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Disulfide Bond Formation System in *Escherichia coli*

Kenji Inaba*

Division of Protein Chemistry, Post-Genome Science Center, Medical Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

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The biological kingdoms have evolved elaborate systems that ensure the catalysis of protein disulfide bond (dsb) formation in the cell. Coexisting in the periplasm of *Escherichia coli* are the DsbA–DsbB disulfide-introducing and DsbC–DsbD disulfide-isomerizing pathways, which promote the oxidative folding of secreted proteins. Recent structural studies of DsbB have illuminated conformational dynamics involved in the effective oxidation of the extremely reduction-prone oxidase, DsbA, as well as the structure of the reaction centre involved in protein Dsb formation *de novo* in conjunction with ubiquinone. Extensive genetic and biochemical analysis has recently provided insight into how DsbD transports electrons from cytosolic thioredoxin to periplasmic DsbC. To a great extent, the molecular mechanisms of the Dsb enzyme system in *E. coli* have been elucidated, and are applicable to the study of protein disulfide formation systems in other organisms.

Key words: crystal structure, disulfide bond, DsbB, DsbD, *E. coli* periplasm.

Abbreviations: PDI, protein disulfide isomerase.

The formation of protein disulfide bonds (Dsbs) is an oxidative reaction that is crucial for the folding and maturation of many secreted and membrane proteins. While almost all organisms, from bacteria to humans, are equipped with dedicated catalytic systems that accelerate this oxidative reaction (*1–4*), the best characterized dsb formation system is the series of *Escherichia coli* Dsb formation enzymes. DsbA is the primary dsb donor in the periplasm of *E. coli*, and catalyses the introduction of dsbs into folding proteins (*5*, *6*), sometimes indiscriminately. DsbB is a cytoplasmic membrane protein that plays a pivotal role in the oxidative system by generating dsbs *de novo* in conjunction with bound ubiquinone (UQ), and then transferring them to DsbA (*7*, *8*). DsbB is thus referred to as the dsb manufacturer in the cell. In addition to the DsbA–DsbB disulfide-introducing pathway, the periplasmic space of *E. coli* contains a disulfide-isomerizing pathway, in which DsbC functions as the major protein disulfide isomerase (PDI) (*9*, *10*), and DsbD, a cytoplasmic membrane protein, maintains DsbC in the reduced state by transferring electrons from thioredoxin present in the cytosol to DsbC in the periplasm (*11*, *12*). Several reviews of the cellular functions and structures of the Dsb enzymes have been published elsewhere (*13–17*). The current review will focus on the latest insights into the molecular mechanisms of the dsb system, particularly DsbB-mediated DsbA oxidation and DsbD-driven electron transport.

*To whom correspondence should be addressed. Tel/Fax: +81-92-642-6433, E-mail: inaba-k@bioreg.kyushu-u.ac.jp

BIOCHEMICAL AND STRUCTURAL PROPERTIES OF DsbA

DsbA is a periplasmic disulfide-introducing catalyst. The expression level of DsbA is controlled by the Cpx two-component system, one of the extracytoplasmic response systems in *E. coli* (*18*). DsbA contains a thioredoxin domain with an active-site cysteine pair (Cys30–Cys33) and an α-helical domain insert (*19*). A deep, hydrophobic groove runs below the cysteine pair, which accommodates a periplasmic loop segment of DsbB during the DsbA–DsbB catalytic cycle (see the next section) (*20*). A wide hydrophobic patch on the other side of the cysteine pair possibly serves to capture substrate proteins. DsbA has strong oxidizing properties, and therefore a strong tendency to be reduced (*21*). The active-site cysteine pair of DsbA has the second highest redox potential ($E'_0 = -120$ mV) among thioredoxin superfamily proteins (*22*).

