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Biochimica et Biophysica Acta 1783 (2008) 520–529

Biochimica et Biophysica Acta BBA

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Review

Structure and mechanisms of the DsbB–DsbA disulfide bond generation machine

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Received 30 September 2007; received in revised form 9 November 2007; accepted 13 November 2007
Available online 26 November 2007

Abstract

All organisms possess specific cellular machinery that introduces disulfide bonds into proteins newly synthesized and transported out of the cytosol. In *E. coli*, the membrane-integrated DsbB protein cooperates with ubiquinone to generate a disulfide bond, which is transferred to DsbA, a periplasmic dithiol oxido-reductase that serves as the direct disulfide bond donor to proteins folding oxidatively in this compartment. Despite the extensive accumulation of knowledge on this oxidation system, molecular details of the DsbB reaction mechanisms had been controversial due partly to the lack of structural information until our recent determination of the crystal structure of a DsbA–DsbB–ubiquinone complex. In this review we discuss the structural and chemical nature of reaction intermediates in the DsbB catalysis and the illuminated molecular mechanisms that account for the de novo formation of a disulfide bond and its donation to DsbA. It is suggested that DsbB gains the ability to oxidize its specific substrate, DsbA, having very high redox potential, by undergoing a DsbA-induced rearrangement of cysteine residues. One of the DsbB cysteines that are now reduced then interacts with ubiquinone to form a charge transfer complex, leading to the regeneration of a disulfide at the DsbB active site, and the cycle can begin anew.

Keywords: Disulfide bond generation; Membrane-bound oxidase; DsbB; Ubiquinone; Menaquinone; DsbA; Crystal structure

1. Introduction

A disulfide bond in a protein is formed by the oxidation of two cysteine residues in close mutual proximity in the three-dimensional structure. This simple chemical reaction can proceed spontaneously by air oxidation and without involvement of any enzymatic catalyst, as demonstrated by the classical experiment of Anfinsen et al. [1]. However, uncatalyzed in vitro oxidation proceeds slowly and does not necessarily occur between the correct combinations of cysteines, leaving a question of whether spontaneous reactions really account for the efficient folding processes expected to occur in vivo. In fact, the group of Anfinsen itself described an activity of an enzyme, protein disulfide isomerase (PDI), that accelerates the in vitro formation and isomerization of protein disulfide bonds [2]. Clear evidence for the notion that disulfide bond formation must be catalyzed in vivo [3] was provided about three decades later by genetic studies of *E. coli*, showing that mutations in a gene *dsbA* result in pleiotropic defects in the formation of disulfide bonds in cell envelope proteins [4,5].

Now it is established that DsbA is the primary disulfide bond donor in the periplasmic space (Fig. 1). DsbA has a thioredoxin domain and an α-helical domain insertion [6], the former of which contains the redox active-site sequence, Cys30–Pro31–His32–Cys33, which has the highest redox potential value (~−120 mV) among all the known thiol–disulfide oxido-reductases [7,8]. Cys30 in the active site has a low pK_a value (~3.5) and is hyper-reactive [9]. While the strongly oxidizing property of DsbA qualifies its role as an efficient disulfide-introducer, its catalytic function can only be assured by a

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doi:10.1016/j.bbamcr.2007.11.006

