Dsbd catalyzed Transport of Electrons across the Membrane of Escherichia coli

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Dsbd proteins catalyze folding and oxidation of polypeptides in the periplasm of Escherichia coli. DsbC reduces wrongly paired disulfides by transferring electrons from its catalytic dithiol motif 99CGYC. Genetic evidence suggests that recycling of this motif requires at least three proteins, the cytoplasmic thioredoxin reductase (TrxB) and thioredoxin (TrxA) as well as the Dsb membrane protein. We demonstrate here that electrons are transferred directly from thioredoxin to DsbD and from DsbD to DsbC. Three cysteine pairs within DsbD undergo reversible disulfide rearrangements. Our results suggest a novel mechanism for electron transport across membranes whereby electrons are transferred sequentially from cysteine pairs arranged in a thioredoxin-like motif (CXXC) to a cognate reactive disulfide.

Disulfide bonds are important features in the folded structure of secreted polypeptides but are generally absent from proteins residing in the cytoplasm (1). Proteins acquire disulfide bonds after entry into the endoplasmic reticulum of eukaryotic cells (2, 3). In bacterial cells, disulfide bond formation occurs in the periplasm, a compartment that is located between the cytoplasmic and outer membranes of Escherichia coli (4, 5). Disulfide bond formation requires oxidation of a pair of cysteine sulfhydryl residues, a reaction catalyzed by bacterial DsbA (6, 7). Electrons generated by oxidative folding are transferred from DsbA to DsbB, an inner membrane protein (8, 9) containing four essential cysteine residues forming two reversible disulfide bonds (10, 11). These disulfide bonds become reduced upon reoxidation of DsbA. DsbB uses quinones or dithiothreitol.

During this rearrangement, the active site98CGYC101 thiol (sulfhydryl) residues of DsbC are oxidized (DsbC-S2). What is the origin of electrons that are required for the proofreading activity of DsbC? Regeneration of reduced DsbC requires the DsbD membrane protein (19) as well as cytoplasmic thioredoxin (TrxA)1 and thioredoxin reductase (TrxB) (20). TrxB transfers electrons from the NADPH coenzyme to oxidized thioredoxin (TrxA-S2) thereby contributing to the reducing environment of the cellular cytoplasm (21, 22). This study examines the specific mechanism whereby DsbD mediates electron transfer from the cytoplasm across the membrane.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—Strains and plasmids used are listed in Table I. Sequences of primers used in this study can be obtained from the authors upon request. Cells were grown in Luria-Bertani (LB) medium (23) at 30 °C using the appropriate antibiotic at the following concentrations: ampicillin, 100 μg/ml; spectinomycin, 50 μg/ml; chloromphenicol, 20 μg/ml; and kanamycin, 50 μg/ml. Induction of Hisα-tagged DsbD using the pSE420 plasmid (Invitrogen) was accomplished by addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 1 mM. Plasmids expressing TrxA-C52S (pTrxA-C52S) and TrxA-C35S (pTrxA-C35S) were obtained from M. Russel (Rockefeller University). Plasmids pRK35 and pCC68 expressing wild-type DsbC and DsbC-C10A were derivatives of plasmids pDM501 and pDM1461 described earlier (14, 15). When needed, mutations were transduced into various backgrounds using P1 bacteriophage as described (23).

**Replacement of DsbD Cysteines with Alanine**—Plasmids encoding DsbD variants with two cysteine residues changed to alanine were generated by site-directed mutagenesis using the method described by Ansaldi et al. (24). Plasmid pDM2200 was used as a template (25). This plasmid is a derivative of pWSK30 (pSC101 replicon) (26) and carries a 3.1-kilobase pair DNA fragment containing the entire coding region for dsbD with upstream sequences. All plasmids were verified by sequencing. For purification of mutant DsbD proteins, clones encoding the full-length dsbD gene (wild-type or mutant) with a C-terminal Hisα tag, Hisα-tagged DsbD, were generated by polymerase chain reaction and cloned into pSE420 (Invitrogen) using EcoRI and BamHI restriction sites as described earlier (25).

**Modification of Sulfhydryl Groups**—Sulfhydryl groups were modified using either 2-aminoethyl methanethiosulfonate hydrobromide (MTSEA) or 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS) reagents. Cells were grown to A950 nm between 0.5 and 1.5 and incubated in the presence of 2.5 mM MTSEA (freshly prepared in water) for 5 min at room temperature. For analytical purposes, samples were precipitated with 7.5% trichloroacetic acid. Precipitates were collected by centrifugation, washed with acetone, spun, and suspended in 0.5 mM Tris-HCl buffer, pH 7.0, containing 4% SDS. Samples were analyzed by 12% SDS-PAGE as well as by Western blot. For purification of the mixed disulfide species, cells were harvested at 4 °C directly after MTSEA treatment. Such pellets were washed and suspended in buffer A (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 10% glycerol). Cells were kept frozen at −80 °C until further use. The redox state of DsbD in vivo was assessed using AMS as described previously (12).