Recently, a number of studies characterizing the structure and function of DsbA homologues from bacteria other than *E. coli* K-12 have been reported (*23*). DsbL from the uropathogenic *E. coli* strain CFT073 is the strongest oxidizing thioredoxin-like protein known to date, with a redox potential of −95 mV (*24*). Several other Gram-negative species encode more than one DsbA homologue. *Neisseria meningitides* contains three DsbA homologues (NmDsbA1–3), one of which (NmDsbA3) has a typical DsbA fold with a hydrophobic patch and a hydrophobic groove near the redox active site, and is oxidized by *E. coli* DsbB as effectively as is *E. coli* DsbA (*25*). In contrast, α-DsbA1 from *Wolbachia pipientis* lacks a hydrophobic groove, and does not interact with *E. coli* DsbB (*26*).

Some Gram-positive bacteria also possess Dsb-like proteins confined in a compartment between the plasma

membrane and the outer cell wall and anchored to the cell surface (*27*). However, *Staphylococcus aureus* and other microorganisms such as *Liesteria monocytogenes* encode a homologue of DsbA but no putative DsbB-like protein, which suggests that in these organisms, DsbA is directly re-oxidized by extracellular oxidants (*28*). Recent analysis using a bioinformatics-based approach suggests that some bacteria carry out dsb formation, but lack DsbB homologues. Rather, they contain bacterial homologues of the eukaryotic enzyme vitamin K epoxide reductase, which has been identified as a novel dsb formation catalyst candidate (*29*). Thus, the bacterial kingdom appears to have developed different oxidative systems composed of different sets of disulfide oxidoreductases, although the presence or absence of DsbA correlates with the presence or absence of DsbB in many cases (*30*).

CONFORMATIONAL DYNAMICS OF DsbB IN DsbA OXIDATION

The central mechanistic aspects of the DsbA–DsbB oxidative system in *E. coli* are (i) how DsbB rapidly converts the oxidizing equivalents of quinone species into protein dsbs and (ii) how DsbB effectively oxidizes DsbA, despite the strong tendency of DsbA to remain in the reduced state. DsbB has a four-helix bundle scaffold composed of four transmembrane helices (TM1–TM4) and two periplasmic loops (P1 and P2). Each of the periplasmic loops contains a pair of essential cysteines, Cys41–Cys44 and Cys104–Cys130, respectively, the latter of which is involved directly in disulfide exchange with DsbA. A chemical scheme of *de novo* dsb generation through the cooperation of DsbB and UQ has been elucidated on the basis of biochemical, crystallographic and theoretical studies of the UQ-neighbouring region of DsbB (*20*, *31*, *32*).

Using a UQ-free preparation of DsbB, standard redox potential values of approximately -210 mV for Cys41–Cys44 and −220 mV for Cys104–Cys130 were determined (*33*). The fact that both cysteine pairs of DsbB have a much lower redox potential than that of DsbA (approximately −120 mV) cannot explain the physiological flow of electrons from DsbA to DsbB. One possibility is that the binding of DsbA induces conformational changes in DsbB that facilitate the uphill transfer of electrons between the two enzymes. The crystal structures of a DsbB (Cys41Ser) variant containing the initial-state Cys104–Cys130 disulfide (*34*) and a DsbB–DsbA intermediate complex covalently linked by Cys104 (DsbB) and Cys30 (DsbA) (*20*, *35*) have been determined. A structural comparison of the two states of DsbB illuminates remarkable conformational transitions in the second periplasmic loop of DsbB during the catalysis of DsbA oxidation (Fig. 1). The Cys104-containing loop segment of DsbB, which is liberated upon complex formation, is accommodated by the deep hydrophobic groove of DsbA (*20*), resulting in the physical separation of Cys104 from its original partner, Cys130 (DsbB). The crystal structures revealed that the distance between C_α atoms of Cys104 and Cys130 is extended from 6.3 Å to 8.9 Å upon the binding of DsbA (*34*). In this geometry, Cys130 seems unlikely to attack the Cys30 (DsbA)–Cys104 (DsbB) intermolecular disulfide in reverse. Thus, the sequestration of Cys130 from the intermolecular disulfide might contribute to the inhibition of backward electron flow from DsbB to DsbA.

