The disulfide bond isomerase DsbC is activated by an immunoglobulin-fold thiol oxidoreductase: crystal structure of the DsbC–DsbD α complex

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The *Escherichia coli* disulfide bond isomerase DsbC rearranges incorrect disulfide bonds during oxidative protein folding. It is specifically activated by the periplasmic N-terminal domain (DsbD α) of the transmembrane electron transporter DsbD. An intermediate of the electron transport reaction was trapped, yielding a covalent DsbC–DsbD α complex. The 2.3 Å crystal structure of the complex shows for the first time the specific interactions between two thiol oxidoreductases. DsbD α is a novel thiol oxidoreductase with the active site cysteines embedded in an immunoglobulin fold. It binds into the central cleft of the V-shaped DsbC dimer, which assumes a closed conformation on complex formation. Comparison of the complex with oxidized DsbD α reveals major conformational changes in a cap structure that regulates the accessibility of the DsbD α active site. Our results explain how DsbC is selectively activated by DsbD using electrons derived from the cytoplasm.

Keywords: disulfide bond isomerase DsbC/electron transporter DsbD/oxidative protein folding/reaction intermediate/thiol oxidoreductase

Introduction

The correct formation of disulfide bonds is important for the folding and function of many secretory and membrane proteins. Organisms from all kingdoms of life have evolved a diverse range of thiol oxidoreductases that efficiently promote the formation of disulfide bonds (Kadokura and Beckwith, 2001). Membrane-embedded electron transport pathways have recently been identified in eukaryotes and bacteria that are essential to maintain the oxidoreductases in the correct active redox state (Fränd et al., 2000).

The periplasm of Gram-negative bacteria contains two complementary pathways that catalyze the formation of disulfide bonds (Debarbieux and Beckwith, 1999; Fabianek et al., 2000). The disulfide bond formation pathway adds new disulfide bonds to folding proteins and

the disulfide bond isomerization pathway rearranges existing disulfide bonds between incorrectly paired cysteines. The components of the two pathways are termed disulfide bond formation proteins (Dsb proteins) (Fabianek et al., 2000; Ritz and Beckwith, 2001).

The disulfide bond formation pathway catalyzes the formation of new disulfide bonds in folding proteins. DsbA (Bardwell et al., 1991; Akiyama et al., 1992) functions by transferring its active site disulfide bond to folding substrate proteins. The electrons DsbA acquires in the process are passed to the inner membrane protein DsbB (Bardwell et al., 1993; Missiakas et al., 1993; Guilhot et al., 1995; Kishigami et al., 1995), which transfers them to the respiratory chain via a quinone cofactor (Kobayashi et al., 1997; Bader et al., 1999).

The catalyzed formation of disulfides is a rapid non-specific process and may result in the formation of incorrect disulfide bonds that trap substrate proteins in non-native conformations (Rietsch et al., 1996; Bader et al., 2000). The disulfide bond isomerization pathway facilitates the rearrangement of these incorrect disulfide bonds, allowing non-native substrates to refold. DsbC (Missiakas et al., 1994; Shevchik et al., 1994; Zapun et al., 1995; Sone et al., 1997) and its homolog DsbG (Andersen et al., 1997; Bessette et al., 1999) are the primary catalysts of disulfide bond rearrangement.

DsbC is a V-shaped homodimeric molecule (McCarthy et al., 2000) with a large uncharged cleft, which is the likely binding site for unfolded substrates. The dimer contains two thioredoxin-fold domains with active site Cys-Gly-Tyr-Cys motifs. The ability of DsbC to rearrange incorrect disulfide bonds requires that the active site cysteines are maintained in the reduced dithiol form, allowing them to attack substrate disulfide bonds. DsbC is exclusively found in the reduced form *in vivo* (Joly and Swartz, 1997; Rietsch et al., 1997).

The transmembrane electron transporter DsbD (Missiakas et al., 1995) activates oxidized DsbC by reducing the active site cysteines (Rietsch et al., 1997; Stewart et al., 1999) using electrons transported from the cytoplasm. DsbD also reduces the isomerase DsbG and CcmG, a protein involved in the reduction of heme-binding cysteines in *c*-type apocytochromes (Sambongi and Ferguson, 1994; Crooke and Cole, 1995; Fabianek et al., 1998, 1999). The rearrangement and removal of incorrect disulfide bonds consequently depend on the transport of electrons from the cytoplasm into the periplasm by DsbD.

DsbD is an inner membrane protein with a three-domain structure (Stewart et al., 1999; Chung et al., 2000; Gordon et al., 2000), consisting of an N-terminal periplasmic domain DsbD α , a central transmembrane domain DsbD β and a C-terminal periplasmic domain DsbD γ . DsbD function can be reconstituted by coexpression of the

separate domains, $\alpha + \beta + \gamma$ (Katzen and Beckwith, 2000). The domains have been proposed to transport electrons sequentially by a cascade of thiol–disulfide exchange reactions. On the cytoplasmic side, thioredoxin donates electrons to the transmembrane domain, DsbD β . DsbD β then transfers the electrons to the periplasmic thioredoxin-like domain DsbD γ , which in turn passes electrons to the active site cysteines of DsbD α . In the final step of the electron transport process, reduced DsbD α specifically interacts with the oxidized form of the three known periplasmic substrate proteins, DsbC, DsbG and CcmG, reducing their active sites (Katzen and Beckwith, 2000; Krupp *et al.*, 2001).

