DsB and DsbB are responsible for disulfide bond formation. DsbA is the direct donor of disulfides, and DsbB oxidizes DsbA. DsbB has the unique ability to generate disulfides by quinone reduction. It is thought that DsbB oxidizes DsbA via thiol disulfide exchange. In this mechanism, a disulfide is formed across the N-terminal pair of cysteines (Cys-41/Cys-44) in DsbB by quinone reduction. This disulfide is then transferred on to the second pair of cysteine residues in DsbB (Cys-104/Cys-130) and then finally transferred to DsbA. We have shown here that the redox potential of the two disulfides in DsbB are −271 and −284 mV, respectively, and considerably less oxidizing than the disulfide of DsbA at −120 mV. In addition, we have found the Cys-104/Cys-130 disulfide of DsbB to actually be a substrate for DsbA in vitro. These findings indicate that the disulfides in DsbB are unsuitable to function as the oxidant of DsbA. Furthermore, we have shown that mutants in DsbB that lack either pair or all of its cysteines are also capable of oxidizing DsbA. These unexpected findings raise the possibility that the oxidation of DsbA by DsbB does not occur via thiol disulfide exchange as is widely assumed but rather, directly via quinone reduction.

The formation of disulfide bonds is a key step in oxidative protein folding. In Escherichia coli, DsbA and DsbB catalyze the formation of disulfides (Dsb stands for DiSulfide Bond) (1). Mutations in either DsbA or DsbB lead to a severe defect in the oxidative folding of many secreted bacterial proteins as well as eukaryotic proteins expressed in the periplasm (2). DsbA is a small periplasmic protein, which possesses an active site disulfide that rapidly reacts with unfolded proteins entering the periplasm, resulting in their oxidation. The disulfide in DsbA is very oxidizing with a redox potential of −120 mV (3). DsbA serves as a powerful relatively nonspecific oxidant. It has the potential of forming incorrect disulfides in proteins that possess more than two cysteines. These miss-oxidation events are thought to be resolved by the disulfide isomerase activity of two other periplasmic disulfide oxidoreductases, DsbC and DsbG.

To be active as a catalyst, DsbA needs to be reoxidized. This is accomplished by an inner-membrane protein called DsbB. The novel catalytic activity of DsbB allows it to reoxidize DsbA by using the oxidizing power of the electron transport system (4–6). Under aerobic conditions, DsbB reoxidizes DsbA and passes the electrons to ubiquinone. From ubiquinone, the electrons are passed on to cytochrome oxidases and then on to molecular oxygen (4, 7). Under anaerobic conditions, electrons are passed to menaquinone and then on to anaerobic electron acceptors. Thus, DsbB plays a central role in disulfide bond formation, because it uses the oxidizing power of quinones to generate disulfides de novo (4, 7).

DsbB is a 21-kDa inner-membrane protein with four transmembrane segments and two periplasmic loops (8). Each loop contains a pair of cysteines, with each pair thought to form a disulfide bond (Fig. 1). DsbB has also been shown to directly bind equimolar quantities of quinone with at least one high affinity-binding site (7).

DsbB has the unique ability to generate disulfides via quinone reduction. The overall reaction scheme is shown below (Scheme 1). Because this is the original source of disulfides in E. coli, identifying the mechanism of DsbB action is important for understanding disulfide bond formation and oxidative protein folding in vivo. A three-step model for the mechanism of DsbB has been proposed, which is depicted in Fig. 2 (5). First, DsbA is directly reoxidized by the C-terminal disulfide (Cys-104/Cys-130) in DsbB. Thus, DsbA is released in an oxidized state and the Cys-104/Cys-130 disulfide is now in a reduced state. In the second step, the Cys-104/Cys-130 disulfide is reoxidized by the disulfide of the first domain (Cys-41/Cys-44), leaving the Cys-104/Cys-130 disulfide in an oxidized state while Cys-41/Cys-44 is in a reduced state. Finally, the Cys-41/Cys-44 cysteine pair is reoxidized by quinone. The disulfide exchange portion of this model was originally proposed by Kishigami and Ito (5). It is primarily supported by the following three findings: 1) When Cys-33 of DsbA is replaced by a serine, a mixed disulfide between Cys-104 of DsbB and Cys-30 of DsbA accumulates (5, 9, 10). This mixed disulfide was assumed to represent a reaction intermediate stabilized by the DsbAC33S mutation, which led the authors to conclude that the Cys-104/Cys-130 disulfide directly reoxidizes DsbA. The actual reoxidation of DsbA by the Cys-104/Cys-130 disulfide was never directly observed (9, 11, 12). 2) The Cys-41/Cys-44 disulfide in DsbB appears to be required for the formation of the Cys-104/Cys-130 disulfide. When a mutant of DsbB in which the Cys-41 and Cys-44 residues have been substituted by serine residues is expressed, the Cys-104/Cys-130 disulfide is found in a reduced state in vivo. In contrast, wild type DsbB is found in a fully oxidized form with both disulfides formed after in vivo thiol trapping (11). From these experiments the authors concluded that the Cys-41/Cys-44 disulfide directly oxidizes the Cys-104/Cys-130 disulfide. This again, has not yet been directly observed (11, 12). 3) The Cys-41/Cys-44 disulfide in DsbB is
resistant to DTT-mediated reduction in vivo as long as electron transport is not impaired (11). From these results it was concluded that the electron transport chain causes the oxidation of the Cys-41/Cys-44 pair of cysteines, presumably directly via quinone. The overall mechanism depicted in Fig. 2 thus involves the formation of a disulfide in the first periplasmic domain via components of the electron transport pathway. This disulfide is then passed on to the cysteines in the second periplasmic domain and then on to DsbA.

