Cell, Vol. 98, 217–227, July 23, 1999, Copyright ©1999 by Cell Press

Oxidative Protein Folding Is Driven by the Electron Transport System

Martin Bader,* Wilson Muse,* David P. Ballou,† Christian Gassner,* and James C. A. Bardwell*‡

*Department of Biology

†Department of Biological Chemistry

University of Michigan

Ann Arbor, Michigan 48109-1048

Summary

Disulfide bond formation is catalyzed in vivo by DsbA and DsbB. Here we reconstitute this oxidative folding system using purified components. We have found the sources of oxidative power for protein folding and show how disulfide bond formation is linked to cellular metabolism. We find that disulfide bond formation and the electron transport chain are directly coupled. DsbB uses quinones as electron acceptors, allowing various choices for electron transport to support disulfide bond formation. Electrons flow via cytochrome *bo* oxidase to oxygen under aerobic conditions or via cytochrome *bd* oxidase under partially anaerobic conditions. Under truly anaerobic conditions, menaquinone shuttles electrons to alternate final electron acceptors such as fumarate. This flexibility reflects the vital nature of the disulfide catalytic system.

Introduction

The proper folding and stability of many proteins depends on the formation of disulfide bonds (Gilbert, 1997). Organisms have developed effective catalysts that ensure the oxidation of cysteines in proteins (Bardwell, 1994; Gilbert, 1997). In *Escherichia coli*, a family of thiol-disulfide oxidoreductases called the Dsb enzymes, for disulfide bond formation, that facilitate disulfide bond exchange in the periplasm of prokaryotes has been identified (Bardwell et al., 1991, 1993; Missiakas et al., 1995; Andersen et al., 1997). To date, six different Dsb proteins have been described in *E. coli* (Bardwell, 1994; Missiakas et al., 1995; Andersen et al., 1997; Fabianek et al., 1998). Of these, only two, DsbA and DsbB, are central in the thiol oxidation reaction. DsbA acts as the direct donor of disulfide bonds in secreted proteins. It is reoxidized in a reaction that requires DsbB in vivo and in vitro (Bardwell et al., 1993; Bader et al., 1998). The remaining proteins, DsbC, DsbD, DsbE, and DsbG, catalyze disulfide isomerization and reduction reactions. DsbC and DsbG appear to catalyze the isomerization of nonnative disulfide bonds (Joly and Swartz, 1997; Bessette et al., 1999) and are maintained in an active reduced form by the inner membrane protein DsbD (Rietsch et al., 1996; Bessette et al., 1999). DsbE acts as a disulfide reductase and plays a role in cytochrome c biogenesis (Fabianek et al., 1998).

‡To whom correspondence should be addressed (e-mail: jbardwel@umich.edu).

An analogous system exists in the endoplasmic reticulum (ER) of yeast. This includes the well-studied protein disulfide isomerase, which catalyzes both the isomerization and formation of disulfide bonds (Gilbert, 1997), and the recently discovered Ero1 protein, which is responsible for maintaining the redox status of protein disulfide isomerase (Frand and Kaiser, 1998; Pollard et al., 1998). The Ero1 protein may thus play a somewhat similar role to the prokaryotic DsbB protein.

DsbA introduces disulfide bonds into folding proteins at a high rate (Zapun et al., 1993). Its active site contains a disulfide bond that is transferred to target proteins in the periplasm via disulfide exchange. DsbA's active site disulfide is the most oxidizing disulfide known, which explains why DsbA functions as an oxidant of thiols rather than a disulfide isomerase or reductant (Wunderlich and Glockshuber, 1993; Zapun et al., 1993). The reasons behind DsbA's very oxidizing nature have been extensively addressed (Grauschopf et al., 1995; Hennecke et al., 1997).

In order to be catalytic, DsbA needs to be reoxidized efficiently. In vivo, DsbA is reoxidized by the inner membrane protein DsbB. Null mutants in *dsbB* are severely defective in disulfide bond formation and accumulate DsbA in a reduced form (Bardwell et al., 1993). That DsbB directly reoxidizes DsbA was confirmed by the isolation of a mixed disulfide cross-link between DsbB and DsbA (Guilhot et al., 1995).