We have recently shown that DsbD α alone can activate DsbC *in vitro* (Goldstone *et al.*, 2001), suggesting that these two proteins interact directly in a thiol–disulfide exchange reaction and that no other periplasmic components are required. The thiol–disulfide exchange reaction can be trapped at the level of the covalent reaction intermediate between DsbC and DsbD α by substituting the reaction partners with their active site mutants, DsbC C101A/S and DsbD α C103A (Goldstone *et al.*, 2001; Krupp *et al.*, 2001). These mutants lack local free cysteines able to resolve the intermolecular disulfide bond that forms between DsbD α Cys103 and DsbC Cys98.

The disulfide bond isomerization pathway requires electrons to be transported into the periplasm, while the disulfide bond formation pathway depends on the transport of electrons out of the periplasm. To maintain the opposite *in vivo* redox states of DsbA and DsbC, the electron transport interactions of the two pathways must be distinguished, although DsbA and DsbC interact with numerous folding substrates. How the electron transport processes are separated, even though both DsbA and DsbC have a thioredoxin fold with very similar biochemical properties, is an intriguing question.

To improve our understanding of the electron transport process associated with disulfide bond isomerization, the crystal structures of oxidized DsbD α , DsbC C101S and the DsbC–DsbD α complex were determined. The structure of the trapped DsbC–DsbD α complex shows DsbD α binding into the central cleft of the V-shaped DsbC dimer. It provides insights into a novel electron transport mechanism across the inner bacterial membrane and suggests a model for DsbC-catalyzed disulfide bond isomerization. Data from *in vivo* genetic analysis employing DsbD α mutants are consistent with the observed interactions between DsbD α and DsbC. The comparison of the reaction intermediate with the structures of DsbC C101S and oxidized DsbD α explains how DsbC is specifically activated by DsbD.

Results and discussion

Overview of the DsbC–DsbD α complex structure

The crystal structure of the DsbC–DsbD α complex shows that DsbD α binds into the central cleft of the V-shaped DsbC homodimer (Figure 1A). Major conformational changes are observed in both molecules, allowing DsbD α to interact with each of the two catalytic domains of the DsbC dimer. The final step of the electron transport process between DsbD α and DsbC is mediated by the interaction of two different types of thiol oxidoreductases.

DsbC belongs to the well characterized family of thioredoxin-like oxidoreductases, while DsbD α is a novel type of oxidoreductase with an immunoglobulin (Ig) fold. The recognition of both DsbC active sites by DsbD α explains the observation that DsbD α selectively interacts with full-length dimeric DsbC, but not with the separate monomeric catalytic domain (Goldstone *et al.*, 2001). Electrostatic interactions in the primary and secondary binding regions suggest a mechanism by which DsbD α recognizes and specifically reduces the oxidized form of DsbC.

DsbD α is a novel thiol oxidoreductase with an Ig fold

The overall structure of DsbD α reveals an Ig fold consisting of a β -sandwich formed by two antiparallel β -sheets and a catalytic subdomain which is inserted in the antigen binding end of the Ig fold (Figure 1B). DsbD α has a classical c-type Ig fold (Bork *et al.*, 1994) with a four-stranded β -sheet (β 1, β 2, β 8 and β 5) that packs against a three-stranded β -sheet (β 4, β 9 and β 12). The catalytic subdomain is inserted in loops 1, 3 and 5 of the Ig fold, forming a five-stranded antiparallel β -sheet. This sheet contains the active site cysteine residues that transport electrons by switching between reduced dithiol and oxidized disulfide bonded forms. The strand β 3 in the center of the catalytic subdomain is flanked on one side by strands β 10 and β 11, which contain the active site cysteine pair. Residue Cys109, the more solvent-accessible active site cysteine is located in strand β 10 and residue Cys103, which forms the active site disulfide bond with Cys109, is on the neighboring strand β 11. The catalytic β -sheet is completed on the opposite side of β 3 by strands β 6 and β 7. These strands together with the connecting loop form a cap structure over the active site as described below. The residues in the catalytic subdomain that surround the active site and the residues of the cap loop are highly conserved (Figure 1D). In a recent paper, Goulding *et al.* (2002) independently report the 1.65 Å structure of oxidized DsbD α , confirming that the α -domain has an Ig fold with a catalytic subdomain.

The DsbD α active site is shielded

In the oxidized form of DsbD α , the disulfide bonded cysteines 103 and 109 are completely shielded from the environment by the active site cap (Figure 1B). The cap residue Phe70 inserts directly over Cys109 with the aromatic side chain forming close van der Waals interactions with the sulfur atom of Cys109. Oxidized DsbD α forms a dimer in the crystal and the dimer contacts are exclusively located in the catalytic subdomain of each monomer. The conserved β 11 strands of two monomers meet in the dimer interface, forming an extended 10-stranded antiparallel β -sheet. The active site Cys109 residues of the two monomers make two backbone hydrogen bonds in the center of the β -sheet. The dimer interface buries 1016 Å² surface area, ~7% of the total molecular surface. Since DsbD α is monomeric in solution (Goldstone *et al.*, 2001), the biological relevance of the DsbD α dimer observed in this crystal structure is unclear. Cap closure and dimerization may function to protect the active site cysteines from non-specific interactions with other periplasmic components.

