Reactivities of Quinone-free DsbB from *Escherichia coli*

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DsbB is a disulfide oxidoreductase present in the *Escherichia coli* plasma membrane. Its cysteine pairs, Cys\(^{41}\)–Cys\(^{44}\) and Cys\(^{104}\)–Cys\(^{130}\), facing the periplasm, as well as the bound quinone molecules play crucial roles in oxidizing DsbA, the protein dithiol oxidant in the periplasm. In this study, we characterized quinone-free forms of DsbB prepared from mutant cells unable to synthesize ubiquinone and menaquinone. While such preparations lacked detectable quinones, previously reported lauroylsarcosine treatment was ineffective in removing DsbB-associated quinones. Moreover, DsbB-bound quinone was shown to contribute to the redox-dependent fluorescence changes observed with DsbB. Now we reconfirmed that redox potentials of cysteine pairs of quinone-free DsbB are lower than that of DsbA, as far as determined in dithiothreitol redox buffer. Nevertheless, the quinone-free DsbB was able to oxidize \(-40\%\) of DsbA in a 1:1 stoichiometric reaction, in which hemi-oxidized forms of DsbB having either disulfide are generated. It was suggested that the DsbB-DsbA system is designed in such a way that specific interaction of the two components enables the thiol-disulfide exchanges in the "forward" direction. In addition, a minor fraction of quinone-free DsbB formed the DsbA-DsbB disulfide complex stably. Our results show that the rapid and the slow pathways of DsbA oxidation can proceed up to significant points, after which these reactions must be completed and recycled by quinones under physiological conditions. We discuss the significance of having such multiple reaction pathways for the DsbB-dependent DsbA oxidation.

Many secretory proteins undergo oxidative folding, in which they acquire intra- or intermolecular disulfide bonds. The periplasmic space of *Escherichia coli* contains a series of Dsb enzymes, which catalyze introduction and isomerization of protein disulfide bonds (1, 2). DsbA is the primary disulfide bond donor having the Cys\(^{30}\)–Cys\(^{33}\) disulfide at the active site. It is kept in the oxidized and active state by a cytoplasmic thiol-dependent electron system. Thus, DsbA was engaged in the formation of the DsbA-DsbB disulfide complex stably. Our results show that the redox potentials of individual cysteine pairs did not differ significantly from those we determined previously. Surprisingly, the quinone-free DsbB was still able to oxidize \(-40\%\) of DsbA by apparent disulfide exchange reactions, whereas other fraction of DsbB was engaged in the formation of the DsbA-DsbB disulfide complex. We discuss how individual elements in this system are integrated into the elaborate multiple mechanisms that ensure effective recycling of the disulfide bond formation reactions, in which quinone molecules play essential roles.

MATERIALS AND METHODS

Preparation of Various Forms of DsbB and DsbA—All the DsbB derivative proteins in this work contained a C-terminally attached His\(_{6}\)-tag as well as Ala and Val substitutions for nonessential DsbB cysteines, Cys\(^{8}\) and Cys\(^{41}\), respectively (4). In the standard preparations, they were overproduced from plasmid in wild-type *E. coli* cells and purified essentially as described previously (14). DsbB thus obtained is referred to as Dsb-
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FIGURE 1. HPLC elution profiles of organic solvent extracts from various DsbB preparations. Materials extracted from DsbB(UQ8) (trace a), DsbB(MK8) (trace b), LS-treated DsbB(UQ8) (trace c), and DsbB(UQ8) (trace d) were separated by reverse phase HPLC. The major peaks in a and b represent UQ8 and MK8, respectively (6). The slight difference in the elution positions of the UQ peaks in a and c was only apparent, because the products under these peaks gave the same mass value of UQ.

B(UQ8) as it contains endogenously bound UQ8. To prepare DsbB without bound quinones (DsbB(ΔQ)), it was overproduced in the ubiA menA double mutant strain, AN384 (15) as described by Inaba et al. (14). We also prepared DsbB with endogenously bound MK8 (DsbB(MK8)) by overproducing it in the ubiA menA mutant strain, TA36, as described by Takahashi et al. (6). DsbA was prepared as described previously (11).

Reduced forms of DsbA and DsbB were prepared as follows. Purified DsbA was reduced by incubation with 20 mM DTT for 30 min, followed by gel filtration (PD-10 Sephadex column, Amersham Biosciences) to remove DTT. DsbB was likewise reduced with DTT but more careful gel filtration steps were required because it is prone to rapid air oxidation in the absence of DTT. Thus, we first equilibrated Bio-Spin resin with degassed buffer (50 mM sodium phosphate pH 8.0, 0.1 M NaCl, 0.1% DDM) containing 20 mM DTT for 30 min, which was then washed thoroughly with degassed DTT-free buffer before loading of a DTT-reduced DsbB preparation. Concentrations of DsbA and of DsbB were determined by the BCA method, using bovine serum albumin as standard.

