

# Paradoxical redox properties of DsbB and DsbA in the protein disulfide-introducing reaction cascade

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**Protein disulfide bond formation in the bacterial periplasm is catalyzed by the Dsb enzymes in conjunction with the respiratory quinone components. Here we characterized redox properties of the redox active sites in DsbB to gain further insights into the catalytic mechanisms of DsbA oxidation. The standard redox potential of DsbB was determined to be  $-0.21$  V for Cys41/Cys44 in the N-terminal periplasmic region (P1) and  $-0.25$  V for Cys104/Cys130 in the C-terminal periplasmic region (P2), while that of Cys30/Cys33 in DsbA was  $-0.12$  V. To our surprise, DsbB, an oxidant for DsbA, is intrinsically more reducing than DsbA. Ubiquinone anomalously affected the apparent redox property of the P1 domain, and mutational alterations of the P1 region significantly lowered the catalytic turnover. It is inferred that ubiquinone, a high redox potential compound, drives the electron flow by interacting with the P1 region with the Cys41/Cys44 active site. Thus, DsbB can mediate electron flow from DsbA to ubiquinone irrespective of the intrinsic redox potential of the Cys residues involved.**

**Keywords:** disulfide bond/DsbB/*Escherichia coli*/redox potential/ubiquinone

## Introduction

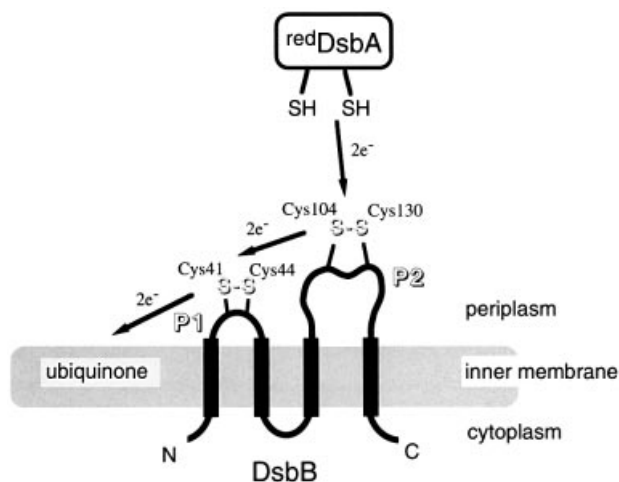
Proteins acquire disulfide bonds in compartments that are separated from the cytosol. In the bacterium *Escherichia coli*, the process of oxidative protein folding occurs in the periplasmic space, where multiple Dsb factors play essential roles (Rietsch *et al.*, 1996; Missiakas and Raina, 1997). A periplasmic enzyme DsbA directly introduces disulfide bonds into newly synthesized proteins upon their translocation across the plasma membrane (Bardwell *et al.*, 1991; Akiyama *et al.*, 1992; Kamitani *et al.*, 1992; Zapun and Creighton, 1994). A cytoplasmic membrane protein DsbB is responsible for the regeneration of oxidized and functional DsbA molecules (Bardwell *et al.*, 1993; Missiakas *et al.*, 1993; Guilhot *et al.*, 1995; Kishigami *et al.*, 1995a). DsbC, another soluble factor, isomerizes disulfide bonds until those of native combinations are established (Zapun *et al.*, 1995; Rietsch *et al.*, 1996; Sone *et al.*, 1997), whereas a membrane protein DsbD maintains the reduced state of DsbC (Rietsch *et al.*, 1997; Chung *et al.*, 2000; Katzen and Beckwith, 2000). Recent studies revealed that eukaryotic cells also possess sophisticated

mechanisms to facilitate oxidative protein folding within the lumen of the endoplasmic reticulum. Protein disulfide isomerase (PDI) is a main factor that directly donates disulfide bonds to proteins (LaMantia and Lennarz, 1993), while two ER membrane-associated proteins, Ero1p and Erv2p, reoxidize PDI to allow the redox reactions to cycle (Frand and Kaiser, 1999; Tu *et al.*, 2000; Sevier *et al.*, 2001).

Whereas these processes invariably involve thiol-disulfide redox reactions, overall oxidizing equivalents must somehow be provided to enable them to proceed. We showed that the oxidizing power for protein disulfide bond formation in *E. coli* is provided by cellular respiratory functions (Kobayashi *et al.*, 1997), and that electron transport components, such as heme and quinones, are required to keep DsbB in a strongly oxidized state (Kobayashi and Ito, 1999). In *in vitro* studies, Bader *et al.* (1999, 2000) showed that ubiquinone is required to directly activate DsbB. Under aerobic conditions, ubiquinone will oxidize DsbB, and the resulting ubiquinol molecules will then be reoxidized by the terminal cytochrome *bd* and *bo* oxidases, with coupled electron transfer to oxygen. Under anaerobic conditions, electrons may go through menaquinone to an alternate final electron acceptor.

The DsbB protein of *E. coli* has four transmembrane segments and two periplasmic regions (Jander *et al.*, 1994). Each periplasmic region contains a pair of essential cysteines; Cys41 and Cys44 in the N-terminal periplasmic loop (P1), and Cys104 and Cys130 in the C-terminal periplasmic domain (P2) (Figure 1). *In vivo* studies using mutants affected in these essential cysteines suggested that the Cys104/Cys130 disulfide bond acts directly to oxidize the Cys30 and Cys33 residues of DsbA (Guilhot *et al.*, 1995; Kishigami *et al.*, 1995b; Kishigami and Ito, 1996). This may then be followed by an intramolecular reaction, in which reduced Cys104 and Cys130 residues are oxidized by the Cys41/Cys44 disulfide bond within DsbB (Kishigami and Ito, 1996). The redox active CXXC motif in DsbB, in the form of Cys41-Val-Leu-Cys44, is oxidized strongly such that it apparently resists a reducing agent, dithiothreitol (DTT), in the membrane-integrated state. This DTT resistance is conferred by the functional respiratory components in the presence of oxygen (Kobayashi and Ito, 1999). *In vitro* assays of DsbB-dependent DsbA oxidation revealed that ubiquinone is required for DsbB to act catalytically, and that the terminal oxidases also contribute to the reaction by recycling the quinone molecules (Bader *et al.*, 1999).