The active form of DsbC (C101S) has a solvent-accessible active site

The overall structure of the disulfide bond isomerase DsbC C101S, which mimics the active reduced form, shows a V-shaped homodimer with a large central cleft. It closely resembles the structure of the oxidized wild-type molecule (McCarthy *et al.*, 2000). Each monomer consists of an N-terminal dimerization domain (1–61) forming the dimer interface and a C-terminal catalytic domain (78–216) with thioredoxin fold (Figure 1C). The two domains are joined by a flexible linker helix that allows for the movement of the domains.

The catalytic domain contains the active site Cys98-Gly-Tyr-Cys101 motif. It catalyzes disulfide bond rearrangement by an attack of the Cys98 thiol group on a substrate disulfide, yielding a covalent DsbC–substrate complex. The intermolecular disulfide is subsequently resolved by a free substrate cysteine, resulting in a substrate with a rearranged disulfide bond. Alternatively, DsbC Cys101 can attack the intermolecular disulfide, yielding oxidized DsbC and a substrate with a broken disulfide bond.

A comparison between the active sites of oxidized DsbC and DsbC C101S shows that the side chain of Cys98 is rotated by $\sim 35^\circ$ towards the solvent in the active form. This rotation exposes an area of $5.5 \text{ \AA}^2$ of the catalytic sulfur atom, making it accessible to substrates. The active reduced form of DsbC is mainly stabilized by hydrogen bonds of the Cys98 thiolate with backbone amide groups at the N-terminus of the active site α -helix.

The DsbC–DsbD α complex reveals two asymmetric binding sites

The crystal structure of the DsbC C101S–DsbD α C103A complex reveals that DsbD α binds into the central cleft of DsbC (Figures 1A, 2A and B). DsbD α interacts with both active sites of DsbC, forming two asymmetric binding sites. DsbD α exclusively binds to the two catalytic domains of the DsbC dimer, and no contacts are observed between DsbD α and the N-terminal dimerization domains of DsbC.

The primary binding site involves interactions between residues surrounding the active sites of both DsbD α and DsbC (Figure 2C and D), and contains the two catalytic cysteine residues, DsbD α Cys109 and DsbC Cys98, which form a disulfide bond between the two molecules (Figure 1A). In the case of DsbD α , the primary binding region lies entirely in the catalytic subdomain and no primary binding site contacts are made with residues in the core of the DsbD α Ig fold. The residues in the primary

binding site are highly conserved in both DsbC and DsbD α .

The secondary binding site includes contacts between the active site region of the second DsbC monomer and residues from strands $\beta 1$ and $\beta 12$ of the DsbD α Ig fold (Figure 2C). The recognition of both DsbC active sites explains why DsbD α selectively forms a complex with full-length dimeric DsbC, but not with the truncated catalytic domain, which is monomeric in solution (Goldstone *et al.*, 2001). On complex formation, $1922 \text{ \AA}^2$ of total accessible surface area are buried, of which two-thirds ($1268 \text{ \AA}^2$) can be attributed to the interactions in the primary binding site and one-third ($654 \text{ \AA}^2$) to the secondary binding site.

The DsbC cleft adjusts to the shape of the binding partner

DsbD α binding induces large conformational changes in DsbC. DsbC assumes a closed conformation with the arms of the V-shaped dimer enclosing the DsbD α molecule. In the closed conformation, both catalytic domains of DsbC, located at the end of the arms, contact DsbD α (Figure 1A). Hinge movements essentially caused by a 30° rotation about the Ψ angle of Leu67 in the DsbC linker helices result in a shift of the catalytic domains by $13 \text{ \AA}$ from their positions in oxidized DsbC and DsbC C101S (Figure 3A). The width of the DsbC cleft between the two arms of the V is reduced to $29 \text{ \AA}$ in the closed conformation. In the open conformation, the width of the binding cleft is $38 \text{ \AA}$, as observed in oxidized DsbC and DsbC C101S (Figure 3A). The flexibility of the molecule allows DsbC to adjust the binding cleft according to the size and shape of the binding partner.

The DsbD α cap controls access to the active site

Large conformational changes were also observed between complexed and oxidized DsbD α . In the DsbC–DsbD α complex, the active site cap is in an open conformation exposing the catalytic sulfur of Cys109 (Figure 3B). Residues Leu54–Gln59 are in a β -strand conformation ($\beta 5$), resulting in an extension of the first Ig fold β -sheet.

After DsbC activation and DsbD α oxidation, the cap assumes a closed conformation, shielding the DsbD α active site. Part of the $\beta 5$ strand separates from the β -sheet, assuming a loop conformation, and the cap structure closes over the active site. The aromatic side chain of Phe70, which is distant from the active site in the DsbC–DsbD α complex, is displaced by $13 \text{ \AA}$ on oxidation and makes