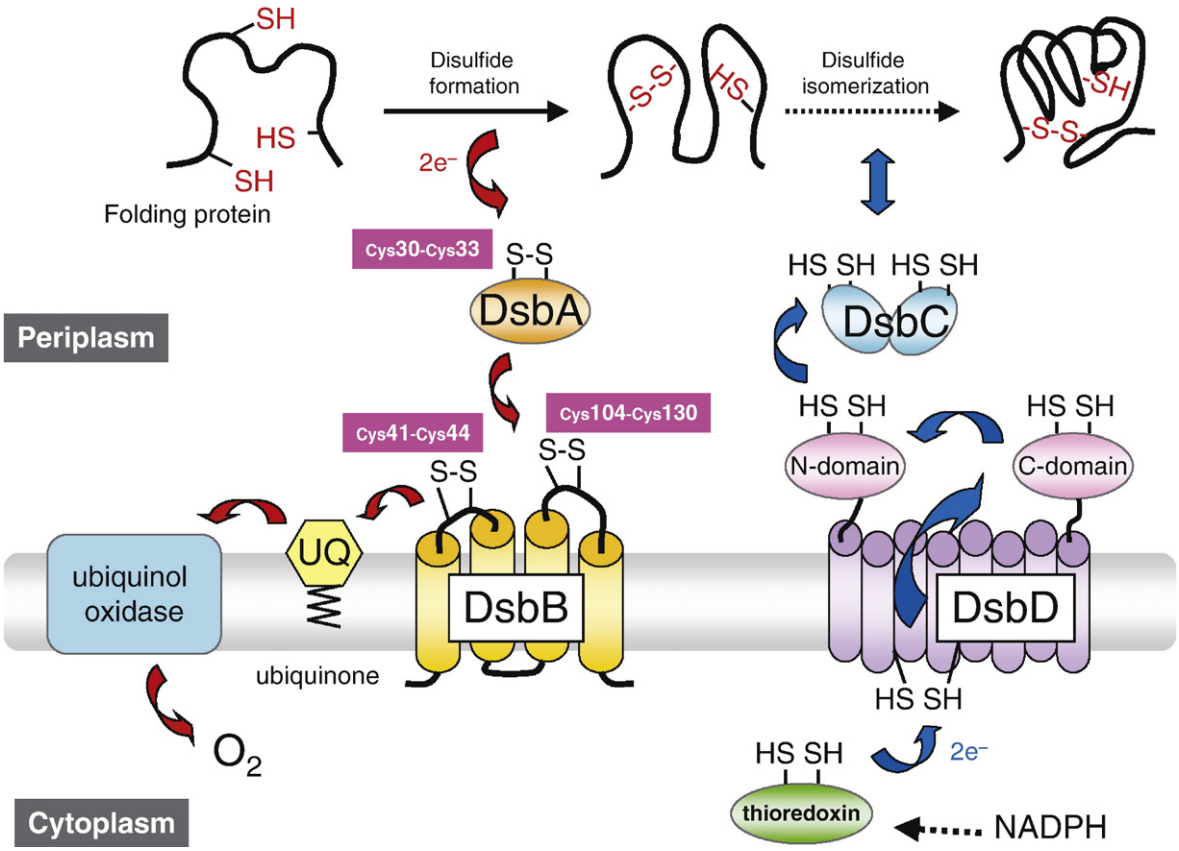

Fig. 1. The major pathway of disulfide bond acquisition for *E. coli* envelope proteins. A disulfide bond is introduced by DsbA and is then isomerized by DsbC to the one between the correct cysteine combination, as required. Red and blue arrows indicate the flow of electrons in the DsbB–DsbA oxidative pathway and the DsbD–DsbC reductive pathway, respectively.

mechanism that keeps this reduction-prone enzyme [10] in the oxidized, active state. This DsbA-activating function is fulfilled by DsbB, which oxidizes the Cys30 and the Cys33 active-site residues of DsbA as they become reduced upon substrate oxidation (Fig. 1) [11,12]. DsbB is integrated into the cytoplasmic membrane by its four transmembrane segments (TM1–TM4). Each of its two periplasmic regions (P1 and P2) contains a pair of cysteines, Cys41 and Cys44 in P1 and Cys104 and Cys130 in P2, that are essential for the functioning of DsbB [13]. Oxidation of DsbA by DsbB in vivo and in vitro requires a respiratory quinone component [14,15]; DsbB in the cell is associated with either ubiquinone (UQ)-8 or menaquinone (MK)-8 [16]. UQ and MK are thought to provide an oxidizing equivalent to the DsbB–DsbA system under aerobic and anaerobic conditions, respectively [15] by oxidizing the Cys41 and the Cys44 residues of DsbB [16–18]. The DsbB–quinone complex can be regarded as the disulfide-generating machine of the *E. coli* cell, which forms the Cys41–Cys44 disulfide bond de novo as the primary disulfide that is donated to DsbA via the Cys104–Cys130 pair of DsbB and then to a wide variety of cellular proteins.

Although the reaction mechanisms of DsbB were investigated extensively in recent years [18–25], these studies have revealed a number of discrepancies regarding the molecular details of the DsbB-mediated disulfide bond generation and oxidation of DsbA, as we summarized previously [24]. One central question on the DsbB–DsbA oxidation system is by what mechanism DsbB is able to oxidize DsbA so selectively and efficiently despite the fact that DsbA itself is an extremely oxidizing protein. The simplest notion would be that quinones directly drive the oxidation cascade [18], as their standard redox potentials, around +110 mV for UQ and −74 mV for MK, are sufficiently higher than that of DsbA. However, our experiments using a completely quinone-free preparation of DsbB revealed that DsbB can oxidize ~0.4 M stoichiometry of reduced DsbA even in the absence of any quinone molecule [24]. Does this mean that the redox potential of DsbB is higher than that of DsbA? Our measurements of the redox potentials of the quinone-free DsbB, using low-molecular weight redox buffers (cysteine/cystine or reduced/oxidized dithiothreitol), yielded values for Cys41–Cys44 and Cys104–Cys130 that were far below the similarly determined redox potential of DsbA [20,24]. Thus, the reaction looks, at least superficially, to be energetically unfavorable. It appears that DsbB is designed such that it somehow gains the ability to oxidize DsbA upon interaction with this specific substrate [19,24].

Another important issue is the mechanism responsible for the generation of the Cys41–Cys44 disulfide bond of DsbB through its interplay with UQ/MK. Unlike the quinone-mediated oxidation of small molecule dithiol compounds, which proceeds

very slowly, that of the DsbB Cys41–Cys44 pair is specific, rapid, and robust [17,18]. The observations that DsbB-bound UQ and MK undergo spectral transitions in a manner dependent on the appearance of a thiolate anion form of Cys44 [16,22] should be taken into account to formulate a scheme of disulfide bond generation by the DsbB–quinone conjugate (see Section 5 for detail).

To achieve a deeper understanding of the above-mentioned puzzles in the DsbB–DsbA system, structural information on DsbB is essential. We have succeeded in determining the long-awaited three-dimensional structure of DsbB in the state of a complex with DsbA and endogenous UQ [26]. In this review, we discuss the structural features of this complex and their bearings on the molecular mechanisms of DsbA oxidation and de novo disulfide bond formation.

2. Crystal structure of the DsbA–DsbB–ubiquinone ternary complex

2.1. Crystallization and model building

DsbB is largely membrane-embedded, having only small hydrophilic regions that are exposed to the aqueous milieu. To overcome the expected and realized difficulty in its crystallographic analysis, we used a disulfide-tethered complex of DsbB with DsbA, which would provide hydrophilic surfaces necessary for the crystal packing. This strategy had additional advantages in that the structure of DsbA had already been established [6], and the complex was likely mimicking an intermediate state of the physiological reaction [27,28]. A complex between DsbA(Cys33Ala) and DsbB(Cys130Ser) was held together with an intermolecular disulfide between Cys30 (DsbA) and Cys104 (DsbB), in which cleavage of the intermolecular disulfide was prevented by the removal of not only Cys33 of DsbA but also Cys130 of DsbB. This construct proved to crystallize to give better X-ray diffraction than complexes having other amino acid substitutions (such as Cys33Ser in DsbA) that we tested.