The ultimate origin of the oxidative power for protein folding is mysterious in both prokaryotic and eukaryotic organisms. Oxidized glutathione was proposed to be the source of the oxidizing equivalents needed to generate disulfide bonds in the ER of eukaryotes (Hwang et al., 1992). Now it appears that glutathione provides reducing, not oxidizing, power to the ER, making the ultimate origin of oxidizing equivalents for protein folding in eukaryotes more of a mystery than ever (Bader et al., 1999; Cuozzo and Kaiser, 1999). In the prokaryote *E. coli*, we proposed that the oxidizing power for disulfide bonds might come from "membrane components interacting with the electron transport chain." We further speculated that "it may be that quinones or certain membrane proteins capable of moving electrons through the membrane are needed to oxidize DsbB" (Bardwell et al., 1993). Recent in vivo experiments provide evidence that disulfide bond formation is indeed linked to the electron transport system. Ito and coworkers found that reduced DsbA accumulates in strains defective in heme or quinone biosynthesis (Kobayashi et al., 1997). They also showed that the Cys41–Cys44 disulfide bond in DsbB is DTT resistant in an oxygen-, heme-, and quinone-dependent manner (Kobayashi and Ito, 1999). Zeng et al. (1998) recently reported that mutants in the ubiquinone biosynthesis exhibit thiol hypersensitivity, a phenotype shared by *dsb* mutations.

To characterize the DsbA–DsbB system in a biochemical manner, we developed an enzymatic assay for DsbB and were able to show that membranes containing overexpressed DsbB catalyze the oxidation of reduced DsbA in vitro (Bader et al., 1998). DsbB's activity in this assay

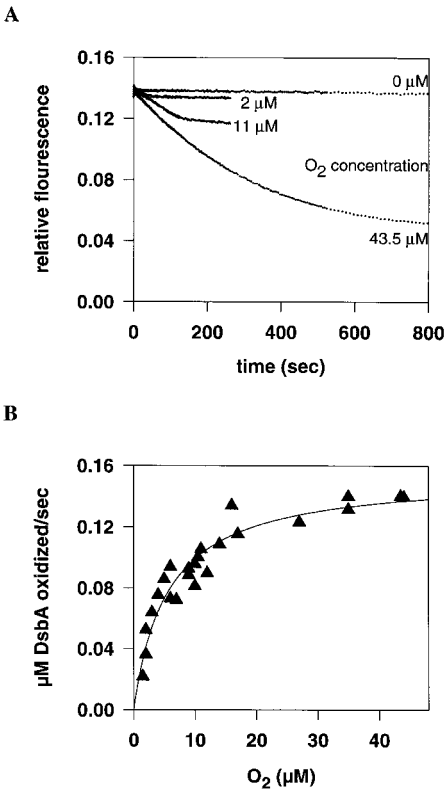


Figure 1. Oxygen Dependence of the DsbA–DsbB Disulfide Catalytic System

(A) Progress curves of DsbA oxidation catalyzed by DsbB at various O_2 concentrations. Oxidized DsbA has a 3.4-fold lower fluorescence than reduced DsbA. Relative fluorescence shown versus time. Reaction of 43.5 μM DsbA and 0.6 μM DsbB at 30°C.

(B) Rate of DsbA oxidation versus O_2 concentration. This figure shows the initial rate of DsbA oxidation from 20–50 s in the reaction of 43.5 μM DsbA catalyzed by 0.6 μM DsbB at 30°C. The O_2 concentration was determined by calculating the amount of DsbA that was oxidized by comparing the fluorescence upon complete oxidation. The O_2 concentration at 50% of the V_{max} is taken to be the K_m.