Identification of DsbB-bound Quinone Species—Quinones were extracted from the DsbB preparations essentially as described by Takahashi et al. (6). The extract was separated by Shim-pack VP-ODS reverse phase column chromatography, using Shimadzu LCMS-2010A, with elution with methyl alcohol-isopropyl alcohol (3:1) and flow rate of 0.2 ml/min. The chromatographic peak of UQ represented the DsbB-bound UQ semiquantitatively, as we used samples of extensive (four times cumulative) hexane extraction (6). Also, we were able to detect consistent mutual alterations of the UQ peak intensities. To establish the peak identities, the peak fractions were subjected to electrospray ionization-mass spectrometry with scanning for masses 900 to 1000 in 1 s.

Fluorescence Measurements—Fluorescence of each DsbB derivative (1 μM) was measured with excitation wavelength of 295 nm and emission wavelengths of 300–400 nm, using a Hitachi F-4500 spectrofluorometer. The buffer used was 50 mM Tris–HCl, pH 8.1 containing 0.1 M NaCl and 0.1% DDM.

Determination of the Redox Potentials of DsbB(ΔQ)—The redox potential values of the active sites in DsbB(ΔQ) were determined as described by Inaba and Ito (11). Briefly, DsbB(ΔQ) (1 μM) was incubated at 30 °C for 3 or 16 h in 50 mM Tris–HCl, pH 8.1 containing 0.1 M NaCl, 0.1% DDM, 100 mM oxidized DTT (Sigma), and different concentrations (1 mM to 5 mM) of reduced DTT. To minimize air oxidation, buffer solutions were thoroughly degassed and flushed with nitrogen before use. After incubation, samples were treated with trichloroacetic acid of a final concentration of 5%. The protein precipitates were washed with cold acetone and then dissolved in buffer containing 50 mM Tris–HCl, pH 7.0, 1% SDS, and 2 mM AMS. Reduced (AMS-derivatized) and oxidized (non-derivatized) forms of DsbB were separated by 12% non-reducing SDS-PAGE, stained with CBB, and quantified by LAS-1000 CCD imaging. The equilibrium constant and the standard redox potential were calculated as described by Huber-Wunderlich and Gockshuber (16).

DsbB(ΔQ)-DsbA Redox Reactions—Procedures described by Inaba and Ito (11) were used to follow disulfide exchange reactions between DsbB(ΔQ) and DsbA, one of which had been reduced immediately before use. In brief, equimolar concentrations (40 μM) of oxidized and reduced proteins were incubated at 30 °C in 50 mM Tris–HCl, pH 8.1, containing 0.1 M NaCl and 0.1% DDM. At specified time points, the reaction was terminated by mixing an aliquot with an equal volume of 10% trichloroacetic acid. Samples were then processed for AMS modification, SDS-PAGE (12%), and CBB staining.

Characterization of the Partially Reduced Forms of DsbB—In the above analysis, the AMS modification caused different degrees of gel mobility shifts of DsbB depending on the numbers and the locations of the alkylated cysteines and of non-modified intramolecular disulfide bonds. The different mobility DsbB species that appeared after reaction with DsbA (bands 1–3 in Fig. 5D) were characterized by PMF analyses. The stained gel bands were excised and subjected to digestions with lysyl endopeptidase and trypsin. Digested peptides were extracted as described by Tie et al. (17) and analyzed by MALDI-TOF mass spectrometry on Voyager-DE STR (Applied Biosystems). The reflector mode was used for peak detections in the mass range of 800–3,100 (m/z), where the monoisoionic mass peaks were measured. For the mass range of 3,000–10,000 (m/z), the linear mode displayed the averaged mass peaks. The program MS-Fit (Protein Prospector) was used for the peptide identification.

RESULTS

Quinone-free DsbB Can Be Prepared by Expressing DsbB in Quinone-deficient Mutant—DsbB is usually prepared as a complex with UQ8 (5). In our previous study (6, 14), we prepared DsbB after its overproduction in different E. coli cells, the wild-type strain, the ubiA mutant defective in UQ biosynthesis, and the ubiA menA double mutant defective in biosynthesis of both UQ and MK (15). Now we directly determined the quinone contents of such preparations. Materials extracted by organic solvent were separated by reverse-phase column chromatography (Fig. 1). DsbB from wild-type cells gave a peak of retention time 12.5 min (Fig. 1, trace a) and that from the ubiA mutant cells gave a peak at 15.5 min (Fig. 1, trace b). Mass spectrometry established the identities of these peaks as those of UQ8 and MK8, respectively (6). Thus, these DsbB preparations were designated as DsbB(UQ8) and DsbB(MK8), respectively. In contrast, DsbB from the double mutant cells yielded no detectable materials that were eluted at these positions (Fig. 1, trace d), justifying the notation of DsbB(ΔQ) for it. The absence of quinone-
nes in our DsbB(\Delta Q) preparations was also confirmed by the lack of NaBH₄-dependent change in A₂75. In the presence of exogenously added UQ1, DsbB(\Delta Q) was fully active in catalyzing DsbB oxidation with kₐₜₚ of 7.0 ± 0.4 mU/mM DsbB/s and Kₘₜₚ for UQ₁ of 2.8 ± 0.5 μM (at pH 8.0; data not shown).