The structure of the isolated DsbB[CSSC] variant with a rearranged Cys41–Cys130 intramolecular disulfide was recently solved by advanced NMR techniques (*36*). The DsbB[CSSC] variant is supposed to mimic DsbB after the release of oxidized DsbA during catalysis. The structure of DsbB[CSSC] suggests that unpaired Cys130 (DsbB), which is generated by the reduction of the Cys104–Cys130 disulfide, approaches the Cys41–Cys44 pair in the P1 loop to form a Cys41–Cys130 disulfide. Biochemical studies in our laboratory showed that this Cys130 approach can occur even during the formation of the DsbA–DsbB binary complex, albeit with significant interaction between the Cys130-neighbouring segment of DsbB and the Phe63–Gly65 loop of DsbA, leading to another (minor) reaction pathway [see ref. (*34*) for more details]. At any rate, the transient formation of a Cys41–Cys130 disulfide seems advantageous to preventing Cys130 from attacking the Cys30 (DsbA)–Cys104 (DsbB) intermolecular disulfide (*37*). In this context, the functional role of the membrane-parallel α-helix in the P2 loop of DsbB is the focus of great interest. This particular α-helix is strikingly amphiphilic and associates peripherally with the outer leaflet of the cytoplasmic membrane (*34*). It also divides the P2 loop such that the mobility of the two cysteines in P2, Cys104 and Cys130, are separately regulated. Indeed, the introduction of charged amino acids into the membrane-facing region of the P2 loop grossly disrupts its association with the membrane, resulting in a severe impairment in the ability of DsbB to oxidize DsbA. It seems reasonable to speculate that the membrane-parallel α-helix of DsbB plays a key role in physiological electron flow, presumably by controlling the position and flexibility of the Cys130-containing loop (*34*). Thus, DsbB undergoes elaborate conformational changes that appear to facilitate the energetically unfavourable transfer of electrons from DsbA to DsbB.

PHYSIOLOGICAL FUNCTION OF DsbC

The process of disulfide rearrangement is essential for the restoration of misfolded proteins with non-native dsbs. This is particularly true for proteins that contain multiple cysteines and are mis-oxidized by DsbA. DsbC, a major PDI in the *E. coli* periplasm, is a V-shaped homodimeric protein with N-terminal dimerization domains and C-terminal thioredoxin domains containing the Cys98–Cys101 active site (*38*). The inner surface of the DsbC cleft is occupied primarily by hydrophobic or uncharged residues that are presumably involved in substrate binding. DsbC exerts isomerase activity only in the reduced state, which is maintained through the activity of the cytoplasmic membrane protein DsbD (see the next section). *E. coli* contains another disulfide isomerase, DsbG, in the periplasm, although its physiological role is unclear. DsbG also has a V-shaped homodimeric structure with a wider hydrophobic cleft than DsbC (*39*), and overexpression of DsbG complements the phenotype of

