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Transmembrane Electron Transfer by the Membrane Protein DsbD Occurs via a Disulfide Bond Cascade

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Summary

The cytoplasmic membrane protein DsbD transfers electrons from the cytoplasm to the periplasm of *E. coli*, where its reducing power is used to maintain cysteines in certain proteins in the reduced state. We split DsbD into three structural domains, each containing two essential cysteines. Remarkably, when coexpressed, these truncated proteins restore DsbD function. Utilizing this three piece system, we were able to determine a pathway of the electrons through DsbD. Our findings strongly suggest that the pathway is based on a series of multistep redox reactions that include direct interactions between thioredoxin and DsbD, and between DsbD and its periplasmic substrates. A thioredoxin-fold domain in DsbD appears to have the novel role of intramolecular electron shuttle.

Introduction

Disulfide bonds are essential for the folding, stability, and function of a large number of proteins. In all organisms, the formation of such bonds is likely a catalyzed process, confined almost exclusively to extracytoplasmic proteins. In *Escherichia coli*, disulfide bond formation takes place in the periplasmic space. In this compartment, cysteines in proteins are kept correctly oxidized by the Dsb system (Rietsch and Beckwith, 1998). Dsb proteins all contain a Cys-X_a-X_b-Cys motif, which is often embedded in a domain termed a thioredoxin-like fold (Martin et al., 1993; Frishman, 1996).

DsbA (Bardwell et al., 1991), a soluble protein with two cysteines located in its thioredoxin-like fold (Martin et al., 1993), oxidizes target proteins by exchanging its single disulfide bond. This exchange leaves DsbA in a reduced form, which is reoxidized by the inner membrane protein DsbB (Bardwell et al., 1993). Compelling evidence that DsbA directly interacts with DsbB was provided by the isolation of the mixed disulfide DsbA–DsbB (Guilhot et al., 1995). Electrons are then transferred from DsbB to terminal electron acceptors in the cytoplasmic membrane (Bader et al., 1999; Kobayashi and Ito, 1999).

The oxidation of envelope proteins with multiple cysteines by DsbA frequently results in the formation of incorrect disulfide bonds. The correction of these mispaired cysteines is dependent on the periplasmic protein DsbC, which catalyzes the rearrangement or isomerization of the disulfide bonds (Rietsch et al., 1996; Joly and Swartz, 1997; Sone et al., 1997). DsbC is a soluble homodimeric protein with thioredoxin-like domains in each monomer (Zapun et al., 1995; Frishman, 1996; McCarthy et al., 2000). The isomerization reaction is probably initiated by attack of the most N-terminal cysteine of one of the active sites of the dimeric DsbC (Cys-98) on a disulfide bond in the substrate protein. This reaction should result in an unstable mixed disulfide between DsbC and its substrate that may be resolved by a nucleophilic attack of an alternate cysteine of the substrate, resulting in a disulfide bond rearrangement in the substrate protein. Alternatively, if the resolution of the complex is the result of the attack of the most C-terminal cysteine of the DsbC active site (Cys-101), the substrate protein exits the reaction in a reduced state, having another chance of being correctly oxidized by DsbA. In this case, DsbC emerges from the reaction with a disulfide bond at its active site and must be reduced to restore its activity (Walker and Gilbert, 1997; Darby et al., 1998).

Reduction of oxidized DsbC relies on the cytoplasmic membrane protein DsbD, which utilizes the thioredoxin pathway in the cytosol as the source of reducing equivalents (Rietsch et al., 1996, 1997). In the absence of DsbD, DsbC is found completely oxidized. Thus, DsbD appears to mediate the transfer of electrons from the cytoplasm into the periplasm. Additionally, DsbD is involved in the reduction of the periplasmic DsbG, a DsbC homolog (Bessette et al., 1999), and is required for the biosynthesis of *c*-type cytochromes (Sambongi and Ferguson, 1994; Crooke and Cole, 1995). The covalent bonds between the vinyl side chains of heme and the thiol groups in the apocytochrome *c* are only established if the cysteines in the apocytochrome are kept reduced (Fabianek et al., 2000). Fabianek et al. (1999) have proposed that DsbD provides the reducing potential for apocytochrome c by recycling the oxidized form of the membrane protein CcmG.

DsbD is composed of three domains: an amino-terminal periplasmic domain, a hydrophobic core consisting of eight transmembrane (TM) segments, and a carboxy-terminal periplasmic domain (Stewart et al., 1999; Chung et al., 2000; Gordon et al., 2000) (Figure 1A). The carboxy-terminal domain contains a Cys-X_a-X_b-Cys motif embedded in an apparent thioredoxin-fold. We had earlier inferred that the amino terminus of the protein constituted an additional TM segment; however, work by Chung et al. (2000) and Gordon et al. (2000) established that this region constituted an amino-terminal signal sequence. (In this paper, we change the numbering system for amino acid residues of DsbD to conform to that of these workers).

We have shown by mutational analysis that the six cysteines conserved among DsbD homologs are required for each of its known in vivo activities (Stewart et al., 1999). One pair of cysteines is located in each of the periplasmic domains, and the third pair appears to lie within TM segments of the hydrophobic core (Figure

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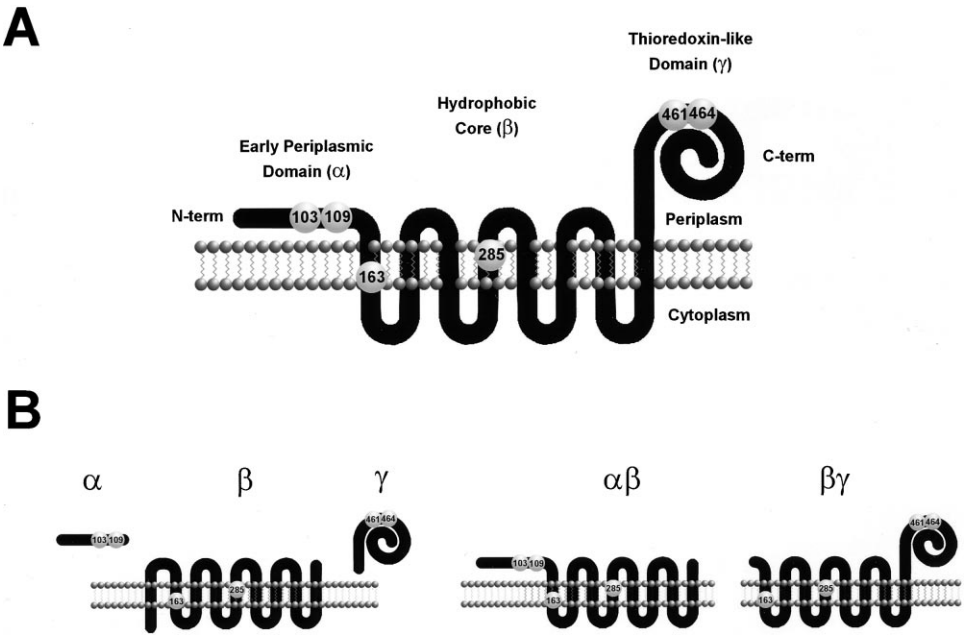

Figure 1. Representation of DsbD and Its Derivatives

(A) Membrane topology of DsbD. Large spheres represent the essential cysteines.

