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Review

nDsbD: a redox interaction hub in the *Escherichia coli* periplasm

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Abstract. DsbD is a redox-active protein of the inner *Escherichia coli* membrane possessing an N-terminal (nDsbD) and a C-terminal (cDsbD) periplasmic domain. nDsbD interacts with four different redox proteins involved in the periplasmic disulfide isomerization and in the cytochrome *c* maturation systems. We review here the studies that led to the structural characterization of all soluble DsbD domains involved and, most importantly, of trapped disulfide intermediate complexes of nDsbD with three of its four redox partners. These results revealed the structural features enabling nDsbD, a 'redox hub' with an immunoglobulin-like fold, to interact efficiently with its different thioredoxin-like partners.

Keywords. Disulfide isomerization, cytochrome *c* maturation, DsbD, DsbC, DsbG, CcmG.

The disulfide bond formation and isomerization pathways of *E. coli*

Disulfide bond formation is a very important post-translational modification of secretory proteins. In Gram-negative bacteria, such as *Escherichia coli*, disulfide bond formation and isomerization are catalyzed in the periplasm by members of the Dsb protein family [1–3]. The two types of reaction, disulfide bond formation and isomerization, are coupled to independent pathways (Fig. 1). The disulfide formation pathway consists of two proteins, DsbA and DsbB. Oxidized DsbA, possessing a thioredoxin-like fold with a helical insertion [4], introduces disulfide bonds to non-native substrate proteins in a random and very fast manner [5, 6]. The inner membrane protein DsbB re-oxidizes DsbA and keeps it in its active state [7]. The two electrons that flow from reduced DsbA to DsbB are then passed from DsbB to ubiquinone-8 from the respiratory chain [7, 8].

DsbA only possesses a very low disulfide isomerase activity. Non-native disulfide bonds introduced by DsbA into substrate proteins are rearranged by DsbC, an efficient periplasmic disulfide isomerase with chaperone activity [9]. DsbC, which does not react with DsbA [10, 11], is kept in its catalytically active, reduced state by the inner membrane protein DsbD [12], which consists of a central transmembrane domain (tDsbD) with eight predicted transmembrane helices, an immunoglobulin-like N-terminal (nDsbD) and a thioredoxin-like C-terminal periplasmic domain (cDsbD) [13–18]. Each of these three domains contains one pair of invariant cysteines, which are crucial for disulfide exchange [13]. DsbD functions as a reductant, receiving its reducing power from cytoplasmic NADPH (reduced nicotinamide adenine dinucleotide phosphate). Two electrons are transferred from NADPH

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via thioredoxin reductase to thioredoxin [19, 20], which passes them on to tDsbD. Electrons are then further transferred via cDsbD to nDsbD [21–23], which can be viewed as a 'redox hub' that distributes electrons to several periplasmic target proteins. Besides DsbC, *in vivo* substrates of nDsbD are DsbG and CcmG, which, like DsbC, both belong to the thioredoxin superfamily [21, 24]. DsbG is a putative DsbC paralogue with so far unknown cellular function, exhibiting low disulfide isomerase activity and chaperone activity [25–27].

The third substrate, CcmG, is a periplasmic, membrane-anchored protein belonging to the cytochrome *c* maturation pathway. CcmG is thought to activate CcmH, which itself reduces apocytochrome *c*, enabling it to become active and incorporate heme [28–30].

In the last few years, the DsbD/DsbC and DsbD/CcmG redox systems have been characterized in detail through structural and functional studies. The present review summarizes the current knowledge regarding the structures and interactions of the various components. The analysis highlights the central role of nDsbD as interaction hub, and in particular the question of how nDsbD specifically interacts with four different components of the disulfide isomerization and cytochrome *c* maturation pathways.