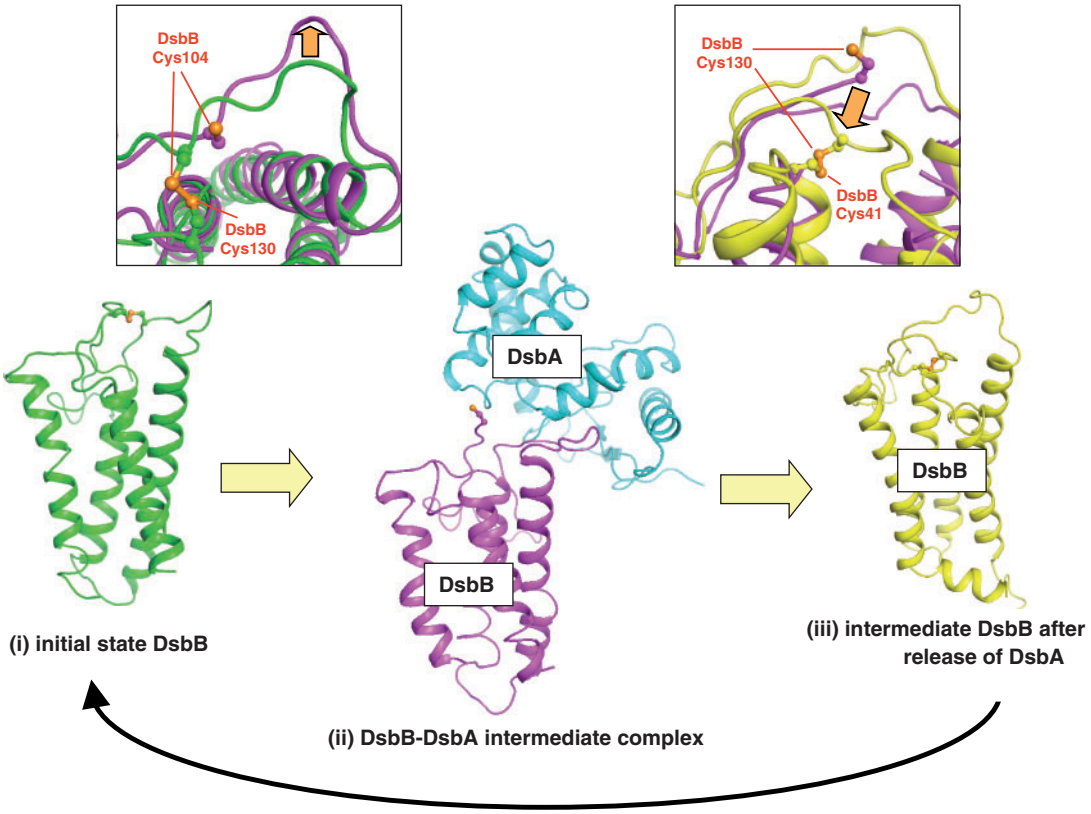

Fig. 1. **Snapshots of three DsbB conformers during DsbB-mediated DsbA oxidation.** Crystal structure of DsbB with the initial-state Cys104–Cys130 disulfide is shown in green (PDB ID: 2ZUQ). In the DsbB–DsbA intermediate complex, DsbA and DsbB are shown in cyan and magenta, respectively (PDB ID: 2ZUP). NMR structure of DsbB with a rearranged Cys41–Cys130 disulfide is shown in yellow (PDB ID : 2K73). The left inset shows the conformational change in the Cys104-containing loop segment of DsbB induced by complex formation with DsbA; the right inset is a close-up of the approach of Cys130 to Cys41 after the release of DsbA to generate a Cys41–Cys130 disulfide. These conformational changes play a key role in effective DsbA oxidation by DsbB.

dsbC$^-$ mutants (*40*), which suggests that it functions as a secondary disulfide isomerase, perhaps with different substrate specificity.

It has been proposed that DsbC can functionally substitute for DsbA as an oxidase. While DsbC accumulates in the oxidized form in a *dsbD*$^-$ strain, DsbC reverts to the reduced form in a *dsbA*$^-$ *dsbD*$^-$ strain, even though DsbD is absent (*41*). These results suggest that in a *dsbA*$^-$ *dsbD*$^-$ background, DsbC is capable of donating a disulfide to folding proteins. Consistent with this notion, the redox potential of DsbC is as high as DsbA (*42*). However, kinetic data shows that the oxidation of DsbC by DsbB is prevented by the homodimeric structure of DsbC (*20*, *43*, *44*). Thus, large kinetic barriers have evolved to guarantee the coexistence of the DsbA–DsbB and DsbC–DsbD redox pathways in the same compartment of the periplasm.