(B) Representation of the DsbD derivatives used in this work. The first TM segment of MalF and the DsbD signal sequence (DsbDss) were fused to β and βγ, respectively, in order to assist them in correctly inserting into the membrane. To export γ to the periplasmic space, the alkaline phosphatase signal sequence (APss) was fused at its N terminus.

1A). These features of DsbD prompted us to propose a model for its activity in which electrons are passed from cytoplasmic thioredoxin (TrxA) to membrane-imbedded cysteines and thence to cysteines in the periplasm (Stewart et al., 1999). In this paper, we report results providing in vivo evidence that DsbD sequentially transfers reducing potential along its three structural domains *directly* from cytoplasmic thioredoxin to periplasmic substrates, and that this transfer involves a cascade of disulfide bond formation and reduction steps.

Results

DsbD Can Be Split into Its Three Structural Domains without Losing Activity

To examine the possibility that disulfide bond oxidation and reduction constitutes the mechanism of electron transfer in DsbD, we had to distinguish different oxidation states of the protein. Furthermore, we sought to analyze the interaction between, on the one hand, cysteines in the different domains of the protein and, on the other hand, TrxA, DsbC, and DsbD by detecting disulfide bonded intermediates between them. However, as DsbD contains six essential cysteines, it appeared difficult to distinguish the oxidation state or interactions between the individual cysteines.

To bypass these difficulties, we chose to split DsbD into smaller polypeptides, each containing no more than two cysteines. The success of this approach requires that simultaneous expression of these polypeptides should restore activity, thus ensuring electron transport from the cytoplasm to the periplasm. DsbD appears, based on the deduced topology, to consist of two periplasmic domains, one at the N terminus comprising approximately 140 amino acids and the second one located at the C terminus, resembling a thioredoxin-like fold (Figure 1A). Both of them are quite conserved among all DsbD homologs. These domains are connected through a hydrophobic central core that shows similarity to CcdA proteins, required for cytochrome *c* biogenesis in both Gram-positive and -negative bacteria (Schiott et al., 1997; Deshmukh et al., 2000). Notably, CcdA and the hydrophobic core of DsbD share a similar membrane topology and contain two essential cysteines located in the lipid bilayer (Deshmukh et al., 2000). Thus, sequence comparisons suggest possible evolution from three separate domains into a single polypeptide chain.

We chose to engineer a set of compatible plasmids that expressed the three domains of DsbD as individual or combined polypeptides. Accordingly, we split DsbD at two different positions: (1) between the early periplasmic domain and the hydrophobic core and (2) between the hydrophobic core and the thioredoxin fold. In this way, we generated constructs encoding five different polypeptides named α, β, γ, αβ, and βγ (see Figure 1B and Experimental Procedures). In each construct, we have replaced the two nonessential cysteines of DsbD with alanines.

To determine whether DsbD is still active when separated into two or three polypeptides, different combinations of its structural domains were analyzed for their ability to function both in the reduction of DsbC, and in the assembly of *c*-type cytochromes. In all cases, we determine the oxidation state of DsbC by acid trapping of cell extracts and alkylation of free cysteines with AMS. The addition of the high molecular weight alkylating

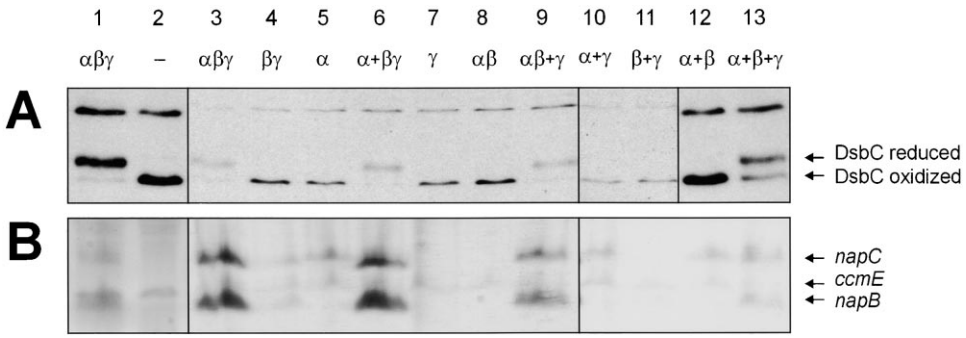

Figure 2. Phenotypes of *dsbD*$^-$ Cells Expressing Different Combinations of DsbD Domains

(A) Western blot showing the in vivo redox state of DsbC. Cells were induced with 0.2% arabinose, harvested at mid-log phase, precipitated with TCA, and subjected to AMS alkylation. DsbC that is reduced becomes alkylated and displays a higher molecular weight than oxidized DsbC. The *dsbD*$^+$ strain FED389 containing no plasmid was used in lane 1. All other lanes use the *dsbD*$^-$ strain FED386 containing different plasmids as follows: (2) no plasmid, (3) pFK093, (4) pFK094, (5) pFK051, (6) pFK094 and pFK051, (7) pFK017, (8) pFK049, (9) pFK049 and pFK017, (10) pFK051 and pFK017, (11) pFK060 and pFK017, (12) pFK051 and pFK060, and (13) pFK051, pFK060, and pFK017.

(B) Heme-stained SDS/PAGE of total proteins. CcmE binds heme through a histidine residue and therefore is not dependent on DsbD. Only NapB should be considered as indicative of DsbD function. Lanes are the same as above.

agent allows ready distinction between the oxidized and reduced form of many proteins including DsbC (Joly and Swartz, 1997; Kobayashi et al., 1997).

Plasmids expressing DsbD derivatives were introduced into a *dsbD* null mutant, and their ability to reduce DsbC was assayed. Nearly all the DsbC from the strain expressing the wild-type DsbD maintained its active-site cysteines in the reduced state (Figure 2A, lanes 1 and 3). However, when any of the three DsbD structural domains was removed from the system, DsbC accumulated in the oxidized state (Figure 2A, lanes 4, 5, 7, 8, 10, 11, and 12). Remarkably, when all the constituents of DsbD were expressed simultaneously as two polypeptide chains (Figure 2A, lanes 6 and 9) or even as three separate proteins (Figure 2A, lane 13), the reduction of DsbC was reestablished. These results indicate that all three domains of DsbD are required, but do not need to be part of a single polypeptide chain to be able to transfer reducing potential from the cytoplasm to DsbC.

The effect of splitting DsbD was also examined by assessing the ability of the separated domains to promote *c*-type cytochrome biosynthesis. The *c*-type cytochromes are normally expressed under anaerobic growth conditions. However, we were able to study cytochrome *c* biogenesis under aerobic conditions by expressing the required genes from an arabinose-inducible promoter inserted upstream from those genes (see Experimental Procedures). In the absence of DsbD, the assembly of the holocytochrome NapB is completely abolished, but some NapC residual assembly activity is still observed (Stewart et al., 1999). Therefore, only the biosynthesis of the holocytochrome NapB should be considered as indicative of DsbD function. As in the analysis of DsbC, the lack of expression of any DsbD domain abolished heme attachment to NapB (Figure 2B, lanes 4, 5, 7, 8, 10, 11, and 12). But again, when the three structural domains were coexpressed as two or three independent polypeptides, holo NapB assembly was rescued (Figure 2B, lanes 6, 9, and 13).