The disulfide isomerase DsbC and its homologue, DsbG

DsbC was the first structurally characterized component of the bacterial protein disulfide isomerization pathway [31]. DsbC is a V-shaped homodimer, with each monomer containing an N-terminal dimerization domain (aa1–aa61) and a C-terminal catalytic domain (aa78–aa216), connected by a linker α-helix. The catalytic domain can be divided into two subdomains. The first one (encompassing aa78–aa122 and aa167–aa216) possesses a thioredoxin-like fold and contains two cysteines (Cys98 and Cys101) that are necessary for activity. The second subdomain (aa123–aa166; α-helices 4 and 5) carries an insertion into the thioredoxin-like fold at the same position as found in DsbA [31]. The overall arrangement of the dimer (Fig. 2a) is such that a large hydrophobic cleft separates the two active sites. McCarthy et al. [31] proposed that this cleft represents the binding site for protein substrates and is required for the chaperone and isomerase activity of DsbC. Dimerization of DsbC is crucial for its isomerase activity [32] and prevents it from interacting with DsbB [33]. In addition, dimerization of DsbC increases its affinity towards its reductant, nDsbD [17, 34], in perfect agreement with the structural finding that each DsbC subunit contributes one interface to the complex with nDsbD.

Recently, also the structure of DsbG, a protein sharing 24% sequence identity with DsbC, was solved [24, 31, 35]. Like DsbC, DsbG exhibits a V-shaped homodimeric structure, but of larger size. This is essentially due to the fact that DsbG has a longer linker helix (2.5 additional turns) between the thioredoxin-like and dimerization domains. In addition, the DsbG dimer possesses a cleft of nearly double the size compared with that of DsbC. The cleft surface of DsbG is also partially hydrophobic,

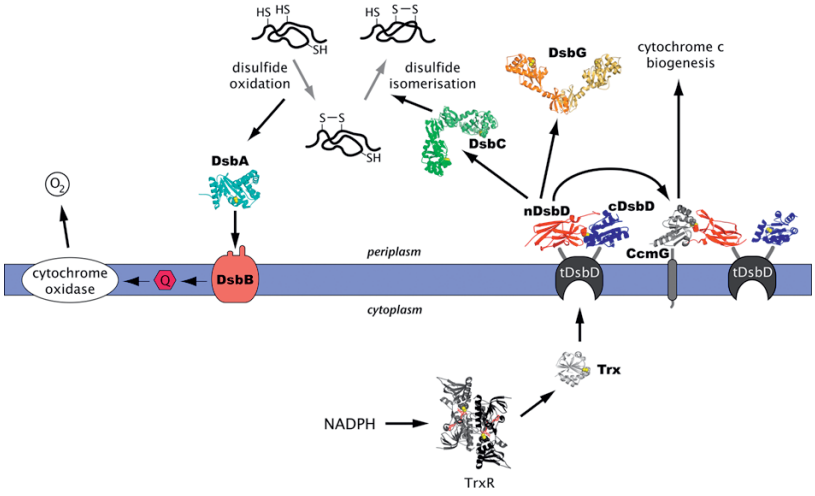

Figure 1. An overview of the *E. coli* Dsb-system. Two electrons are transported from cytoplasmic NADPH via thioredoxin reductase (dark grey and black; PDB-code: 1CL0 [44]) and thioredoxin (light grey; 2TRX [45]) to tDsbD. tDsbD transports the electrons through the membrane via periplasmic cDsbD (blue; 1VRS [10] and 2FWE [39]) to nDsbD (red; 1VRS [10] and 1Z5Y [40]). nDsbD functions as a hub protein and passes the electrons on to either DsbC (green; 1EEJ [31]; disulfide isomerization pathway) or CcmG (grey; 1Z5Y [40]; cytochrome *c* maturation) or DsbG (orange; 1v58 [35]). DsbC isomerizes non-native disulfide bonds to native disulfide bonds. DsbA (cyan; 1DSB [4]) randomly oxidizes unfolded polypeptide chains and transports the electrons to DsbB (pink) that itself gives them via quinone (claret-red) and cytochrome oxidases to molecular oxygen. A flow of two electrons is indicated by black arrows.