We also attempted to remove UQ8 from a DsbB(UQ8) preparation by the LS treatment procedures of Grauschopf et al. (13). Although peaks of early retention times, presumably some impurities, were decreased by the LS treatment, the UQ peak was not at all affected by this treatment (Fig. 1, compare trace c with a). The peak identity was confirmed by its mass of 728 Da exactly corresponding to that of UQ8. It was not appropriate to repeat washings with LS because such manipulations led to the formation of DsbB aggregates. Thus, we were unable to obtain UQ₈-free DsbB by LS treatment, although it remains unsettled why Grauschopf et al. (13) did not detect UQ by the NaBH₄-dependent absorbance change with their preparations.

Absence of the Redox-dependent Fluorescence Changes for Quinone-free DsbB—We were able to reproduce the observation (13) that DTT treatment of DsbB (DsbB(UQ8) in our case) resulted in a significant increase of tryptophan fluorescence (Fig. 2A, broken line). However, we found that DsbB(\Delta Q) exhibited the higher fluorescence without any DTT treatment (Fig. 2B, continuous line). DsbB(\Delta Q) was in the fully oxidized state (Fig. 2I, lane 3), which was reduced with DTT (Fig. 2I, lane 4) without any further increase in the fluorescence intensity (Fig. 2B, broken line). Similarly different behaviors were observed between the UQ-bound and the UQ-free forms of DsbB(\[CSS\]) (Fig. 2, C and D). However, DsbB(\[SSCC\]) no longer responded to DTT (Fig. 2E), in agreement with the previous observation (13). Because the \[SSCC\] variant does not actively interact with UQ (10–12), the results obtained above collectively suggest that bound UQ was somehow responsible for the DTT-induced fluorescence increase observed for DsbB(UQ8). A simplest interpretation may be that the bound UQ quenches the intrinsic tryptophan fluorescence of DsbB and that the DTT treatment of the DsbB-UQ complex reduces UQ, possibly leading to dissociation of the resulting ubiquinol from DsbB. In support of the UQ-mediated quenching mechanism, addition of increasing concentrations of UQ1 to the DsbB(\Delta Q) sample led to the progressive reduction of fluorescence (Fig. 2M).

The fluorescence intensity of DsbB(\[SSCC\])(UQ8) was low (Fig. 2E), comparable to that of the wild-type DsbB(UQ8) without DTT treatment. In contrast, DsbB(\[SSCC\])(\Delta Q), irrespective of DTT treatment, exhibited the higher fluorescence intensity comparable to the DTT-induced value of wild-type DsbB(UQ8) (Fig. 2F). Similar results were obtained with \[CSS\] forms of these proteins (Fig. 2, G and H). These results suggest that the reduction of UQ was mediated by the Cys₄⁴ and Cys₄⁴ residues of DsbB, in the absence of which UQ remained oxidized and active in quenching the fluorescence. At any event, our results indicate that the DTT-induced fluorescence increase observed for DsbB(UQ8) does not represent the redox state change of DsbB itself. UQ molecules on DsbB behave like a static fluorescence quencher, although the quenching mechanism remains unclear due to lack of structural information on DsbB. The fluorescence decrease induced by quinone analogues was also observed previously for other ubiquinone oxidoreductases (18).

Redox Properties of DsbB Cysteines in the Absence of Quinones—As shown above, the fluorescence intensity changes observed for DsbB(UQ8) do not report changes in the DsbB protein itself, although they could be useful to assess the redox reactivity of the DsbB-UQ combina-