Fig. 1. Crystal structures of the DsbC–DsbD α complex, oxidized DsbD α and DsbC C101S. (A) Structure of the DsbC–DsbD α complex. DsbD α (red) binds into the proposed substrate binding cleft of the V-shaped DsbC dimer (blue and green). The intermolecular disulfide bonds formed between the active site sulfur atoms of DsbC (Cys98) and DsbD α (Cys109) are shown as yellow spheres. DsbC assumes a closed conformation on DsbD α binding with hinge movements in both linker helices. (B) Structure of the N-terminal domain of the oxidized transmembrane electron transporter DsbD. The catalytic subdomain (blue) of DsbD α containing the active site is inserted at the antigen binding end of the Ig fold (red). The active site cysteines Cys103 and Cys109, represented by yellow spheres, form a disulfide bond in oxidized DsbD α . The active site is shielded by a cap that includes strands $\beta 6$ and $\beta 7$ and the loop between them. (C) The structure of the isomerase DsbC C101S in the open conformation. Each DsbC monomer consists of an N-terminal dimerization domain connected to a C-terminal catalytic domain by a linker helix. The catalytic domains have a thioredoxin fold that contains the active site cysteine residues. Figures were generated with MOLSCRIPT (Esnouf, 1999), GRASP (Nicholls *et al.*, 1991) and RASTER3D (Merritt and Bacon, 1997). (D) Multiple sequence alignment of 10 bacterial DsbD α sequences from SwissProt (Bairoch and Apweiler, 2000) using CLUSTALX (Thompson *et al.*, 1997). The secondary structure of DsbD α is indicated above the sequences. Conserved active site and cap residues are shown below the alignment.

close van der Waals interactions with the active site disulfide.

In addition to this, several side chains of DsbD α (Phe11, Phe70, Tyr71 and Phe108) that form a complementary binding surface for DsbC in the complex (Figure 2C) change orientation on DsbD α oxidation.

The active sites interact in the conserved primary binding site

The primary binding site accounts for the majority of the interactions between DsbC and DsbD α and is highly conserved. The interactions observed are between either hydrophobic or uncharged polar groups, with the notable

Fig. 2. DsbC–DsbD α binding interactions. (A) DsbD α (red) binds into the central cleft of DsbC and contacts the catalytic domains of both monomers (blue and green). The orientation of the DsbC–DsbD α complex is that of Figure 1A. (B) Top view of the DsbC–DsbD α complex. (C) Primary and secondary binding surfaces of DsbD α . The two binding sites on DsbD α are revealed by removing DsbD α from the complex shown in (B) and rotating it by 180° around a horizontal axis. The primary binding site residues interacting with the blue DsbC monomer are shown in blue and listed on the left. The DsbD α residues of the secondary binding site are shown in green and listed on the right. (D) Primary and secondary binding surfaces of DsbC. The blue and green DsbC monomers are removed from the complex shown in (B) and rotated by approximately $\pm 40^\circ$ about a vertical axis to reveal the DsbD α binding surfaces shown in red. Residues in the primary binding site group around the active site sulfurs (yellow) of both DsbC and DsbD α . Secondary binding site residues (green and red) are located in the opposite DsbC active site and the Ig subdomain of DsbD. Figures were generated with PYMOL.

exception of DsbD α Asp68 and DsbC Arg125. The lifted active site cap of DsbD α reveals an aromatic pocket consisting of residues Tyr40, Tyr42, Phe70 and Tyr71, which surround the central negatively charged Asp68 (Figure 4A). The DsbC active site (Thr97, Cys98, Gly99), and in particular the highly conserved aromatic side chain of Tyr100, bind into the DsbD α pocket, and the OH groups of DsbC Tyr100 and DsbD α Tyr40 form a hydrogen bond. DsbC Cys98 is positioned just above the strand β 11 of DsbD α , which contains Cys109 (Figure 4A). The active site residue Cys109 forms a disulfide bond with the DsbC active site residue Cys98 and it makes two hydrogen bonds with the conserved *cis*-proline loop (Gly181–Pro183) of DsbC.

The positive dipole of the DsbC active site α -helix interacts electrostatically with the complementary negative charge of DsbD α Asp68 via a tightly bound water molecule. The DsbD α active site β -strand and the adjacent loops (Ala106–Pro112) contact the region surrounding the DsbC active site (Pro194–Tyr196, Gly181–Pro183 and Arg125–Gln126).

Fig. 3. Conformational changes during the thiol–disulfide exchange reaction between DsbC and DsbD α . (A) Ribbon presentation of the DsbC dimer showing the open (white) and closed (blue and green) conformation of the molecule. In the open conformation, the sulfur atoms (yellow spheres) of the two DsbC active site Cys98 are 38 Å apart. DsbC assumes a closed conformation on binding to DsbD α and the hinge movements observed in the DsbC linker helices result in the reduction of the distance between the active sites to 29 Å in the closed form. (B) Representation of the open (red) and shielded (white) form of the DsbD α active site. In the open form observed in the DsbC–DsbD α complex, the opening of the active site cap facilitates access to the DsbC binding pocket. In the shielded oxidized form of DsbD α , the binding pocket is protected from the environment by Phe70, which makes close van der Waals interactions with the active site disulfide (yellow). Phe70 moves 13 Å from its position in oxidized DsbD α .

The second DsbC active site interacts with residues of the Ig fold

The DsbC–DsbD α interactions in the secondary binding site group around the active site α -helix (Cys98, Gly99, Tyr100 and Lys103) of the second DsbC catalytic domain. The DsbD α residues involved in binding are Asp21 and Phe22 in the first strand β 1, and Pro118 and Ser120 at the end of the last strand β 12 of the molecule.