In spite of the remarkable recent progress, as discussed above, a number of questions remain concerning the catalytic mechanisms of the DsbB actions. First, the mode of interaction between quinones and DsbB should be clarified beyond what has been proposed in recent studies



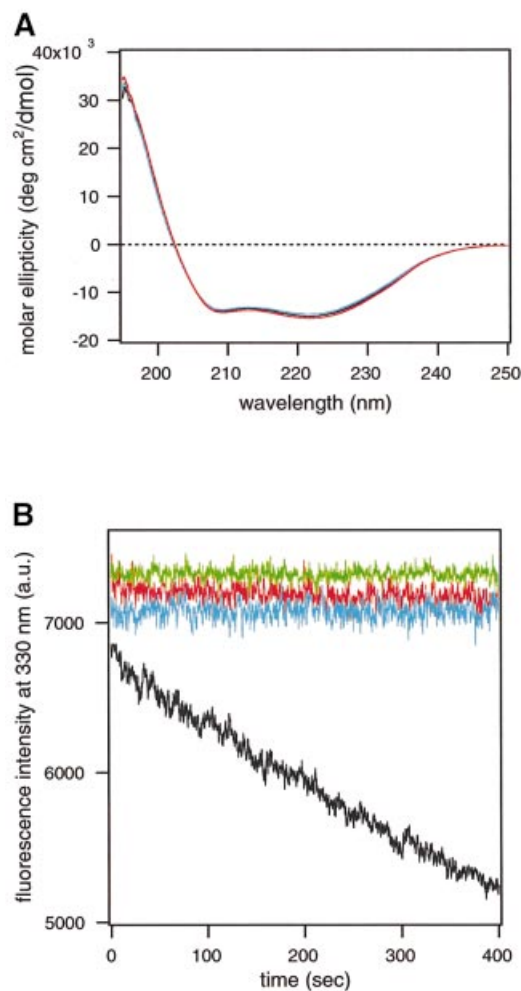
**Fig. 1.** Proposed electron pathway during reoxidation of DsbA by DsbB in conjunction with ubiquinone. Reduced DsbA presumably interacts with and is reoxidized by the P2 region of DsbB. The transient product of the reduced DsbB should be rapidly reoxidized for reactivation by concerted actions of ubiquinone coupled with the intramolecular electron shuttle from the P2 to P1 region.

(Kadokura *et al.*, 2000; Kobayashi *et al.*, 2001; Xie *et al.*, 2002). Secondly, the interdomain interaction and electron transfer mechanism within the DsbB molecule should be clarified. Finally, molecular interaction and electron transfer between DsbB and DsbA should be studied. To gain insights into these problems and to understand how the electron transfer reactions mediated by DsbB are electrochemically regulated, it is a prerequisite to characterize basic parameters, such as redox properties of the two crucial periplasmic domains, P1 and P2, of DsbB. The primary objective of this study was to determine the redox potential of the two redox active cysteine pairs in DsbB and to use these parameters as well as those of mutationally altered DsbB variants to understand the molecular mechanisms of DsbA oxidation by DsbB. We found that the redox potential difference between DsbA and DsbB is apparently reversed with regard to the physiological flow of electrons.

## Results

### **Properties of DsbB variants having only one pair of essential cysteines**

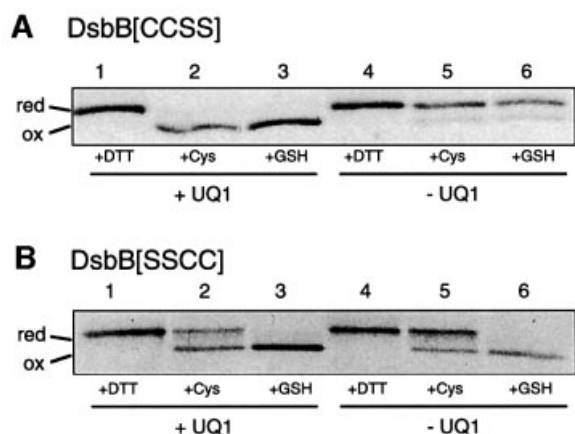
DsbB is an integral membrane protein with two redox active periplasmic domains. It does not show a large redox-dependent change in spectrophotometric or fluorometric properties. These features do not make it straightforward to determine individual redox potential values of the reactive domains. To circumvent the difficulty, we constructed DsbB variants, in which either of the two essential cysteine pairs had been mutated to serine. Thus, only Cys41 and Cys44 in the P1 domain remain in a construct of DsbB[CCSS], and only Cys104 and Cys130 in the P2 domain remain in a construct of DsbB[S SCC]. Previous studies showed that, among the six cysteine residues in DsbB, Cys8 and Cys49 are dispensable (Jander *et al.*, 1994), and that DsbB[S SCC] retains its ability to interact with DsbA (Kobayashi and Ito, 1999; Kobayashi *et al.*, 2001), while DsbB[CCSS] retains its ability to be



**Fig. 2.** (A) CD spectra in the far UV region of oxidized wild-type DsbB (black), DsbB[CCSS] (blue) and DsbB[S SCC] (red). Spectra were recorded in 50 mM sodium phosphate (pH 8.0), 0.1 M NaCl and 0.1% *N*-dodecyl- $\beta$ -D-maltoside. Sample concentration was 5  $\mu$ M. (B) DsbA reoxidations catalyzed by wild-type DsbB (black), DsbB[CCSS] (blue), DsbB[S SCC] (red) and a mixture of DsbB[CCSS] and DsbB[S SCC] (green). Reactions were followed by fluorometric monitoring of the redox state of DsbA at 30°C, as described in the Materials and methods.

oxidized strongly by respiratory functions of the cell (Kobayashi and Ito, 1999). Measurements of the far UV circular dichroism (CD) spectra revealed no significant difference among wild-type DsbB, DsbB[CCSS] and DsbB[S SCC], all of which exhibited an essentially-identical spectrum characteristic of proteins with predominantly  $\alpha$ -helical secondary structure (Figure 2A). It was suggested that the mutations of Cys to Ser at the active sites did not extensively affect the overall structure of DsbB[CCSS] or DsbB[S SCC]. Taken together, these mutant forms of DsbB probably retain characteristic reactivities ascribable to the P1 and P2 domains, respectively.