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4 DsbB variants having cysteine to serine substitutions at positions 41, 44, 104, and/or 130 are indicated by C for cysteine and S for serine for these positions in the above order (for instance, DsbB[\[CSS\]] for a variant with positions 41 and 44 mutated).
FIGURE 2. Effects of DTT on tryptophan fluorescence of DsbB(UQ8) and of DsbB(∆Q). A–H, fluorescence spectra with excitation at 295 nm were recorded in the absence (solid line) or presence (broken line) of 10 mM DTT for the indicated derivatives of DsbB. I–L, redox states were assessed by AMS-modifiability for the DsbB samples that were used for the fluorescence measurements. The samples used in A–H were trichloroacetic acid-precipitated and processed for AMS modification, SDS-PAGE (12%) and staining with CBB. M, saturable fluorescence decrease of DsbB(∆Q) by UQ1 addition. Fluorescence intensities at 330 nm were measured with 1 μM DsbB([CCCC](UQ8)] in the presence of varying concentrations of externally added UQ1, and plotted against the ratios of [UQ1]/[DsbB]. F₀ denotes the fluorescence intensity of DsbB([CCCC](∆Q)) in the absence of UQ1.
much slower and insignificant (Fig. 4B). These results are similar to those obtained previously using quinone-bound or LS-treated DsbB preparations (11–13). The rapid reverse reaction between DsbA and DsbB Cys\textsubscript{104}-Cys\textsubscript{130} is consistent with the observed redox potential differences between DsbA and DsbB as well as with the fact that Cys\textsubscript{104}-Cys\textsubscript{130} pair of DsbB directly interacts with DsbA (21). In contrast, the Cys\textsubscript{41}-Cys\textsubscript{44} pair does not directly interact with DsbA (11–13). We also noted that DsbA only slowly oxidized DsbB\textsubscript{[CSSC]/H9004Q} (Fig. 4C).

Forward Direction Redox Reactions between DsbA and Quinone-free DsbB—We then followed the forward (physiological) direction of DsbA-DsbB\textsubscript{[H9004Q]} reactions using the reduced form of DsbA. Reactions can be true also for its action against the wild-type DsbB. Thus, it is possible that Cys\textsubscript{104}-Cys\textsubscript{130} is first and directly oxidized, producing the band 2 species (having oxidized Cys\textsubscript{104}-Cys\textsubscript{130} as shown below), and that subsequent thiol-disulfide exchange within DsbB resulted in the equally populated band 1 (having oxidized Cys\textsubscript{41}-Cys\textsubscript{44} as shown below) and band 2 products. In contrast to this equimolar reaction, in which complete oxidation of DsbB\textsubscript{[ΔQ]} was undetectable, use of a DsbA/DsbB molar ratio of two resulted in the rapid and complete oxidation of a majority of DsbB\textsubscript{[ΔQ]} molecules (Fig. 4D, band 3 in lanes 5–7), although some components, half-oxidized DsbB species, the DsbA-DsbB disulfide-linked complex and oxidized DsbA were also visible in this reaction (Fig. 4D, lanes 5–7).

**FIGURE 3.** Redox equilibrium of DsbB(ΔQ) with DTT. A, DsbB(ΔQ) derivatives with indicated Cys to Ser substitutions were equilibrated for 3 h with the series of redox buffers of varying [DTT\textsubscript{red}]/[DTT\textsubscript{ox}] ratios and then separated into the reduced (red; AMS-modified) and oxidized (ox; non-modified) forms. B, results in A were quantified, and the proportions of the reduced form are plotted against [DTT\textsubscript{red}]/[DTT\textsubscript{ox}] ratios, with curve fitting as described by Inaba and Ito (11). The symbols denote [CCSS]ΔQ (circle), [SSCC]ΔQ (square), and [CSSC]ΔQ (triangle), respectively. C, intrinsic redox potential values of the DsbB cysteine combinations determined using DTT redox buffer are shown along with those of DsbA and DTT. D, wild-type DsbB(ΔQ) was examined as in A. Band 1 and band 2 indicate hemi-oxidized species of DsbB.
between reduced DsbA and oxidized DsbB[SSCC][ΔQ] was inefficient but small extents of DsbA oxidation and accompanying DsbB[SSCC] reduction as well as formation of the DsbA-DsbB complex were noticeable (Fig. 5A). Neither DsbB[CCSS][ΔQ] nor DsbB[CSSC][ΔQ] was able to oxidize DsbA significantly or to form the intermolecular disulfide with DsbA (Fig. 5, B and C). In contrast to the above cysteine variants, the wild-type DsbB[CCCC][ΔQ] rapidly reacted with reduced DsbA, converting ~40% of DsbA into the oxidized form and ~5–10% into the DsbA-DsbB complex. DsbB itself was converted into the two half-reduced forms, band 1 and band 2 (Fig. 5D, lanes 1–4). These reaction products were in equilibrium since the protein patterns did not change significantly between the 1 min and the 30 min reaction times examined (Fig. 5D, lanes 2 and 4). This reaction appears to represent stoichiometric thiol-disulfide exchanges, in which an initial complex formed between reduced DsbA and oxidized DsbB leads to rapid equilibration of the reaction components involved.