As with the primary binding site, a negatively charged residue (Asp21) interacts with the positive dipole of the N-terminus of the active site α -helix (Figure 4B). The

Fig. 4. Interactions of the two DsbC active sites with DsbD α residues. (A) Stereo diagram of the primary binding site showing the DsbC active site interacting with DsbD α . The primary binding surface of DsbD α is shown colored according to the calculated electrostatic potential using GRASP. Negative charges are colored red and positive charges are in blue. Important DsbC residues (Ile96–Leu104, Gly181–Val185) are shown in blue ball-and-stick and cartoon representation. DsbC residues are labeled in black and DsbD α residues in green. DsbC Tyr100 binds into an uncharged pocket adjacent to the DsbD α active site Cys109, which forms a disulfide bond with DsbC Cys98 and hydrogen bonds to the *cis*-proline loop, Thr182–Pro183. (B) Stereo diagram of the secondary binding site. The electrostatic surface of the DsbD α secondary binding site is presented with DsbD α residues labeled in green. The DsbC active region is shown in green ball-and-stick and ribbons representation with yellow labels. DsbD α Asp21 interacts with the DsbC active site Cys98 and Gly99, while Tyr100 packs against DsbD α Phe22.

following Phe22 interacts with the conserved Tyr100 of the DsbC active site (Figure 4B) by π – π stacking of the aromatic side chains in an edge-to-face orientation.

The secondary binding site is approximately half the size of the primary binding site and several water molecules are located in the interface region. Despite the low sequence conservation of DsbD α residues in the secondary binding region, the specific interactions with the second DsbC active site, which is highly conserved among the DsbC homologs, suggest that this binding site is of physiological importance. The existence of two DsbC binding sites on the DsbD α molecule explains why DsbD α preferentially interacts with dimeric DsbC, rather than with monomeric DsbC (Goldstone *et al.*, 2001).

Recognition of inactive oxidized DsbC by DsbD α

The positive dipole at the N-terminus of the DsbC active site α -helix is one of the major factors stabilizing the reduced form of the catalytic Cys98 and lowering the pK_a

of the cysteine sulfur atom (Kortemme and Creighton, 1995; Zapun *et al.*, 1995). In reduced DsbC, the negatively charged deprotonated sulfur atom (Cys98-S[−]) neutralizes the positive dipole of the active site α -helix, but in the oxidized form (Cys98-S-S-Cys101) the uncharged disulfide bond has little effect on the helix dipole.

We propose that DsbD α exploits the changes in the charge distribution of the active site to distinguish between oxidized and reduced forms of DsbC. In the DsbC–DsbD α complex, two negatively charged Asp residues (Asp21 and Asp68) from DsbD α interact with the positive dipoles of each of the two DsbC active site α -helices. In activated DsbC, the active sites cysteines are reduced and the thiolate groups of the Cys98 residues introduce negative charges at the N-termini of the active site α -helices. This results in a charge repulsion between the DsbC active sites and the DsbD α residues Asp21 and Asp68, decreasing the binding affinity of DsbD α for the reduced form of the disulfide bond isomerase. In this model, the interactions

Fig. 5. *In vivo* redox state of DsbC and DsbD α derivatives. (A) Cells (*dsbD*[−], *trxA*[−]) expressing the designated derivatives were induced with 0.2% arabinose, harvested at mid-log phase, precipitated with TCA and subjected to AMS treatment. Proteins were separated by SDS–PAGE and visualized by western blotting using antibodies against DsbC. DsbD α derivatives used in lanes 3–8 are mutated in amino acids in the primary binding site. The amino acids mutated in the DsbD α derivative used in lane 9 correspond to the secondary binding site. (B) As above, but using antibodies against DsbD α .

between DsbC and DsbD α primarily result from the large binding surfaces, but the binding affinity is modulated by the charge repulsion. DsbD α would preferentially interact with fully oxidized DsbC, followed by DsbC with one oxidized and one reduced active site, and the binding affinity would be weakest for fully reduced DsbC.

***In vivo* characterization of the DsbC–DsbD α interactions**

To validate the significance of the observed interactions between DsbC and DsbD α , the function of mutant proteins was investigated by determining their *in vivo* redox state using reconstituted two- or three-piece DsbD systems (Katzen and Beckwith, 2000). Ten amino acid substitutions corresponding to the primary (Figure 2C, F11A, Y40A, Y42A, D68R, F70R, Y71A, Q101N and F108A) and the secondary (Figure 2C, D21R and F22R) binding sites were chosen and incorporated individually into a His₆-tagged derivative of DsbD α .

Although the steady thiol redox state of DsbD α ranged between 50 and nearly 100% oxidized, depending on the mutation, no differences in the DsbC redox state were observed. We reasoned that stronger perturbations of DsbD α might cause a clearly observable effect on DsbC reduction. Pairs of mutations that alter the same binding site were combined and their effects on DsbC reduction were analyzed by employing the three separate domains $\alpha + \beta + \gamma$ in the context of a *dsbD*[−], *trxA*[−] background strain. (This analysis is possible in a *trxA*[−] background because the overexpression of the DsbD domains generates enough reduced DsbD α to sustain DsbC reduction.)

DsbD α variants harboring two mutations in the primary binding site have modest effects on the redox state of DsbC (Figure 5A, compare lanes 3–8 with 2). However, the combination of two mutations of the secondary binding site showed a profound effect on the DsbC redox state, comparable to that seen when any domain of DsbD (in this case DsbD γ) is removed from the system (Figure 5A, compare lanes 1 and 9). All DsbD α mutants show less propensity to be reduced by DsbD γ compared with wild-type DsbD α (Figure 5B, compare lanes 3–9 with 2). This

is not surprising since DsbD γ is believed to have a thioredoxin-like fold, so it is conceivable that both DsbD γ and DsbC interact with a similar set of DsbD α residues. Since the molar ratio of DsbD α /DsbC in the three-piece system is much larger than the DsbD γ /DsbD α ratio, only a very small proportion of DsbD α is needed to be reduced in order to activate DsbC.

