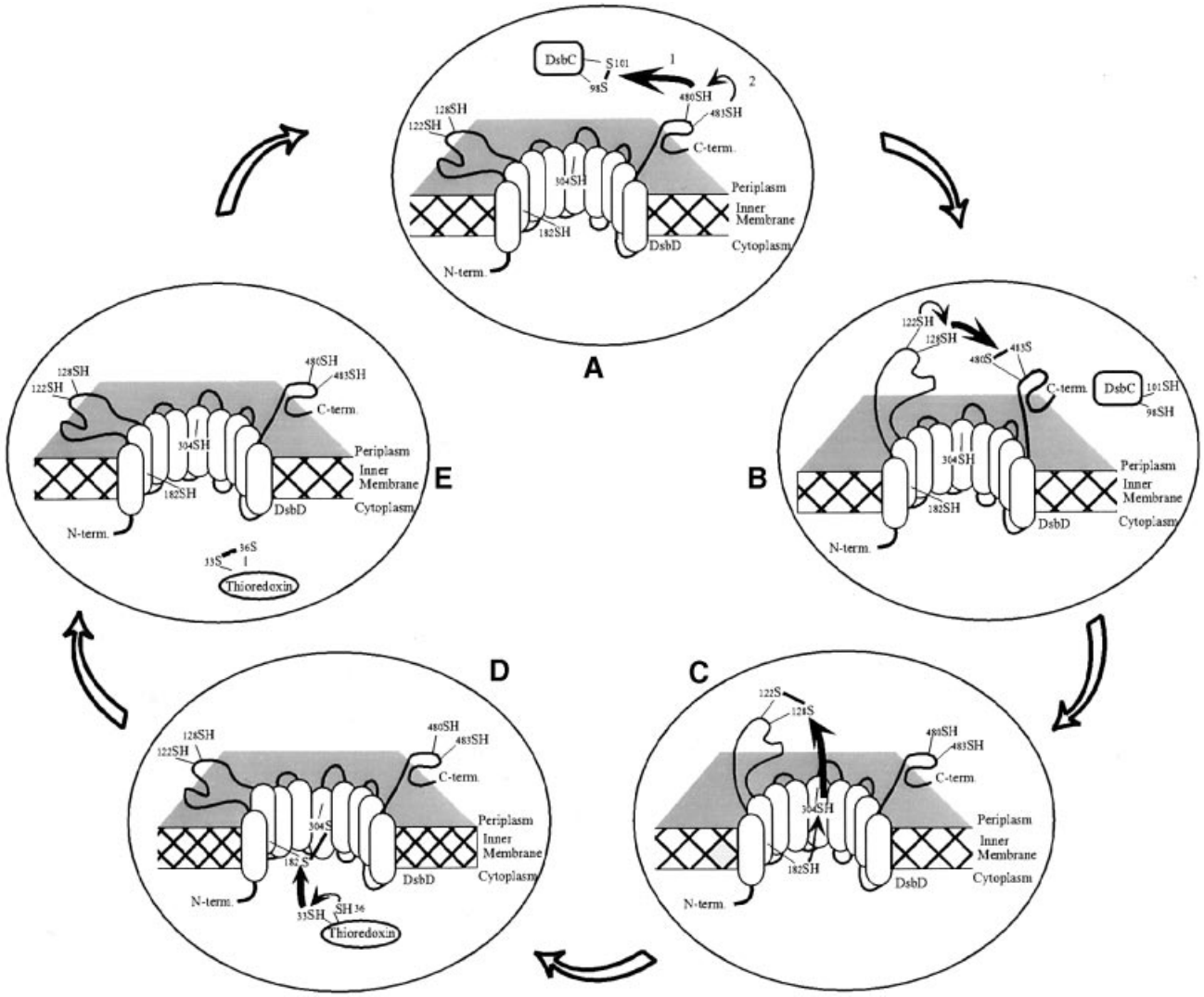

Fig. 6. Model for the mechanism of transduction of electrons across the membrane by DsbD. This model presumes the passing of electrons along cysteine residues within DsbD to transduce disulfide-reducing potential into the periplasm. See Discussion for a detailed description.