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Electron Transfer across the Membrane

** EXPERIMENTAL STRATEGY—** Genetic analyses suggest that to elucidate the mechanism of electron transfer to periplasmic DsbC, we sought to capture reaction intermediates as an inter-molecular disulfide between DsbD and DsbC.

**A Stable Mixed Disulfide between DsbD and DsbC—** The overall topology of DsbD has been previously shown by constructing fusions of DsbD membrane-spanning segments to the mature part of alkaline phosphatase (25, 30, 31). Two of these studies have revealed that both the N- and C-terminal domains of DsbD (each ~10 kDa in size) are positioned in the periplasm (Fig. 1A), suggesting that DsbD is initiated in the secretory pathway via a cleavable signal sequence prior its insertion in the cytoplasmic membrane (25, 31). Both N- and C-terminal domains harbor two pairs of cysteines, Cys103–Cys109 and Cys461–Cys464, respectively (Fig. 1A). The two domains are connected by eight transmembrane (TM) segments containing three cysteine residues, Cys103, Cys107, and Cys167, and Cys167–Cys464, respectively (Fig. 1A). The presumed disulfide bond is dispensable for electron transfer. In contrast, alanine substitution of cysteine residues at any one of the other cysteines (C109, C2, C4, C5, C6, C8) abolishes DsbD-mediated electron transfer, causing accumulation of DsbC-S2 (25, 30). When examined by immuno blotting of cellular extracts, none of the DsbD substitution mutants formed a stable intermolecular disulfide (Fig. 1B). Thus, this intermediate can be resolved rapidly by the attack of the second thiol, Cys101 of DsbC.

**RESULTS**

**Electron Transfer between TrxA and DsbD**

**Electron Transfer across the Membrane**

**TABLE I**

**Bacterial strains and plasmids**

| Strains | Relevant characteristics | Source or Ref. |
|---------|-------------------------|---------------|
| K38 | HfrC tonA22 garB10 ompF relA1 phoA6 | M. Russel (40) |
| A307 | K38 trxA14- Kan | (40) |
| A333 | A307 carrying plasmid pTrxA_C285S | (40) |
| A334 | A307 carrying plasmid pTrxA_C285S | (40) |
| DM2355 | K38 dsbD–Isp | (25) |
| DM2441 | K38 dsbD–Isp dsbC: Tn10 Kan | This study |

**Plasmids**

| Plasmids | Source or Ref. |
|----------|---------------|
| pSE420 | ColE1-based vector, trc promoter, AmpR | Invitrogen (41) |
| pHS3399 | pBR322 replicon, CmR | (26) |
| pWSK30 | pSC101-based vector AmpR | (26) |
| pDM2200 | pWSK30 carrying a 3.1-kilobase pair cutA1 dsbD–PstI-EcoRV DNA fragment | (25) |
| pJC222 | Derivative of pDM2200 encoding DsbD_C103A/C109A | (25) |
| pJC223 | Derivative of pDM2200 encoding DsbD_C103A/C109A | (25) |
| pJC415 | Derivative of pDM2200 encoding DsbD_C163A/C282A | (25) |
| pJC416 | Derivative of pDM2200 encoding DsbD_C163A/C282A | (25) |
| pJC234 | Derivative of pDM2200 encoding DsbD_C285A/C285A | (25) |
| pJC27 | Derivative of pDM2200 encoding DsbD_C461A/C68 | (25) |
| pJC284 | Derivative of pDM2200 encoding DsbD_C461A/C68 | (25) |
| pCC58 | Derivative of pDM2200 encoding DsbD_C58/C58 | (25) |
| pRK35 | pHSG399 encoding wild-type DsbC | This study |
| pCC68 | pHSG399 encoding DsbD_C101A | This study |
| pCC550 | pSE420 coding for wild-type His-tagged DsbD | This study |
| pCC51 | pSE420 coding for the His-tagged DsbD | This study |

**Protein Purification—** Wild-type DsbD (pJC50), DsbD_C103A/C109A, and DsbD_C285A (pCC15) with an appended C-terminal His tag were expressed by cloning the structural genes into pSE420 (Invitrogen). Cells expressing either one of the various His-tagged DsbD proteins were grown to OD280 = 1 and incubated for 2 h with 1 mM isopropyl-1-thio-β-D-galactopyranoside. Prior to lysis by French pressure (p.s.i. 14,000), cells were incubated with 2.5 mM MTSEA for 5 min at room temperature. Cleared cell extracts were centrifuged at 100,000 × g for 45 min at 4 °C. Membrane proteins in the sediments were suspended in 50 mM Tris-HCl, 150 mM NaCl, 2% octylglucoside, and 10 mM imidazole, pH 7.5. Insoluble material was removed by centrifugation prior to affinity purification of the various His-tagged DsbD proteins on nickel-nitrilotriacetic acid.