Altogether, our data indicate that these truncated proteins assemble in the membrane into a functional entity that, as in the wild-type situation, acts in two separate pathways: DsbC reduction and cytochrome *c* biogenesis.

Electrons Are Sequentially Transferred from One Domain to Another

The functional expression of the separated polypeptide domains of DsbD suggests that all the thiol-disulfide exchange reactions occurring in this three-component system mimic those that take place in the intact molecule. If the set of reactions involves the transfer of a disulfide bond from one domain to another, as has been suggested for DsbB (Kishigami and Ito, 1996), it should be possible to establish the sequential order in which these players participate, using this system. The simplest way to study this question is to disrupt the electron pathway by taking one domain away from the system, and then to assess the redox status of the remaining components. Those domains that act at a step later than that of the removed component should be unable to receive reducing potential, and thus would remain in their oxidized form. In contrast, those domains that accomplish their function at a step prior to that of the removed component would accumulate in the reduced form or, alternatively, their redox state would remain unchanged.

As a prerequisite for this analysis, we should be able to distinguish the oxidized and reduced isoforms of the constituent domains α, β, and γ. In other words, the apparent molecular weights of the reduced polypeptides should be shifted when derivatized with AMS. We found that the alkylated and nonalkylated isoforms of α are clearly distinguishable on SDS/PAGE (compare lanes 1 and 3 in Figure 3A). This shift was due to the alkylation of free cysteines since the reduction per se did not affect the mobility of the protein (Figure 3A, lane 2). The AMS treatment also shifted β to a higher molecular weight position (Figure 3B, compare lanes 1, 2, and 3). However, only a fraction of the molecules were alkylated (Figure 3B, lanes 3). The accessibility to AMS might be partly restricted due to the high hydrophobicity

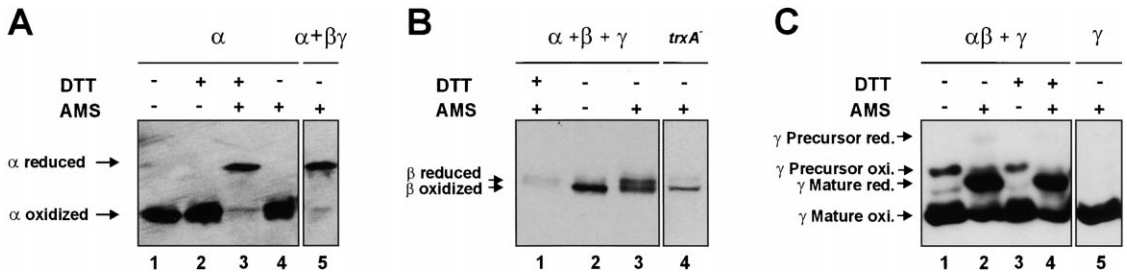

Figure 3. In Vivo Redox State of the DsbD Derivatives

(A) Cells expressing the designated DsbD derivatives were subjected to reduction by DTT, TCA precipitation, and AMS treatment as indicated. Proteins were separated by SDS/PAGE and visualized by Western blotting using antibodies raised against the α peptide. Oxidized and reduced standards were generated without AMS alkylation, or subjected to reduction by 40 mM DTT before precipitation. The strain background was FED126. The following plasmids were used: pFK051 (lanes 1, 2, 3, and 4), pFK051, and pFK094 (lane 5).

(B) Western blot developed using antibodies anti-Myc-c epitope (they recognize the β-Myc-c fusion peptide). The strain background used in lane 4 was FED161. All other lanes used FED126. All the strains contained plasmids pFK051, pFK060, and pFK017.

(C) Western blot developed using antisera against the γ peptide. The strain background was FED126. The following plasmids were used: pFK049 and pFK017 (lanes 1, 2, 3, and 4), and pFK017 (lane 5).

of this polypeptide. Remarkably, in a *trxA*$^-$ background, the band corresponding to the reduced isoform of β disappeared (Figure 3B, lane 4), suggesting either that: (1) the two cysteines are engaged in a disulfide bond or (2) these residues become inaccessible to AMS alkylation for other reasons.

The expression of the fusion APss-γ yielded mainly three bands (Figure 3C, lane 1). Their mobilities were not altered by reduction with DTT (Figure 3C, lane 3). The main bands probably correspond to the unprocessed and mature forms of γ, while the third band likely represents a degradation product. Only a small proportion of the unprocessed form was accessible to alkylation, even by treating the cells with DTT. However, a vast fraction of the mature form shifted to a higher molecular weight upon AMS treatment (Figure 3C, lanes 2 and 4), indicating that its cysteines were in the dithiol form. Notably, in the absence of αβ, essentially all the mature γ migrated as the oxidized form (Figure 3C, lane 5).

In summary, all these results indicate that the reduced and oxidized forms of α, β, and γ are clearly distinguishable. The composite polypeptides αβ and βγ, bearing four cysteines each, were not included in the analysis since the oxidation state of specific cysteines could not be unequivocally determined.

With these results in hand, we aimed to determine whether the electrons follow a sequential pathway among these domains. When α, β, and γ were simultaneously expressed from compatible plasmids, all of their active cysteines were partly accessible to the alkylating reagent AMS (Table 1). Interestingly, when two of the domains were fused into a single molecule (e.g., αβ and βγ), the remaining polypeptide became more accessible to AMS (Table 1 and data not shown). It may be that when DsbD is split into two polypeptides instead of three, the system works more efficiently, and the rereduction of the separate domain is achieved faster.

When the plasmid expressing γ is not included in the three-component system, the redox state of β remains unchanged. However, α becomes completely oxidized (Table 1). Similarly, in cells expressing the two-component system, α and βγ, essentially all α is in the reduced state, but α is completely oxidized when βγ is removed (Figure 3A, lanes 4 and 5). This result indicates that γ is required for the reduction of α. In other words, in the pathway of transfer of electrons from cytoplasmic thioredoxin through DsbD, γ acts before α but later than β.

When α is removed from the three-component system, the redox state of the remaining peptides remains unchanged (Table 1). This finding is consistent with the previous result, suggesting that α is the last destination of the electrons in DsbD before being transferred to the periplasmic substrates. In the absence of α, there is no acceptor for the electrons that have been passed into DsbD from thioredoxin. Finally, in the absence of β, the rest of the components become completely oxidized (Table 1), implying that β is involved in the earliest step in transferring electrons from thioredoxin and that it, in turn, transfers those electrons to the periplasmic domains.