Kadokura and Beckwith (12) recently investigated the mechanism of DsbB and propose a more complicated model of disulfide exchange within DsbB that is different from the model suggested by Ito and coworkers. To obtain this model, the authors split DsbB into two fragments (α and β), each fragment containing one pair of cysteines. DsbB-α contains the Cys-41/Cys-44 pair and DsbB-β contains the Cys-104/Cys-130 pair. When coexpressed, these two fragments can reconstitute DsbB activity in vivo. The authors isolated a disulfide-bonded ternary complex between DsbA, DsbB-β, and DsbB-α. These results could not be explained by the original model and led the authors to propose that the Cys-104/Cys-130 disulfide does not directly reoxidize DsbA itself, but cooperates with the Cys-41/Cys-44 disulfide in a more complex mechanism of thiol disulfide exchange that results in the oxidation of DsbA. These results suggest that the originally proposed model of DsbB function needs to be revisited.

Key to both models is the assumption that the stable mixed disulfide bond that can be observed between the DsbACys-33 mutant and DsbB (9, 10), and between DsbB-α and DsbB-β (12), represent normal intermediates in the reaction mechanism. However, reaction intermediates generally are highly unstable. Therefore, the more stable the mixed disulfide is, the less likely it is that it represents a real intermediate (13). When studying disulfide exchange reactions within a multicysteine protein, removal of one cysteine may not completely block disulfide exchange, leading to the accumulation of the intermediate normally present prior to the block. Instead, the unstable reaction intermediates have the tendency to rapidly rearrange to the most stable configurations. Work on the folding pathway of bovine pancreatic trypsin inhibitor has shown that so called “reaction intermediates” can as easily be products that lie off the folding pathway as real intermediates (13, 14).

In addition, if DsbB functions via a single obligatory disulfide exchange pathway, then removal of any one cysteine within DsbB should inactivate the pathway. Although the cysteines within DsbB are clearly important for the activity of DsbB, they appear not to be absolutely essential. Jander et al. (1994) carefully analyzed the phenotype of strains that contain mutations in cysteines of DsbB (8). They noted that strains containing mutations in Cys-44 or Cys-104 show a greater defect in disulfide bond formation in vivo than strains containing mutations in Cys-41 or Cys-130. Additionally, Kishigami and Ito report that in vivo, residues Cys-41 and Cys-44 of DsbB appear to be “dispensable to a certain extent” (5). Because none of the cysteines in DsbB appear to be absolutely essential, it now seems prudent to consider roles for these cysteines other than in direct disulfide exchange with DsbA. They could be involved in structural disulfides, metal, or other cofactor binding sites or in changing the reactivity of the quinone bond to DsbB or in the ability of DsbB to exchange oxidized quinone for reduced quinone. Cysteines, of course, have many functions within proteins apart from being involved in catalytic disulfides. Methanol dehydrogenase from Methyllobacterium is a quinone binding protein that contains a disulfide near the active site that remarkably, like DsbB, is formed between two adjacent cysteines. This disulfide, although important for the catalytic activity of the enzyme, “does not appear to function as a redox component of the mechanism. Rather it may function in the stabilization or protection of the free radical semiquinone form of the prosthetic group from solvent at the entrance to the active site.” (15) These considerations and our desire to understand the mechanism of DsbB, which is central to the creation of disulfide bonds in the cell, made it clear that further study of this reaction mechanism is necessary. The results presented here suggest that DsbB can directly form disulfides in DsbA de novo in a reaction that does not depend upon direct thiol disulfide exchange.

EXPERIMENTAL PROCEDURES

Protein Purification and Preparation—All proteins were purified as described previously (4, 7). In brief, DsbA was purified from periplasmic extracts of the DsbA-overproducing strain JCB607 via anion exchange chromatography using a 5-ml HiTrap Q-Sepharose HP column. Periplasmic extracts were loaded onto the column equilibrated with 10 m M MOPS/NaOH, pH 7.0, and DsbA was eluted using a 500-ml gradient from 10 m M MOPS/NaOH, pH 7.0, to 10 m M MOPS/NaOH, 500 m M NaCl, pH 7.0. Fractions containing DsbA were reduced with 20 m M DTT, pooled, and dialyzed against 2 × 4 liters of 0.1 m M EDTA. The DsbA protein was then concentrated, aliquoted, and stored at −80 °C. Mutant DsbB derivatives that just contain the Cys-41/Cys-44 cysteine pair where the other cysteines are substituted with serine (abbreviated DsbB[CCSS] or a DsbB[SSCC] derivative that just contains the Cys-104/Cys-130 pair and a cysteine free derivative of DsbB, called DsbB[SSSS], 32707