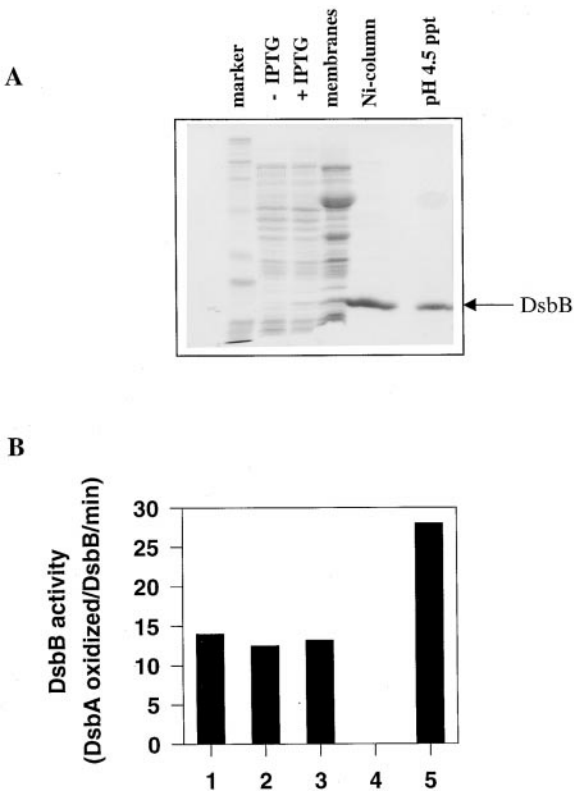


Figure 2. Purification of DsbB

(A) SDS-PAGE analysis of DsbB purified from the DsbB overexpression strain WM76. Membranes were prepared as described in Experimental Procedures. DsbB was purified from n-Dodecyl-β-D-maltoside solubilized membranes over nickel affinity and a pH 4.5 dialysis followed by centrifugation. This DsbB was 97% pure.

(B) DsbB's activity was monitored (1) in membranes (2) after purification over nickel-NTA and (3) after pH 4.5 dialysis. DsbB's activity disappears (4) after a hydroxyapatite column. This DsbB was >99% pure, as monitored by scanning stained gels. Inclusion of n-Dodecyl-β-D-maltoside extracts from *dsbB* null membranes (JCB819) (5) restores DsbB's ability to oxidize DsbA. Detergent-solubilized membranes were present at a total protein concentration of 10 μg/ml and did not affect DsbA's redox state in the absence of DsbB (not shown). The assay mixture consisted of 50 mM sodium phosphate (pH 6.0), 300 mM NaCl, 0.5 mM EDTA, and 10 μM of $DsbA^{red}$. Activities were derived from the initial, linear fluorescence decrease upon oxidation of DsbA. The concentration of DsbB in membranes was determined as described in Experimental Procedures.

strongly depends on the presence of oxygen, implying that oxygen can serve as the final electron acceptor. We investigated if other components present in the membrane are necessary for DsbB's activity. We find that DsbB uses components of the electron transport system to drive its reoxidation. DsbB can be efficiently reoxidized by ubiquinone, which in turn is reoxidized by cytochrome terminal oxidases that pass electrons on to oxygen.

Results

In Vitro Reconstitution of a Disulfide Catalytic Machine

Disulfide bonds are directly introduced into folding proteins by the DsbA protein. DsbA is then reoxidized by an inner membrane protein called DsbB. We had previously shown that DsbB in a total membrane fraction is catalytically active in reoxidizing a 3000-fold excess of DsbA in an oxygen-dependent reaction (Bader et al., 1998). This reoxidation reaction in vitro was followed by monitoring the loss of fluorescence as DsbA is oxidized. Oxidized DsbA has a 3.4-fold lower tryptophane fluorescence than reduced DsbA (Zapun et al., 1993). Thus, oxidation of DsbA by DsbB causes an easily measurable decline in fluorescence over time. We purified DsbB to 97% homogeneity (see below) and found it to be fully active at oxygen concentrations >30 μM. At lower oxygen concentrations, the reaction between DsbB and DsbA gradually slowed and then stopped before reaching completion, verifying that oxygen was required in this reaction. Under anaerobic conditions achieved by the extensive use of oxygen-scrubbing agents, no reoxidation of DsbA occurred (see Figure 1A). By plotting the initial slope of the reaction between DsbB and DsbA

Table 1. Purification of DsbB-Activating Factor #1

	Total Protein (mg)	DsbB Reactivation (DsbB Activity/ μg Protein)
dsbB null membranes	352	2.9
Dodecyl-Maltoside extract	304	5.6
Ammonium sulfate fraction	41	14.5
Q Sepharose pool	6	40.8
Hydroxyapatite pool	0.5	90.6
PBE-94 pool	0.2	127.1

Fractions from each purification step were tested in the fluorescence assay for their ability to reactivate DsbB that had been purified over both a nickel column and a hydroxyapatite column. The total protein concentration of the pooled fractions was determined and used to express DsbB's reactivation (DsbB activity/μg protein). A 44-fold enrichment of the DsbB-activating protein was accomplished by the established purification procedure.

against the oxygen concentration, we were able to estimate the apparent K_m for oxygen of this reaction to be ~5 μM (Figure 1B). This K_m is about 50-fold lower than the concentration of oxygen in aqueous solutions exposed to air (~240 μM). The very high affinity of this disulfide catalytic system for oxygen implies that it should still be able to function under rather anaerobic conditions.