The use of increased concentrations of reduced DsbA did not result in the production of the completely reduced species of DsbB[ΔQ] (Fig. 5D, lanes 5–7). It is thus inferred that the half-oxidized forms of DsbB, the products initially formed, are no longer active in oxidizing DsbA effectively. The reaction shown in Fig. 4D, lanes 5–7, and that shown in Fig. 5D, lanes 5–7, were symmetrical with respect to the redox states of DsbA versus DsbB[ΔQ] with the same numbers of cysteine residues involved. The distributions of the final products in these two experiments were very similar, consistent with their equilibrated states. Thus, despite the lower redox potentials of DsbB cysteines as determined in the DTT redox buffer, DsbB can equilibrate with reduced DsbA to oxidize it to a significant extent even in the absence of quinone molecules.

**Two Hemi-oxidized DsbB Species Equilibrate with Each Other in the Absence of Quinones**—In the above analyses, we observed the generation of the two partially oxidized/reduced species having different SDS-PAGE mobilities (bands 1 and 2 in Figs. 3–5). We determined identities of these protein species by mass spectrometry. Proteins extracted from bands 1, 2, and 3 regions of the gel in Fig. 5D were subjected to digestions with trypsin and lysyl endopeptidase under non-reducing conditions and then to MALDI-TOF mass spectrometry (Fig. 6). The mass peaks that coincided with expected cysteine-containing DsbB peptides are highlighted in red in Fig. 6 and these peptide fragments are compiled and identified as shown in TABLE ONE. The 1095.5-Da peak, observed commonly with band 1 and band 2, corresponded to the Pro40–Arg48 fragment, in which Cys41 and Cys44 had not been AMS-modified (Fig. 6A). Thus, this fragment should have contained the internal Cys41–Cys44 disulfide bond. This 1095.5-Da fragment was greatly diminished for band 2, being consistent with a notion that Cys43 and Cys46 in band 2 had mostly been reduced. Although we did not detect a peak that corresponded to the Pro40–Arg44 fragment having two cysteines alkylated with AMS of 510 Da (22), this failure might have been due to an ionization/driftling interference by the two closely positioned anionic AMS moieties. Instead, the band 2 was found to retain the Cys104–Cys130 disulfide bond; both band 2 and band 3 but not band 1 generated a prominent peak of 4711.3 Da (Fig. 6C), corresponding to the complex of two peptides, Gly84–Arg109 and Trp119–Arg133, that were linked by the Cys104–Cys130 disulfide bond. Although all the three bands contained the individual Gly84–Arg109 (3049.38 Da) and Trp119–Arg133 (1663.80) peptides as well (Table I), we assume that significant portions of these peptides were generated by MALDI-induced cleavages of disulfide and AMS-Cys bonds (17).

Band 1 was suggested to have reduced Cys104 and Cys130, for the following reasons. First, it never generated the 4711.3-Da fragment discussed above. Secondly, it generated two unique peaks of 2174.8 Da and 1663.8 Da.
3559.3 Da (Fig. 6, B and C), which correspond to the AMS-modified forms of Trp^{115}–Arg^{133} (containing Cys^{130}) and Gly^{84}–Arg^{139} (containing Cys^{134}), respectively. Taken all together, we conclude that band 1 and band 2 represent hemi-oxidized forms of DsbB containing, respectively, the Cys^{41}–Cys^{44} and the Cys^{104}–Cys^{130} disulfide bonds.

As expected, band 3 is the fully oxidized form of DsbB. We have not detected a DsbB species having the Cys^{41}–Cys^{130} interloop disulfide bond in these experiments. We believe that this disulfide bond did exist in the DsbA-DsbB disulfide complex that was visible in small amount throughout the reaction (Fig. 5D and Refs. 14 and 20), but not in the more abundantly produced partially reduced DsbB species. In any case, the partially reduced DsbB represented hemi-oxidized species having either of the constitutive disulfide bonds in DsbB. These results and the results shown in Figs. 4 and 5 suggest that reduced DsbA is first oxidized by Cys^{104}–Cys^{130} to produce band 1 species, which is equilibrated immediately with band 2 species.

**DISCUSSION**

In this work we succeeded in preparing quinone-free DsbB from cells incapable of synthesizing UQ and MK, but not by the detergent washing procedures reported by Grauschopf et al. (13). In contrast to their results, our UQ-free DsbB preparations did not show fluorescence increase upon reduction with DTT. The results of our fluorescence measurements revealed that the DTT-dependent increase is a characteristic of DsbB that bears UQ. Thus, DsbB(DQ) exhibited the higher level of tryptophan fluorescence independently of DTT, suggesting that UQ is responsible for the decreased fluorescence observed for the “oxidized” DsbB(UQ8); UQ actually quenched the fluorescence intensity of DsbB(DQ) when supplemented to it.