*In vitro* functionality of the DsbB variants was assayed by monitoring the fluorescence changes associated with DsbA oxidation, as described by Bader *et al.* (1998). In contrast to wild-type DsbB, which exhibited ubiquinone-dependent activity to oxidize DsbA, neither DsbB[CCSS], DsbB[S SCC] nor their mixture exhibited any detectable

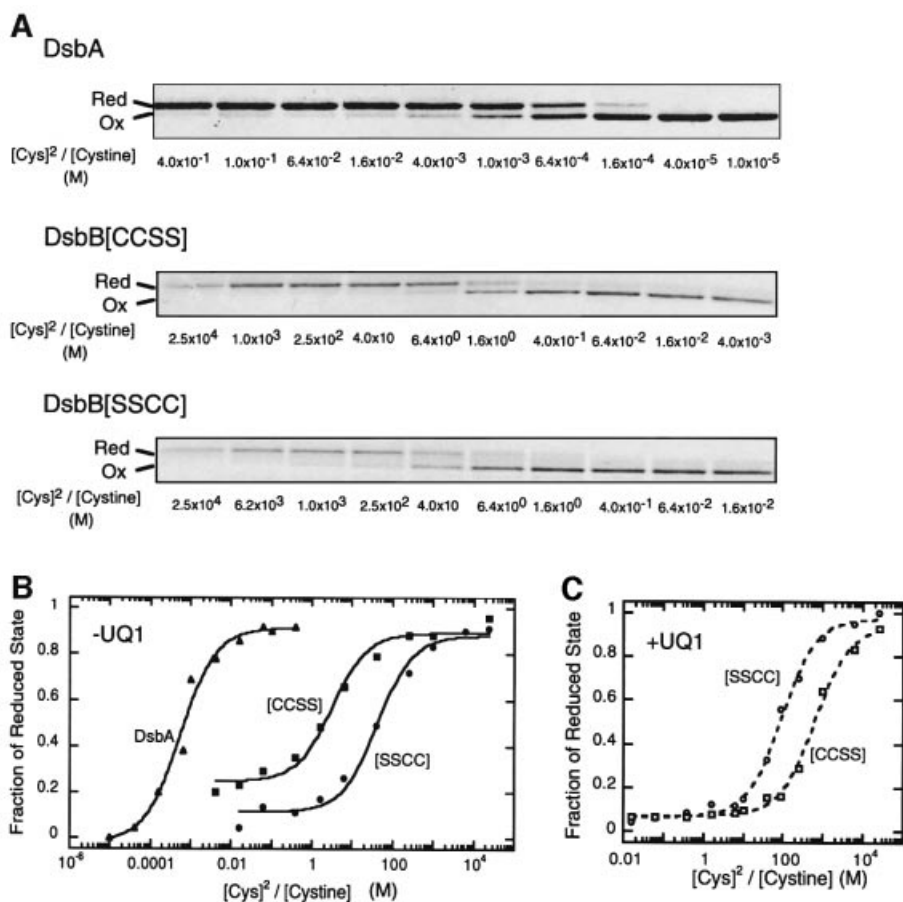


**Fig. 3.** Redox states of DsbB[CCSS] (A) and DsbB[SsCC] (B) in the presence of low molecular weight reducing agents. Oxidized DsbB[CCSS] and DsbB[SsCC] were incubated with 10 mM DTT, L-Cys or GSH for 3 h at 30°C in 50 mM sodium phosphate (pH 8.0), 0.1 M NaCl and 0.1% *N*-dodecyl- $\beta$ -D-maltoside. For samples 1–3, UQ-1 was added to a concentration of 20  $\mu$ M. Redox reactions were then terminated with 7.5% TCA, and free thiols were subjected to alkylation with AMS. Modification with AMS (~0.54 kDa) resulted in a visible mobility shift of the protein upon SDS–PAGE.

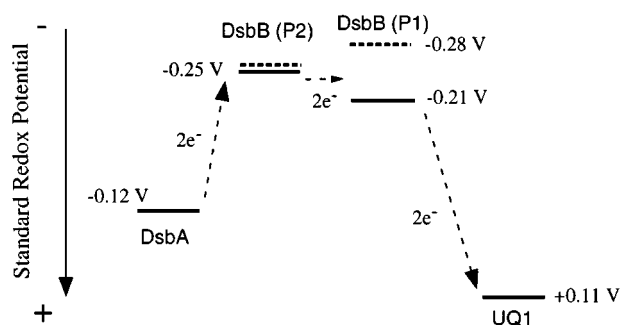
activity of DsbA oxidation (Figure 2B). Thus, the mutant proteins are inactive in the overall reactions, in which the integrity of both the P1 and P2 domains is essential. The lack of complementation between the two mutant forms is consistent with the involvement of an intramolecular electron transfer between the P1 and P2 regions in the DsbA–DsbB catalytic cycle (Kishigami and Ito, 1996).

#### **Reactivities of Cys41/Cys44 and Cys104/Cys130 active sites with low molecular weight reducing agents**

The redox potential of a cysteine pair in a protein can be determined by quantifying the steady state ratios of their reduced and oxidized forms in the presence of excess redox buffer of defined redox compositions. This requires that the cysteine residues are accessible by the buffer constituents. Although glutathione redox buffer, reduced glutathione (GSH)/oxidized glutathione (GSSG), is commonly used for this type of determination, we found that DsbB has an unusually low reactivity with GSH. In experiments shown in Figure 3, purified preparations of DsbB[CCSS] and DsbB[SsCC] were incubated with either GSH, cysteine or DTT, and then treated with trichloroacetic acid, which quenched the thiol–disulfide reactivity



**Fig. 4.** Redox equilibria of wild-type DsbA, DsbB[CCSS] and DsbB[SsCC] with cysteine/cystine at pH 8.0 and 30°C. (A) After equilibration in redox buffers of varying  $[Cys]^2/[Cystine]$  ratios for 3 h, all the samples were treated with 7.5% TCA, followed by alkylation with AMS. Reduced and oxidized proteins were separated by 12% SDS–PAGE under non-reducing conditions. (B) Fractions of proteins in the reduced state were estimated after Coomassie Blue staining, using a LAS-1000 image reader. The plots were interpreted by equation 1, and  $K_{eq}$  values were determined (see Materials and methods). (C) Experiments were carried out in the presence of an equimolar amount of externally added UQ-1. The curves for UQ-1-bound DsbB[CCSS] and DsbB[SsCC] are represented by broken lines.



**Fig. 5.** Intrinsic redox potential values of the DsbB redox active sites in relation to those of DsbA and UQ-1. The standard redox potential values of DsbA and DsbB were calculated from equation 2 on the basis of their redox equilibria with cysteine/cystine at pH 8.0 and 30°C (Figure 4). The broken lines represent the standard redox potential of DsbB in the presence of UQ-1.

and denatured the proteins. Protein precipitates were then dissolved in buffered SDS containing 4-acetoamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS), which alkylates -SH groups (Vestweber and Schatz, 1988; Uchida *et al.*, 1995; Kobayashi *et al.*, 1997). Reduced and oxidized forms of DsbB, DsbB[CCSS] or DsbB-[SSCC] can be distinguished by SDS-PAGE (Kobayashi and Ito, 1999), since AMS modification of a pair of cysteines increases the molecular mass of the protein by ~1080 Da.