**RESULTS**

**Experimental Strategy—** Genetic analyses suggest that to maintain DsbC, DsbG, and DsbE in a reduced state, electrons must flow from cytoplasmic NADPH to the periplasm (18, 25, 27). This process is dependent on TrxB, TrxA, and DsbD.

Electrons flow from NADPH to TrxA (22, 28). TrxA is a 10 kDa in size) are positioned in the periplasm; these two thiolate cysteines are essential for electron transfer. In contrast, alanine substitution of cysteine residues at any one of the other cysteines (C109, C2, C4, C5, C6, C8) abolishes DsbD-mediated electron transfer, causing accumulation of DsbC-S2 (25, 30). When examined by immunoblotting of cellular extracts, none of the DsbD substitution mutants formed a stable intermolecular disulfide between DsbD and DsbC appears to be unstable and may be resolved rapidly by the attack of the second thiol, Cys101 of DsbC.

**To test this possibility, E. coli strains expressing DsbD_C101A and various DsbD cysteine substitution mutants were examined. An inter-molecular intermediate was observed in cells expressing DsbD_C101A and DsbD_C101A (Fig. 1C) whereas alanine substitution of any other cysteine of DsbD did not produce a mixed disulfide (Fig. 1B). To characterize the nature of the mixed disulfide intermediate was observed in cells expressing DsbD_C101A and DsbD_C101A (Fig. 1C) whereas alanine substitution of any other cysteine of DsbD did not produce a mixed disulfide (Fig. 1B). To characterize the nature of the
Expressing DsbC C101A or wild-type DsbC. Proteins were precipitated with nickel-nitrilotriacetic acid and eluted with imidazole. The eluate was incubated either with (+) or without (−) 5 mM DTT, separated on 12% SDS-PAGE, and analyzed by immunoblotting. Proteins in lanes 1 and 2 were reacted with antibodies against DsbD (α-DsbD), whereas proteins in lanes 3–5 were reacted with antibodies against DsbC (α-DsbC). Purified DsbC (lane 5) was used as a control for the mobility of DsbC C101A on SDS-PAGE.

**Component of the intermolecular disulfide with DsbD.** To identify the corresponding cysteine residue of DsbD, we analyzed DsbD variants harboring alanine substitutions at Cys103 as well as at other cysteine residues. DsbDC103A/C109A failed to form the intermolecular disulfide with DsbCC101A (Fig. 1C; lanes labeled C1 and C2 for DsbDC103A/C109A). All other combinations of alanine substitutions produced the mixed disulfide species (data not shown). Thus, electron transfer between DsbD and DsbC involves the formation of a disulfide between cysteine 109 of DsbD and cysteine 98 of DsbC. Further, the intermolecular disulfide is resolved by the Cys109 thiol of DsbD, generating the intramolecular cystine Cys109-Cys109 (C1 and C2) as well as reduced DsbC-(SH)2.