According to the DsbD topology, β is the only domain

Table 1. Transfer of Reducing Potential among the DsbD Derivatives

	Redox State		
Polypeptides Expressed	α	β[a]	γ[a]
α + β +γ	partly reduced	partly reduced	partly reduced (50% reduced[b])
αβ + γ	—	—	partly reduced (75% reduced[b])
α + βγ	reduced	—	—
α + β	oxidized	partly reduced	—
β + γ	—	partly reduced	partly reduced
α + γ	oxidized	—	oxidized
α + β + γ (*trxA*$^-$)	partly reduced	oxidized	oxidized

[a] Cysteine accessibility to AMS might be partly impaired. The real amount of reduced polypeptides might be larger.
[b] Estimated from Western blot signal.

of DsbD that bears two cysteines localized to TM segments in the cytoplasmic membrane (Stewart et al., 1999; Chung et al., 2000; Gordon et al., 2000). Since one of these cysteines, Cys-163, may lie close to the cytoplasmic face of the membrane, it was not surprising to find that β is the starting point of the pathway. Consequently, it is predicted that β would be oxidized in a background defective in the thioredoxin/thioredoxin reductase system. As expected, β and γ are completely oxidized in a *trxA* background (Table 1, Figure 3B, lane 4). The fact that α is partly reduced in this mutant background was not unexpected. The concomitant overexpression of active thiol proteins like β and γ leads to the accumulation of reducing potential in the membrane. This may account for the rereduction of α, which ultimately allows reduction of DsbC. Indeed, we have found that the overexpression of the intact DsbD protein allows reduction of DsbC in the absence of TrxA (not shown).

In summary, our results strongly imply the following electron pathway: thioredoxin → β → γ →α → DsbC. The detection of the mixed disulfides α–DsbC and thioredoxin–βγ, described in the following sections, further supports this model.

Cysteine 109 in the α Domain of DsbD Mediates the Reduction of DsbC

Genetic studies have shown that electrons are passed via TrxA to DsbD and via DsbD to DsbC (Rietsch et al., 1996, 1997; Stewart et al., 1999; Chung et al., 2000; Gordon et al., 2000). However, these studies do not indicate whether this pathway involves direct interactions between these proteins or whether other intermediates are involved. Detection in vivo of DsbD–DsbC and DsbD–TrxA mixed disulfide complexes would indicate that DsbD interacts directly with these two proteins. Therefore, we sought to detect these complexes and, further, to identify the cysteines in DsbD that mediate these interactions.

We initiated these studies using the three separate polypeptides α, β, γ. To avoid artifactual thiol-disulfide exchange reactions, cells were TCA-precipitated and free thiols were alkylated with AMS. Immunoblots, developed using anti-DsbC antibodies, revealed the presence of the reduced form of DsbC, some nonspecific cross-reacting bands, and a band of an apparent molecular weight of approximately 40 kDa (indicated with an asterisk) (Figure 4A, lane 1). This latter band disappeared upon the addition of DTT (Figure 4A, lane 2), suggesting that it represented a mixed disulfide. Anti-α antibodies recognized the same band, which also disappeared by reducing the sample (Figure 4B, lanes 1 and 2). Western blots using either anti-c-Myc (when the β domain was tagged with the c-Myc epitope) or anti-γ antibodies failed to reveal a band at the corresponding position (not shown).

Four lines of evidence suggest that the apparent complex seen in the gels in Figure 4 represents the mixed disulfide α–DsbC. First, the apparent molecular weight of this complex correlates well with the sum of the molecular weights of α and DsbC (37.7 kDa). Second, the complex reacts with anti-DsbC and anti-α antibodies. Third, the band disappears upon the addition of a reductant. And finally, the band's presence depends on simultaneous expression of DsbC (see Figure 4, lanes 3 and 4) and a thiol-active form of α (see below). These results support the model proposed above, in which α represents the ultimate step of the pathway before the electrons are transferred from DsbD to periplasmic substrates.

To identify the active cysteine that participates in the formation of this mixed disulfide, we mutated each of the codons specifying the essential cysteines of DsbD and examined the ability of the altered DsbD molecules to form a disulfide bridge with DsbC. Mutating any cysteine of β or γ resulted in the elimination of the α–DsbC complex (Figure 4, lanes 9 to 16). According to our model (see previous section), mutations of cysteines in β or γ should disrupt the electron flow among domains, leaving α completely oxidized, and thus unable to form a disulfide bond with target proteins.

In contrast to β and γ, alteration of the two cysteines in α resulted in different effects. While the C109A mutation totally abolished the formation of the mixed disulfide with DsbC, the C103A replacement enhanced the formation of the complex (Figure 4, lanes 5 to 8). Here, we also observed additional bands, which may represent other mixed disulfides involving the α peptide (Figure 4B, lanes 5 and 6).

Interestingly, mutations C103A and C109A completely destabilized α, which was no longer detectable on Western blots (Figure 4B, lanes 5 to 8). This effect was not peculiar to the cys→ala replacements as C103Y and C109Y mutations had exactly the same consequences (data not shown).

These results strongly suggest that Cys-109 is the active cysteine that reacts with DsbC. Further, the role of Cys-103 may be to resolve this mixed α–DsbC disulfide, resulting in a changed arrangement of disulfide linkages in both proteins.

Identification of a Mixed Disulfide between α and CcmG

Fabianek et al. (1999) have proposed that electrons are transferred from DsbD to the apocytochrome *c* via the membrane proteins CcmG and CcmH. This transfer might involve the formation of the intermediate DsbD–CcmG. To test this model and to determine whether Cys-109 is also involved in this interaction, we assessed the ability of α_{C103A} to form a mixed disulfide with CcmG. We presumed, in these experiments, that α_{C103A}, bearing only one cysteine, would be able to establish mixed disulfides in the absence of β and γ. We used a C-terminal His_6-tagged derivative of α_{C103A}, as this version was considerably more stable than the untagged α.

The expression of α_{C103A}-His_6 in *dsbD*$^-$ cells triggered the formation of several mixed disulfides (Figure 5A). Notably, when *ccmG* was induced, as a part of the *ccm* operon, we were able to distinguish an additional complex of approximately 30–35 kDa that reacted with both CcmG and α antibodies (Figure 5, lanes 3, 4, 9, and 10). This mixed disulfide was absent in strains unable to express CcmG, due to an in-frame deletion of the gene (Figure 5, lanes 5 and 6) or to repression of the entire *ccm* operon (Figure 5, lanes 1 and 2). A null mutation in *dsbC* did not afftet the formation of the complex, indicating that DsbC is not required for the interaction between DsbD and CcmG (Figure 5, lanes 9 and 10). Again, several findings indicate that this complex is the

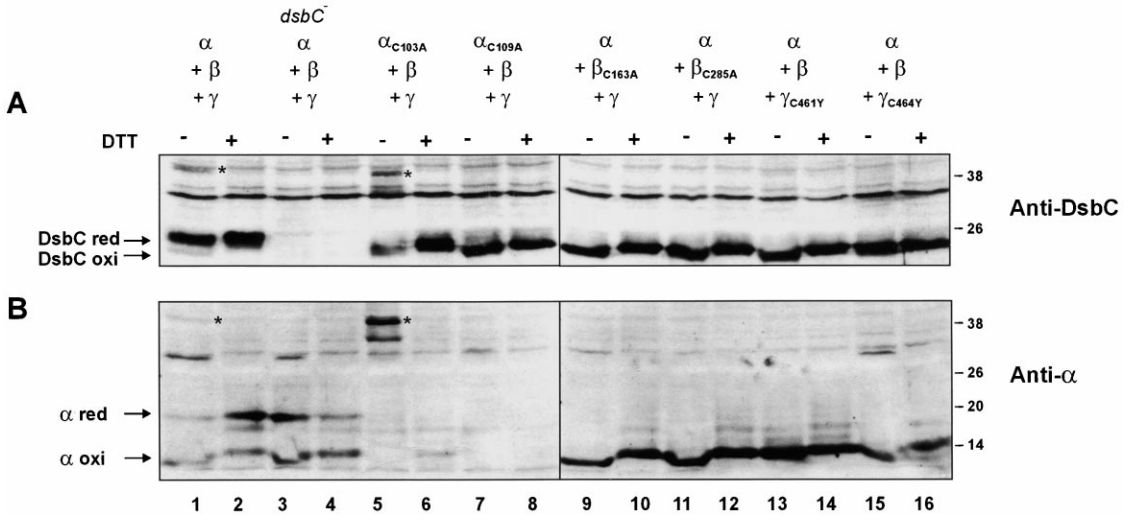

Figure 4. Analysis of the Formation of a Complex between DsbD Derivatives and DsbC