DsbB[CCSS] was reduced after incubation with any of the reducing agents (Figure 3A, lanes 4–6). However, it was found that DsbB[SSCC] was only reduced with DTT or Cys (Figure 3B, lanes 4 and 5), but not with GSH at a concentration of 10 mM (Figure 3B, lane 6) or 100 mM (data not shown). We confirmed that GSH reduced only one of the two disulfide bonds in wild-type DsbB (data not shown). Thus, the Cys104/Cys130 disulfide bond is GSH-resistant, suggesting that the cysteine residues in the P2 domain are inaccessible by GSH. Previously, it was shown that the Cys41/Cys44 disulfide was refractory to reduction by DTT when DsbB was integrated into the membrane with functional respiratory components (Kobayashi and Ito, 1999). We now examined reactivities of the DsbB derivatives in the presence of a limited concentration (20  $\mu$ M) of ubiquinone-1 (UQ-1). As expected, DsbB-[SSCC] was essentially unaffected by UQ-1 (Figure 3B, lanes 1–3). Consistent with the previous results, detergent-solubilized DsbB[CCSS] was no longer DTT-resistant, even in the presence of UQ-1 (Figure 3A, lane 1), but, interestingly, the DsbB[CCSS]-UQ-1 combination now resisted the Cys and GSH actions (Figure 3A, lanes 2 and 3).

#### Determination of intrinsic redox potential for the Cys41/Cys44 and Cys104/Cys130 active sites

The above results indicated that GSH/GSSG redox buffer is inappropriate for the determination of the intrinsic standard redox potential of DsbB. We therefore determined the redox potential values for the active sites in DsbB from their redox equilibrium constants ( $K_{eq}$ ) with cysteine/cystine at pH 8.0 and 30°C. To validate our methodology, we first determined the redox potential for DsbA (Figure 4A, top panel and B, leftmost curve). The  $K_{eq}$  value and standard redox potential of DsbA were

determined to be  $(5.9 \pm 1.1) \times 10^{-4}$  M and  $-0.12$  V, respectively. The redox potential thus obtained was close to the literature values (Grauschopf *et al.*, 1995; Huber-Wunderlich and Glockshuber, 1998), which were obtained using GSH/GSSG redox buffer and the fluorescence intensity change of DsbA upon reduction. We then used DsbB[CCSS] and DsbB[SSCC] to determine the individual redox potential values for the P1 and P2 active sites in DsbB (Figure 4A, bottom two panels and B, right two curves). The  $K_{eq}$  value and standard redox potential of DsbB[CCSS] (Cys41/Cys44 in the P1 region) were  $3.2 \pm 0.8$  M and  $-0.21$  V, respectively (Figure 5). Those of DsbB[SSCC] (Cys104/Cys130 in the P2 region) were  $(4.1 \pm 0.9) \times 10$  M and  $-0.25$  V, respectively (Figure 5). Similar redox potential values were obtained using DTT<sup>red</sup>/DTT<sup>ox</sup> redox buffer (data not shown).

It is interesting to note that the intrinsic redox properties of DsbA and DsbB do not explain the directionality of their redox reactions, specifically for the Cys104/Cys130 active site in the P2 region, which is believed to act as an oxidant against the active site of DsbA, and has proved to have a much lower redox potential than the Cys30/Cys33 active site in DsbA.

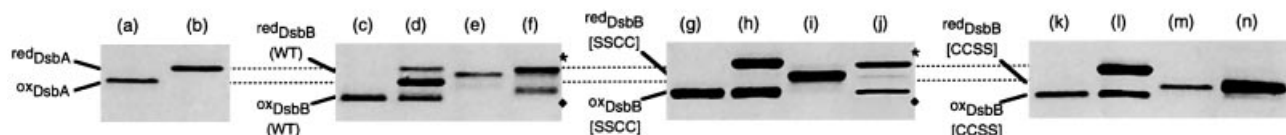
#### Apparent redox potential values in the presence of ubiquinone

The results presented above raised the possibility that ubiquinone binding might alter the effective redox potential of DsbB. The absorption spectrum of UQ-1 in solution was slightly blue-shifted upon addition of either DsbB, DsbB[CCSS] or DsbB[SSCC], indicative of transfer from aqueous to more hydrophobic environments (data not shown). Thus, all of the DsbB proteins are capable of accommodating UQ-1 molecules. The redox potential determination assays were repeated in the presence of an equimolar amount of UQ-1. The redox values for DsbB[SSCC], the P2 model protein, were not significantly altered in the presence of UQ-1 (Figure 4C); the  $K_{eq}$  value and standard redox potential value were  $(9.2 \pm 0.9) \times 10$  M and  $-0.26$  V, respectively (Figure 5).

In contrast, UQ-1 binding significantly affected the apparent redox property of DsbB[CCSS], the P1 model protein. The apparent redox equilibrium curve for DsbB-[CCSS] was strikingly shifted toward higher [Cys]<sup>2</sup>/ [Cystine] ratio regions (Figure 4C), indicating that the P1 region of DsbB, at least superficially, possesses a much lower redox potential in the presence of UQ-1 than in its absence. The values for  $K_{eq}$  and redox potential were now  $(6.3 \pm 0.7) \times 10^2$  M and  $-0.28$  V, respectively (Figure 5).

#### Intrinsic redox interactions between DsbA and DsbB

We prepared oxidized and reduced forms of DsbA, DsbB, DsbB[CCSS] and DsbB[SSCC], and examined redox reactions between DsbA and DsbB derivatives in their equimolar mixtures (Figure 6). The reaction between reduced DsbA and oxidized DsbB resulted in partial oxidation of DsbA (lane d). Although this is the supposedly physiological reaction, it did not go to completion; we believe that this reaction was due to endogenously bound ubiquinone molecules, which were present in the DsbB preparation at ~20% stoichiometry (data not shown).



**Fig. 6.** Disulfide exchange reactions between DsbA and DsbB derivatives. All the samples were incubated at 30°C for 1 h in 50 mM sodium phosphate (pH 8.0), 0.1 M NaCl and 0.1% *N*-dodecyl- $\beta$ -D-maltoside, before treatment with 7.5% TCA and alkylation with AMS. Lane (a), oxidized DsbA; (b), reduced DsbA; (c), oxidized DsbB(WT); (d), reduced DsbA + oxidized DsbB(WT); (e), reduced DsbB(WT); (f), oxidized DsbA + reduced DsbB(WT); (g), oxidized DsbB[SCCC]; (h), reduced DsbA + oxidized DsbB[SCCC]; (i), reduced DsbB[SCCC]; (j), oxidized DsbA + reduced DsbB[SCCC]; (k), oxidized DsbB[CCSS]; (l), reduced DsbA + oxidized DsbB[CCSS]; (m), reduced DsbB[CCSS]; (n), oxidized DsbA + reduced DsbB[CCSS]. The asterisks on lanes (f) and (j) indicate DsbA that was reduced upon mixing with an equimolar amount of reduced DsbB, while the diamonds represent the other reaction product (i.e. concomitantly produced DsbB with the oxidized P2 domain).