**Electron Transfer between TrxA and DsbD—E. coli lacking thioredoxin (ΔtrxA) accumulate oxidized DsbC (20, 25) and DsbD (Fig. 3A).** Both cysteines of the dithiol motif of thioredoxin (32CGPC35) are required for electron transfer, as either mutant TrxA C32S or mutant TrxA C35S accumulated oxidized DsbD (Fig. 3A). Substitution of TrxA C35S but not TrxA C32S produced a spectrum of disulfide-linked intermolecular intermediates (Fig. 3B). Thus, substitution of the second cysteine of thioredoxin with serine (TrxA C35S) results in a mutant protein capable of attacking numerous cytoplasmic disulfides but unable to resolve the mixed disulfide intermediates. Although cells expressing TrxA C35S accumulated DsbD in an oxidized state, a mixed disulfide between DsbD and the mutant thioredoxin was not detected (data not shown). Thus, similar to DsbC, the proposed disulfide intermediate between thioredoxin and DsbD appears to be unstable and may be reduced either by DsbD itself or by other cytoplasmic redox factors. To explore this possibility, cells expressing TrxA C35S and DsbD variants with single cysteine to alanine substitutions were analyzed by electron transfer between TrxA and DsbD. Indeed, incubation with DTT resolved the 80-kDa intermolecular disulfide and resulted in the appearance of a 24-kDa polypeptide (Fig. 2). α-DsbD, but not α-DshD, bound to the 24-kDa protein, indicating that this species represents DsbC C101A released from its disulfide linkage with DsbDC103A. Incubation with the reducing agent dithiothreitol (DTT) resolved the 80-kDa mixed disulfide and formed the intermolecular disulfide (Fig. 2). The intermolecular disulfide of DsbDC103A with a C-terminal His6 tag was expressed together with DsbDC101A in the E. coli strain DM2441 (K38 ΔdsbD ΔdsbC). Crude cellular extracts were subjected to affinity chromatography on nickel-nitrilotriacetic acid and eluted with imidazole. The eluate was incubated with (+) or without (−) 5 mM DTT, separated on 12% SDS-PAGE, and analyzed by immunoblotting. Proteins in lanes 1 and 2 were reacted with antibodies against DsbD (α-DsbD), whereas proteins in lanes 3–5 were reacted with antibodies against DsbC (α-DsbC). Purified DsbC (lane 5) was used as a control for the mobility of DsbC C101A on SDS-PAGE.

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antibodies raised against DsbD (α-DsbD). Wild-type thioredoxin (WT strain K38) harbors an active site dihior motif (CXXC). However, the first cysteine residue (TrxA<sub>WT</sub>, strain A333) or the second cysteine residue (TrxA<sub>C35S</sub>, strain A334) is substituted with serine in the mutant thioredoxin proteins. Strain A307 carries a deletion of the thioredoxin gene (ΔtrxA). Treatment with AMS (12) slows the mobility of reduced DsbD compared with oxidized DsbD on SDS-PAGE. Both sulfhydryls of the active site dihior of thioredoxin are needed to reduce oxidized DsbD. TrxA<sub>D</sub> proteins. Strain A307 carries a deletion of the thioredoxin gene (ΔtrxA<sub>D</sub>). DsbD and DsbDC<sub>285A</sub> were purified by affinity chromatography from MTSEA-treated cell extracts and examined by immunoblotting with antibodies against DsbD (α-DsbD). Wild-type thioredoxin (WT strain K38) harbors an active site dihior motif (CXXC). However, the first cysteine residue (TrxA<sub>WT</sub>, strain A333) or the second cysteine residue (TrxA<sub>C35S</sub>, strain A334) is substituted with serine in the mutant thioredoxin proteins. Strain A307 carries a deletion of the thioredoxin gene (ΔtrxA). Treatment with AMS (12) slows the mobility of reduced DsbD compared with oxidized DsbD on SDS-PAGE. Both sulfhydryls of the active site dihior of thioredoxin are needed to reduce oxidized DsbD. TrxA<sub>D</sub> proteins. Strain A307 carries a deletion of the thioredoxin gene (ΔtrxA<sub>D</sub>). DsbD and DsbDC<sub>285A</sub> were purified by affinity chromatography from MTSEA-treated cell extracts and examined by immunoblotting with antibodies against DsbD (α-DsbD). Wild-type thioredoxin (WT strain K38) harbors an active site dihior motif (CXXC). However, the first cysteine residue (TrxA<sub>WT</sub>, strain A333) or the second cysteine residue (TrxA<sub>C35S</sub>, strain A334) is substituted with serine in the mutant thioredoxin proteins. Strain A307 carries a deletion of the thioredoxin gene (ΔtrxA). Treatment with AMS (12) slows the mobility of reduced DsbD compared with oxidized DsbD on SDS-PAGE. Both sulfhydryls of the active site dihior of thioredoxin are needed to reduce oxidized DsbD. TrxA<sub>D</sub> proteins. Strain A307 carries a deletion of the thioredoxin gene (ΔtrxA<sub>D</sub>). DsbD and DsbDC<sub>285A</sub> were purified by affinity chromatography from MTSEA-treated cell extracts and examined by immunoblotting with antibodies against DsbD (α-DsbD). Wild-type thioredoxin (WT strain K38) harbors an active site dihior motif (CXXC). However, the first cysteine residue (TrxA<sub>WT</sub>, strain A333) or the second cysteine residue (TrxA<sub>C35S</sub>, strain A334) is substituted with serine in the mutant thioredoxin proteins. Strain A307 carries a deletion of the thioredoxin gene (ΔtrxA). Treatment with AMS (12) slows the mobility of reduced DsbD compared with oxidized DsbD on SDS-PAGE. Both sulfhydryls of the active site dihior of thioredoxin are needed to reduce oxidized DsbD. TrxA<sub>D</sub> proteins. Strain A307 carries a deletion of the thioredoxin gene (ΔtrxA<sub>D</sub>). DsbD and DsbDC<sub>285A</sub> were purified by affinity chromatography from MTSEA-treated cell extracts and examined by immunoblotting with antibodies against DsbD (α-DsbD). Wild-type thioredoxin (WT strain K38) harbors an active site dihior motif (CXXC). However, the first cysteine residue (TrxA<sub>WT</sub>, strain A333) or the second cysteine residue (TrxA<sub>C35S</sub>, strain A334) is substituted with serine in the mutant thioredoxin proteins. Strain A307 carries a deletion of the thioredoxin gene (ΔtrxA).![](https://i.imgur.com/3.png)
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The active site thiolate of the CXXC motif attacks the disulfide between cysteines 103 and 109 (C103C109) and the dithiol motif (C98CGYC) via the formation of a disulfide bond. The final electron transfer step involves reduction of the active site thiolate by thioredoxin-like motifs.