![Fig. 1. Predicted membrane topology of DsbB. DsbB is a 20-kDa inner membrane protein that is predicted to have four transmembrane helices and two periplasmic loops, with each loop containing a disulfide that is required for catalytic activity (4, 5, 8–10). In addition, DsbB has been shown to bind quinone with at least one high affinity-binding site (21, 22). Residues that are believed to be involved in quinone binding are from both periplasmic loops and are shaded gray. DsbB also has two additional non-essential, non-conserved cysteines that have been eliminated by substitution as shown to prevent aggregation during purification (4).](image)
DsB Catalyzes Disulfide Bond Formation de Novo

Fig. 2. Current model for the mechanism of DsbB. DsbB maintains DsbA in an oxidized state in vivo via its novel ability to generate disulfides in DsbA by reducing quinones. A three-step model for the mechanism of DsbB has been proposed and is depicted here (5). 1, reduced DsbA is reoxidized by the Cys-104/Cys-130 disulfide of DsbB, releasing DsbA in an oxidized state and the Cys-104/Cys-130 disulfide of DsbB in a reduced state; 2, the Cys-104/Cys-130 disulfide is then reoxidized by the Cys-41/Cys-44 disulfide, releasing the Cys-104/Cys-130 disulfide in an oxidized state and the Cys-41/Cys-44 in a reduced state; 3, finally, the Cys-41/Cys-44 disulfide is regenerated via quinone reduction. The arrows show the direction of electron flow, which is opposite that of disulfide flow.

have been previously described (4). His-tagged DsbB(CCSS), DsbB(SSCC), and DsbB(SSSS) were purified like His-tagged DsbB according to Bader et al. (4, 7).

AMS/Gel-shift Experiments—Components of the DsbA/DsbB pathway were incubated at room temperature in 50 mM sodium phosphate, 300 mM NaCl, 0.5 mM EDTA, 0.1% n-dodecyl-β-D-maltoside for the specified time periods. The proteins were then precipitated with 12% trichloroacetic acid that contained a 40 μg/ml bovine serum albumin carrier and pelleted at 15,000 rpm in a Taylor Scientific 162/4VS microcentrifuge 20 min at 4 °C. Pellets were then washed with acetone and modified with an AMS trap solution (10 mM 4-acetamido-4'-maleimidystilbene-2,2'-disulfonic acid (AMS), 100 mM Tris, 1 mM EDTA, pH 7.7) as described previously (16, 17). The proteins were then subjected to non-reducing 14% Tris-glycine SDS-PAGE (Novex) and visualized by a rapid Coomassie Blue stain (18). When required, the DsbB or DsbA proteins were reduced with 25 mM DTT on ice for 30 min. The DTT was then removed via gel filtration using Amersham Biosciences PD10 columns.

Redox Potential Measurements—To determine the redox potential of our mutants, purified DsbB was incubated with mixtures of oxidized and reduced DTT. To establish that these reactions were at equilibrium, the incubation reactions were performed for both 1 and 15 h and showed identical results. Samples were precipitated with trichloroacetic acid (AMS), the incubation reactions were performed for both 1 and 15 h and showed identical results. Samples were precipitated with trichloroacetic acid and modified with AMS as described above. Quantification of band intensities was performed using National Institutes of Health IMAGE 1.62. The curves were generated using SigmaPlot. The redox potentials were calculated from the equilibrium constant with DTT as previously described (19).

RESULTS

Predictions of the Thiol-disulfide Model—The model for the mechanism of DsbB that is shown in Fig. 2 (5) makes a number of specific predictions about the reactivity of the various components of the pathway when mixed and about the redox properties of the cysteine pairs in DsbB. First, when oxidized quinone is mixed with DsbB, it should directly oxidize the Cys-41/Cys-44 cysteine pair. This disulfide can then be transferred to the Cys-104/Cys-130 cysteine pair. Second, DsbB variants that contain only the Cys-104/Cys-130 disulfide should be sufficient to oxidize DsbA in a stoichiometric fashion. In addition, mutants in DsbB lacking the Cys-104/Cys-130 pair of cysteines or all of the cysteines should be inactive in oxidizing DsbA, even in a stoichiometric fashion. Third, the Cys-41/Cys-44 and the Cys-104/Cys-130 disulfides formed within DsbB should both be substantially more oxidizing than the active site disulfide of DsbA (~120 mV) and less oxidizing than ubiquinone (+110 mV). Given that electron transport occurs down an electrochemical gradient, one should be able to rank the redox potentials of these components from the most oxidizing to the least oxidizing in the order: ubiquinone → Cys-41/Cys-44 (DebB) → Cys-104/Cys-130 (DsbB) → Cys-104/Cys-130 (DsbB) → DsbA. Finally, DsbA should be unable to oxidize DsbB.