**Table I.** Redox properties,  $pK_a$  values of the Cys30 and Michaelis–Menten parameters for wild-type DsbA and the active site variants

	CXXC motif	$pK_a$ of Cys30 <sup>a</sup>	$E'_0$ DsbA (V) <sup>b</sup>	$k_{cat}$ <sup>c</sup> (nM UQ-1/nM DsbB/s)	$K_m$ for DsbA <sup>c</sup> ( $\mu$ M)
DsbA (wild-type)	CPHC	3.28 $\pm$ 0.09	-0.12 $\pm$ 0.01	4.6 $\pm$ 0.2	6.0 $\pm$ 0.6
DsbA (PDI-type)	CGHC	3.71 $\pm$ 0.05	-0.15 $\pm$ 0.01	3.0 $\pm$ 0.3	10.4 $\pm$ 2.4
DsbA (Trx-type)	CGPC	6.21 $\pm$ 0.04	-0.21 $\pm$ 0.01	4.0 $\pm$ 0.3	4.9 $\pm$ 0.9

<sup>a</sup>The values are based on previous data by Huber-Wunderlich and Glockshuber (1998).

<sup>b</sup>The standard redox potentials ( $E'_0$  DsbA) of the active site were determined as shown in Figure 4.

<sup>c</sup>These parameters were obtained from Michaelis–Menten analysis for UQ-1 reduction in the presence of wild-type DsbB (5 nM) and UQ-1 (10  $\mu$ M) at pH 8.0 (see Materials and methods).

In contrast, the reverse reaction, between reduced DsbB and oxidized DsbA, occurred quantitatively, producing reduced DsbA and half-oxidized DsbB, in which only one of the two cysteine pairs was oxidized (lane f). The DsbB cysteines that participated in the above reaction should have been Cys104 and Cys130, since nearly quantitative reactions occurred between reduced DsbB[SCCC] and oxidized DsbA (lane j). However, the ‘forward reaction’ of the DsbA–DsbB[SCCC] combination did not occur (lane h). DsbB[CCSS] did not react with DsbA in either direction (lanes n and l).

These results confirm biochemically that the P2 domain cysteines can directly undergo the redox interaction with the DsbA active site. However, the reaction only proceeded in the ‘reverse’ direction without the P1 domain cysteines or ubiquinone, consistent with our results that the intrinsic redox potential of the P2 region in DsbB is lower than that of DsbA. No direct redox interaction has been observed between DsbA and P1 region of DsbB.

#### Effects of CXXC motif alterations on the reaction parameters of the ubiquinone-driven DsbA oxidation by DsbB

To understand the functional significance of the reversed redox potential difference between DsbA and DsbB, we studied ubiquinone-coupled DsbA oxidation activity of DsbB (Bader *et al.*, 2000). Contribution of the redox potential property of the DsbA active site was first examined. For this purpose, its CXXC motif mutants were used. The X-X dipeptide in the CXXC motif is a main determinant of the redox property of DsbA (Huber-Wunderlich and Glockshuber, 1998). Thus, DsbA derivatives having the X-X sequence of wild-type DsbA (Pro-His), of PDI (Gly-His), and thioredoxin (Trx; Gly-Pro) were purified, reduced and subjected to the reaction with DsbB in the presence of UQ-1. Their standard redox potential values were also determined (Table I). The

$E'_0$  DsbA value was lowered from the wild-type value of -0.12 to -0.21 V by the Trx-type alteration (CGPC), and to -0.15 V by the PDI-type alteration (CGHC). Accordingly, the  $pK_a$  value of the Cys<sup>30</sup> residue should have been altered by the mutations (Huber-Wunderlich and Glockshuber, 1998; Table I). Reactions with DsbB were allowed at pH 8.0, well above the highest  $pK_a$  value of Cys<sup>30</sup> in the DsbA variants, which should be fully thiolated (Huber-Wunderlich and Glockshuber, 1998). Interestingly,  $k_{cat}$  values of the ubiquinone reduction (and hence DsbA oxidation) were not significantly affected by the DsbA active site mutations (Table I). Thus, the alleviation in the redox potential difference did not result in accelerated reaction kinetics. The mutational alterations of the CXXC motif did not greatly affect either the  $K_m$  value of DsbB against DsbA (Table I). These results indicate that redox potential *per se* of the active site in DsbA is not crucial for its ubiquinone-driven oxidation by DsbB.

We then examined DsbB variants with altered Cys41-X-X-Cys44 motifs in the P1 domain. Thus, the Val42-Leu43 dipeptide of wild-type DsbB was replaced by Pro-His (DsbA-type), Gly-His (PDI-type) or Gly-Pro (Trx-type) (Kobayashi *et al.*, 2001). The redox potential measurements showed that these substitutions only slightly lowered the values for the Cys41/Cys44 active site (Table II;  $E'_0$  DsbB(P1)). While the DsbA-type and PDI-type CXXC alterations lowered the  $k_{cat}$  value ~3-fold, the Trx-type alteration did so ~50-fold. All the variants retained the wild-type affinity with UQ-1 (Table II;  $K_m$  for UQ-1). Also, they exhibited normal CD spectra (data not shown). Thus, the DsbA oxidation activity can be compromised strikingly without significant change in the intrinsic redox potential of the CXXC motif or in the affinity to ubiquinone. It was also noted that the DsbA-type and PDI-type variants of DsbB had  $K_m$  values for DsbA 2- to 3-fold higher than the wild-type DsbB protein (Table II;  $K_m$  for DsbA).

**Table II.** Redox properties and Michaelis–Menten parameters for wild-type DsbB and the active site variants

	CXXC motif in the P1 region	$E'_{0\text{DsbB(P1)}}$ (V)	$k_{\text{cat}}^{\text{b}}$ (nM UQ-1/nM DsbB/s)	$K_{\text{m}}$ for DsbA <sup>b</sup> ( $\mu\text{M}$ )	$K_{\text{m}}$ for UQ-1 <sup>c</sup> ( $\mu\text{M}$ )
DsbB (Wild-type)	CVLC	$-0.21 \pm 0.01$	$4.6 \pm 0.1$	$6.0 \pm 0.7$	$1.2 \pm 0.2$
DsbB (DsbA-type)	CPHC	$-0.25 \pm 0.02$	$1.7 \pm 0.1$	$14.3 \pm 2.3$	$1.3 \pm 0.2$
DsbB (PDI-type)	CGHC	$-0.26 \pm 0.04$	$1.4 \pm 0.2$	$20.3 \pm 4.7$	$1.7 \pm 0.3$
DsbB (Trx-type)	CGPC	$-0.24 \pm 0.02$	$9.4 \pm 0.4 \times 10^{-2}$	$7.2 \pm 0.9$	$1.3 \pm 0.3$

<sup>a</sup>The standard redox potentials ( $E'_{0\text{DsbB(P1)}}$ ) of the P1 active site in the DsbB variants were determined by constructing the DsbB[CCSS] proteins with the altered CXXC motif. The experimental method was the same as used for DsbB[SSCC] and DsbB[CCSS] (see Figure 4).