The crystals of DsbA(Cys33Ala)–DsbB(Cys130Ser) yielded a diffraction data set to ~3.7 Å resolution, insufficient to reveal side chains unequivocally. In addition, a part of the periplasmic loop of DsbB was devoid of electron density presumably due to its mobility. The approach we used to build up an overall structure of DsbB from such low-resolution diffraction data was called the “methionine-marking method”. In this approach, we introduce or remove a methionine by mutation. Crystallization of a selenomethionine version of the mutant reveals the position of the mutation site by the anomalous scattering signal of selenium. This method, though somewhat time- and labor-consuming, gave informative assignment in most cases, as the crystallizing conditions that had been worked out for the wild-type protein were mostly applicable to the mutants and the selenium signals could be identified even in data of relatively low (~6.0 Å) resolution. We suggest that the methionine-marking method may have general applicability to trace the peptide backbone in electron density maps of limited resolutions or quality.

2.2. Structural features

The solved structure of DsbB contains four transmembrane helices (TM1−TM4) arranged into a four-helix bundle configuration (Fig. 2a). The second periplasmic loop has an additional short helix running parallel to the membrane and probably interacting peripherally with the lipid bilayer (Fig. 2a). The segment flanked by the horizontal helix and TM4 is devoid of electron density, suggesting that this region has some mobile or unstructured property. A UQ molecule endogenously bound to DsbB is seen around the N-terminal part of TM2, in the interior of the four-helix bundle scaffold (see Section 5).

The structure of the DsbA portion in the complex is almost identical, within the resolution limit of the present analysis, to the structures previously determined for the reduced and oxidized DsbA protomers [29], indicating that DsbA does not undergo gross conformational changes upon interaction with DsbB. DsbA has a long and deep hydrophobic groove and a wide hydrophobic patch flanking the redox active site. While the hydrophobic groove and the hydrophobic patch of DsbA are assumed to play roles in binding unfolded substrate proteins [6], a recent study with thioredoxin shows a mode of substrate-recognition by a thioredoxin domain [30]. Remarkably, our structure reveals that a Pro100–Cys104–Phe106 portion of the second periplasmic loop of DsbB is accommodated in the hydrophobic groove of DsbA (Fig. 2b), in which Cys104 of DsbB is forming an intermolecular disulfide bond with Cys30 of DsbA. Whereas the surface of the DsbA groove is highly hydrophobic [6], the DsbB segment captured there is not particularly enriched with hydrophobic residues. The mechanism and specificity of the DsbA–DsbB recognition and its relationship to the more promiscuous substrate-recognition by DsbA are left for future studies.

3. Reaction mechanism of DsbB-mediated DsbA oxidation

If the redox potential of isolated DsbB, as determined from its equilibrium with low-molecular-weight redox compounds, were absolute and unchangeable, then the ability of quinone-free DsbB to oxidize DsbA significantly could not be explained in terms of the thermodynamic principle. The crystal structure of the DsbB–DsbA complex has revealed that DsbB actually undergoes a structural transition when it forms the disulfide-linked complex with DsbA, thus providing an excellent structural basis to resolve the above-mentioned apparent paradox [26]. As discussed in the preceding section, the region around Cys104 of DsbB is drawn into the DsbA groove near the active site. Our assignment of the position of residue 130 indicates that it is located in the proximity of the Cys41–Cys44 region in the complex. Thus, the distance between Cys104 and Cys130 is far longer than the disulfide bond-forming range. Although only limited information is available about the structure of DsbB in isolation [31], it is well established that the resting state of DsbB is completely oxidized, containing both the Cys41–Cys44 and the Cys104–Cys130 disulfide bonds [17,24]. The different Cys104–Cys130 distances in resting DsbB vs. in the DsbB–DsbA complex indicate that the