The low level fluorescence observed with the SSCC variant of DsbB(UQ8) even in the presence of DTT implies that UQ8 remained oxidized and associated with DsbB in the absence of its Cys^{41} and Cys^{44}. We suggest that rapid reduction of UQ8 on DsbB is mediated by the Cys^{41}/Cys^{44} pair of cysteines. In other words, DTT can be oxidized effectively by the quinone-coupled reactivity of DsbB Cys^{41}–Cys^{44}, in agreement with our earlier contentions that Cys^{41}–Cys^{44} is kept oxidized strongly by respiratory components of the cell and that DsbB can be regarded as a respiration-coupled oxidase acting against DTT (10). The strongly oxidizing redox potential value, ~−69 mV, estimated by Grauschopf et al. (13) must be regarded as that of the Cys^{41}/Cys^{44}-coupled UQ8 on DsbB. It is notable that this value appeared to be only obtained by the spectroscopic measurement. The above reevaluation nicely explains the physiological reactivity of the DsbB-UQ complex.

In this work, we determined parameters of the redox equilibrium between DsbB(DQ) and DTT, using the AMS gel shift assay. The redox potentials thus determined fall within the similar range as those obtained previously for the DsbB(UQ8)-cysteine redox equilibrium. These results reconfirm that both Cys^{41}–Cys^{44} and Cys^{104}–Cys^{130} of DsbB are much less oxidizing than Cys^{30}–Cys^{33} of DsbA. This was not caused by the use of variants lacking either pair of cysteines, since wild type DsbB(DQ) also underwent the redox equilibrium generating the two hemi-oxidized species at similar DTTred/DTTox ratios as the variants lacking either cysteine pair underwent oxidation/reduction equilibrium.

To our surprise, DsbB(DQ) was still capable of oxidizing ~40% of DsbA in a 1:1 reaction. Two species of partially reduced DsbB were consequently generated, which were identified by the PMF analyses to have either of the Cys^{41}–Cys^{44} and Cys^{104}–Cys^{130} disulfide bonds. These two species are rapidly generated and populated almost equally for prolonged time. This is consistent both with the notion that they rapidly equilibrate with each other and with the similar redox potentials that we determined for Cys^{31}–Cys^{44} and Cys^{104}–Cys^{130}. What property of DsbB might account for its ability to oxidize much more oxidizing DsbA molecules? It might be possible that the redox potential values...
obtained using the small molecule redox buffer (DTTred/DTTox or cysteine/cystine) do not represent the situation, in which DsbB interacts specifically with DsbA. One obvious difference between DTT and DsbA is the fact that the former will access non-selectively to both of Cys41–Cys44 and Cys104–Cys130 but the latter will interact specifically with Cys104–Cys130 of DsbB (11–13). As suggested by the rapid intramolecular equilibration of the cysteine pairs we observed, the other disulfide (Cys41–Cys44) could then serve as another two electron acceptor through equilibration with Cys104–Cys130.

Moreover, the specific association between DsbA and DsbB may induce local conformational modulations of the complex, thereby affecting their intrinsic redox properties in the direction that facilitates the electron flow from DsbA to DsbB. Recently, Sevier et al. (23) discussed that the region around the Cys104–Cys130 cysteine pair in DsbB might be unstructured and flexible and thereby recognizable by the substrate binding site of DsbA. Such a property is well fitted to our proposal of DsbA-induced conformational and redox modulations of DsbB. The functional importance of the second periplasmic domain in DsbB is also suggested by the low but appreciable level of formation of oxidized DsbA and reduced DsbB[SSCC] upon equilibrium of DsbB[SSCC](ΔQ) with DsbA (Figs. 4A and 5A).

In effect, the equilibrium state we observed between DsbB(ΔQ) and DsbA in the Fig. 4 and the Fig. 5 experiments represent the "physiological redox potential" of DsbB, in which the partner molecule, DsbA rather than small molecule DTT or cysteine, participate as an essential player. Although detailed structural information on DsbB and its complex with DsbA is required to obtain any definitive solution to the paradox of the DsbB-DsbA reaction that overcomes the apparent redox potential barrier, such reactivity should have been made possible by the design of nature that allows the structural arrangements for cooperative actions of the six essential cysteines that participate in this system. A preferential route of electron flow could be created by quaternary association of protein components (24) and this could well be accompanied by altered redox properties of individual components.