<sup>b</sup>These parameters were obtained from Michaelis–Menten analysis for UQ-1 reduction in the presence of DsbB (5–200 nM) and UQ-1 (10  $\mu\text{M}$ ) at pH 8.0 (see Materials and methods).

<sup>c</sup>The  $K_{\text{m}}$  values for UQ-1 were obtained from Michaelis–Menten analysis for UQ-1 reduction in the presence of DsbB (5–200 nM) and reduced wild-type DsbA (10  $\mu\text{M}$ ) at pH 8.0 (see Materials and methods).

## Discussion

DsbB showed different redox reactivities with different small reducing agents. While DsbB[CCSS] is reducible with any of GSH, Cys and DTT in the absence of UQ-1, DsbB[SSCC] did not react with GSH. Also, the latter protein was not completely reduced with 10 mM Cys. The lower reactivity of the Cys104/Cys130 active site may be explained in terms of its low standard redox potential ( $-0.25$  V). Indeed, it is completely reduced with DTT (standard redox potential,  $-0.33$  V). However, the standard redox potential value for GSH/GSSG ( $-0.24$  V) is lower than that for Cys/cystine ( $-0.20$  V). Therefore, unreactivity of Cys104/Cys130 against GSH may be partly due to some steric or electrostatic incompatibility of GSH–P2 active site interactions. Bader *et al.* (2000) pointed out that DsbB lacks the ability to oxidize GSH.

Striking redox coupling between Cys41/Cys44 and UQ-1 was observed in our assays. The apparent redox potential of the Cys41/Cys44 pair is lowered notably in the presence of UQ-1. Two interpretations are possible to account for this UQ-1 effect. First, UQ-1 may act as an oxidant against the P1 active site, leading to immediate oxidation of Cys41 and Cys44. The reduced component of Cys41/Cys44 is thus eliminated from the equilibrium. Secondly, UQ-1 may exert allosteric-like effects on DsbB such that the Cys41/Cys44 active site becomes more reducing. Although these two possibilities are not mutually exclusive, the second possibility should be considered more seriously. Indeed, the concentration of Cys or GSH used in this assay (10 mM) by far exceeds that of UQ-1 (20  $\mu\text{M}$ ). Even if UQ-1 acted as an oxidant against DsbB, the oxidized form of UQ-1 should have been consumed by the reaction cycles. Furthermore, the extent of the UQ-1-dependent redox potential lowering (Figure 4C) did not significantly change when UQ-1 concentration was increased 10-fold (UQ-1:DsbB[CCSS] molar ratios of 1 to 10) (data not shown). This result indicates that excess UQ-1 molecules do not affect the redox property of the P1 active site further. Thus, it seems reasonable to conclude that stoichiometric binding of UQ-1 to DsbB renders the Cys41/Cys44 pair more reducing and refractory to reduction with Cys or GSH.

The extent of this redox potential lowering is insufficient to make DsbB resist a more reducing agent, DTT; DsbB[CCSS] was reducible with DTT even in the presence of UQ-1. However, our previous results demon-

strated that DsbB[CCSS] was refractory to reduction with DTT, provided that it is integrated into the *E.coli* cytoplasmic membrane with functional respiratory components and that air oxygen is available (Kobayashi *et al.*, 1999). Under the latter conditions, ubiquinone could continuously act as an oxidant against the P1 active site of DsbB and keep it oxidized.

In contrast to the P1 active site, the redox property of the P2 domain was not significantly affected by addition of UQ-1. This means that the redox potential of the P2 domain remains lower than that of DsbA in the presence of UQ-1. Being consistent with a more reducing character of the P2 domain than DsbA, a disulfide-bond exchange reaction occurred between oxidized DsbA and reduced DsbB[SSCC], but not in the other direction. Similar observation was made in our previous *in vivo* study. Deprivation of protoheme or ubiquinone/menaquinone transiently generated a reduced form of DsbB and then allowed the reverse electron transfer from DsbB to DsbA (Kobayashi *et al.*, 1997). Without the aid of the respiratory chain, electrons predominantly flow from DsbB to DsbA.

How does the DsbA oxidation reaction go forward despite the unfavorable redox potential differences (Figure 5)? Since any of Cys41, Cys44, Cys104 and Cys130 are indispensable for the oxidation activity of DsbB, it is unlikely that electrons bypass the Cys41/Cys44 and Cys104/Cys130 pairs to reach ubiquinone. Also, the transmembrane proton electrochemical potential is not essential for the electron transfer from DsbA to DsbB in the cell, because the DsbA–DsbB oxidative system can be reconstituted *in vitro* with solubilized DsbB and ubiquinone (Bader *et al.*, 1999, 2000). Regeneration of oxidized DsbA is essentially driven by the strong oxidizing power of ubiquinone. In fact, the higher redox potential of ubiquinone (+0.11 V) seems to be suited to oxidize DsbA ( $-0.12$  V) (Bader *et al.*, 1999). Perhaps DsbB, which has high affinity for both DsbA and ubiquinone, is optimized as an electron flow pathway between the two, and the intrinsic redox potential of each domain, *per se*, is less important.

This notion is strongly supported by the results for DsbA and DsbB mutants with the altered CXXC motif. Although the redox potential of the Trx-type mutant of DsbA was close to that of the P2 domain, it was oxidized by DsbB at almost the same rate as wild-type DsbA. PDI-type DsbA, whose redox potential was slightly lower than that of wild-type DsbA, was oxidized more slowly than the

wild type. These results indicate that the reversal of the redox potential values between DsbA and the P2 domain of DsbB is not a rate-limiting factor in this catalytic cycle.