(A) *dsbD*⁻ cells containing plasmids encoding the designated proteins were grown and processed as before. Proteins were separated by SDS/PAGE and visualized by Western blotting using antibodies against DsbC. The strain background used in lanes 3 and 4 was FED266. For all other lanes, the strain FED126 was used. The following plasmids were used: pFK051, pFK053, and pFK017 (lanes 1 to 4), pFK068, pFK053, and pFK017 (lanes 5 and 6), pFK069, pFK053, and pFK017 (lanes 7 and 8), pFK051, pFK70, and pFK017 (lanes 9 and 10), pFK051, pFK071, and pFK017 (lanes 11 and 12), pFK051, pFK053, and pFK128 (lanes 13 and 14), and pFK051, pFK053, and pFK129 (lanes 15 and 16). The asterisks indicate the complex DsbC–α. The molecular weights of protein markers (in kDa) are indicated on the right.

(B) Same as above but using antibodies against the α peptide.

mixed disulfide α–CcmG: (1) the apparent molecular weight of the complex was in the expected range (32.7 kDa); (2) its presence depended on the concomitant expression of CcmG and α; (3) both CcmG and α antibodies recognized the same band; and (4) the complex disappeared when the reductant DTT was added.

Finally, the immunoblot pictured in Figure 5A indicated that α_{C103A} is capable of forming mixed disulfides with additional components. Whether these molecules truly represent undiscovered DsbD substrates remains to be investigated.

DsbD Directly Interacts with TrxA via Cysteine 163

Our model for electron flow through the domains of DsbD predicts that DsbD would interact with the electron donor in the cytoplasm via one of the two essential cysteines of its hydrophobic core β. To assess whether TrxA is the direct electron donor and to identify the DsbD residue mediating this interaction, we employed the strategy described above—replacement of essential cysteines and trapping of the mixed disulfide.

We found it difficult to detect mixed disulfides that involved β as a single polypeptide. Perhaps the fusion of a ninth TM segment at its N terminus slightly alters the structure of this domain, diminishing its affinity for its partner. Therefore, we decided to use the more efficient two-piece system composed of α and the fused βγ domains. We observed a mixed disulfide of approximately 50 kDa reacting with TrxA and γ antibodies when either none of the essential cysteines were exchanged, or

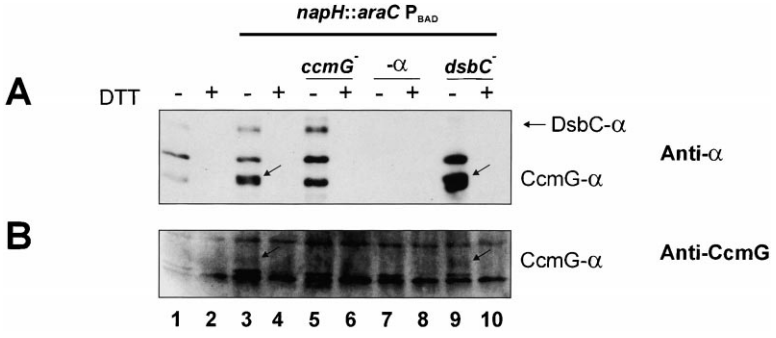

Figure 5. Western Blot Analysis of Complex Formation between α and CcmG

(A) The plasmid pFK090, encoding the reactive peptide α_{C103A}-His_6, was introduced into the strains described below with the exception of those of lanes 7 and 8, which carry no plasmid. Cells were grown and processed as described. Proteins were separated by SDS/PAGE and visualized by Western blotting using antibodies against the α peptide. The following strain backgrounds were used: FED126 (*dsbD*⁻, lanes 1 and 2), FED386 (*dsbD*⁻, *napH*::*araC* P_{BAD}, lanes 3, 4, 7, and 8), FED387 (*dsbD*⁻, *napH*::*araC* P_{BAD}, *ccmG*⁻, lanes 5 and 6), and FED390 (*dsbD*⁻, *napH*::*araC* P_{BAD}, *dsbC*⁻, lanes 9 and 10). The tilted arrows indicate the mixed disulfide CcmG–α.

(B) Same as above but using antisera against CcmG.

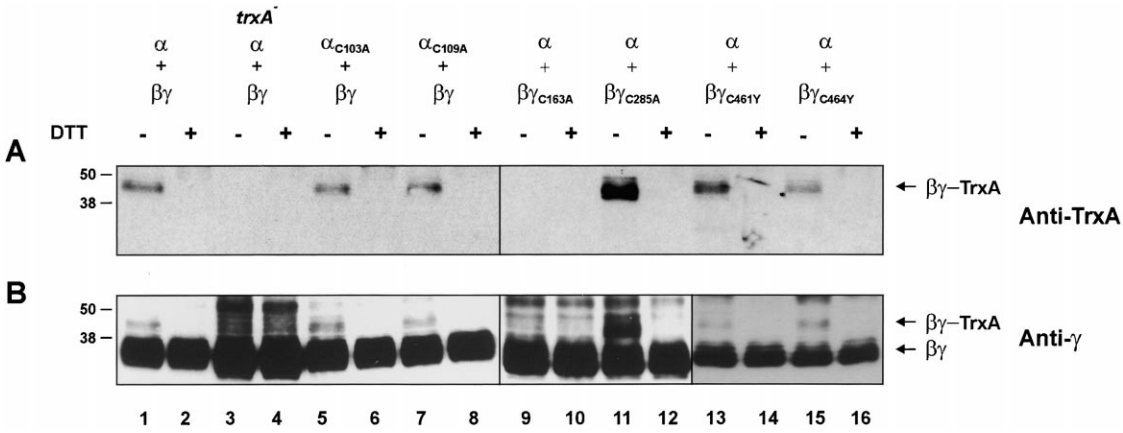

Figure 6. Analysis of the Mixed Disulfide Formation between DsbD Derivatives and TrxA

(A) *dsbD*⁻ cells expressing the designated DsbD derivatives were grown and processed as indicated before. Western blots were developed using antisera against TrxA. The strain background used in lanes 3 and 4 was FED161. All other lanes use the strain FED126. The following plasmids were used: pFK086 and pFK094 (lanes 1 to 4), pFK090 and pFK094 (lanes 5 and 6), pFK091 and pFK094 (lanes 7 and 8), pFK086 and pFK100 (lanes 9 and 10), pFK086 and pFK101 (lanes 11 and 12), pFK086 and pFK102 (lanes 13 and 14), and pFK086 and pFK103 (lanes 15 and 16). The molecular weight of protein markers (in kDa) are indicated on the left.