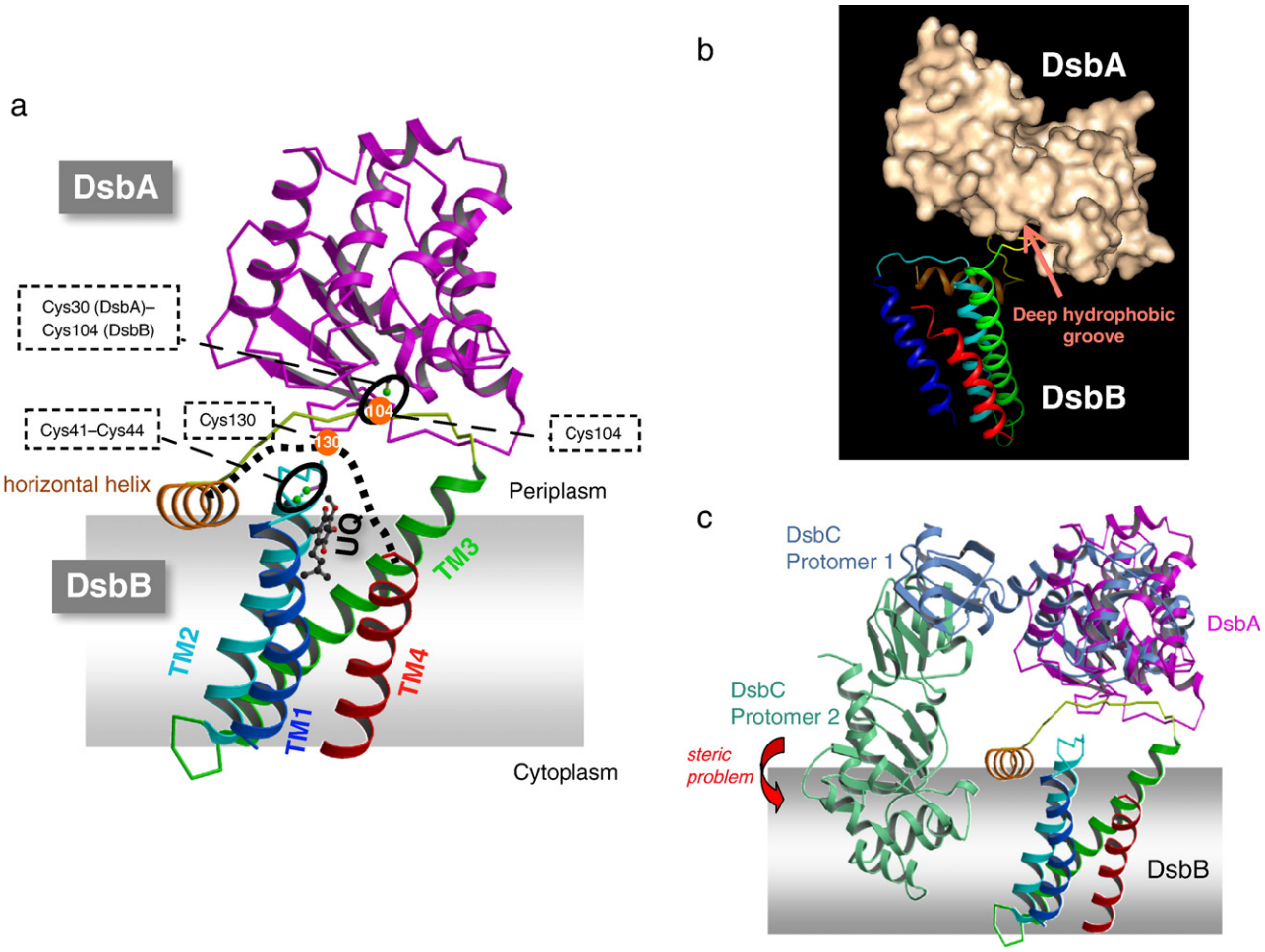

Fig. 2. Crystal structure of the DsbA–DsbB–UQ complex. The back born arrangement is shown in (a), in which UQ molecule bound to DsbB is shown by ball-and-stick representation. The dotted line indicates a mobile loop of DsbB that connects the horizontal helix and TM4. To show the DsbA–DsbB contact clearly, another view is shown in (b), in which the molecules are rotated by 90° around a vertical axis and DsbA is shown by space-filling representation. The extended DsbB segment from Pro100 to Phe106 is drawn into the deep hydrophobic groove of DsbA, where the Cys30(DsbA)–Cys104(DsbB) intermolecular disulfide bond is accommodated. (c) A hypothetical DsbB–DsbC complex, in which the thioredoxin domain of a DsbC protomer (protomer 1, sky blue) is superimposed on that of DsbA (magenta) such that the rmsd between the backbones of these two thioredoxin domains is minimized.

complex formation is accompanied by a cysteine-relocating conformational change in DsbB [26]. The separation of Cys104 and Cys130 is likely brought about by the accommodation of the Cys104-containing DsbB peptide into the deep groove of DsbA (Fig. 2b). It is also noted that the bulky side chain of Met64 of DsbA intervenes between these two DsbB cysteines. In addition, Cys130 may have moved toward the short P1 loop.

What does the DsbA-induced cysteine relocation in DsbB contribute to the physiological reaction? Our detailed analyses of the DsbB-mediated DsbA oxidation reactions in vitro revealed the occurrence of two pathways, rapid and slow [16,22,24]. We believe that the whole reaction is initiated by a nucleophilic attack on the Cys104–Cys130 disulfide of DsbB by the hyper-reactive Cys30 residue of DsbA (Fig. 3). It is conceivable, in view of the large redox potential difference between these two individual proteins, that some conformational change has taken place in DsbB upon its encounter with DsbA. Such a conformational change might well occur before the actual formation of the intermolecular disulfide bond. After its formation, the reaction pathway diverges. If the Cys30–Cys104 intermolecular disulfide is attacked by Cys33 of DsbA, the reaction is committed to the rapid pathway, in which DsbA is immediately oxidized with concomitant reduction of Cys104 and Cys130. This is then followed by rapid disulfide–dithiol exchanges between the 104–130 and the 41–44 cysteine pairs within DsbB. Our results show that this rapid pathway can proceed up to this point even in the absence of any quinone molecules [24]. In the normal reaction, quinone rapidly oxidizes the 41–44 cysteine pair, resulting in the oxidation of Cys104 and Cys130 as well, due to the equilibration between the two hemi-oxidized states. Thus, the role of quinone in the rapid pathway is to recycle DsbB back to the fully oxidized state, enabling its catalytic turnover.

While the rapid pathway predominates, a minor fraction enters the slow pathway, which is triggered by a Cys130 attacking the 41–44 cysteine pair in the same DsbB molecule. This results in the formation of a version of the DsbA–DsbB complex that was first proposed by Kadokura and Beckwith to be the physiological intermediate of the system [19]; it contains the Cys30(DsbA)–Cys104(DsbB) intermolecular disulfide as