synthesized DsbD, with all of the cysteines in the reduced state, utilizes the cysteine at position 480 in the thioredoxin-like active site to form mixed disulfides with certain oxidized substrates such as DsbC, DsbG or components of the cytochrome *c* assembly pathway (Figure 6A). This mixed disulfide is resolved by the attack of C483 and the resultant C480–C483 disulfide bond in the thioredoxin domain is subsequently reduced via attack of one of the cysteines in the N-terminal periplasmic domain of DsbD, either C122 or C128 (Figure 6B). Reduction of the resulting C122–C128 disulfide bond then begins through the nucleophilic attack of C304, which is near the periplasmic face of the membrane, and is completed when the cysteine residue at C182, near the cytoplasmic side of the membrane, attacks the disulfide bond formed with C304 (Figure 6C). This leaves a disulfide bond between C182 and C304, possibly within the plane of the membrane (Figure 6D). Since, from the topological analysis, we can not define the exact positions of C304 and C182 within (or near) the membrane, we can not specify how much of a perturbation of the topology this disulfide bond would cause. Finally, cytoplasmic thioredoxin reduces the disulfide bond in DsbD (Figure 6D), returning DsbD to the reduced state (Figure 6E). These steps bear similarities to the reverse process that occurs in the re-oxidation of DsbB, where it is thought that a disulfide bond is passed from the first periplasmic domain of that protein to cysteines in the second periplasmic domain, which then transfer their oxidizing potential to DsbA (Jander *et al.*, 1994; Guilhot *et al.*, 1995; Kishigami *et al.*, 1995; Kishigami and Ito, 1996).

In this model, we have assumed that the cysteines of the thioredoxin domain are responsible for the initial step in the reduction of DsbC. Alternatively, it is possible that the C122,C128 cysteine pair carry out this first step, and that the cysteines of the thioredoxin domain act later, transferring the disulfide bond to the C304,C182 pair.

We recognize that a variety of other models may explain our findings. Certain of the cysteines may be involved in disulfide bonds that play a structural rather than a functional role in DsbD. Some of the cysteines may be portions of metal binding sites. Other membrane components may be involved in the process, as components of the electron transfer apparatus are in the case of DsbB (Kobayashi *et al.*, 1997; Zeng *et al.*, 1998; Bader *et al.*, 1999; Kobayashi and Ito, 1999). It is possible that amino acids

in addition to cysteines may contribute to the movement of electrons along the membrane-embedded portions of DsbD. Topological or conformational changes other than those involving disulfide bonds may contribute to the activity of this protein.

Nevertheless, our proposed model lends itself to some potential tests. First, predictions can be made about the oxidation state of the various cysteine residues as they exist in different genetic backgrounds. For example, in mutants lacking thioredoxin, most of the cysteines may be joined in disulfide bonds. Secondly, it may be possible to identify mixed disulfide intermediates between thioredoxin and DsbD, and between one of the cysteines of the thioredoxin domain of DsbD and other cysteines in the protein. By using mutants lacking the first cysteine of the active site of DsbA, it was possible to detect and characterize the nature of a mixed DsbB–DsbA disulfide complex (Guilhot *et al.*, 1995; Kishigami *et al.*, 1995). Preliminary studies using this approach with DsbD have identified a disulfide-bonded complex between the cytoplasmic thioredoxin 1 and the non-thioredoxin domain of DsbD. The specific cysteine of this domain involved in the complex has yet to be identified (F.Katzen and J.Beckwith, unpublished results). Finally, studies on the topology of the protein using protease treatment in spheroplasts may detect topological differences in DsbD expressed in different genetic backgrounds (e.g. missing thioredoxin or mutant for certain cysteines). Approaches to these questions are currently being pursued in our laboratory.

Finally, our results indicate that the previously proposed transcriptional and translational start sites for *dsbD* are incorrect. It is clear that the translation of the ORF that codes for DsbD begins at least 76 codons upstream from that suggested by Missiakas *et al.* (1995). This conclusion is based on the expression of a series of AP fusions to DsbD, on the molecular weights of these fusion proteins, on the failure to observe significant expression from the later start sites, on the conservation in DsbD homologs across species of this longer ORF and on the presence of a transmembrane domain in our proposed ORF that is necessary for deducing the proper topology of the protein.