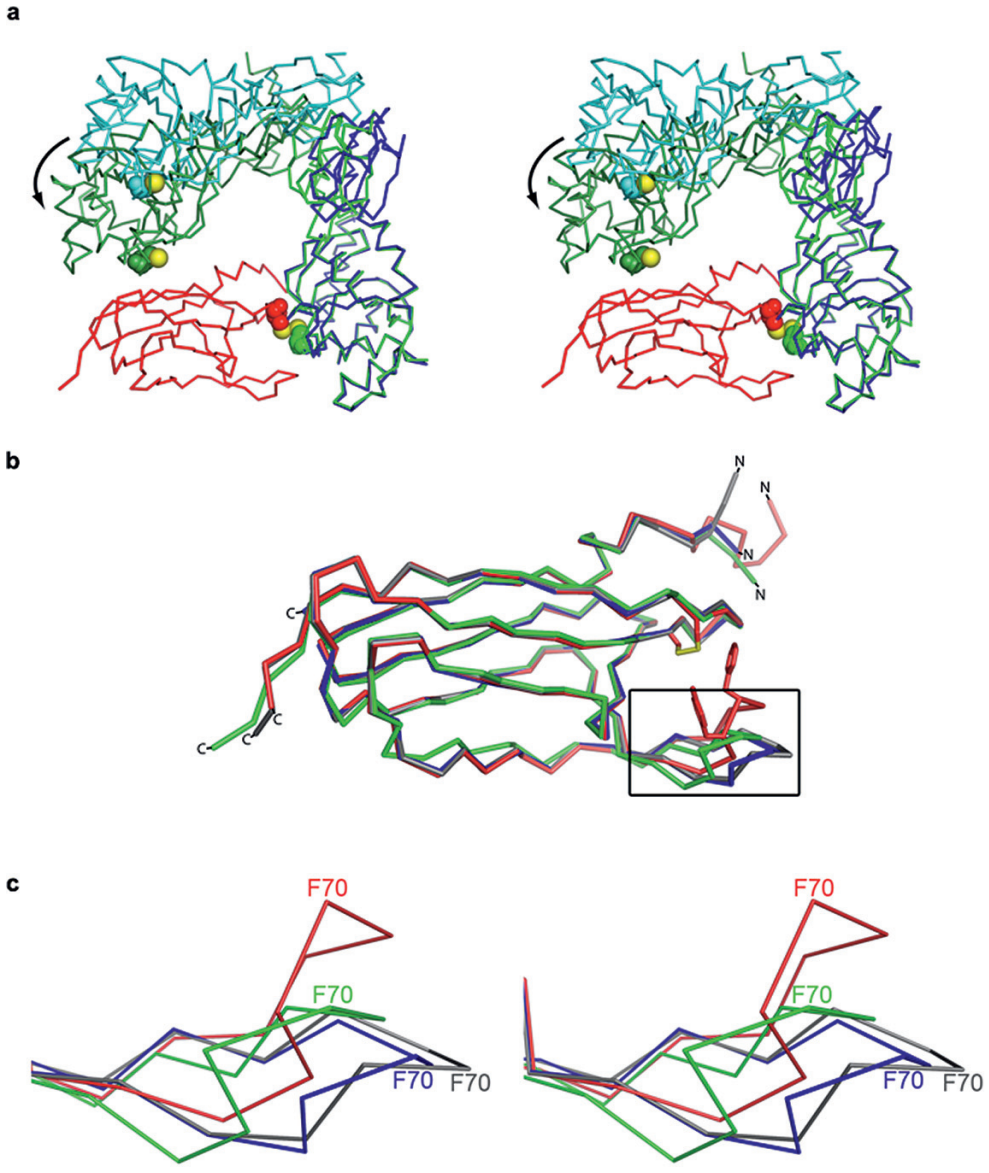

Figure 2. (*a*) Uncomplexed dimeric DsbC (blue and cyan; 1EEJ [31]) undergoes a large conformational change upon complex formation with nDsbD (light and dark green (DsbC) and red (nDsbD); 1JZD [17]). The solvent-accessible cysteines are depicted in spacefill representation with the S_γ atom coloured in yellow. (*b*) Superposition of the different nDsbD structures available. The C_α trace of uncomplexed nDsbD is depicted in red, the Cys103–Cys109 disulfide bond and the Phe70 and Tyr71 side chains (which shield the disulfide bridge) appear in stick representation (1L6P [16]). The C_α trace of nDsbD in nDsbD-SS-DsbC is coloured in green (1JZD [17]), the one of nDsbD in nDsbD-SS-cDsbD in blue (1VRS [10]) and the one of nDsbD in nDsbD-SS-CcmG in grey (1Z5Y [40]). (*c*) Zoomed stereoview (frame in Fig. 2b), showing the differences in the opening of the nDsbD cap-loop region. In nDsbD-SS-cDsbD (blue) and nDsbD-SS-CcmG (grey) the cap-loop undergoes a clear opening compared with uncomplexed nDsbD (red), whereas in nDsbD-SS-DsbC the less opened cap-loop (green) is laterally shifted.

but also features negatively charged patches that are not found in DsbC [35].

nDsbD: activator of the disulfide bond isomerase DsbC

Initial bioinformatics studies on DsbD suggested that its cDsbD domain might belong to the thioredoxin-like superfamily, due to the presence of a typical CXXC active site motif and due to the sequence similarity of cDsbD to the thioredoxin-like domains of protein disulfide isomerase (PDI) [36–38]. The central region of the DsbD sequence could be readily assigned by bioinformatics and experimental topological analyses to a transmembrane domain with eight predicted transmembrane helices [13–15]. In contrast, the topology of nDsbD remained unclear in the absence of a crystal structure. The sequence of