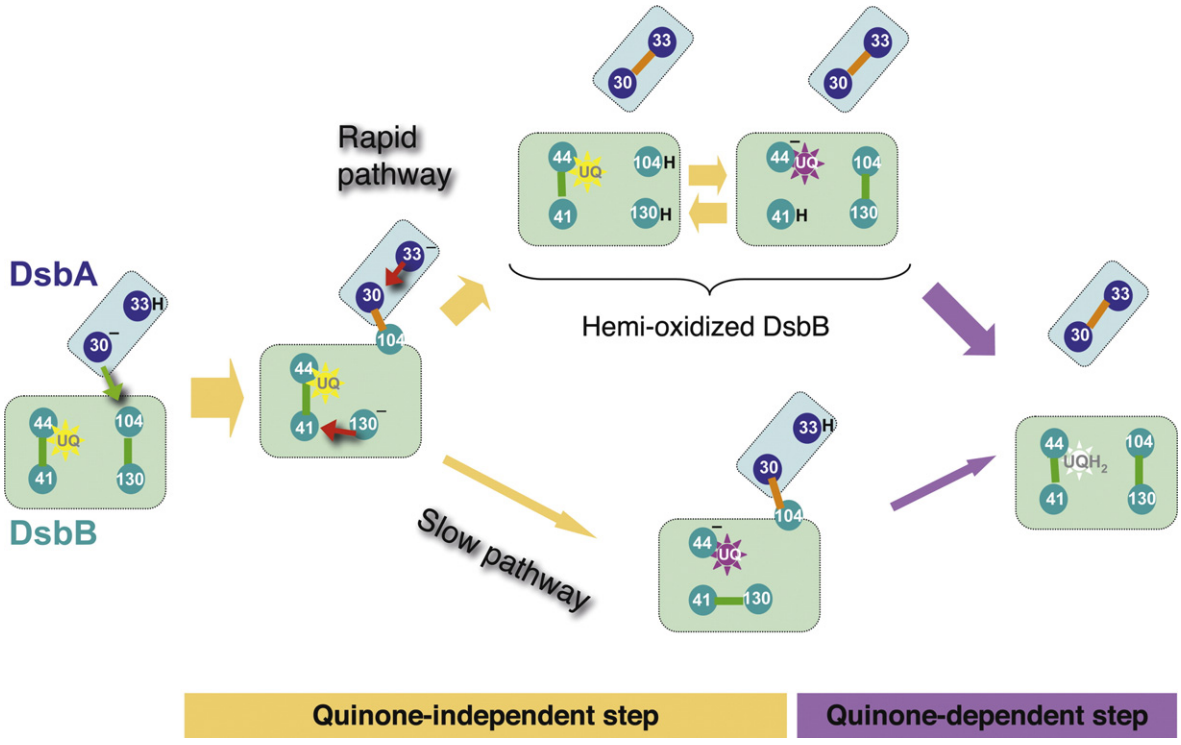

Fig. 3. DsbB-catalyzed DsbA oxidation proceeds through two alternative pathways. The rapid pathway proceeds through direct dithiol–disulfide exchanges between the active-site cysteines. The slow pathway proceeds through resolution of the disulfide-linked DsbA–DsbB complex having the rearranged Cys130–Cys41 disulfide. The red arrows indicate nucleophilic attacks that initiate respective pathways. The reaction can proceed up to the steps indicated by yellow arrows even without the aid of quinone, whereas those indicated by purple arrows require quinone. The end products of the quinone-coupled physiological reactions are the oxidized forms of both DsbA and DsbB. Note that the DsbB-bound quinone forms a charge transfer complex with the Cys44 thiolate during the course of both pathways (see the text for details).

well as the rearranged Cys130–Cys41 intramolecular disulfide. This intermediate is resolved slowly by quinone-driven and concerted reactions, in which fully oxidized species of both DsbA and DsbB are produced.

We envisage that the distance between Cys104 and Cys130 is a crucial element that determines whether the reaction can proceed in the forward direction or not. As the crystal structure of the DsbA–DsbB complex has revealed, Cys130 in the complex is separated from Cys104 such that it cannot attack Cys104, which is now engaged in the intermolecular disulfide bond. In this way, the backward resolution of the complex to reverse the reaction can be prevented. The consequent prolonged existence of the DsbA–DsbB complex should be a prerequisite for the next event to occur: initiation of the rapid pathway by Cys33 attacking Cys30 in DsbA or initiation of the slow pathway by Cys130 attacking Cys41 in DsbB (Fig. 3, red arrows). Whereas DsbA is immediately oxidized in the rapid pathway, Cys130 is further "sequestered" in the form of Cys130–Cys41 disulfide in the slow pathway until resolved by the quinone-driven resolution. It is conceivable that the slow pathway functions as a fail-safe mechanism to prevent a reverse electron flow [24].

These considerations lead to a notion that, upon interaction with DsbA, DsbB must change its effective redox potential to a value at least comparable with the redox potential of DsbA. The redox potential values estimated on the basis of the DsbB's reactivities with small molecule redox compounds do not account for the specific reactivity that DsbB exhibits against its substrate, DsbA. Specific interaction between these two proteins leads to a conformational change in DsbB and modulation of their redox equilibrium in such a way to enable unidirectional electron flow from DsbA to DsbB. Thus, DsbB is designed as a strong and selective oxidant against DsbA but not other proteins.

4. Structural basis that precludes oxidation of DsbC by DsbB

While DsbA appears to introduce disulfide bonds into newly translated and translocated polypeptides quite rapidly in vivo, this oxidation system must cooperate with the disulfide isomerization system to achieve the native disulfide proteome of the cell. DsbC is the major disulfide isomerase in *E. coli*, which must be kept reduced by the membrane-integrated DsbD protein in conjunction with the thioredoxin-based reducing system in the cytosol [3,32,33] (see Fig. 1). DsbC must not be oxidized by DsbB. A structural feature of DsbC that distinguishes it from DsbA, a monomeric enzyme, is that DsbC forms a V-shaped homo-dimer [34]. Superimposition of the thioredoxin domain of one of the DsbC protomer onto the DsbB–DsbA complex in a way to minimize RMSD results in a clash of the other protomer with the membrane surface (Fig. 2c). Our modeling, taken together with the result of Bader et al. [35] that a monomerized mutant of DsbC can substitute for DsbA,

suggests that the above-mentioned steric problem prevents the dimeric, wild-type DsbC from interacting with the membrane-integrated DsbB enzyme. On this structural basis the DsbA–DsbB oxidative pathway and the DsbC–DsbD reductive pathway can coexist in the same cellular compartment without undergoing significant crosstalk. Co-evolution of the DsbB and DsbD pathways [36] might have been the nature's strategy to achieve this structural elaboration.

5. Molecular mechanism of disulfide bond generation on DsbB

UQ-binding site on DsbB had not been determined unequivocally until our crystallographic studies. The electron density map of the DsbA–DsbB–UQ ternary complex revealed a disk-like electron density near the N-terminal end of TM2, which we have concluded to represent the head group of endogenous UQ8 [26]; the isoprenoid chain is invisible at this resolution (Fig. 4a). This assignment is based on the difference Fourier map between the data from UQ8-containing and the UQ-free crystals (Fig. 4b). The UQ ring is in the vicinity of the Cys41–Cys44 disulfide, the product of oxidation by UQ.