We have shown previously that DsbA oxidation by DsbB(UQ8) or by DsbB(MK8) can follow either of two parallel pathways, fast and slow (Refs. 6 and 14; see Fig. 7). A major fraction of the 1:1 reaction between reduced DsbA and oxidized DsbB(ΔQ) followed the rapid pathway, in which oxidized DsbA and two species of hemi-oxidized DsbB, having either of Cys41–Cys44 and Cys104–Cys130, were generated. Notably, DsbB was never reduced fully even when substantial fraction of DsbA remained in the reduced form in the 2:1 reaction. Thus, neither of the

FIGURE 6. Peptide mass fingerprints of the DsbB species produced after redox equilibrium with DsbA. Bands 1–3 in the Fig. 5D experiment were subjected to lysyl endopeptidase and trypsin digestions and MALDI-TOF mass analysis. Spectra are shown for mass (m/z) ranges of 960–1170 (A), 1570–2350 (B), and 3000–4800 (C). Cysteine-containing peptides are highlighted in red. See also TABLE ONE and text for the peak identities.
hemi-oxidized DsbB species is able to oxidize DsbA. These results, taken together, suggest that the fast reaction requires the fully oxidized initial state of DsbB and may proceed by direct and unidirectional thiol transfer from the Cys30/Cys33 pair of DsbA to the Cys104–Cys130 disulfide that is followed by reversible thiol-disulfide exchange between Cys104–Cys130 and Cys41–Cys44 (21). In this context, similar stability of these disulfides (after separation from DsbA) would be an element important for the reactivity of DsbB. In the normal quinone-coupled situation, however, the equilibrium will never be achieved because of constant quinone-mediated oxidation of the Cys41–Cys44 pair, driving the reaction constantly forward (Fig. 7, upper).

The slow pathway of DsbA oxidation proceeds through the disulfide-linked DsbA–DsbB complex, which resolves slowly. DsbB(H9004Q) is shown in this study to be able to undergo both pathways up to the points (hemi-oxidized states or the intermolecular complex) after which quinones become essential. Note that under physiological conditions UQ (or MK) is constitutively associated with DsbB presumably in a 1:1 ratio, until it is reduced to ubiquinol (or menaquinol). The end results in the quinone-coupled reactions are the regeneration of both DsbA and DsbB as their oxidized and active forms. The commitment steps for the two pathways are proposed to be in the nucleophilic attacks (shown by red broken arrows) by Cys33 of DsbA (rapid pathway) and by Cys41 of DsbB (slow pathway) of respective target cysteines (DsbA Cys30 and DsbB Cys41, respectively). See “Discussion” for more details. Note that DsbB-bound quinones in the slow pathway assume the electronic transition states that are induced by reduced Cys44 (Refs. 6 and 14; shown in pink). The rapid pathway may also involve a brief quinone transition (6).

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**TABLE ONE**

DsbB-derived peptide ions in PMF analysis of the DsbB species produced after equilibration with DsbA

| Peptides | Measured mass m/z | Expected mass m/z |
|----------|-------------------|-------------------|
| Phe4–Arg12 | 1020.54 | 1020.52 |
| Phe109–Lys118 | 1144.61 | 1144.60 |
| Tyr78–Arg93 | 1618.84 | 1618.84 |
| Trp117–Arg132 | 1663.80 | 1663.79 |
| Val150–Lys40 | 1681.04 | 1681.04 |
| Gly84–Arg109 | 3049.38 | 3049.57 |
| Pro100–Arg109 | 1095.52 | 1095.53 |
| Complex of Gly84–Arg109 & Trp117–Arg132 | 4711.37 | 4711.43 |
| Observed only with band 1 | | |
| Trp117–Arg132 | 2174.84 | 2174.00 |
| Gly84–Arg109 | 3559.32 | 3558.06 |

a Having the Cys41–Cys44 disulfide bond.
b Linked by the Cys30–Cys130 disulfide bond.
c Cys130 modified with AMS.
d Cys104 modified with AMS.