Some of these interactions are promiscuous, e.g., recognition of the DsbD Cys103-Cys109 disulfide by the dithiol motifs of DsbC, DsbE, and DsbG or interaction of DsbC, TrxA, and DsbA with folding substrates. However, the active site thiolate cannot resolve disulfides within another thioredoxin-like domain presumably because electrons cannot be favorably transferred to any one of these molecules. For example, DsbA does not recognize the DsbD Cys103-Cys109 disulfide. This interaction would otherwise result in a wasteful flow of electrons between cytoplasmic, membrane, and periplasmic compartments. Oxidative protein folding catalyzed by the DsBA-DsbB system can be regarded as electron transfer from the periplasm to the membrane. It involves only two thioredoxin-like reactions: electrons flow from the substrate to the dithiol of DsBA to DsbB Cys104-Cys130 disulfide and then to the dithiol of DsbB and finally to the electron transport system.

Electron transfer from the cytoplasm to the periplasm requires a sequence of three thioredoxin-like reactions: 1) [Cys32]TrxA–[Cys163]DsbD, 2) [Cys285]DsbD–[Cys461]DsbD, and 3) [Cys109]DsbD–[Cys98]DsbC. Thus, it appears that DsbD has evolved as a fusion of two unique peptide configurations harboring disulfides tethered to a thioredoxin-like domain.

DsbD-mediated electron transfer across the cytoplasmic membrane of *E. coli* has been studied by several investigators (25, 30, 31). Stewart et al. (30) have examined the membrane topology of DsbD and were the first to propose a mechanism for sequential electron transfer. In the Stewart model, the thiocysteine within the C-terminal thioredoxin-like motif of DsbD attacks the disulfide of periplasmic DsbC, another thioredoxin-like motif. Electrons are transferred from the membrane-embedded dithiol of DsbD to the N-terminal disulfide and finally to the C-terminal periplasmic domain. Thioredoxin within the cytoplasm of *E. coli* attacks the membrane-embedded disulfide of DsbD and is recycled by thioredoxin reductase.

Our hypothesis presumes a conformational change of DsbD as the membrane-embedded disulfide/cysteines (C163 and C285) must be accessible to cytoplasmic thioredoxin and to the periplasmic domains of DsbD. If the distance between C163 and C285 were similar to the distance between the inner and outer leaflets of the cytoplasmic membrane, the conformational change would have to span a distance of 30 nm. Thus, disulfide bond formation would require mass movements of DsbD transmembrane domains. Our future work will aim to characterize these events.

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**FIG. 5. Model for DsbD-mediated transfer of electrons across the cytoplasmic membrane.** TrxA (NADPH) donates electrons to the active site dithiol motif of TrxA. TrxA resolves the disulfide between cysteines 163 and 285 of DsbD (C3 and C5). Cysteines 163 and 285 donate electrons to the thioredoxin motif of DsbD thereby reducing the dithiol at cysteines 461 and 464 (C6 and C7). The reduced dithiol attacks the disulfide at cysteines 103 and 109 (C1 and C2), which transfer electrons to the oxidized dithiol motif of DsbC. The square-shaped box indicates the presence of a thioredoxin-like fold and dithiol motif. Electron transfer occurs as a sequence of disulfide rearrangements between cysteine pairs arranged in a thioredoxin-like motif (CXXC) with a cognate reactive disulfide.
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