As touched upon briefly in the Introduction, the DsbB-bound quinones assume anomalous electronic states that exhibit a pink color (λ_{max} = ~510 nm) in the case of UQ and a violet color (λ_{max} = ~550 nm) in the case of MK [16,22]. The spectral transitions occur under a variety of conditions. First, they occur transiently during both the rapid and the slow reaction pathways of DsbA oxidation. Second, they occur constitutively for DsbB variants in which Cys44 is unpaired. The latter situations include the complex between DsbB and a DsbA variant lacking Cys33, in which the rearranged Cys130–Cys41 disulfide is formed at the expense of the Cys44 reduction (see the preceding section). The pH-dependence of the transition suggests the involvement of a deprotonated thiolate form of Cys44 [22]. Titration of a ubiquinone derivative, UQ1, to the complex between quinone-free DsbB and DsbA (Cys33Ser) indicates that the spectral transition is saturable at a 1:1 UQ-to-DsbB molar ratio. In view of the critical role played by the Cys44 thiolate and the involvement of a single UQ molecule, it is

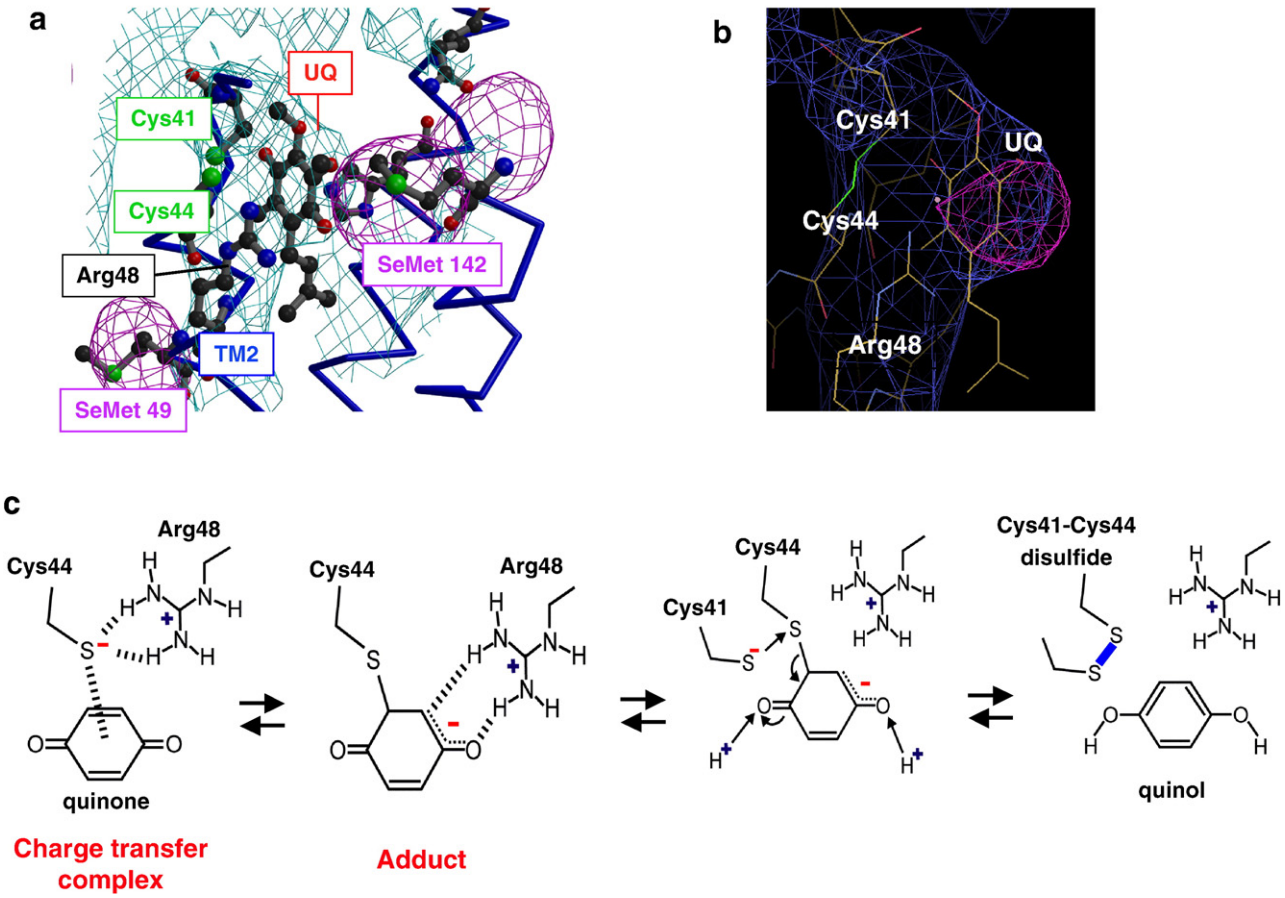

Fig. 4. The disulfide-generating reaction center of DsbB. (a) The electron density map near the N-terminus of TM2, revealing the head group of UQ, the Cys41–Cys44 disulfide and Arg48. For the sake of clarity, ribbons and sticks are placed to show these residues on the basis of the reference selenium signal from SeMet49. (b) Difference Fourier map calculated from the diffraction data of the UQ-bound and UQ-free crystals. The electron density difference, shown in magenta, represents the density assignable as that of UQ. (c) A chemical scheme of disulfide bond generation on DsbB. The Cys44 residue is predicted to form a charge transfer complex as well as its adduct form with UQ, which are stabilized through electrostatic interaction with Arg48. The S–C covalent linkage of the Cys44–quinone adduct is then subject to a nucleophilic attack by Cys41, resulting in the generation of the Cys41–Cys44 disulfide bond. The oxidative power of quinone is thus transformed into a protein disulfide bond at the quinone-coordinating reaction center of DsbB shown in (a).

unlikely that the spectral transition is accounted for by a quinhydrone-type stack of two quinone rings that was reported to occur in isolated wild-type DsbB [23]; the same research group now reports that the UQ spectral change involves multiple steps, one depending directly on DsbB's interaction with DsbA and the other representing following processes occurring within the DsbB molecule [37].