Materials and methods

Bacterial strains and growth conditions

Strains used are listed in Table I. Strain DHB4 was used for the topology analysis (Boyd *et al.*, 1987). Cultures were generally grown in NZ-amine at 37°C, with antibiotic selection to maintain the plasmids (200 μg/ml ampicillin, 50 μg/ml spectinomycin, 10 μg/ml chloramphenicol). For the simultaneous analysis of AP activity and pulse labeling, strains were grown overnight in minimal M63 media with 0.2% glucose, then subcultured 1:100 into fresh M63 plus glucose. For the analysis of the cysteine mutants in the reduction of DsbC, strain RI242 was used. This strain contains the null mutation *dsbD*::TnCam. To determine the *in vivo* redox state of DsbC and the steady-state level of the DsbD mutants, cells were grown overnight in NZ-amine (5 ml, at 37°C with aeration). The overnight cultures were then sub-cultured 1:100 into NZ-amine or NZ-amine with 0.1% glucose and 0.1 or 0.2% arabinose. The subcultures were grown to an OD_{600} of ~0.5 (measured in a Spectronic 20D).

For the analysis of the heme groups of the *c*-type cytochromes, we used the wild-type strain MC1000 and *dsbD* null strain FED126. This strain contains an internal in-frame deletion of 536 codons of the *dsbD* gene. We replaced the deleted DNA sequences by an *Eco*RI site, following the protocol devised by Link *et al.* (1997). For this purpose, we employed the primers DsbDΔ1b, DsbDΔ1f, DsbDΔ2b and DsbDΔ2f (Table II).

Fusion construction

To determine the topology of DsbD, portions of the *dsbD* gene were fused to a portion of the *phoA* gene (missing its signal sequence). The plasmid pDHB5747 is a pBR-based, ampicillin-resistant vector that contains a fusion of the *malF* gene to *phoA*. Fragments of *dsbD* were cloned into this vector in the place of the *malF* gene. To accomplish this, the vector was digested with *Mlu*I and *Bsp*EI (there are two *Bsp*EI sites in the vector, however, one is blocked by methylation), removing the *malF* sequence. The *dsbD* fragments were generated by PCR from pDipZ2 with appropriate restriction sites encoded in the primers (Table II). The 5′ primer (FUSF) contains a restriction site for *Bss*HI, which leaves an overhang compatible with *Mlu*I. The 3′ primers (DSF0, DSF1–10) contain a *Bsp*EI site, positioned such that a translational fusion between *dsbD* and *phoA* is created when the fragment is cloned into the vector. The vector and PCR fragments were then ligated together; the ligation was subsequently electroporated into DHB4.

Alkaline phosphatase activity and expression rates of the fusions

Mid-log cultures in M63 medium (as described above) were split into two aliquots, one of which was assayed for AP enzymatic activity, and the other was radiolabeled and immunoprecipitated with anti-AP antibodies and anti-OmpA antibodies (as an internal control). Both procedures were performed as described previously, as were the calculations of AP activity relative to expression rate (San Millan *et al.*, 1989; Boyd *et al.*, 1993). In addition, the amount of AP expressed was normalized to the amount of OmpA expressed (as an internal control) taking into account the relative number of methionine residues in each fusion and OmpA.

Production of antibodies to DsbD

A fragment of the *dsbD* gene, from codon 442 to the stop codon, was generated by PCR using the primers DtrxUP and DtrxDN(f) (Table II). This fragment was cloned into the T7 expression vector pET-26b(+) (Novagen) using the restriction enzymes *Nco*I and *Bam*HI. The resulting construct (pEJS70) expressed the *pelB* signal sequence of the vector fused to the thioredoxin-like domain of DsbD, in turn fused to the His_6 tag from the vector. The His-tagged thioredoxin-like domain was then purified over a nickel column and eluted with 20 mM imidazole. The purified protein was inoculated into two rabbits by Covance (Denver, PA). The resulting antisera were affinity-purified to the thioredoxin-like domain of DsbD, which was immobilized on a nitrocellulose membrane. The purified antisera were subsequently used to develop Western blots at a dilution of 1:300.