nDsbD does not contain the typical CXXC thioredoxin motif, but a CX_5C motif instead. In 2002, two groups independently solved the structure of nDsbD: Goulding et al. [16] determined the crystal structure of the unliganded protein, while shortly after Haebel et al. [17] published a comprehensive structural study on the kinetically stabilized, mixed disulfide complex between nDsbD and the DsbC homodimer (nDsbD-SS-DsbC).

Unlike the other Dsb proteins, namely DsbA, DsbC, CcmG and cDsbD, which all possess a thioredoxin-like fold, nDsbD features an immunoglobulin-like fold consisting of a β-sandwich formed by two antiparallel β-sheets. The active site cysteines Cys103 and Cys109 connect two neighbouring β-strands in the oxidized state of nDsbD. In unliganded nDsbD, the so-called cap-loop region (residues Asp68–Gly72) protects the active site cysteines from the environment and assumes a closed conformation, disfavouring illegitimate redox reactions (Fig. 2b, c) [16, 17].

Upon formation of a mixed disulfide between nDsbD and DsbC, the cap-loop of nDsbD opens (Fig. 2b, c), and the V-shaped DsbC dimer adopts a more closed conformation to form the nDsbD-SS-DsbC complex (Fig. 2a). As already mentioned, the complex possesses two interfaces. In the first one (primary interface), Cys109 of nDsbD, forms an intermolecular disulfide bond with Cys98 of one DsbC monomer. The second subunit of DsbC forms a secondary, asymmetric binding interface with the opposite side of nDsbD. The active site of the second DsbC subunit is involved in the secondary binding interface, but it is not covalently linked to nDsbD, which does not possess any cysteines in addition to those of the active site [17].