Model for selective activation of oxidized dimeric DsbC

The structure of the DsbD α –DsbC complex demonstrates that the transport process is mediated by a thiol–disulfide exchange reaction between DsbC and DsbD α . The electron transport reaction is initiated by binding of reduced DsbD α into the substrate binding cleft of oxidized DsbC. The binding induces major conformational changes and DsbC assumes a closed conformation allowing interactions between the two DsbC catalytic domains and DsbD α . The interaction with both catalytic domains explains the preferential binding of DsbD α to the dimeric form of DsbC, as observed in our previous *in vitro* studies (Goldstone *et al.*, 2001).

We propose a model where DsbD α distinguishes oxidized from reduced DsbC by interacting with the positive dipoles of the two active site α -helices in oxidized DsbC. The open conformation of the DsbD α active site cap exposes the DsbC binding pocket and the catalytic residue Cys109 of DsbD α . Our results show that the four sulfur atoms of the catalytic residues involved in the thiol–disulfide exchange reaction are appropriately aligned for the following nucleophilic attack of DsbD α Cys109 on the active site disulfide of oxidized DsbC. In the course of the reaction, Cys109 forms an intermolecular disulfide bond with Cys98 of DsbC. The intermediate disulfide bond is resolved by the nucleophilic attack of the free Cys103 from DsbD α , yielding reduced DsbC and oxidized DsbD α . In this model, the DsbC–DsbD α complex dissociates because of the charge repulsion between the DsbD α residue Asp68 and the Cys98 thiolate of reduced DsbC.

The overall reduction of DsbC involves a series of electron transfers across the inner membrane mediated by proteins that consistently alternate between thioredoxin family members (trx) and non-family members: thio-reductase to thioredoxin (trx) to DsbD β to DsbD γ (trx) to DsbD α to DsbC (trx).

The structure of the DsbC–DsbD α complex also suggests how the electron transfer reactions of the disulfide bond formation and isomerization pathways are isolated. On one hand, DsbC dimerization increases its affinity for the two independent binding sites on DsbD α , while protecting it from oxidation by DsbB (Bader *et al.*, 2001). On the other hand, steric hindrance due to the extended helical domain of DsbA, as observed in the superpositioned structures of DsbA and the DsbC–DsbD α complex, prevents DsbA from being unspecifically reduced by DsbD.

Implications for the isomerase function of DsbC

The crystal structure of the DsbC–DsbD α complex can serve as an initial model for the interactions of the disulfide bond isomerase DsbC with folding substrate proteins. The interactions between DsbC and DsbD α include a total of 13 aromatic residues (nine in the primary binding site). The DsbC residue with the largest contribution to both binding sites is the conserved Tyr100 found in the active site motif. A random mutagenesis screen of the active site dipeptide for increased isomerase activity has revealed the preference of an aromatic residue (Tyr/Phe) in this location (Bessette *et al.*, 2001). This result is consistent with the structure of the DsbC–DsbD α complex, where residue Tyr100 inserts into an uncharged pocket next to the catalytic cysteine (Cys109) of DsbD α (Figure 4A). The complex is further stabilized by the hydrogen bonds of Cys109 with the conserved *cis*-proline loop of DsbC.

During disulfide bond isomerization, similar interactions may occur. DsbC Tyr100 is likely to interact with aromatic or hydrophobic residues exposed by misfolded substrate proteins bound to the uncharged central cleft of DsbC. The *cis*-proline loop orients the substrate disulfide for the nucleophilic attack by Cys98. The intermolecular disulfide formed between Cys98 of DsbC and the substrate can subsequently be resolved either by a free substrate cysteine or by Cys101 of DsbC, resulting in the isomerization or breakage of the substrate disulfide. Substrate refolding is further facilitated by the chaperone activity of DsbC.

The structures of the complex and of oxidized DsbD α demonstrate the principal ability of DsbC to catalyze the rearrangement of disulfide bonds even if they are buried in the final structure. During refolding, conformational rearrangements in the substrate are expected. The comparison of complexed with uncomplexed DsbC shows that the substrate binding cleft can adjust to the shape of the binding partner, allowing DsbC to interact with a broad range of substrate proteins in different conformations.

Conclusion

In this paper, we provide structural insights into the final step of a novel transmembrane electron transport process in *Escherichia coli* and discuss the implications of our results for the process of disulfide bond isomerization during oxidative protein folding. We trapped an electron

transport intermediate and determined its crystal structure and those of the two reaction products, oxidized DsbD α and reduced DsbC, which is in this case represented by DsbC C101S.

The structure of the DsbC–DsbD α complex shows that the electron transport is mediated by two different families of thiol oxidoreductases. DsbC belongs to the thioredoxin-fold oxidoreductases, while DsbD α is a new type of thiol oxidoreductase with an Ig fold. Our findings represent the first structure of a complex between thiol oxidoreductases of different families. Similar thiol–disulfide exchange reactions between alternating families of thiol oxidoreductases appear to be the common mode of electron transfer in related pathways (Katzen and Beckwith, 2000), i.e. the bacterial DsbA/DsbB pathway and the eukaryotic PDI/Ero1 pathway (Frand and Kaiser, 1998; Tu *et al.*, 2000). The approach used (trapping mixed disulfides) and the structural features observed are essential for further elucidating the mechanism of transfer between such families. Furthermore, proteins with an Ig fold and appropriately positioned cysteines can now be regarded as candidates for novel oxidoreductases in genome searches for proteins exhibiting oxidoreductase activity, both in prokaryotes and eukaryotes.