Directed mutation of the cysteine residues

DsbD contains eight cysteines. The first six (at positions 13, 122, 128, 182, 301 and 304) were mutated individually. The last two, in the thioredoxin-like domain, were mutated as a pair (480,483). Two methods were employed to change the cysteine residues in DsbD to alanines. The first method employed the Altered Sites kit from Promega, following the directions of the manufacturer. The cysteines at positions 13, 122, 128 and 182 were mutated to alanine by this procedure using primers DC13A, DC122A, DC128A and DC182A. This method was not able to generate mutations in cysteine 301 or 304, nor the double mutation in cysteines 480 and 483. These mutations were subsequently generated by a PCR-based method, termed crossover PCR or PCR stitching. Two PCR products were generated. The first product was generated from a primer at the start of *dsbD* (D43Up) and one at the point of the desired mutation (D301Anti, D304Anti, D48XAnti). The second product was produced from a primer at the end of *dsbD* (D43Dn) and one at the point of mutation (D301Sense, D304Sense, D48XSense). Each of these pairs of products overlapped each other by 20 or more bases. A third PCR, where these two products prime each other, is used to create one product that is the full length of the gene, incorporating the new mutation. In the same reaction, this full-length product is amplified by primers at the 5′ and 3′ ends (D43Up, D43Dn). Table II lists the oligonucleotide primer sequences.

The mutated versions of *dsbD* were cloned by PCR into the vector pBAD43. This vector has a pSC101 origin of replication from pAM238 and a spectinomycin-resistance gene. The vector contains the arabinose BAD promoter with an optimized Shine–Dalgarno sequence and the gene for the AraC regulatory protein to regulate expression (from the vector pBAD24; Guzman *et al.*, 1995). The coding region of the *dsbD* gene was cloned immediately downstream of the promoter into the *Nco*I and *Hin*dIII restriction sites.

All of the mutations were verified by DNA sequencing.

Table I. Strains and plasmids used in this research

Strain or plasmid	Relevant genotype and features	Source or reference
Plasmids		
pDHB5747	Amp^r pBR-based vector with *phoA* gene	laboratory collection
pEJS49–60	12 plasmids based on pDHB5747, with the DsbD–AP fusions 0–11	this work
pBAD43	Spc^r pSC101-based vector with arabinose regulation and an optimized Shine–Dalgarno sequence	D.Weiss and D.Ritz
pEJS92	pBAD43 with wild-type *dsbD*	this work
pEJS93	pBAD43 with *dsbD*-C13A	this work
pEJS94	pBAD43 with *dsbD*-C122A	this work
pEJS87	pBAD43 with *dsbD*-C128A	this work
pEJS88	pBAD43 with *dsbD*-C182A	this work
pEJS89	pBAD43 with *dsbD*-C301A	this work
pEJS95	pBAD43 with *dsbD*-C304A	this work
pEJS96	pBAD43 with *dsbD*-C480A, C483A	this work
pDipZ2	Amp^r (pBR-based) with 3 kb fragment of the DsbD region	Rietsch *et al.* (1996)
pEC66	Cam^r (pACYC184), 9.5 kb *Nco*I fragment with *napBC* and *ccmABCDEFGH*	Fabianek *et al.* (1999)
Strains		
DHB4	Δ(*ara-leu*)7697 *araD*139 Δ*lacX*74 *galE galK rpsL phoR* Δ(*phoA*)PvuII Δ*malF*3 *thi*	Boyd *et al.* (1987)
MC1000	*araD*139 (*araABC-leu*)7679 *galU galK* Δ(*lac*)X74 *rpsL thi*	laboratory collection
RI89	MC1000 *phoR* Δ*ara714 leu*$^+$	Rietsch *et al.* (1996)
RI242	RI89 *dsbD*::mini-Tn*10*Cam1	Rietsch *et al.* (1996)
FED126	MC1000 Δ*dsbD*	this work