Different modes of interaction in nDsbD-SS-cDsbD and nDsbD-SS-DsbC

The C-terminal domain of DsbD has also been structurally characterized. With a C_α-rmsd of 1.54 Å for 91 out of 105 residues, its structure shows a high similarity to *E. coli* thioredoxin and it functions as a stiff electron shuttle between tDsbD and nDsbD [18, 39]. Upon complex formation of nDsbD and cDsbD (nDsbD-SS-cDsbD), cDsbD exhibits only one significant conformational change: the main chain carbonyl of Thr529, forced by van der Waals interactions exerted by Phe11 and Phe108 of nDsbD [39], rotates by about 100º. The cap-loop region of nDsbD adopts an even more open conformation than that observed for nDsbD-SS-DsbC (Fig. 2b, c). By superimposing the nDsbD molecules of the two complexes, the relative orientations of the thioredoxin-like folds of cDsbD and DsbC, respectively, differ by around 20º. In the nDsbD-SS-cDsbD complex, cDsbD shares only one binding interface with nDsbD compared with the two interfaces found in nDsbD-SS-DsbC. The nDsbD-SS-cDsbD interface and the primary interface of nDsbD-SS-DsbC have a similar size of around 1300 $Å^2$, but the total binding interface area of nDsbD-SS-DsbC is about 1.5-fold larger due to the second interface. These findings imply a lower affinity of nDsbD to cDsbD than to DsbC, which can, however, be compensated by high effective concentrations of nDsbD and cDsbD in the context of the full-length protein.

The bimolecular reduction of nDsbD by cDsbD and DsbC by nDsbD are both very fast, with rate constants of $1.1 \cdot 10^5\ M^{-1}s^{-1}$ and $3.9 \cdot 10^6\ M^{-1}s^{-1}$ [10].

Modelling of the nDsbD-SS-DsbG complex on the basis of the known structure of DsbG and the primary interface between nDsbD and DsbC showed that the cleft in DsbG, in principle, was wide enough to allow two copies of nDsbD to form a mixed disulfide complex with each subunit of DsbG without steric clashes. Whether both subunits of DsbG can be reduced simultaneously by two molecules of DsbD *in vivo* remains unknown, but the analysis indicates that a secondary interface between nDsbD and the second subunit of DsbG is unlikely.

nDsbD reduces CcmG: branching to another redox pathway

CcmG is the third interaction partner for which a structure in a mixed-disulfide complex with nDsbD is known [40]. High-resolution crystal structures of uncomplexed CcmG had been described earlier for the Gram-negative bacterium *Bradirhyzobium japonicum* and the related protein of the Gram-positive *Mycobacterium tuberculosis* [41, 42]. *B. japonicum* CcmG possesses a thioredoxin-like fold, enriched by an $\alpha\beta$-insertion (after β-strand 2) that is required for *c*-type cytochrome maturation. Additionally, an N-terminal extension links the transmembrane anchor helix to the thioredoxin-like domain [41]. With a low rmsd value of 1.4 Å (on C_α-atoms), *B. japonicum* CcmG is very similar to *E. coli* CcmG (from the nDsbD-SS-CcmG complex), and only the seven-residue-shorter N-terminal extension adopts a different conformation [40].

nDsbD interacts with CcmG in a manner similar to what it does with cDsbD: (i) the relative orientation of the thioredoxin-like domains is the same and (ii) the cap-loop region opens in a similar way upon complex formation (Fig. 2b, c), but exhibits more flexibility as judged from the features of the electron density in the crystal structure and from the temperature factors of the refined atomic model. An important feature found neither in the nDsbD-SS-cDsbD nor in the nDsbD-SS-DsbC complex is a substantial involvement of N-terminal nDsbD residues (Ser9 and Phe11) in the binding interface. These residues form

a hydrogen-bond network with Tyr141 of CcmG (a highly conserved residue among different species), the phenolic ring of which fits in a hydrophobic pocket formed by residues of CcmG and nDsbD. In contrast, cDsbD and DsbC do not have a tyrosine at that position and do not specifically interact with these N-terminal nDsbD residues [40].