MECHANISM OF DsbD-DRIVEN ELECTRON TRANSFER ACROSS THE MEMBRANE

Recent genetic and biochemical approaches have helped clarify the mechanism of DsbD-mediated electron flow. Reducing equivalents are transferred first from thioredoxin to the transmembrane (β-) domain of DsbD, and then successively to the DsbD C-terminal thioredoxin-like (γ-) domain, the DsbD N-terminal immunoglobulin-like (α-) domain and then DsbC (Fig. 2) (*45*). Measurement of the redox potentials of each active site indicates that DsbD-mediated electron flow is thermodynamically favourable (*46*, *47*). It remains to be elucidated, however, how the β-domain of DsbD (DsbDβ) transfers electrons across the membrane. DsbDβ is composed of eight transmembrane helices (TM1–TM8) and does not contain cofactors, such as heme, metal centres, FAD or quinone (*46*). Instead, two essential cysteine residues, Cys163 and Cys285, are present in the transmembrane region of DsbD, and most likely contribute directly to electron transport activity. In fact, two mixed disulfide complexes, Trx1-DsbDβ(Cys163) and DsbDβ(Cys285)–DsbDγ, were detected when the three isolated structural domains of DsbD (α, β and γ) were co-expressed in *E. coli* (*45*). These results suggest that Cys163 and Cys285 are exposed to the aqueous milieu of the cytoplasmic and periplasmic spaces, respectively.

Extensive analysis using cysteine-scanning mutagenesis and alkylating agents has demonstrated that the C-terminal halves of the TM1 and TM4, which contain Cys163 and Cys285, respectively, are accessible by a membrane-impermeable cysteine-alkylating agent (*48*).

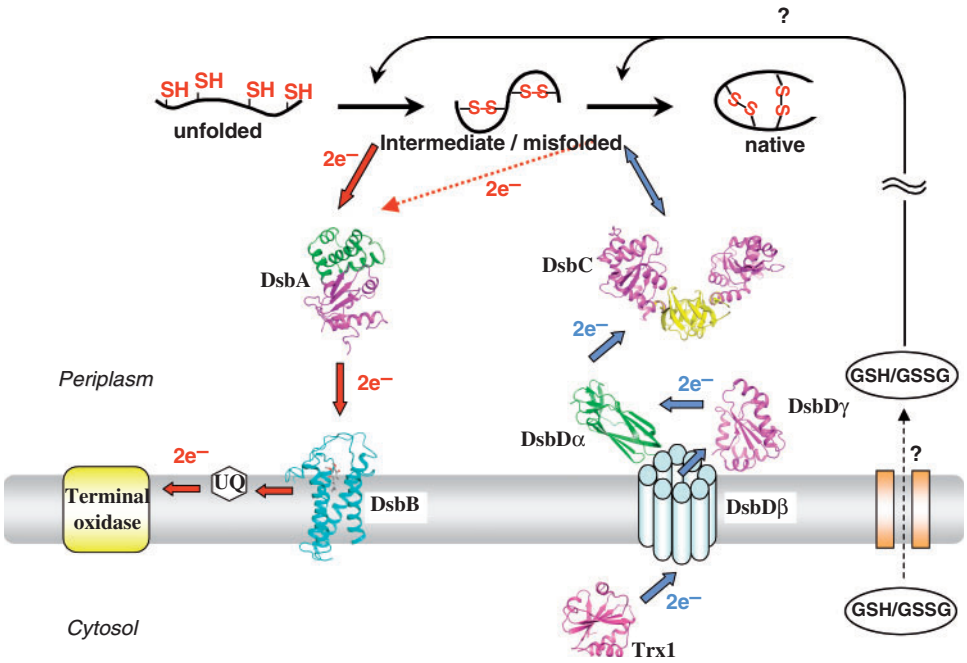

Fig. 2. **Electron flow through the disulfide-introducing (red) and disulfide-isomerizing (blue) pathways in *E. coli*.** dsb enzymes involved in the oxidative system are shown as ribbon representations. The only unknown structural component is the transmembrane domain of DsbD (DsbDβ). The thiredoxin domains of the dsb enzymes are shown in magenta. PDB ID codes correspond to DsbA (1DSB), DsbB (2ZUQ), DsbC (1EFJ), DsbDα (1JPE) and DsbDγ (2FWG). Oxidized and reduced glutathione possibly contributes to oxidative protein folding, either directly or indirectly.