We wanted to determine if DsbA, DsbB, and O_2 are the only components required to catalyze oxidative protein folding in prokaryotes. DsbB purified to ~97% homogeneity exhibited a specific molar activity similar to DsbB present in total membrane fractions (Figure 2), hinting that no other components were necessary.

Most cofactors that are involved in the transfer of electrons to oxygen, such as hemes or flavins, absorb strongly in the range of 400–600 nm. To investigate if such cofactors were bound to our purified DsbB, we examined its visible absorption spectrum. Surprisingly, far too little absorption was observed in this range to account for stoichiometric amounts of a bound cofactor such as a heme or a flavin. However, a very small amount of absorption at ~400 nM could be detected (data not shown). This led us to consider the possibility that an amount of an enzyme undetectable on gels but capable of catalytically reoxidizing DsbB might actually be present in our 97% pure "photographically homogeneous" DsbB preparation. If this presumptive DsbB reoxidant was as active in reoxidizing DsbB as DsbB was in reoxidizing DsbA, a 99.6% pure DsbB preparation would be necessary to ascertain that potential enzymatic reoxidants were purified away. In fact, additional purification of DsbB over a hydroxyapatite column rendered DsbB incapable of catalytically oxidizing DsbA (Figure 2B, bar 4). Activity could be fully restored by addition of membranes prepared from DsbB null mutants (Figure 2B, bar 5). DsbB null mutant membranes in the absence of DsbB show no activity in reoxidizing DsbA (Bader et al., 1998). This clearly demonstrated that DsbB had not been irreversibly inactivated and strongly suggested that an additional factor present in membranes was needed to complete this disulfide catalytic system. We called this additional component DsbB-activating factor.

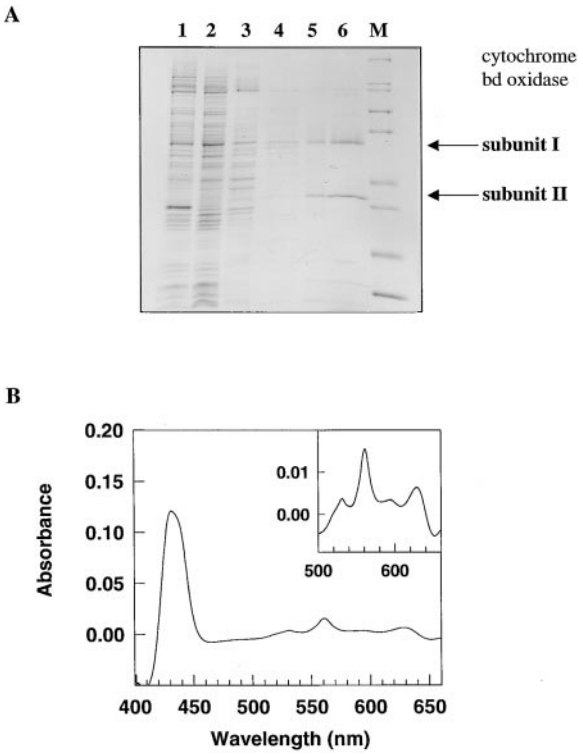


Figure 3. SDS-PAGE and Spectral Analysis of the DsbB-Activating Factor

(A) Pooled fractions that reactivate DsbB in vitro were subjected to 14% SDS-PAGE. Lanes: 1, JCB 819 membrane extract; 2, n-Dodecyl-β-D-maltoside extract; 3, ammonium sulfate fraction; 4, Q Sepharose pool; 5, hydroxyapatite pool; 6, PBE-94 pool; M, molecular weight standard (200, 116, 97, 66, 55, 37, 31, 22, 14 kDa). Two predominant bands can be identified in lane 6 corresponding to the apparent molecular weight of 50 kDa and 30 kDa.

(B) The purified DsbB-activating factor was reduced with sodium dithionite and the reduced minus oxidized spectrum recorded. The spectrum clearly identifies the presence of cytochrome *bd* oxidase with a significant peak at 628 nm typical for heme d in the difference spectrum. The inset shows the identical difference spectrum using an expanded absorbance scale.