Which Disulfide in DsbB Is Oxidized by Quinone?—When DsbB reoxidizes DsbA, it passes the electrons directly on to quinone as part of its catalytic cycle (4, 7). We began our investigation into the mechanism of DsbB by seeking to determine which disulfide bond is directly formed by oxidized quinone. To do this, we used AMS/gel-shift experiments with a mutant of DsbB that only contained the first pair of cysteines (Cys-41/Cys-44), the second pair (Cys-104/Cys-130) were substituted with serines (abbreviated DsbB(CCSS)) or with the mutant DsbB(SSCC) that only contained the second pair of cysteines (Cys-104/Cys-130). Both of these proteins as well as the cysteine-free mutant DsbB(SSSS) used later, contained 50–70% quinone bound after purification, similar to the amount found to be bound to wild type DsbB. Because residues from both the first and second periplasmic domains of DsbB have been shown to be involved in quinone interaction (20–22), the observed retention of normal quinone content suggests that these mutations have not globally disrupted the fold of DsbB.

DsbB(CCSS)red and DsbB(SSCC)red were incubated at room temperature in the absence of oxidized quinone. The reaction was stopped by trichloroacetic acid precipitation followed by 4-acetamido-4'-maleimidystilbene-2,2'-disulfonic acid (AMS), modification. AMS is a maleimide derivative that specifically, rapidly, and irreversibly alkylates free thiols by the addition of a ~0.5-kDa moiety per thiol group (17). The resulting increase in mass of the modified protein can easily be visualized on non-reducing SDS-PAGE gels. This technique allows one to follow the redox status of a thiol-containing protein. Using this technique we found that quinone is able to oxidize the Cys-41/Cys-44 disulfide in DsbB and was unable to interact with the Cys-104/Cys-130 cysteine pair.

To show that the oxidation of the Cys-41/Cys-44 disulfide pair is not due to air oxidation, we incubated 10 μM DsbB(CCSS)red and DsbB(SSCC)red in the absence of quinone for either 10 min (Fig. 3a, lanes 2 and 3 and Fig. 3b, lanes 2 and 3), and up to 60 min (data not shown) and we observed no spontaneous oxidation. A 2-fold molar excess of oxidized quinone (20 μM of Q0C10) added to DsbB(CCSS) oxidized the protein completely within less than 1 min (Fig. 3a). In contrast DsbB(SSCC) remained reduced after 10 min (Fig. 3b) or even after 60 min (data not shown) in the presence of oxidized quinone.

These results show that the Cys-41/Cys-44 motif in DsbB is...
the oxidation of both disulfide bonds within 1 min of incubation (Fig. 3c). Because both disulfides of wild type DsbB are formed when quinone is added and because we showed previously that only the Cys-41/Cys-44 disulfide of DsbB is directly oxidized by quinone (Fig. 3, a and b), we conclude that the Cys-41/Cys-44 disulfide needs to be formed to allow for the oxidation of the Cys-104/Cys-130 disulfide. The simplest way that this could happen is for the Cys-41/Cys-44 disulfide to be transferred directly to the second pair of cysteines in DsbB, thereby oxidizing them. We favor this interpretation of the data, although it is conceivable that the formation of the first disulfide increases the reactivity of the quinone bound to DsbB in such a way that the quinone is then able to also directly oxidize the Cys-104/Cys-130 disulfide.

Our results show that \( Q_0C_{10} \) is able to completely oxidize wild type DsbB, despite the fact that only the Cys-41/Cys-44 cysteine pair of reduced DsbB can be directly oxidized by quinone. Our finding, that quinone is able to oxidize Cys-41/Cys-44, which in turn is capable of oxidizing the Cys-104/Cys-130 disulfide, shows that DsbB can self-oxidize and is consistent with the model for DsbB action as shown in Fig. 2.

Which Disulfide in DsbB Is Capable of Oxidizing DsbA?—Next we wanted to determine which disulfide in DsbB is capable of oxidizing DsbA. We incubated 20 \( \mu M \) DsbA\( ^{red} \) with either 10 \( \mu M \) DsbB\[CCSS\] (wild type) or the same cysteine pair mutants DsbB[CCSS]\^{ox} or DsbB[SSCC]\^{ox}, and the cysteine-less mutant DsbB[SSSS] in the absence of added quinone, and followed the redox status of both DsbA and DsbB over time. We observed that wild type DsbB and all mutants of DsbB, including the cysteine-free variant, are able to oxidize DsbA\( ^{red} \) (Fig. 4, a–d). This shows that the cysteines in DsbB are not essential for its ability to oxidize DsbA. DsbA, which was added in an approximate 2-fold excess over DsbB, was only partially oxidized, suggesting that DsbB is acting in a stoichiometric fashion. To confirm that this was a stoichiometric process and not a very slow catalytic process, we simply reduced the amount of DsbB 5-fold to 2 \( \mu M \) but incubated five times longer. Under these conditions, DsbA was only partially oxidized and the amount did not increase over time. This indicated that, in the absence of added quinone, wild type DsbB as well as all the cysteine mutants act in a stoichiometric fashion (Fig. 4e). This oxidation of DsbA cannot be due to disulfide transfer between DsbB and DsbA, because the disulfide bonds of wild type DsbB and the CSSS and SSCC mutants of DsbB remain in an oxidized state throughout the time-course. In addition, the cysteine-free mutant of DsbB was at least as capable as wild type DsbB in oxidizing DsbA. If it is not the disulfides in DsbB that oxidize DsbA, then what is? We considered three possibilities: (a) a transition metal contaminant or other small molecule present in the solution oxidizes DsbA, (b) more interestingly, that the quinone bound to DsbB directly oxidizes DsbA in a stoichiometric fashion, or (c) that a small amount of chromosomally encoded wild type DsbB protein present in the DsbB mutant overproducing strains may have copurified with the DsbB mutants.