According to our quantum chemistry simulation, the spectral anomaly of UQ likely represents a charge transfer complex between UQ and the Cys44 thiolate, which is stabilized by a positively charged guanidinium group of a highly conserved arginine residue, Arg48 [25]. The functional importance of this charge transfer phenomenon is corroborated by genetic studies showing that Arg48 is indeed important for the UQ transition [25] as well as for the full ability of DsbB to bind UQ [38]. Also, the normal arrangement of the Cys44–Arg48 interval is important for the spectral change of UQ [22,25] as well as for the in vivo quinone-coupling of DsbB [39]. The theoretical work further suggests that a covalent adduct can be formed between UQ and Cys44, which is accompanied by a new electrostatic interaction with Arg48, and that the covalent Cys44–UQ bond induces and receives a nucleophilic attack by Cys41, thus generating the Cys41–Cys44 disulfide bond (Fig. 4c). In the slow pathway reaction, the Cys44–UQ adduct may induce more complex processes of oxidative disulfide rearrangements that propagate to the DsbA cysteines, details of which are left for future studies.

The above scheme of disulfide bond generation is in excellent agreement with the DsbB–UQ structural arrangement revealed by the crystallographic study. The UQ-interacting residues, Cys41, Cys44, and Arg48, of DsbB are all lined up on the same side of TM2 as the head group of UQ (Fig. 4a). This geometry would allow Cys44 to engage in the charge transfer interaction with UQ through the aid of Arg48. This area, where these pivotal elements meet, can be regarded as the reaction center for the generation of disulfide bonds. Although a single-round molecular event of quinone-independent disulfide transfer from DsbB to DsbA is possible as we have already discussed, the steady state flow of disulfides should originate from the Cys41–Cys44 disulfide that is generated in the reaction center. This disulfide is first donated to DsbA via the Cys104–Cys130 disulfide and then relayed further onto diverse protein species.

6. Mechanisms of disulfide bond generation in eukaryotic cells

In eukaryotic cells, disulfide bonds are introduced primarily in the endoplasmic reticulum (ER), where several oxido-reductases reside [40–42]. They include PDI, a soluble protein having two thioredoxin domains [43], and Ero1p, a membrane-associated and FAD-containing oxidase acting on PDI [44–47]. Ero1p homologs exist in all eukaryotes including plants [48]. Erv2p is a yeast protein belonging to a distinct protein family [49] with an Ero1-like function [46]. Thus, an electron flow path seems to operate in the order of folding protein PDI–Ero1p/Erv2p–FAD, in which electrons may be accepted ultimately by molecular oxygen [50] or other electron acceptors [51]. Sometimes, these processes are accompanied by the production of hydrogen peroxide and, hence, oxidative stresses [51,52]. Ero1p and Erv2p can be regarded as functional homologues of DsbB in that they convert oxidizing equivalents stored in a respiration-related compound to protein disulfides, which are to be donated further to another client-oriented oxido-reductase. However they do differ from DsbB in amino acid sequences, in the mode of membrane association (peripheral vs. integral) and the nature of the cofactors (soluble vs. membrane-embedded).

The crystal structures of Ero1p and Erv2p [53,54] led Sevier et al. [55,56] to predict that the core catalytic domains of these enzymes have structural arrangements that are similar to that of DsbB. That this is indeed the case has been shown by the crystal structure of DsbB in complex with DsbA [26]. Thus, they all possess a four-helix bundle scaffold, within which a redox cofactor (UQ or FAD) is embraced (Fig. 5). A Cys-X-X-Cys

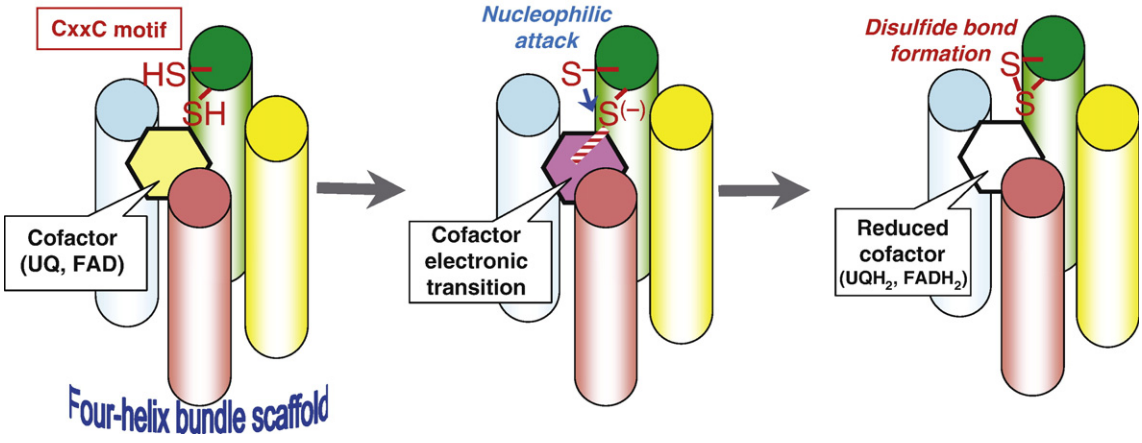

Fig. 5. Common architectures and chemical principles of the disulfide bond-generating enzymes from prokaryotes to eukaryotes. Shown is a proposed mechanism of protein disulfide bond generation operating in the core regions of the UQ- and FAD-dependent disulfide-generating enzymes, which commonly assume the four-helix bundle configuration. A CxxC motif is located at the N-terminal region of one α-helix, near which a cofactor resides and serves as an electron acceptor. Electronic transition of the cofactor takes place through its interaction with one of the cofactor–proximal cysteines, leading to the induction of the disulfide-generating nucleophilic attack by the other member of the cysteine pair.

motif is present near the cofactor, as its disulfide-generating target, whereas another pair of cysteines in an unstructured region functions dynamically to shuttle the generated disulfide from the active site to the client-oriented oxidase. Moreover, this "shuttle disulfide" might serve as a substrate selectivity filter, blocking the access of cellular proteins or small redox compounds to the redox active site [57]. The oxidase activity of Ero1p is subject to redox-homeostatic regulation by still different disulfides ("regulatory disulfides"), which feed-back controls the oxidase activity of the enzyme by sensing the redox states of the ER [52].