Even more drastic effects on catalytic turn-over were observed when the CXXC motif of DsbB was altered. The Trx-type DsbB mutant showed an ~50-fold lower  $k_{\text{cat}}$  value than the wild-type DsbB. This mutant also lacked *in vivo* functionality; reduced DsbA markedly accumulated in the cell expressing the Trx-type DsbB mutant (Kobayashi *et al.*, 2001). The other two mutations also diminished significantly the DsbA oxidation activity. These results imply the functional importance of the Val42-Leu43 sequence flanked by Cys41 and Cys44. Since all the DsbB variants retained high affinity to ubiquinone, the functional impairments might not be ascribed to a defect in the redox coupling between the P1 active site and ubiquinone. Also, the alteration in the redox property of the P1 active site alone cannot explain the extremely lowered  $k_{\text{cat}}$  value of Trx-type DsbB mutant. Probably, it is not a redox potential change, but a local conformation change that obstructs the P2 to P1 electron flow. The occurrence of an intramolecular disulfide exchange reaction was suggested by the observation that a DsbB Cys44Ser variant forms an aberrant intramolecular disulfide bond that was formed presumably between Cys41 and a P2 cysteine (Kishigami and Ito, 1996). The Val-Leu to Gly-Pro change in the Trx-type DsbB mutant causes serious structural perturbations on the vicinity region of the protein. In agreement with this notion, random mutation analyses of the CXXC DsbB motif showed that Pro at the second X position is incompatible with the functionality of DsbB (Y.Takahashi, unpublished data). We suggest that the polypeptide backbone around or within the CXXC motif is particularly important for the proposed intramolecular disulfide exchange between P1 and P2.

Our previous results for insertion or deletion mutagenesis also pointed out the functional importance of the P1 loop. The insertion of 31 amino acids into the region C-terminally adjacent to the CXXC motif abolished the oxidation activity of DsbB (Kobayashi *et al.*, 2001). Also, deletion of a single amino acid from this region reduced DsbB and rendered it inactive in the cell (Kobayashi *et al.*, 2001). The P1 region is involved not only in the redox coupling with ubiquinone, but also in the disulfide donation to the P2 domain, leading us to conclude that the structural or chemical nature of this region is one of the key factors affecting the functionality of DsbB.

Functional and biological significance of the reversed redox potential values between DsbA and DsbB remains an exciting unsolved problem. The difficulty in reduction of the active Cys pairs in DsbB, paradoxically, may act advantageously to keep the enzyme constantly active and to transmit the oxidizing power; the reduced form of DsbB should be inactive. Also, the low redox potential may determine the reaction specificity of DsbB. If DsbB had as high redox potential as DsbA, it would have been a more oxidizing catalyst that oxidizes periplasmic substrate proteins directly without mediating DsbA. This catalytic system may be designed to utilize specific interactions between reduced DsbA and oxidized DsbB, coupled with the oxidizing power of ubiquinone. Further genetic, biophysical and structural studies on the disulfide

exchange reactions between DsbA and DsbB will reveal new mechanisms of electron flow that are needed for protein folding in the extracytoplasmic space.

## Materials and methods

### Plasmids encoding DsbB-His<sub>6</sub>-Myc and its derivatives

DsbB-His<sub>6</sub>-Myc and its mutant derivatives in this work were over-expressed from respective genes placed under the isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG)-inducible *lac* promoter on pUC119. The *HindIII*-*KpnI* *dsbB*-*his<sub>6</sub>*-*myc* fragment of pSS51 (Kishigami and Ito, 1996) was cloned into the multi-cloning site of pUC119. Site-directed mutagenesis was carried out using the QuikChange mutagenesis kit from Stratagene with appropriate sets of primers. To prevent the disulfide-linked oligomerization of DsbB, non-essential cysteines, Cys8 and Cys49, were mutated (Bader *et al.*, 1999). The mutant DsbB protein with Cys8Ala and Cys49Val alterations is referred to simply as DsbB in this paper. The final plasmids were all confirmed for the sequences of the open reading frames.

The DsbB variants with altered CXXC motifs were constructed by replacing the Val42-Leu43 sequence with Pro-His (DsbA-type; 5'-CTGAAACCTTGCCCGCACTGTATTTATGAACGCGTCGCG-3'), Gly-His (PDI-type; 5'-CTGAAACCTTGCGGTCAGTATTATGAACGCGTCGCG-3') and Gly-Pro (Trx-type; 5'-CTGAAACCTTGCGTCCGTGTTATTTATGAACGCGTCGCG-3'), respectively, using the sets of primers indicated in parentheses and their complementary counterparts.

### Plasmids encoding DsbA and its derivatives

The *dsbA* plasmid, pSS18, was constructed by cloning the *EcoRI*-*HindIII* fragment of the pUC19-derived *dsbA*<sup>+</sup> (Kanaya *et al.*, 1994) into the multi-cloning site of pMW119 provided by Nippon Gene. Plasmids for DsbA variants with altered CXXC motifs were also constructed using the QuikChange mutagenesis kit; the mutagenic primers used were 5'-TCTTTCTTCTGCGGTCGCTATCAGTTTGAAGAAGTT-3' (Trx-type) and 5'-TCTTTCTTCTGCGGTCAGTATCAGTTTGAAGAAGTT-3' (PDI-type) as well as their complementary strands.

### Bacterial strains and growth conditions

The plasmids encoding DsbB and its derivatives were introduced into *E. coli* strain SS141 (MC4100 *dsbB::kan5/F'lacI<sup>q</sup> lacZ<sup>+</sup>Y<sup>+</sup>A<sup>+</sup> lacPL8*; Kishigami *et al.*, 1995a). Plasmid-bearing cells were grown aerobically at 37°C on L medium containing 100  $\mu$ g/ml of ampicillin, induced with 0.5 mM IPTG at a Klette unit of 0.6 and harvested 4 h after induction. DsbA and its derivatives were overexpressed using *E. coli* strain AK2168 (AD16 *dsbA::Cm*; a gift from Akio Kihara, Hokkaido University) harboring the plasmid. Cells were grown at 37°C on L medium containing 100  $\mu$ g/ml of ampicillin, induced with 0.5 mM IPTG at a Klett unit of 0.6 and harvested 4 h after induction.