The first possibility seems unlikely since (a) EDTA was present in the incubation mixture, which should act to chelate free metals; (b) the oxidizing activity is heat-labile; and (c) DsbA is very slowly oxidized by metal-mediated air oxidation in a catalytic fashion (3). To test for the presence of other small molecules that might stoichiometrically oxidize DsbA, we concentrated the DsbB preparation, using a 10,000-Da cutoff Centricon spin column and showed that the material that flows through this spin column is completely inactive in DsbA oxidation (data not shown). We decided it was also important to test directly whether oxidized quinones, free in solution, could oxidize DsbA. So, we

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**Fig. 3.** Quinone is capable of rapidly oxidizing DsbB[CCSS] and DsbB[CCCC] but not DsbB[SSCC]. AMS/gel-shift experiments were used to analyze the oxidation of DsbB by quinone. DsbB[CCSS] (a), DsbB[SSCC] (b), and DsbB[CCCC] (c) were reduced with DTT, and the DTT was then removed via gel filtration. 10 \( \mu M \) of the reduced protein was then incubated in the absence of added quinone at room temperature for up to 10 min to assess the extent of spontaneous oxidation. To these reduced proteins we then added 20 \( \mu M \) oxidized \( Q_0C_{10} \) and followed their redox state over time. At the indicated time points, the proteins were precipitated with trichloroacetic acid, modified with AMS, and subjected to non-reducing SDS-PAGE. a, reduced DsbB[CCSS] did not show any spontaneous oxidation after 10 min of incubation (lane 3) but was rapidly oxidized by the addition of \( Q_0C_{10} \) (lanes 4–6). b, reduced DsbB[SSCC] did not show any spontaneous oxidation after 10 min (lanes 2 and 3) and remained reduced despite the addition of \( Q_0C_{10} \). c, reduced DsbB[CCCC] did not show any spontaneous oxidation after 10 min but was rapidly and completely oxidized upon addition of \( Q_0C_{10} \).
analyze the ability of DsbB[CCSS] (a), DsbB[CCCC] (b), DsbB[SSCC] (c), and DsbB[SSSS] (d) to oxidize DsbA. 20 μM reduced DsbA was incubated at room temperature for 60 min to assess the extent of spontaneous oxidation. 20 μM reduced DsbA was then incubated with 1 μM DsbB[CCSS] (a), DsbB[SSCC] (b), DsbB[CCCC] (c), and DsbB[SSSS] (d), and the redox states of DsbA and DsbB were monitored over time. All time points were obtained by trichloroacetic acid-precipitating the proteins, modifying with AMS, and subjecting them to non-reducing SDS-PAGE as before. The ratio of oxidized to reduced protein was then quantified using IMAGE 1.62 software. The data were plotted as shown in Fig. 5a, (c), and DsbB[SSSS] (d) to oxidize DsbA. 20 μM reduced DsbA was incubated at room temperature for 60 min to assess the extent of spontaneous oxidation. 20 μM reduced DsbA was then incubated with 10 μM DsbB[CCSS] (a), DsbB[SSCC] (b), DsbB[CCCC] (c), and DsbB[SSSS] (d), and the redox states of DsbA and DsbB were monitored over time. All time points were obtained by trichloroacetic acid-precipitating the proteins, modifying with AMS, and subjecting them to non-reducing SDS-PAGE as before. In all controls DsbA showed no spontaneous oxidation over time (a–d, lane 2). All of the forms of DsbB used here were able to oxidize DsbA (a–d, lanes 3–5). AMS/gel-shift experiments were then used to assess the extent of DsbA oxidation by the various forms of DsbB. c, 2 μM DsbB[CCSS], DsbB[SSCC], DsbB[CCCC], and DsbB[SSSS] was incubated with 20 μM reduced DsbA, and we monitored the redox state of DsbA over time using trichloroacetic acid precipitation, AMS modification, and non-reducing SDS-PAGE as before. The gels were then analyzed with IMAGE 1.62 (National Institutes of Health) to determine the ratio of oxidized DsbA over total DsbA. This was plotted against the time of incubation and showed that the DsbB is working stoichiometrically. f, 40 μM reduced DsbA was incubated in buffer containing 50 μM Q_{C10} for 60 min, and we observed no spontaneous oxidation (lane 1). 40 μM reduced DsbA was then incubated with 10 μM DsbB[CCSS] (lanes 3 and 4), DsbB[SSCC] (lanes 6 and 7), DsbB[CCCC] (lanes 9 and 10), and DsbB[SSSS] (lanes 12 and 13) with and without 50 μM Q_{C10} for 10 min. The ability of DsbB[CCSS] to oxidize DsbA is improved by the addition of Q_{C10}, whereas the other forms of DsbB remain unaffected by the addition of Q_{C10} (compare lanes 3 and 4 with lanes 6 and 7, 9 and 10, and 12 and 13).