It appears likely that DsbDβ has an hourglass-like structure with an inverted symmetry, in which the C-terminal sides of Cys163 and Cys285 are cytoplasmic-proximal and periplasmic-proximal, respectively, to allow DsbDβ to communicate with both cytosolic thioredoxins and periplasmic DsbDγ (Fig. 3). One expects that there are dynamic conformational changes in DsbDβ that accompany changes in its redox state. However, recent results of cysteine-scanning mutagenesis reported by Cho and Beckwith (*49*) demonstrated that the accessibility of single-Cys replacements in TM1 and TM4 of DsbDβ to alkylation agents is largely unchanged upon reduction of the Cys163–Cys285 pair, which indicates the conformation of this domain is rather static between the oxidized and reduced forms. Nevertheless, significant structure-based switching mechanisms may be in place to open up the channel, alternatively allowing a direct interaction between oxidized DsbDβ and cytoplasmic thioredoxin (Trx1), and between reduced DsbDβ and periplasmic DsbDγ. High resolution structures of the Trx1-DsbDβ(Cys163) and DsbDβ(Cys285)–DsbDγ complexes are eagerly awaited to clarify this critical issue.

INVOLVEMENT OF GLUTATHIONE IN OXIDATIVE PROTEIN FOLDING IN *E. Coli*

Glutathione (GSH) is a ubiquitous compound that serves as the main redox buffer in the cell (*50*). GSH is synthesized in the cytosol and maintains this compartment as a reducing environment. The presence of GSH in the periplasm and its functional role in oxidative protein folding are as yet unclear. Recently, Eser *et al*. (*51*) showed that glutaredoxins exported to the periplasm can catalyse dsb formation in a *dsbA*$^-$ strain, and that mutations in the GSH biosynthesis pathway abolish this oxidative activity. The source of oxidizing power for glutaredoxin-mediated dsb formation is most likely oxidized glutathione, GSSG, which, in turn, suggests the presence of GSH in the *E. coli* periplasm. However, mutation of the ATP-binding cassette-type transporter CydDC, which mediates GSH transport across the *E. coli* cytoplasmic membrane (*52*), does not affect the ability of exported glutaredoxins to promote dsb formation in the periplasm. Thus, the real physiological role of GSH in oxidative protein folding in the periplasm is not clear. DsbA can function as an oxidase independently of GSH, and DsbB oxidizes GSH poorly. In addition, extracellular GSH can diffuse into the periplasm through outer membrane porins. Thus, the significance of the GSH/GSSG ratio in redox regulation in the periplasm remains an essential issue that requires further investigation.

DISULFIDE BOND FORMATION IN THE CYTOSOL

The cytoplasm of most organisms is a highly reducing environment, in which protein cysteines are maintained in thiol/thiolate form. However, dsb formation of cytosolic proteins is encountered in cells infected with large double-stranded DNA viruses, leading to the proper assembly and maturation of the viruses (*53*). Some thermophiles also seem equipped with a disulfide-introducing system to increase the thermodynamic stability of intracellular proteins (*54*). Several protein disulfide oxidoreductases (PDO) unique to the disulfide-rich thermophiles