### Protein purification

Purification of DsbB was performed essentially as described by Bader *et al.* (1998, 1999). Membrane fractions were solubilized in 50 mM sodium phosphate buffer (pH 8.0) containing 0.3 M NaCl and 1% *N*-dodecyl- $\beta$ -D-maltoside with gentle stirring on ice for 1 h. After removal of insoluble materials by centrifugation at 100 000 g for 60 min, the supernatant was loaded onto a Ni-NTA agarose column (Quiagen) pre-equilibrated with the same buffer, followed by washing with 50 mM sodium phosphate (pH 8.0) containing 0.3 M NaCl, 0.1% *N*-dodecyl- $\beta$ -D-maltoside and 20 mM imidazole. DsbB was then eluted with 200 mM imidazole in otherwise the same buffer. Peak DsbB fractions were pooled and applied to a 5 ml hydroxyapatite column (Bio-Rad) pre-equilibrated with 50 mM sodium phosphate (pH 8.0), 0.3 M NaCl and 0.02% *N*-dodecyl- $\beta$ -D-maltoside. The column was then washed with 50 mM sodium phosphate (pH 6.2), 0.1 M NaCl and 0.02% *N*-dodecyl- $\beta$ -D-maltoside. DsbB was eluted with a linear 60 ml gradient of 50–500 mM sodium phosphate (pH 6.2), containing 0.1 M NaCl and 0.02% *N*-dodecyl- $\beta$ -D-maltoside. UV spectrum measurements after reduction with sodium borohydride (NaBH<sub>4</sub>) (Bader *et al.*, 2000) indicated that the purified preparations of wild-type DsbB and DsbB[CCSS] contained ~0.2 mol equivalent of endogeneously bound quinone. No bound quinones were detected for the purified preparation of DsbB[SSSC].

DsbA and its variants were purified from the periplasmic extracts by DEAE-Sephacrose CL-6B column chromatography and Superdex S-75 gel filtration (Pharmacia) as described by Akiyama *et al.* (1992). The

purity was at least 95% as judged from Coomassie Blue-stained patterns after SDS-PAGE.

#### Preparation of reduced DsbA and DsbB

Purified DsbA was reduced by incubation with 20 mM DTT for 30 min on ice. DTT was then removed by gel filtration on PD-10 Sephadex columns (Amersham).

Preparation of reduced DsbB required more careful sample manipulations because of its rapid air oxidation upon removal of DTT. Thus, we first suspended the PD-10 resin with anaerobic buffer solution containing 0.1 U/ml of protocatechuic acid dioxygenase and 2 mM protocatechuic acid (Bull and Ballou, 1981). This suspension was allowed to stand in a column overnight, which was washed thoroughly with degassed buffer (50 mM sodium phosphate pH 8.0, 0.1 M NaCl, 0.1% *N*-dodecyl- $\beta$ -D-maltoside) before loading of 20 mM DTT-reduced DsbB.

#### Measurement of CD spectra

CD spectra in far UV region of wild-type DsbB and its variants were recorded using Jasco J-700. Sample concentration was 5  $\mu$ M and the light path of the cuvette was 1 mm. The buffer used in this measurement was 50 mM sodium phosphate (pH 8.0), 0.1 M NaCl and 0.1% *N*-dodecyl- $\beta$ -D-maltoside.

#### Activity assays

The DsbB activity to oxidize DsbA were assayed by monitoring fluorescence intensity of DsbA, which is attenuated upon oxidation (Bader *et al.*, 1998). Fluorescence measurements at the excitation wavelength of 295 nm and the emission wavelength of 330 nm were carried out using a Hitachi F-4500 spectrofluorimeter. The concentration of reduced DsbA in this assay was 10  $\mu$ M, while that of wild-type DsbB and its variants was 10 nM. The reaction was allowed at 30°C in 50 mM sodium phosphate buffer (pH 8.0) containing 0.1 M NaCl, 0.1% *N*-dodecyl- $\beta$ -D-maltoside and 10  $\mu$ M UQ-1.

For Michaelis-Menten analysis, we followed the DsbB activities to reduce UQ-1 (coenzyme Q1; Sigma) using a Beckman DU 640 spectrophotometer (Bader *et al.*, 2000; Kadokura *et al.*, 2000). Reduction of ubiquinone is accompanied by a decrease in its absorbance at 275 nm. The assay was performed at 30°C in a reaction mixture composed of 50 mM sodium phosphate (pH 8.0), 0.1 M NaCl and 0.1% *N*-dodecyl- $\beta$ -D-maltoside. The rates of the ubiquinone reduction (nM UQ-1/nM DsbB/s) were calculated from the initial linear absorbance decrease and the extinction coefficient difference between oxidized and reduced forms of UQ-1 ( $\Delta\epsilon_{275} = 12.25$  mM/cm).

#### Determination of redox potential values

The redox potential values of the active sites in DsbA and DsbB were determined as follows. Oxidized DsbA or DsbB (1  $\mu$ M) was incubated for 3 h at 30°C in 50 mM sodium phosphate (pH 8.0) containing 0.1 M NaCl, 0.1% *N*-dodecyl- $\beta$ -D-maltoside, 10  $\mu$ M cystine (Nacalai) and different concentrations of L-cysteine (10  $\mu$ M–500 mM; Nacalai). To minimize air oxidation, buffer solutions were thoroughly degassed and flushed with nitrogen. After incubation, samples were treated with trichloroacetic acid (TCA) of a final concentration of 7.5% to denature and precipitate the proteins as well as to avoid any subsequent redox reactions. The protein precipitates were collected by centrifugation (16 000 g for 10 min at 4°C), washed with cold acetone, and dissolved in modification buffer containing 50 mM Tris-HCl (pH 8.0), 1% SDS and 20 mM AMS. Reduced and oxidized forms of the proteins were then separated by 12% SDS-PAGE (without any reducing agents), and stained with Coomassie Blue. The ratio of the reduced and oxidized forms under each cysteine/cystine redox condition was quantified after LAS-1000 CCD imaging.

The equilibrium constant,  $K_{eq}$ , was calculated according to equation 1:

$$R = ([Cys]^2/[Cystine]) / \{K_{eq} + ([Cys]^2/[Cystine])\} \quad (1)$$

where  $R$  is a fraction of the reduced proteins at equilibrium. The standard redox potential was calculated from the Nernst equation (2), using a value of  $-0.20$  V of the Cys/Cystine redox couple ( $E_0'$  Cys/Cystine) (Szajewski and Whitesides, 1980; Wunderlich *et al.*, 1993).

$$E_0'_{DsbA \text{ or } DsbB} = E_0'_{Cys/Cystine} - (RT/2F) \ln K_{eq} \quad (2)$$

where  $F$  and  $R$  are the Faraday constant and the gas constant, respectively.

#### Redox reactions between DsbA and DsbB

Disulfide exchange reactions between DsbA and DsbB under stoichiometric reaction conditions were investigated by alkylation with AMS and separation of the proteins on SDS-PAGE (12%). Equimolar amounts of

oxidized and reduced proteins were incubated at 30°C for 1 h in 50 mM sodium phosphate (pH 8.0), 0.1 M NaCl and 0.1% *N*-dodecyl- $\beta$ -D-maltoside. They were TCA-precipitated and processed as described in the preceding section.

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## Note added in proof

Kadokura and Beckwith (2002) demonstrated that P1 and P2 domains of DsbB indeed interact through a Cys41–Cys130 disulfide bond.