To see if the ability of DsbB to oxidize DsbA was changed by the addition of quinone, the same reactions were also carried out in the presence of 50 μM added quinone (Fig. 4f and data not shown). The DsbA red concentration was 40 μM, and 10 μM DsbB was used. When quinone was added, wild type DsbB fully oxidized DsbA very rapidly and to completion. Oxidation was complete even within 1 min using 2 μM DsbB[CCCC] (data not shown). For the DsbB[CCSS], DsbB[SSCC], and DsbB[SSSS] mutants, the oxidation of DsbA was improved only very slightly or not at all (see Fig. 4f). The slight improvement may be due to saturation of the quinone-binding site of DsbB.

Although not essential for its activity, the cysteines of DsbB appear to be very important for the catalytic activity of DsbB, because mutants that removed one or both pairs of cysteines in DsbB were only able to oxidize DsbA in a stoichiometric fashion. These results suggest that the cysteines in DsbB may be important for the ability of DsbB to turn over oxidized quinone but may not be directly involved in disulfide exchange with DsbA.

What Is the Redox Potential of Each Disulfide in DsbB?—To further characterize the Cys-41/Cys-44 and Cys-104/Cys-130 disulfides, we decided to measure their redox potential. We were unable to establish equilibrium with either of these two disulfides using mixtures of oxidized and reduced glutathione, suggesting that these disulfides may be inaccessible to this reagent (data not shown). This is a somewhat surprising property for disulfides that are thought to oxidize DsbA via thiol-disulfide exchange. We were, however, able to reach equilibrium with the more reactive redox agent DTT. We incubated 1 μM DsbB[SSCC] in various ratios of oxidized and reduced DTT for 1 or 15 h at 30 °C. Both incubation periods gave the same results showing that we had reached equilibrium. The protein was then trichloroacetic acid-precipitated, modified with AMS, and subjected to non-reducing SDS-PAGE as before. The ratio of oxidized to reduced protein was then quantified using IMAGE 1.62 software. The data were plotted as shown in Fig. 5a,
and we calculated the equilibrium constant ($K_{ox}$) of the reaction between this disulfide in DsbB and DTT to be 0.12. From the redox potential of DTT ($-312$ mV), we were able to calculate the redox potential of the Cys-104/Cys-130 disulfide to be $-284$ mV by using the Nernst equation as previously described (19). This makes it much less oxidizing than the disulfide of DsbA, which has a redox potential of $-120$ mV. The Cys-104/Cys-130 disulfide in DsbB thus appears to have redox properties that make it very unsuitable for reoxidizing DsbA. The redox potential of the Cys-41/Cys-44 disulfide despite its redox potential may imply that the Cys-41/Cys-44 pair of cysteines is even more inaccessible than the Cys-104/Cys-130 pair.

To directly determine if these disulfides within DsbB could be oxidized by DsbA, a reaction opposite from that predicted in the model shown in Fig. 2, we used AMS/gel-shift experiments. We incubated $20 \mu M$ DsbAox with either $10 \mu M$ DsbB[CCSS]red or DsbB[SSCC]red and attempted to oxidize the disulfides in DsbB using DshAox. From these experiments we observed that it is possible for DsbA to directly oxidize the Cys-104/Cys-130 cysteine pair in DsbB[SSCC] (Fig. 6a) indicating that DsbA might recognize this cysteine pair as a substrate in vitro. DsbA, however, was unable to oxidize the first disulfide in DsbB (data not shown). That oxidized DsbA is incapable of oxidizing the Cys-114/Cys-44 disulfide despite its redox potential may imply that the Cys-114/Cys-44 pair of cysteines is even more inaccessible than the Cys-104/Cys-130 pair.

To verify that DsbA can oxidize the Cys-104/Cys-130 disulfide in DsbB and that the ability of DsbA to oxidize DsbB[SSCC] is not simply an artifact of the absence of the first pair of cysteines, we decided to test whether DsbA could oxidize the second disulfide of wild type DsbB. We incubated $10 \mu M$ wild type DsbBred with $20 \mu M$ DsbAox and followed the interaction using AMS/gel-shift analysis (see Fig. 6b). Within less than 1 min of incubation the reduced band of DsbB disappeared, suggesting that DsbA is indeed capable of recognizing the Cys-114/Cys-130 cysteine pairs in DsbB as a substrate for oxidation.