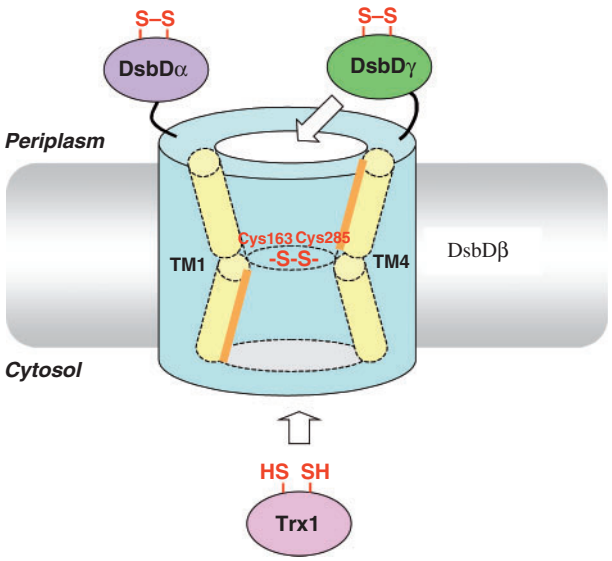

Fig. 3. **Structure model of the transmembrane domain of DsbD (DsbDβ).** The model shows the putative hourglass-like architecture of DsbDβ, in which the redox active site, a Cys163 and Cys285 pair, resides at the site that can be accessed by Trx1 present in the cytosol and DsbDγ present in the periplasm. Bold orange lines represent the water-exposed C-terminal halves of TM1 and TM4. This figure is originated from Cho *et al.* (*48*) and shown with slight modification.

have been identified, among which, the *Py. furiosus* PDO has been characterized structurally and biochemically (*55*). Although the specific roles of PDO family proteins and the source of their oxidative/reductive power has not been fully elucidated, it is possible, by analogy with the eukaryotic protein disulfide isomerase in the endoplasmic reticulum (ER), that they have dual functions in the cytoplasm, as oxidases and isomerases.

Even in mesophilic eubacteria, some cytosolic proteins undergo dsb formation, particularly when they are subjected to oxidative stress (*56*) caused by reactive oxygen species (ROS) generated in the cell. To counteract oxidative stress, organisms activate numerous pathways that detoxify ROS and repair cellular damage. For example, several transcription factors and molecular chaperones are activated through the formation of intra or intermolecular dsbs. One such protein, OxyR, is a redox-sensing transcription factor that activates a regulon of more than 20 antioxidant genes. The DNA-binding activity of OxyR is tightly regulated by the redox state of its essential cysteines (*57*). However, the mechanism of regulation of OxyR activity *in vivo* remains unclear. There is some evidence that the formation of a Cys199–Cys208 disulfide is necessary and sufficient for the activation of OxyR (*57*), but it has also been suggested that activity is controlled solely through a variety of oxidative modifications to Cys199 (*58*).

Hsp33 is a redox switch protein that functions as a molecular chaperone, depending on the oxidative state of its essential cysteines (*59*). Of note, the chaperone activity of Hsp33 is dramatically decreased by DTT, and can be fully restored by oxidized GSH or hydrogen peroxide. In its active, oxidized form, Hsp33 contains two dsbs, Cys232–Cys234 and Cys265–Cys268, whereas in the inactive, reduced form, these four cysteine residues coordinate a tightly associated zinc ion (*60*). Thus, dsb formation is transduced into biological effects through induced conformational changes in regulatory proteins or enzymes in order to maintain cellular redox homeostasis.

CONCLUDING REMARKS

The molecular details of the dsb formation/isomerization system of *E. coli* have recently been clarified by genetic, biochemical and structural studies. It is remarkable that each dsb enzyme possesses unique structural and redox features that collectively ensure the physiological flow of electrons. Strikingly, the functions of two cytoplasmic membrane proteins, DsbB and DsbD, seem to involve well-designed conformational changes in response to binding to their cognate binding partner, facilitating the transfer of electrons unidirectionally along specified pathways. In this context, high-resolution structures of the transmembrane region of DsbD, the sole unsolved structural domain among the Dsb enzymes of *E. coli*, will be required for more precise descriptions of DsbD-mediated electron transport. The accumulated fundamental knowledge of the Dsb system in *E. coli* to date can be applied to the study of the more complicated dsb formation system in the ER of eukaryotic cells, in which more redox enzymes are involved and their activities are tightly regulated according to the redox environment. We expect that further advances in the field of thiol-based redox biology will shed more light on the molecular mechanisms of 'protein quality control in the cell', one of the most exciting issues in present-day molecular cell biology.

CONFLICT OF INTEREST

None declared.

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