Table II. Primers used in this work

Name	Sequence (5′ to 3′)
FUSF	tactgcgcgccccgggtactctgggtgttgccct
DSF0	aaattccggacgtcccggcgcgtcgaataat
DSF1	ctgtccggacgatcgcggtaaatctcgct
DSF2	tcttccggagcggtgggctgctcttgctg
DSF3	gtatccggagctctggcagtggagagccg
DSF4	tgatccggagggtgctgtagcgccgcctg
DSF5	ctgtccggaccctgttggcgattgctcatc
DSF6	acttccggattcccgctttgggcgatatac
DSF7	gcttccggagttttgacttgttccatccacg
DSF8	agctccggacgtaatccccatacatcacc
DSF9	agttccggacgcatccagccgcgtttagc
DSF10	cactccggatgctttaacagcgccacatcttg
DSF11	tagtccggacgatcgcgcaaatgtgcgctga
DtrxUP	acgcccatggctcaaactcagacgcatctcaa
DtrxDN(f)	caagggatccggttggcgatcgcgcaaat
D43Up	cacagagaattcaccatggctcaacgcatctttacgctg
D43Dn	gcaagtaagcttcacggttggcgatcgcgcaaatg
DC13A	ctgatcctgctacttgccagcacttccgtttttgcc
DC122A	gtcacctaccagggcgctgctgatgccggtttct
DC128A	gctgatgccggtttcgcttatccgcccagaaac
DC182A	gtatcgcctttacgccagccgtgctgccaatgtac
D301Sense	cgattgccggactgatcgcttcaccatgcaccaccg
D301Anti	cggtggtgcatggtgaagcgatcagtccggcaatc
D304Sense	gactgatctgttcaccagccaccaccgcaccgcttag
D304Anti	ctaagcggtgcggtggtggctggtgaacagatcagtc
D48XSense	tagatctttatgccgactgggccgtcgccgctaaagagtttgagaaataca
D48XAnti	tgtatttctcaaactctttagcggcgacggcccagtcggcataaagatcta
DsbDΔ1b	gaggaattcccggcgcgtcgaataatc
DsbDΔ1f	gagggatccttgctggcggtctggact
DsbDΔ2b	gagaagcttacttcgccactgtgttga
DsbDΔ2f	gaggaattcaaccgtgaacgacacttg

Determination of the in vivo redox state of DsbC

To assay the function of the cysteine mutants of DsbD, the ability of these mutants to reduce DsbC *in vivo* was assayed. The redox state of DsbC was visualized by alkylating the free cysteine residues with the high-molecular-weight compound AMS (4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid disodium salt, mol. wt 536.44; from Molecular Probes, Eugene, OR). This agent can only alkylate free thiols and therefore only reacts with the reduced form of the protein. Alkylation of free cysteines retards the mobility of the protein on an SDS–polyacrylamide gel by an amount roughly corresponding to the added mass. AMS alkylation was performed as described in Joly and Swartz (1997), except that the acetone wash after TCA precipitation was omitted. Instead, the acid was neutralized when the pellet was resuspended in 15 mM AMS and sample buffer by the addition of Tris–HCl pH 8.0 at a concentration of 0.67 M. The samples were separated on a 12% SDS–polyacrylamide gel and were then blotted to nitrocellulose. Washing, incubation and detection of the blot followed the ECL Western blotting kit protocol (Amersham). Anti-DsbC antibody (Joly and Swartz, 1997) and secondary antibody (donkey anti-rabbit, horseradish peroxidase-linked; Amersham) were both used at 1:5000.

Determination of c-type cytochrome heme binding

Either MC1000 or FED126 cells, containing each of the plasmids of the DsbD cysteine variants together with pEC66, were grown as described above. Cultures (1 ml) were pelleted and resuspended in 20 μl of non-denaturing sample loading buffer. After SDS–PAGE, covalently bound heme in *c*-type cytochromes was visualized in-gel as described by Thomas *et al.* (1976).

Determination of the steady-state level of the DsbD mutants

Strains containing the mutants of DsbD were grown in rich media (NZ-amine, Difco) in the absence of induction as well as in the presence of a glucose–arabinose inducing mixture (0.1% each). Western blots were performed using the antibody to DsbD described above. The Western protocol was performed as described above.

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