Thus neither the Cys-41/Cys-44 disulfide nor the Cys-104/Cys-130 disulfide in DsbB is capable of oxidizing DsbB[SSCC] and partially oxidizing DsbB[CCCC] but not DsbB[CCSS]. AMS/gel-shift experiments were used to determine if DsbA is capable of oxidizing the second disulfide in DsbB. DsbB[SSCC] (a), DsbB[CCCC] (b) were reduced on ice with DTT, and the DTT was then removed via gel filtration. 10 $\mu M$ of the reduced protein was then incubated at room temperature for 10 min to assess the extent of spontaneous oxidation. To these reduced proteins we then added $20 \mu M$ oxidized DsbA and followed their redox state over time. All time points were obtained by trichloroacetic acid-precipitating the protein, modifying with AMS, and subjecting them to non-reducing SDS-PAGE.

FIG. 5. Redox potential of DsbB[CCSS] and DsbB[SSCC]. The redox potentials of DsbB[CCSS] (a) and DsbB[SSCC] (b) were measured by incubating both proteins in various ratios of reduced and oxidized DTT. At equilibrium the protein was then trichloroacetic acid-precipitated, modified with AMS, and subjected to non-reducing SDS-PAGE. The gels were then analyzed and quantified using IMAGE 1.62, and curves were generated. a, we obtained a $K_{ox}$ of 0.12 for DsbB[SSCC] with DTT, which yields a redox potential of $-284$ mV; b, we obtained a $K_{ox}$ of 0.05 for DsbB[CCSS] with DTT, which yields a redox potential of $-271$ mV.

FIG. 6. DsbA is capable of oxidizing DsbB[SSCC] and partially oxidizing DsbB[CCCC] but not DsbB[CCSS]. AMS/gel-shift experiments were used to determine if DsbA is capable of oxidizing the second disulfide in DsbB. DsbB[SSCC] (a), DsbB[CCCC] (b) were reduced on ice with DTT, and the DTT was then removed via gel filtration. 10 $\mu M$ of the reduced protein was then incubated at room temperature for 10 min to assess the extent of spontaneous oxidation. To these reduced proteins we then added $20 \mu M$ oxidized DsbA and followed their redox state over time. All time points were obtained by trichloroacetic acid-precipitating the protein, modifying with AMS, and subjecting them to non-reducing SDS-PAGE. a, reduced DsbB[SSCC] showed no spontaneous oxidation after 10 min (lanes 3 and 4). Following the addition of oxidized DsbA, DsbB[SSCC] was rapidly oxidized by DsbA (lane 5). b, reduced DsbB[CCCC] showed no spontaneous oxidation after 60 min (lane 2). Following the addition of oxidized DsbA, DsbB[CCCC] was partially oxidized very rapidly (lanes 3–6).

To verify that DsbA can oxidize the Cys-104/Cys-130 disulfide in DsbB and that the ability of DsbA to oxidize DsbB[SSCC] is not simply an artifact of the absence of the first pair of cysteines, we decided to test whether DsbA could oxidize the second disulfide of wild type DsbB. We incubated $10 \mu M$ wild type DsbBred with $20 \mu M$ DsbAox and followed the interaction using AMS/gel-shift analysis (see Fig. 6b). Within less than 1 min of incubation the reduced band of DsbB disappeared, suggesting that DsbA is indeed capable of recognizing the Cys-114/Cys-130 cysteine pairs in DsbB as a substrate for oxidation.

Thus neither the Cys-41/Cys-44 disulfide nor the Cys-104/Cys-130 disulfide in DsbB is capable of oxidizing DsbB[SSCC] and partially oxidizing DsbB[CCCC] but not DsbB[CCSS]. AMS/gel-shift experiments were used to determine if DsbA is capable of oxidizing the second disulfide in DsbB. DsbB[SSCC] (a), DsbB[CCCC] (b) were reduced on ice with DTT, and the DTT was then removed via gel filtration. 10 $\mu M$ of the reduced protein was then incubated at room temperature for 10 min to assess the extent of spontaneous oxidation. To these reduced proteins we then added $20 \mu M$ oxidized DsbA and followed their redox state over time. All time points were obtained by trichloroacetic acid-precipitating the protein, modifying with AMS, and subjecting them to non-reducing SDS-PAGE. a, reduced DsbB[SSCC] showed no spontaneous oxidation after 10 min (lanes 3 and 4). Following the addition of oxidized DsbA, DsbB[SSCC] was rapidly oxidized by DsbA (lane 5). b, reduced DsbB[CCCC] showed no spontaneous oxidation after 60 min (lane 2). Following the addition of oxidized DsbA, DsbB[CCCC] was partially oxidized very rapidly (lanes 3–6).
We investigated the mechanism of DsbB. Our results presented here indicate that a revision in the model given in Fig. 2 may be in order. We were able to show that DsbB[CCSS] and DsbB[CCCC] are completely and rapidly oxidized by quinone, whereas DsbB[SSCC] is not. This is consistent with the last two steps of the model in Fig. 2 (Fig. 3, a–c). In this way DsbB appears to interact with quinone as the model in Fig. 2 predicts. However, when we examined the oxidation of DsbA by DsbB, our results were in stark disagreement with the model. We were able to show that DsbB[CCSS], DsbB[SSCC], and DsbB[SSSS] are all capable of oxidizing DsbA in a stoichiometric fashion, indicating that the cysteines are not absolutely required for DsbA oxidation as the model in Fig. 2 requires. In addition, our results show that the redox potential of the disulfide pairs in DsbB are actually less oxidizing than those of DsbA, not more oxidizing as required by the model, and that the Cys-104/Cys-130 disulfide in DsbB. Then the Cys-104/Cys-130 disulfide is oxidized either via a thiol disulfide exchange reaction with the Cys-41/Cys-44 disulfide as shown or directly by quinone. B, direct oxidation. Quinone bound to DsbB directly oxidizes DsbA. Neither disulfide within DsbB participates in thiol disulfide exchange with DsbA. Instead, these disulfides are required at some other step of the catalytic cycle such as quinone exchange. The redox potentials of the quinone and the disulfides in DsbB and DsbA are shown. The arrows show the direction of electron flow.