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**FIGURE 7. Two reaction pathways in DsbB-catalyzed DsbA oxidation.** The rapid pathway proceeds through direct disulfide exchanges between the active site cysteines, while the slow pathway proceeds through the disulfide-linked DsbA–DsbB complex, which resolves slowly. DsbB(H9004Q) is shown in this study to be able to undergo both pathways up to the points (hemi-oxidized states or the intermolecular complex) after which quinones become essential. Note that under physiological conditions UQ (or MK) is constitutively associated with DsbB presumably in a 1:1 ratio, until it is reduced to ubiquinol (or menaquinol). The end results in the quinone-coupled reactions are the regeneration of both DsbA and DsbB as their oxidized and active forms. The commitment steps for the two pathways are proposed to be in the nucleophilic attacks (shown by red broken arrows) by Cys33 of DsbA (rapid pathway) and by Cys41 of DsbB (slow pathway) of respective target cysteines (DsbA Cys30 and DsbB Cys41, respectively). See “Discussion” for more details. Note that DsbB-bound quinones in the slow pathway assume the electronic transition states that are induced by reduced Cys44 (Refs. 6 and 14; shown in pink). The rapid pathway may also involve a brief quinone transition (6).
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quinone reduction. This slow reaction is initiated by the formation of the intermolecular Cys104–Cys30 bond between DsbB and DsBA, resulting in the liberation of reduced Cys44, which in turn triggers a disulfide rearrangement within DsbB to form a Cys41–Cys130 interloop disulfide (20) and consequent reduction of Cys44. Furthermore, the reduced Cys44 induces the electronic transition of bound UQ, leading to the resolution of the Cys104–Cys30 bond, regeneration of all the disulfide bonds in this system and reduction of UQ (14). We showed here that the redox potential, and thus the stability, of the Cys41–Cys130 disulfide is comparable to that of Cys104–Cys130 (Fig. 3). This feature might enable the formation of the DsBA-DsbB disulfide without being attacked backward by Cys130, which is now engaged in the disulfide-linkage with Cys41. Stability of Cys130 might have been tuned within a certain range, which is compatible both with the above-mentioned “ratchet” function and with the unidirectional resolution that reinitializes the system. We believe that the completion of a cycle in this mode of reaction is coupled with the transition and electron acceptance by UQ (Fig. 7, lower). This is consistent with our present analysis using quinone-free DsbB, in which the DsBA-DsbB complex remained unresolved.

Interesting questions that remain are why this system has two alternative pathways and how they are selected. We believe that both modes of reactions are initiated by a nucleophilic attack of the Cys104–Cys30 disulfide by Cys44 of DsBA, which is known to have unusual low pKa value and to be hyper-reactive (25). This leads to the formation of the DsBA (Cys33)-DsbB (Cys44) complex and reduction of DsBA Cys33 and DsbB Cys130. Our results indicate that this event occurs without quinones. Although Cys33 is known to be of low reactivity (25), reactivity of Cys130 of DsbB is unknown. We suggest that the fate of the DsBA-DsbB complex will be determined by which of the following events takes place first; (i) Cys44 of DsBA attacks the Cys30–Cys44 intermolecular disulfide to form the Cys30–Cys130 disulfide of DsBA and to reduce Cys104 and Cys130, or (ii) Cys30 attacks the Cys41–Cys44 disulfide to form the interloop Cys41–Cys130 disulfide and to reduce Cys44. Obviously, case (i) will lead to the rapid pathway (Fig. 7, upper). Case (ii) will result in the slow pathway of DsBA oxidation, in which the DsBA-DsbB complex persists until resolved by the concerted actions of quinone-Cys44 conjugate and Cys33 (Fig. 7, lower). Assuming that DsBA Cys33 and DsbB Cys44 are geometrically separated, the resolution process must be complex such that it involves multiple disulfide rearrangements and proceeds slowly. At any event, the choice between the slow and the rapid pathways will depend on the race between Cys33 and Cys130 with respect to their ability of attacking nucleophilically the respective target disulfides (Fig. 7).

Our present results using the quinone-free reaction components indicate that the rapid pathway predominates. Probably, Cys33 is still more reactive than Cys130. While a simplest scenario would be that the choice is stochastic, another possibility is that there is some regulatory mechanism that affects the pathway selection according to certain physiological variations. For instance, if Cys33 of DsBA is somehow occluded then the alternative slow pathway becomes in operation for slow but more directly quinone-driven resolution of the complex. Such a mechanism might be useful to effectively recycle DsbB by the powered clearing of disabled DsBA molecules.

We have revealed that DsbB is not intrinsically more oxidizing than DsBA. Otherwise, DsbB itself might be channeled to oxidize diverse substrates. Instead, nature’s design might have been to preserve DsbB of limited quantity in the membrane as a DsBA-specific oxidant. Multiple devices are installed into this system to enable DsbB to oxidize even more oxidizing molecules of DsBA. First, as suggested here, specific interaction between DsbB and DsBA transforms redox properties of the cysteine residues involved such that the DsbB Cys104–Cys130 disulfide can be exchanged directly with the Cys276/Cys333 disulfide of DsBA. Second, the resulting hemi-oxidized DsbB loses the reactivity with DsBA, thus endowing the directionality in the exchange reaction. Third, the above process is followed by rapid intramolecular disulfide equilibrium enabling rapid recycling of the system in the presence of quinones that oxidizes the Cys41–Cys44 disulfide bond. Fourth, the alternative slow pathway enables slow but directly quinone-coupled reaction, possibly as a failsafe mechanism. Finally and most importantly, DsbB is coupled with quinone molecules that provide the oxidizing power to cycle the catalytic turnover. Strikingly, quinone seems to act by more than one mechanism to ensure the above objectives. Although our analyses of quinone-free DsbB yielded a number of important predictions as stated above, they must be examined by structural determination of DsbB as well as its complex with DsBA.

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