(B) Same as above but using antisera against γ.

when cysteines other than C163 were replaced (Figure 6, lanes 1, 2, 5–8, and 11–16). The complex practically disappeared when C163 was mutated (Figure 6, lanes 9 and 10). Since the complex depends on TrxA expression (Figure 6, lanes 3 and 4), and the apparent molecular weight was approximately the sum of the weights of βγ and TrxA (59 kDa), we postulate that this complex represents the mixed disulfide TrxA–βγ.

Similar results were obtained using the complementary two-piece system "αβ + γ" (not shown).

The complex with thioredoxin was more abundant when cysteine 285 was mutated (Figure 6, lanes 11 and 12). The simplest explanation for this observation is that the formation of this mixed disulfide is due to an attack on oxidized thioredoxin by cysteine 163. This would be simply the reverse reaction of the normal pathway for passage of electrons across the membrane. The absence of C285 may prevent, then, the resolution of the disulfide-bonded complex. A prediction of this hypothesis is that the formation of the complex thioredoxin–$\beta\gamma_{C285A}$ should be enhanced in a strain where oxidized thioredoxin is present in high amounts. To test this prediction, we determined the amount of the complex in a thioredoxin reductase mutant (*trxB*⁻). Consistent with our proposal, we observed a substantial increase in the amount of the thioredoxin–$\beta\gamma_{C285A}$ complex (Figure 7, compare lanes 7 and 8 with 5 and 6), suggesting that the reverse thiol reaction is occurring.

Nevertheless, in the wild-type situation, of the thioredoxin–DsbD pair, it is thioredoxin which is the reductant. Therefore, a variant of thioredoxin mutated in the less reactive cysteine (cysteine 36) should still be able to form the mixed disulfide with βγ. In agreement with this prediction, in cells expressing the variant $TrxA_{C36Y}$, the thioredoxin–βγ complex can be readily detected (Figure 7, lanes 1 to 4).

Altogether, these results suggest that thioredoxin *directly* transfers reducing potential to DsbD via cysteine 163.

Discussion

In this paper, we describe a novel mechanism of electron transfer across a membrane. Our results support the proposal that the *E. coli* cytoplasmic membrane protein DsbD utilizes at least two thiol redox reactions among its structural domains to transfer electrons from cytoplasmic thioredoxin to substrate proteins localized in the periplasm. The process involves the passage of reducing equivalents among three domains of DsbD; each domain contains a pair of cysteines essential for these reactions. The finding that cytoplasmic TrxA forms a mixed disulfide with DsbD via the cysteine located at position 163 indicates a direct interaction between these two proteins (Figure 8). The reducing equivalents generated by this reaction are then used to reduce the thioredoxin-like domain γ, and finally transferred to the catalytic domain α. The α domain then utilizes the active cysteine at position 109 to form a mixed disulfide with an oxidized periplasmic substrate such as DsbC. The mixed disulfide is probably resolved by a nucleophilic attack of cysteine 103 that results in release of the substrate in its reduced state.

Our results are consistent with a mechanism in which the reducing equivalents are passed from cytoplasmic thioredoxin ultimately to DsbD substrates simply by a series of steps requiring only reduction of intramolecular disulfide bonds. For instance, the disulfide-bonded intermediate between TrxA and the β portion of DsbD may be the result of the reduction of a disulfide bond between cysteines at positions 163 and 285. Although we have not been able to demonstrate this directly, the fact that these cysteines become inaccessible to AMS in a *trxA* background is consistent with this hypothesis. Similar steps would explain the passage of electrons from the β portion of DsbD to the thioredoxin-like domain, γ, and thence to the catalytic domain of DsbD, α.

The proposal that the TrxA–DsbD–DsbC pathway involves a direct cascade of disulfide bond reduction

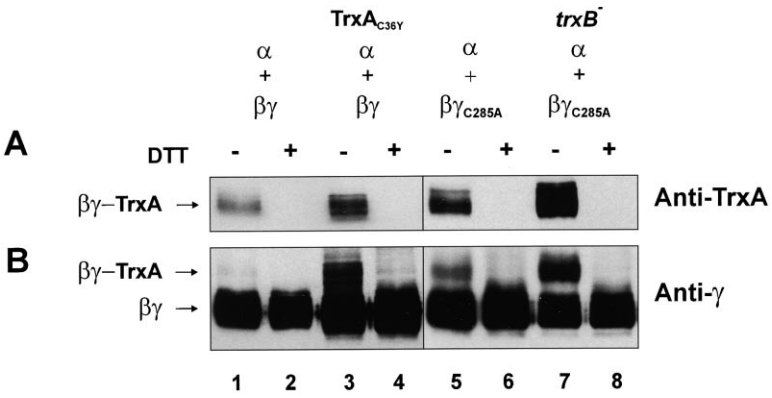

Figure 7. Formation of the Complex thioredoxin–βγ in Cells Deficient in the thioredoxin/thioredoxin Reductase System

(A) *dsbD*$^-$ cells expressing the designated DsbD derivatives were grown and processed as indicated before. Western blots were developed using antisera against TrxA. The strain background used in lanes 3 and 4 was FED161, while lanes 7 and 8 used strain FED513. All other lanes use the strain FED126. The following plasmids were used: pFK086 and pFK094 (lanes 1 and 2), pFK086, pFK094, and pFK145 (lanes 3 and 4), and pFK86 and pFK101 (lanes 5–8).

(B) Same as above but using antisera against γ.

steps raises a tantalizing question about the roles of cysteines 163 and 285. How do these residues become accessible both to cytoplasmic thioredoxin and to the electron acceptor on the opposite side of the cytoplasmic membrane? We can imagine at least two ways in which this might take place. First, Cys-163 and Cys-285 could be positioned in a channel-like structure that can be accessed from both sides of the lipid bilayer. Second, the reduction of the Cys-163-Cys-285 disulfide bond may cause a perturbation of the topology of DsbD such that the free sulfhydryl moves to a position closer to the periplasmic face of the membrane. There, it may become available to promote further transfer of reducing potential. Alternatively, our proposal for the disulfide bond cascade may be incomplete. In addition to interactions between cysteines, small molecules such as quinones, as in DsbB (Bader et al., 1999), could account for intermediate steps in the passage of reducing equivalents between domains of DsbD.

Many thiol-redox active proteins considered as reductants can, under the appropriate conditions, function as oxidants, and vice versa. For example, thioredoxin 1, which is normally a very important cytoplasmic reductant, can act as an oxidant, promoting disulfide bonds when the conditions of the cytoplasm change (Stewart et al., 1998), or when it is exported to the oxidizing environment of the periplasm (Debarbieux and Beckwith, 1998, 2000). DsbC, which normally acts as a reductant or as a thiol-disulfide isomerase, can operate as an oxidant in the absence of a functional DsbD (Rietsch et al., 1996). These and other examples indicate that in any of these thiol-redox reactions between two different proteins, the reaction can proceed in either direction. Our results suggest that this is also the case for DsbD. In the absence of a cytoplasmic source of reducing potential, DsbD can initiate a nucleophilic attack on the normally reducing TrxA. Whether or not this reaction ever occurs in wild-type cells remains to be studied.