FAD associated with Ero1p or Erv2p undergoes spectral changes during their reduction by model substrates such as thioredoxin, generating absorbance shoulders around 600 nm [49,51]. Although this phenomenon (blue semiquinone formation) could represent one-electron transfer from a thiolate anion (Cys355 in the case of Ero1p) to a nearby isoalloxazine ring of FAD, its significance to the reaction mechanism is unclear [49]. Thiolate–FAD charge transfer complexes have been described in some other FAD-linked thiol oxidases, including an egg white sulfhydryl oxidase [58] and Erv1p in the intermembrane space of mitochondria [59]. The disulfide bond on the FAD–Erv1p complex is formed in a manner coupled with the respiratory chain [60] and then donated to Mia40, which in turn facilitates import of its substrates, small Tim proteins and Cox proteins, by trapping them in mixed disulfide complexes [61]. Earlier characterizations of the chemistry of flavin–cysteine interplay in several flavoenzymes also showed that during the catalytic cycles they transiently give rise to a cysteine–FAD charge transfer complex as well as their covalent linkage involving the C (4a) of the isoalloxazine ring [62–67]. A positively charged base is commonly located in the redox active site of these enzymes, which stabilizes the thiolate anion that engages in the charge transfer and adduct-forming interaction with FAD. Thus, similarity can be found between the flavoenzymes and DsbB both in the mode of thiolate–cofactor interaction and its electrostatic stabilization (Fig. 5).

7. Concluding remarks

As we have seen here, recent years have witnessed remarkable progress in our structural and chemical understanding of the enzymes involved in protein disulfide bond formation. Now we are approaching the atomic level description of the electron flow pathways involved in the process. However, it is also true that there are a number of enigmas still awaiting clarification. For instance, what is the significance of the rapid and slow pathways that DsbB appears to follow? Are they somehow controlled according to the redox-related cellular physiology? So far, reactivities of this enzyme have only been examined in detergent solubilized states but they should also be examined in the physiological, membrane-integrated state. For instance, quinone binding to DsbB could be more specific and efficient when the reaction components are all embedded in the lipid phase. The possibility remains that this enzyme forms a complex with other membrane proteins such as quinol oxidases. Important basic knowledge still to be acquired is how reduced quinol may exchange with oxidized quinone when DsbB is fully reoxidized. Although we have determined the location of UQ on DsbB, the molecular details of DsbB–UQ interaction require higher resolution structures, including those bearing the charge transfer state of UQ.

DsbA seems to bind a wide variety of unfolded substrate proteins in a manner that its Cys30–Cys33 disulfide receives two successive nucleophilic attacks by two cysteines on the substrate. In contrast, the reduced form of DsbA recognizes specifically the Cys104 region of DsbB and pulls this DsbB region into the deep groove; Cys30 of DsbA now acts as a nucleophile to initiate reduction of DsbB. Although the hydrophobic groove of DsbA is involved in the latter recognition [26], the binding site for general substrates has not unequivocally been determined. Essential structural elements that distinguish between the two binding modes will be uncovered when structures are determined also for DsbA–substrate complexes.

One of the mechanistically most important issues is the structure of DsbB in isolation, which will reveal the actual conformational change that is induced upon complex formation with DsbA. In addition, higher resolution structures of DsbB and its complex with DsbA are eagerly awaited. Information on the side chain atoms will be beneficial to uncover the mechanism of DsbB even more precisely, to which local conformation changes around the UQ-binding region as well as the DsbA-binding region and accompanied modulations of pK_a values of key cysteine residues will contribute.

Many of the questions discussed above should also apply to the eukaryotic cysteine oxidation systems. Although not fully covered by this review, eukaryotic cells possess several different oxido-reductases in the ER. In addition to the mitochondrial Erv1, a virus-encoded Erv-1 family oxidase and its downstream factors function to generate disulfide bonds in the otherwise reducing cytosol [68]. Substrate specificities and interrelationships of these factors as well as their regulation should be clarified. Whereas even a single enzyme, PDI, exhibits multiple cellular roles [69], higher eukaryotes have multiple PDI-family members presumably having different substrate specificities and different partner proteins [70]. The function of PDI might be determined by its redox states. In this context, regulation of the Ero1p and Erv2p activities [52] would be of prime interest, along with the question of whether there are any other regulators of PDI redox states. The oxidative folding system could impart oxidative stress to the compartment [42] and its regulation should be important to maintain redox homeostasis [52]. In order to control the cellular quality, the disulfide-introducing enzymes must be in concert with the physiological and global balance, such that levels of the total protein disulfide bonds and the accompanying reactive oxygen species are set adequately. Although the disulfide metabolism is based on simple, two-electron oxidation/reduction reactions involving two thiol groups, it is intrinsically relevant to the higher order defense systems against various redox stresses [71]. We can expect much out of molecular-level disulfide biology.

Acknowledgements

We thank Satoshi Murakami, Mamoru Suzuki, Atsushi Nakagawa, Eiki Yamashita, Kengo Okada and Tomitake Tsukihara for their help and cooperation in the crystallographic studies. We are also grateful to Shigehiko Hayashi for the fruitful collaboration in the theoretical chemistry of DsbB–quinone interaction. This work was supported by PRESTO (to K. Inaba) and CREST (to K. Ito) from the Japan Science and Technology Agency and grants from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan (No. 19687005 to K. Inaba and No. 14037231 to K. Ito) and from the Special Coordination Funds for Promoting Science and Technology of MEXT (to K. Inaba).

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