**Fig. 7. The direct oxidation model for the mechanism of DsbB.** The mechanism of reoxidation of DsbA by DsbB can be split into two processes, a self-oxidation step and a step where DsbB directly oxidizes DsbA. A, self-oxidation. Quinone directly oxidizes the Cys-41/Cys-44 disulfide in DsbB. Then the Cys-104/Cys-130 disulfide is oxidized either via a thiol disulfide exchange reaction with the Cys-41/Cys-44 disulfide as shown or directly by quinone. B, direct oxidation. Quinone bound to DsbB directly oxidizes DsbA. Neither disulfide within DsbB participates in thiol disulfide exchange with DsbA. Instead, these disulfides are required at some other step of the catalytic cycle such as quinone exchange. The redox potentials of the quinone and the disulfides in DsbB and DsbA are shown. The arrows show the direction of electron flow.
DsbB Catalyzes Disulfide Bond Formation de Novo

required for DsbB to be able to exchange oxidized quinone for reduced quinone. Reduced DsbB, in the presence of oxidized quinone, is also able to directly form disulfides within itself. DsbB thus acts to catalyze its own oxidation. The Cys-41/Cys-44 disulfide within DsbB is formed first, and this enables the formation of the Cys-104/Cys-130 disulfide. DsbB thus generates disulfides directly in DsbA and within itself; both catalytic events are dependant on quinone reduction. Thus DsbB has two substrates for its disulfide quinone reductase activity.

The observation that cysteineless DsbB does reoxidize DsbA in vitro does not prove the absence of disulfide exchange between the cysteines of DsbB and DsbA in the catalytic cycle of DsbB. The conditions used in our experiments might conceivably allow a direct but non-physiological transfer of electrons from DsbA to the quinone bound to DsbB without the necessity of the disulfides of DsbB. We note that the single turnover reactions, in the absence of added quinone, even with oxidized wild type DsbB are also rather slow, compared with the rate for wild type seen in the presence of 20 μM oxidized quinone, a concentration equivalent to that of the DsbA and only 2-fold higher than that of DsbB. Thus the presence of free quinone, or quinone loosely associated with DsbB, may be necessary to drive the reaction so that it can proceed rapidly. Wild type DsbB is purified in a form where its cysteines are fully oxidized and it contains 50–70% oxidized quinone bound. Thus, one might expect it to be capable of rapidly oxidizing DsbA whether this oxidation is due to direct thiol disulfide exchange or direct quinone reduction. The fact that it is slow but becomes very rapid upon the addition of 20 μM quinone suggests that quinone addition relieves some blocked step in the catalytic cycle. The strong overall thermodynamic drive provided by the 230-MV difference between the redox potentials of the added ubiquinone at +110 mV and the redox potential of DsbA at −120 mV could conceivably be sufficient to overcome the redox barrier provided by the Cys-41/Cys-44 disulfide at −271 mV or the Cys-104/Cys-130 disulfide at −284 mV. However, this would be a very unusual situation. Disulfide exchange and electron transfer reactions follow a regular trend from low potential centers to high potential centers, so that each step in the reaction goes downhill in terms of free energy. This has been shown to be the case in many electron transport systems, including the hemes of the bacterial photosynthetic reaction center (23). However, in the folding pathway of bovine pancreatic trypsin inhibitor there have been instances recorded where a stable disulfide is converted to a less stable disulfide (14). There is, however, no absolute necessity that each step in the pathway be strongly energetically favorable. Rare instances have been reported where electron transport might possibly occur upward through an energy gradient (24–26). In the most definitive example we are aware of, a 50-MV uphill electron transport reaction was found to occur in an R264K mutation protein of Rhodopsuedo-

REFERENCES