Our data suggest that DsbD uses a single mechanism to reduce all of its substrates in the periplasm. This interpretation is supported by the fact that proteins with diverse functions, such as DsbC and CcmG, form mixed disulfides with DsbD via the cysteine at position 109. We presume that DsbD also uses this residue to interact with the DsbC homolog DsbG. Our results contradict previous suggestions (including our own) that the thioredoxin-moiety of DsbD is the domain that transfers electrons to substrates (Crooke and Cole, 1995; Missiakas et al., 1995; Stewart et al., 1999; Chung et al., 2000). While this proposal seemed reasonable at the time, perhaps this rather complex pathway of disulfide bond reduction indicates that two thioredoxin-folds (those of DsbD and DsbC in this case) cannot interact with each other. In fact, there are no examples in the literature of such interactions.

In our model, the thioredoxin-like domain appears to have the unusual role of shuttling reducing potential between two different domains of a protein. Although there are examples in which active thioredoxins and their cognate reductases are combined in one hybrid polypeptide, as is the case of the thioredoxin system of *Mycobacterium leprae* (Wieles et al., 1995), or the *Salmonella typhimurium* AhpF protein (Poole et al., 2000), the thioredoxin domain in DsbD appears to behave as a flexible thiol-active arm that connects two different functions of a protein: the electron translocase activity of β and the thiol-disulfide reductase role of α. Our in vivo data, however, do not indicate the number of molecules of DsbD needed for the transfer of a pair of electrons.

The DsbD homolog CcdA, which in some organisms is required for the biogenesis of *c*-type cytochromes (Schiott et al., 1997; Deshmukh et al., 2000), consists of only the hydrophobic domain we have termed β, raising the question of why two additional periplasmic domains are required for the activity of DsbD. It is tempting to speculate that DsbD evolved by acquiring α and γ. Per-

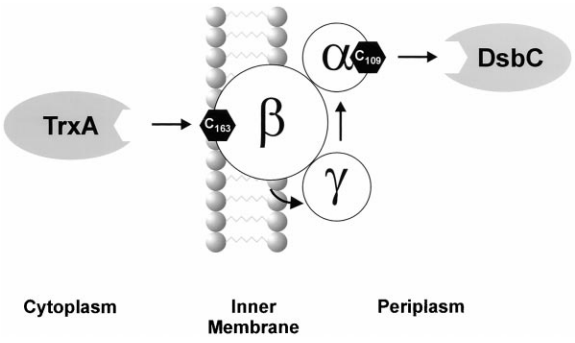

Figure 8. Scheme of the Electron Transport via DsbD

The arrows indicate the direction of the flow of electrons. For simplicity, only DsbC, representing a periplasmic substrate, is shown; and only the first and last cysteines of DsbD involved in the pathway are pictured.

Table 2. Strains and Plasmids Used in This Work

Strain or Plasmid[a]	Relevant Genotype or Features
Strains	
AD494[b]	*trxB*::Kan^r
CAG12177[c]	*zeh-298*::*Tn*10
EC29[d]	Δ*ccmG*
FED126[e]	MC1000 Δ*dsbD*
FED161	FED126 Δ*trxA*
FED266	FED126 *dsbC*::mini-Tn*10* Kan^r
FED338	FED126 Δ*ccmG zeh-298*::*Tn*10
FED386	FED126 *napH*::*araC* P_{BAD}
FED387	FED338 *napH*::*araC* P_{BAD}
FED389	MC1000 *napH*::*araC* P_{BAD}
FED390	FED386 *dsbC*::mini-*Tn*10 Kan^r
FED513	FED126 *trxB*::Kan^r
MC1000[f]	*araD*139 (*araABC-leu*)7679 *galU galK* Δ(*lac*)X74 *rpsL thi*
STL117[g]	*dsbC*::mini-Tn*10* Kan^r
WP570[f]	Δ*trxA*
Plasmids	
pBAD18[h]	Arabinose regulation, pBR-based, ampicillin^r
pBAD33[h]	Arabinose regulation, pACYC184-based, chloramphenicol^r
pBAD43[e]	Arabinose regulation, pSC101-based, spectinomycin^r
pET-26b(+)[i]	T7 expression vector, C-terminal His_6 tag
pFK017	pBAD43 with APss-γ
pFK029	pET-26b(+) with α
pFK049	pBAD18 with αβ
pFK051	pBAD33 with α
pFK053	pBAD18 with MalF′-β
pFK060	pBAD18 with MalF′-β-c-Myc
pFK068	pBAD33 with α_{C103A}
pFK069	pBAD33 with α_{C109A}
pFK070	pBAD18 with MalF′-β_{C163A}
pFK071	pBAD18 with MalF′-β_{C285A}
pFK086	pBAD33 with α-His_6
pFK090	pBAD33 with α_{C103A}-His_6
pFK091	pBAD33 with α_{C109A}-His_6
pFK093	pBAD18 with DsbD
pFK094	pBAD18 with DsbDss-βγ
pFK100	pBAD18 with DsbDss-βγ_{C163A}
pFK101	pBAD18 with DsbDss-βγ_{C285A}
pFK102	pBAD18 with DsbDss-βγ_{C461Y}
pFK103	pBAD18 with DsbDss-βγ_{C464Y}
pFK128	pBAD43 with APss-γ_{C461Y}
pFK129	pBAD43 with APss-γ_{C464Y}
pFK145	pBAD43 with $TrxA_{C36Y}$
pLD47[h]	Modified *malF* gene
pLMD104[j]	TrxA under the control of the *phoA* promoter
pLMD60[j]	APss-6aa-TrxA

[a] Except where indicated, the source of the strain or plasmid is the present work.
[b] Derman et al., 1993, [c] Singer et al., 1989; [d] Fabianek et al., 1998; [e] Stewart et al., 1999; [f] Lab Collection; [g] Rietsch et al., 1997; [h] Guzman et al., 1995; [i] Novagen; [j] Debarbieux and Beckwith, 1998.

Table 3. Primers Used in This Work

Name	Sequence (5′ to 3′)
DsbD-24F	GAGGGTACCACACGGAGACACAGATTA
DsbD+63B	GAGCTGCAGTTCTCCCAGTACATCTTC
BADFOR	CTGACGCTTTTTATCGCAAC
DsbD-7C	GAGTCTAGACTACACAGGCTGTGGCGCTGC
DsbD-TagF	GAGCCATGGGACGTTCACAATTT
DsbD-TagR	GAGAAGCTTTTGCTGCGGAACAGACAC
DsbD-8	GGTATGGCCAGCAGCAAGAGCAGCCCAC
BADREV	ACCGCTTCTGCGTTCTGATT
DsbD_Myc-tag	CTCTCTAGACTACAGCAGATCTTCTTCAGA AATCAGTTTCTGTTCAAGTGGGCGCACGC TAACCAATGCCG
DsbD-4	GGAGAATTCCAGGATTGGGCATTTGGT
DsbD-4C	GTGTCTAGATTTCCCACTGCAAGTGTC
DsbD-12	CCCACCGCGCAATTGCC
DsbD-12C	AAATTGTGAACGTCCCGGCG
DsbD-5C	GAGTCTAGATCAAAGTGGGCGCACGCT AAC
Ccm1F	ATCTGCTGGCGAAGAAAC
Ccm1B	GGAGGTCGACCAGCACGAAGAACTGACA
Ccm2F	GGAGGGTACCTTGCACGGCAACTACAGC
Ccm2B	GGAGTCTAGACTGCGGCGTGTCAATAAC

haps α is capable of interacting with a wider range of substrates than those of CcdA, making DsbD a more versatile protein. The fact that in many cases, a gene encoding a thioredoxin-like protein is located adjacent to *ccdA* (Page et al., 1997) might reflect earlier stages in its evolutionary process.