Purification and Identification of the DsbB-Activating Factor

To purify this additional component, we used highly purified and thus inactive DsbB. We screened membrane fractions for their ability to activate DsbB. The rapid fluorescence assay (Bader et al., 1998) was used to measure stimulation of DsbB activity by the membrane fractions. Membranes prepared from the *dsbB*::kan5 null mutant JCB819 were used as a source of the DsbB-activating factor. The membrane fraction was resolved using a series of four steps (ammonium sulfate precipitation, Q Sepharose, hydroxyapatite, and chromatofocusing chromatography; see Table 1) to obtain a homogeneous sample that could activate DsbB. After the final chromatographic step, only two proteins with apparent molecular weights of 30 kDa and 50 kDa were visible after SDS-PAGE (Figure 3A). The N-terminal sequence of the 30 kDa protein was found to correspond to subunit II of the *E. coli* cytochrome *bd* terminal oxidase. The 50 kDa protein gave the sequence VEL, with the V starting

at position 5 of the protein. Despite the fact that this sequence is very short, only a single *E. coli* protein has VEL at the appropriate position: subunit I of the cytochrome *bd* oxidase. These results implicate cytochrome *bd* oxidase as the missing factor required for DsbB's activation in vitro.

The spectral properties of the purified DsbB-activating complex strongly supported our conclusion that the DsbB-activating component is indeed cytochrome *bd* oxidase (Figure 3B). The difference spectrum of reduced versus oxidized cytochrome *bd* oxidase shows five distinct peaks at 429 nm, 532 nm, 560 nm, 594 nm, and 628 nm (Miller and Gennis, 1983). This was identical to the difference spectrum obtained for reduced versus oxidized DsbB-activating factor (Figure 3B). These peaks are characteristic for the heme b (560 nm, 532 nm, 594 nm) and the heme d (628 nm) prosthetic groups in cytochrome *bd* oxidase (Jünemann, 1997).

To prove that cytochrome *bd* oxidase indeed corresponds to our DsbB-activating factor, we purified cytochrome *bd* oxidase to >95% purity from an overproducing strain of *E. coli* (GO105/pTK1) according to Kaysser et al. (1995). GO105/pTK1 contains a plasmid-overproducing cytochrome *bd* oxidase and a null mutation in the genes for cytochrome *bo* oxidase. This mutation eliminates any potential contamination with the cytochrome *bo* oxidase, the second terminal oxidase in *E. coli*. For the purification of cytochrome *bd* oxidase, we followed only the protein to heme ratio at 280/412 nm, not DsbB activating ability. The activity of the purified cytochrome *bd* oxidase was then tested for its ability to reactivate DsbB. The specific activity of cytochrome *bd* oxidase was found to be identical to the DsbB-activating factor that had been purified from *dsbB* null membranes. These results let us conclude that cytochrome *bd* oxidase is indeed identical to the DsbB-activating factor.

Cytochrome *bd* oxidase is one of two terminal oxidases in *E. coli* that catalyze the transfer of electrons from ubiquinone to molecular oxygen (Kita et al., 1984; Puustinen et al., 1991). We have previously shown that molecular oxygen is the final acceptor for electrons that are generated in the oxidation of folding proteins (Bader et al., 1998). Since cytochrome *bd* oxidase appears to be the DsbB-activating factor, we propose that electrons flow from DsbB to oxygen via this heme-containing cytochrome *bd* oxidase. This also explains the puzzling observation that purified DsbB apparently lacks the prosthetic groups that are often used in the transfer of electrons to oxygen.

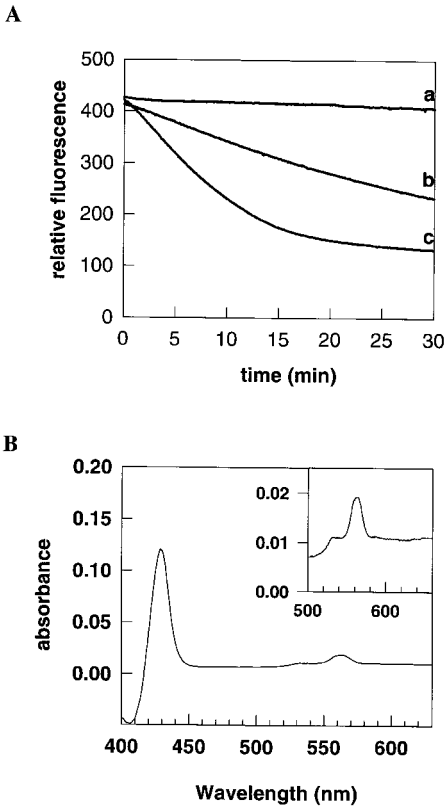


Figure 4. Both Cytochrome *bd* and *bo* Terminal Oxidases Restore DsbB's Activity In Vitro