The results presented also provide a first model for the interaction of the chaperone/protein disulfide bond isomerase DsbC with misfolded (misoxidized) substrate proteins. Previous evidence suggested that DsbD α exists in an unstable, unfolded state when it is fixed in the ‘reduced’ conformation by mutation of either of its cysteines (Katzen and Beckwith, 2000). Substrates with exposed hydrophobic patches are recognized by the uncharged cleft of DsbC and the two active Cys-Xaa-Xbb-Cys motifs catalyze the rearrangement of incorrect disulfide bonds. The concurrent refolding of the substrate is facilitated by the chaperone activity of DsbC.

Materials and methods

Plasmids

Plasmid pFK119 encodes the cytoplasmic variant of DsbD α C103A with an N-terminal His₆ tag (Goldstone *et al.*, 2001). The dsbC mutant C101S without signal sequence was amplified from DsbCC101S-pET22 using the primers 5'-GCTTTGCCATGGCTGATGACGC-3' and 5'-CTAGTT-ATTGCTCAGCGGTGGCAGC-3', and cloned into *Nco*I- and *Bam*HI-linearized pProEX HT (Life Technologies). The gene was inserted downstream of a His₆ tag and a tobacco etch virus (TEV) protease cleavage site. Plasmid sequences were verified by DNA sequencing.

Protein expression, purification and complex preparation

DsbC C101S and native DsbD α were expressed and purified as described previously (McCarthy *et al.*, 2000; Goldstone *et al.*, 2001). His₆-DsbC C101S was expressed in *E. coli* BL21 DE3 cells and purified by immobilized metal affinity chromatography (IMAC) using a 5 ml Hi-Trap Chelating column (Amersham Pharmacia) pre-loaded with nickel. The His₆ tag was removed by TEV digestion (50 mM Tris–HCl pH 8.0, 0.5 mM EDTA and 2 mM β -mercaptoethanol) and the protein purified by IMAC. His₆-DsbD α C103A was prepared as described previously (Goldstone *et al.*, 2001) and the His₆ tag was removed by TEV (Life Technologies) digestion.

The complex between DsbC C101S and DsbD α C103A was formed as described previously (Haebel *et al.*, 2001) and the complex formation reaction was monitored by non-reducing SDS–PAGE. The DsbC C101S–DsbD α C103A complex was purified by size-exclusion chromatography using a Superdex75 column (Amersham Pharmacia).

Table I. Data collection and refinement statistics

Data collection	DsbC C101S	DsbC–DsbD α	DsbD α
Radiation source	SSRL BL9-2	ESRF ID29	SSRL BL9-2
Wavelength (Å)	0.98	0.98	0.98
Space group	$P2_12_12_1$	$P4_12_12$	$C222_1$
Unit cell (Å ³)	$59.4 \times 78.3 \times 95.6$	$68.9 \times 68.9 \times 230.3$	$53.1 \times 54.6 \times 102.5$
Resolution (Å)	1.9–30	2.3–30	1.9–30
No. of reflections	182 932	239 773	70 473
No. of unique reflection	34 245	25 225	12 214
Completeness (%) ^a	98.6 (95.2)	97.6 (97.5)	95.5 (84.7)
R_{sym} ^{a,b}	4.0 (41.8)	6.7 (22.6)	3.9 (19.0)
Average I/σ	28.4	7.4	31.2
Refinement statistics			
Resolution (Å)	1.9–30	2.3–30	1.9–30
No. of reflections	33 417	24 849	11 540
No. of atoms	3518	4350	1023
DsbC	3271	3280	
DsbD α		946	936
Water molecules	247	124	87
R factor	0.20	0.23	0.22
R_{free} factor	0.25	0.29	0.25
R.m.s.d. bonds (Å)	0.005	0.012	0.006
R.m.s.d. angles (°)	1.2	1.5	1.3
Average B factors (Å ²)	33.6	43.3	30.3

^aNumbers in parentheses indicate values in the highest resolution shell.

^b $R_{\text{sym}} = \Sigma |I - \langle I \rangle| / \Sigma I$, where I is the observed intensity and $\langle I \rangle$ is the average intensity of symmetry-related reflections.

Crystallization, data collection and processing

Purified DsbC C101S was dialyzed into 10 mM HEPES pH 7.0 and concentrated to 28 mg/ml. Crystals were grown by the hanging drop vapor diffusion method using microseeding. Crystals grew after 3–5 days from 25% PEG 550 MME and 100 mM Tris–HCl pH 9.0. After crystal dehydration (McCarthy *et al.*, 2000), diffraction data for DsbC C101S crystals were collected under cryogenic conditions on beamline BL9-2 at the SSRL ($\lambda = 0.98$ Å). Data were processed using DENZO and SCALEPACK (Otwinowski and Minor, 1997). The crystals belong to the space group $P2_12_12_1$ with $a = 59.4$ Å, $b = 78.3$ Å and $c = 95.6$ Å, and the asymmetric unit contained two molecules.