We point out that other laboratories have reported somewhat different results regarding the essentiality of the different cysteines of DsbD (Chung et al., 2000; Gordon et al., 2000). However, our previous results (Stewart et al., 1999), the extensive results with the cysteine mutants reported here, and our own attempts to replicate results from these other laboratories (data not included) do not support their conclusions.

The novel mechanism of transport that we report in this paper for DsbD may not be restricted to prokaryotes. Frand et al. (2000) proposed that certain members of the PDI family might be devoted to the isomerization of disulfide bonds in proteins of the endoplasmic reticulum of eukaryotic cells, which, as in the case of DsbC, should be maintained in their reduced state. These authors postulated two models that account for the source of reducing potential. For one thing, the reduced glutathione, imported from the cytosol, might interact with those enzymes. Alternatively, a membrane-bound PDI-homolog with multiple cysteines, such as Eps1 (Wang and Chang, 1999), might reduce those enzymes by transferring reducing equivalents from the cytosol. Whether or not Eps1 is a truly eukaryotic DsbD analog remains to be studied.

The approach we have used to elucidate the mechanism of electron transfer through DsbD relied on the ability to separate this protein into three domains that together carried out the reactions of the intact DsbD polypeptide. This striking ability of the separated components to functionally interact may be true for other membrane proteins with both membrane-imbedded and soluble domains. While, in our case, the evidence from cross-species comparisons for evolutionary separation of these domains provoked our interest in the possibility of working with fragments of the protein, the principles underlying this reassembly of a functional protein may be widely relevant. They may also allow more ready genetic and structural analysis of the mechanisms of interaction of domains of a membrane protein.

Experimental Procedures

Strains and Media

Strains used in this work are listed in Table 2.

E. coli cells were grown in NZ-amine (Rietsch et al., 1996) at 37°C, with the appropriate antibiotics.

To stimulate cytochrome *c* maturation under aerobic conditions, the P_{BAD} promoter together with the *araC* gene were introduced into the chromosome of different strains disrupting the *napH* gene, driving the expression of the genes *napB* and *napC* and the *ccm* gene region. In brief, the ClaI-KpnI fragment from pBAD18, containing *araC* and the P_{BAD} promoter, was ligated in between two otherwise adjacent fragments that had been previously amplified employing the primer pairs ccm1F-ccm1B and ccm2F-ccm2B (Table 3), and digested with AccI and KpnI, respectively. The product was used to replace its chromosomal counterpart following an established protocol (Link et al., 1997).

Plasmid Constructions

The parental plasmid pFK093 was obtained by amplifying a DNA fragment using primers DsbD-24F and DsbD + 63B (Table 3), which was cloned in between the KpnI and PstI sites of pBAD18. Both nonessential cysteines in the leader peptide and at position 282 were replaced by alanines as described below. All the DsbD derivatives described in this work carry alanine residues at these positions.

A fragment encoding the α domain of DsbD was amplified by PCR from pFK093 using the primers BADFOR and DsbD-7C, digested with the enzymes KpnI and XbaI, and cloned into the corresponding sites of pBAD33, resulting in plasmid pFK051. Alternatively, a BlpI-PvuI fragment of pFK029 (filled in at its 5′ end) was cloned into pFK051, previously digested with SalI and PvuI, and appropriately filled in. The resulting construct, pFK086, expressed α fused to a His_6 tag at its C terminus.

To express β, preserving its native topology, the first TM segment of MalF was fused to its N terminus. A fragment encoding β was PCR amplified from pFK093 using the primers DsbD-8 and BADREV, digested with the enzymes MscI and HindIII, and subsequently cloned into the corresponding sites of pLD47 (which encodes the first TM segment of MalF). The resulting construct, pFK053, was then used as template for a PCR reaction, employing primers BADFOR and DsbD_Myc-tag. The product was digested with XbaI and EcoRI and cloned into the corresponding sites of pBAD18. The resulting plasmid, pFK060, expressed MalF-β fused to the c-Myc epitope at its C terminus.

To export γ to the periplasmic space, a fragment encoding γ was amplified using the primers DsbD-4 and DsbD-4C, digested with XbaI and EcoRI, and cloned into the corresponding sites of pLMD60 (which encodes the APss). Next, the BsrGI-XbaI fragment encoding APss-γ was removed and cloned into pBAD43 (previously digested with Acc65I and XbaI), yielding pFK017.

The plasmid pFK049, which expresses the αβ peptide, was obtained by amplifying a fragment using the primers DsbD-24 and DsbD-5C, and cloning it in between the KpnI and XbaI sites of pBAD18.

To generate the βγ-expressing plasmid, a fragment from pFK093 was amplified using the diverging phosphorylated primers DsbD-12 and DsbD-12C. The product was self ligated, yielding the plasmid pFK094. The cleavage of the DsbDss may occur between the original alanine and glycine, now located approximately fifteen residues before the first TM segment.

The plasmid pFK145 that expresses $TrxA_{C36Y}$ was constructed by cloning a BsrGI-XbaI DNA fragment from pLMD104 into pBAD43, which was previously digested with Acc65I and XbaI.

When indicated, the cysteines of the described constructs were mutated either to alanine or to tyrosine using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) as directed by the manufacturer. All the constructions were verified by DNA sequencing.

Antibodies

Anti-c-Myc and anti-TrxA rabbit polyclonal antisera were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and from Sigma (St. Louis, MO), respectively. Anti-γ and anti-DsbC antibodies were already described (Joly and Swartz, 1997; Stewart et al., 1999). To obtain antisera against α, a DNA fragment was generated using the primers DsbD-TagF and DsbD-TagR, and cloned into the NcoI-HindIII sites of the vector pET-26b(+) (Novagen, Madison, WI) yielding pFK029. The fusion protein α-His_6 was purified over a nickel column, and used to raise antibodies in rabbit (Covance, Denver, PA). Anti-CcmG antisera were a gift from Linda Thöny-Meyer.

Thiol-Redox State Analyses

To analyze the formation of mixed disulfides and to determine the in vivo redox state of the proteins, free thiols were acid trapped and alkylated with the high molecular mass reagent AMS (4-acetamido-4′maleimidylstilbene-2,2′-disulfonic acid, Molecular Probes, Eugene, OR) as described (Stewart et al., 1999). When indicated, DTT was added to the samples at a final concentration of 40 mM. The visualization of *c*-type cytochrome in-gel was performed as indicated (Stewart et al., 1999).

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