The hub function of nDsbD

nDsbD is a very interesting subject for analysis of protein-protein interactions, since it has been structurally characterized both in the unliganded state and in complex with three of the four known *in vivo* partners (cDsbD, DsbC and CcmG). In the three complexes (and considering only the primary interface for nDsbD-SS-DsbC), around 90% of the nDsbD interface residues (contacting the active site of the partner protein) are the same. In all three cases the interface is rather small (area around 1350 Å^2), and mediates only a small number of interactions. The adaptability of nDsbD to three different targets results from at least three structural features: (i) it possesses a very flexible cap-loop region, which assumes a closed conformation in the absence of a protein ligand. Upon formation of the mixed disulfide, the cap-loop region adopts a more open conformation, adapting to the partner protein. (ii) For DsbC recognition, nDsbD employs a second, flat binding interface on its side. In this case, interactions are of non-covalent nature and include one salt bridge. (iii) The N-terminal segment of nDsbD plays a role in the recognition of CcmG: it is involved in a hydrogen-bonded network and in hydrophobic interactions with Tyr141 of CcmG, a residue not conserved in the two other partners of nDsbD (it corresponds to a Ser180 in DsbC and to a Leu508 in cDsbD).

In contrast to nDsbD, two of the three thioredoxin-like target proteins of nDsbD [cDsbD and CcmG, based on a comparison between CcmGs of *E. coli* (complex) and *B. japonicum*] undergo very small conformational changes upon formation of the mixed disulfide complex with nDsbD [10, 40]. In complexed DsbC, the only conformational change involves the relative orientation of the two subunits and leaves the thioredoxin domain unaffected [17].

Structural analysis of the three complexes prompted Rozhkova et al. [10] and Stirnimann et al. [40] to propose that nDsbD, in order to form mixed disulfides with its various target proteins, must be able to undergo substantial movements relative to tDsbD. A likely orientation for two membrane-linked complexes, nDsbD-SS-cDsbD and nDsbD-SS-CcmG, relative to membrane was proposed [10, 40]. Based on that assumption, nDsbD has to substantially change its orientation relative to the membrane upon changing partner from cDsbD to CcmG (nDsbD turned 'up-side down'; see Fig. 3, panels A and C). Upon formation of the complex with the non membrane-bound DsbC, nDsbD has to assume yet a different orientation to allow a proper interaction with both interfaces of DsbC (Fig. 3). The above observations underline the key function of nDsbD as a redox hub in the periplasm of Gram-negative bacteria.

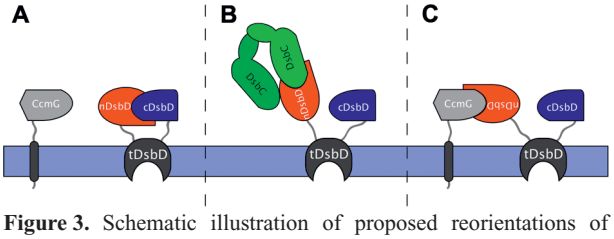

Figure 3. Schematic illustration of proposed reorientations of nDsbD to fulfil its 'redox hub' function [10, 40]. Panel *A* shows the proposed orientation in the nDsbD-SS-cDsbD complex; panels *B* and *C* depict possible orientations in the nDsbD-SS-DsbC and nDsbD-SS-CcmG complexes, respectively.

Concluding remarks

All known periplasmic proteins interacting with nDsbD have been structurally characterized, as have the complexes of nDsbD with those partners, with the single exception of nDsbD-SS-DsbG. In spite of these successes, the transmembrane domain (tDsbD), to which nDsbD is anchored, remains elusive not only from a structural but also from a functional point of view: it is still unclear how electrons are transported through the membrane and how cytoplasmic thioredoxin or periplasmic cDsbD interact with tDsbD [43]. Thus, important research goals for the future are the structure determination of tDsbD and its complexes with soluble partners (cDsbD and thioredoxin), as well as the structure determination of the nDsbD-SS-DsbG complex.

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