Oxidized DsbD α (20 mg/ml) was crystallized from 23% PEG4000, 0.1 M NaCH₃CO₂ pH 4.6, 0.1 M (NH₄)₂SO₄ using the hanging drop method. Diffraction data for oxidized DsbD α were collected under cryogenic conditions on beamline BL9-2 at the SSRL ($\lambda = 0.98$ Å). Data were processed with DENZO and SCALEPACK, revealing that DsbD α crystallized in the space group $C222_1$ with $a = 53.1$ Å, $b = 54.6$ Å and $c = 102.5$ Å, and one molecule in the asymmetric unit.

The DsbC C101S–DsbD α C103A complex was crystallized using the hanging drop method and streak seeding. The precipitant buffer contained 25% PEG5 k MME, 0.2 M NaCl, 0.05 M (NH₄)₂SO₄, 5% glycerol, 0.2 M NaCH₃CO₂ pH 4.9. Needle-shaped crystals were fragmented and dehydrated prior to flash cooling in liquid nitrogen (Haebel *et al.*, 2001). Diffraction data were collected at 100 K on beamline ID29 at the ESRF ($\lambda = 0.98$ Å). The complex crystallized in space group $P4_12_12$ with $a = b = 68.9$ Å and $c = 230.3$ Å, and contained one complex molecule consisting of a DsbC homodimer and a DsbD α monomer in the asymmetric unit. Data were processed using MOSFLM and SCALA (CCP4, 1994; Powell, 1999).

Crystallographic statistics are summarized in Table I.

Structure determination and refinement

The structure of DsbC C101S was solved by molecular replacement using AMORE (CCP4, 1994) with native DsbC [Protein Data Bank (PDB) access code 1eej] as the search model. DsbC C101S was built using program O (Jones *et al.*, 1991) and refined with CNS (Brünger *et al.*, 1998) using a maximum likelihood target function with bulk solvent correction. After the addition of water molecules, the refinement with 1.9 Å data converged at an R factor = 0.220 and R_{free} = 0.246. The model contains residues A1–A215, B1–B216 and 247 water molecules. The side chains of residues 157–162 are poorly defined. The model shows

excellent stereochemistry with all residues except Asp129 in most favored and additionally allowed regions of the Ramachandran plot.

The structure of the DsbC C101S–DsbD α C103A complex was determined by molecular replacement using MOLREP (Vagin and Teplyakov, 2000) at 4.5 Å resolution with one DsbC C101S monomer as search model. Two copies of DsbC C101S were found in the asymmetric unit. Rigid body refinement using CNS with each DsbC C101S monomer divided into two separate domains resulted in a model with R factor = 0.450 and R_{free} = 0.485. ARP/wARP (Perrakis *et al.*, 1999) helped to remove model bias and produced a high quality electron density map at 2.3 Å that allowed the building of DsbD α C103A in the program O. The structure was refined with CNS using simulated annealing with maximum likelihood target function and torsion angle dynamics. The final model refined to a final R factor = 0.234 and R_{free} = 0.293. The structure contains residues A4–A215 and B1–B214 of the DsbC C101S dimer, residues C8–C125 of Dsb α C103A and 124 water molecules. The side chains of the DsbC loop 158–164 and the side chains of the last two C-terminal residues of DsbD α are poorly defined in the density. The model has good stereochemistry and the DsbD α residue Asp79, which is in a disallowed region of the Ramachandran plot, assumes a γ -loop conformation.

The structure of oxidized DsbD α was determined by molecular replacement with MOLREP using the structure of DsbD α C103A from the DsbC–DsbD α complex as search model. The structure was updated using ARP/wARP and refined to a final R factor = 0.216 and R_{free} = 0.254 in CNS. The final model contains residues 10–125 and 87 water molecules and has excellent stereochemistry. Residue Asp79 is located in a γ -loop, as observed in complexed DsbD α . The coordinates for DsbC C101S (PDB accession code 1jzo), oxidized DsbD α (PDB accession code 1jpe) and the DsbC C101S–DsbD α C103A complex (PDB accession code 1jzd) were deposited at the PDB.

In vivo thiol redox-state analysis

To determine the *in vivo* redox state of DsbD α and DsbC, strains FED126 (MC1000 $\Delta dsbD$) or FED161 (FED126 $\Delta trxA$) carrying the corresponding DsbD derivatives were grown and processed as described previously (Katzen and Beckwith, 2000). 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).

DsbD α , DsbD β , DsbD γ and DsbD $\beta\gamma$ derivatives were expressed from plasmids pFK086, pFK060, pFK017 and pFK094, respectively (Katzen and Beckwith, 2000). When indicated, corresponding amino acids were

mutated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) as directed by the manufacturer. All the constructions were verified by DNA sequencing.

Acknowledgements

We thank G.Leonard and G.Sainz for assistance with data collection on the ESRF beamline ID29. A.Scheidig kindly facilitated access to beamline BW7B at DESY and P.Ellis provided assistance on beamline BL9-2 at the SSRL. P.W.H. is supported by a Boehringer Ingelheim Fonds doctoral scholarship. D.G. is the recipient of a FRST Bright Future Scholarship and F.K. is a Charles A.King Trust fellow. J.B. is an American Cancer Society Research Professor and work in the J.B. laboratory is supported by Grant GM55090 from the NIH. Research in the Metcalf laboratory is funded by the New Zealand Health Research Council and the Auckland Medical Research Foundation.

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Received May 28, 2002; revised July 16, 2002;
accepted July 29, 2002