(A) The reoxidation of reduced DsbA (10 μM) was followed in the presence of 60 nM two column purified DsbB. The ability of different membrane preparations (prepared from strains DS187, GL101, and DS253) to restore DsbB's catalytic activity was tested. DS187 (Δ*cyd*::cam) is a strain of *E. coli* that lacks the genes for cytochrome *bd* oxidase. GL101 (*cyo*::kan) is a strain that lacks the genes for cytochrome *bo* oxidase. DS253 (Δcyd::*cam* Δcyo::*kan*) is a strain that lacks both cytochrome *bo* and *bd* oxidases. Fluorescence measurements were performed in the presence of *cyd cyo* null membranes (a), *cyd* null membranes (b), and *cyo* membranes (c). While membranes containing either cytochrome *bd* or *bo* terminal oxidase are able to reactivate DsbB, no reoxidation of DsbA can be detected if membranes from the double mutant (DS253) are added. The final concentration of total membrane protein added was 10 μg/ml.

(B) Reduced minus oxidized difference spectra of DsbB-activating factor #2 that corresponds to cytochrome *bo* terminal oxidase. The inset shows the absorbance from 500 to 650 nm using an expanded absorbance scale.

Both *E. coli* Terminal Oxidases Reactivate DsbB

As a genetic test to determine if cytochrome *bd* oxidase is the only protein component present in *E. coli* membranes that can effect the reoxidation of DsbB, we tested membranes prepared from DS187 Δ*cyd*::cam for their ability to reoxidize DsbB. This strain contains a deletion in the cytochrome *bd* oxidase operon. Surprisingly, membranes prepared from this *cyd* null mutant strain were still able to reactivate highly purified, inactive DsbB (Figure 4A). These results suggested that these membranes contain a second, alternate DsbB-activating factor. As a control, we showed that reoxidation of DsbA was strictly dependent on the presence of both DsbB and membranes; in the absence of DsbB, these membranes were not able to directly reoxidize DsbA. This second DsbB-activating factor was purified using two chromatographic steps from *E. coli* DS187 lysates by analyzing individual fractions for their ability to reactivate DsbB.

E. coli contains two terminal oxidases, cytochrome *bd* oxidase and cytochrome *bo* oxidase (Miller and Gennis, 1983; Kita et al., 1984). Since cytochrome *bd* oxidase was capable of acting as a DsbB-activating factor, we suspected that this second DsbB-activating factor was the alternate terminal oxidase, cytochrome *bo* oxidase. Cytochrome *bo* oxidase has a diagnostic difference

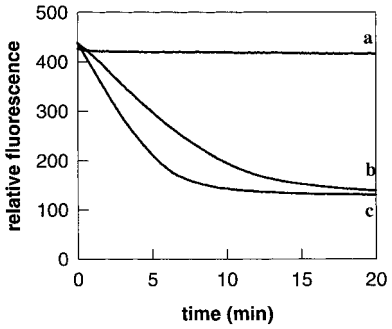


Figure 5. Reconstitution of the Disulfide Catalytic System with Purified Proteins

Cytochrome *bo* and *bd* oxidases were purified from overexpressor strains GO103/pJRhisA and GO105/pTK1, respectively. The concentration of cytochromes *bo* and *bd* were 10 nM, while reduced DsbA was at 10 μM and DsbB at 20 nM. Fluorescence measurements: a, reduced DsbA, DsbB; b, reduced DsbA, DsbB, cytochrome *bo* oxidase; c, reduced DsbA, DsbB, cytochrome *bd* oxidase.

spectrum for the reduced minus the oxidized form with peaks at 530 and 562 nm. The second DsbB-activating activity has a difference spectrum identical to that of cytochrome *bo* oxidase, suggesting that this second DsbB-activating factor is indeed cytochrome *bo* oxidase (Figure 4B).

When membranes prepared from a strain (DS253) containing null mutations in both the cytochrome *bo* and *bd* oxidase genes were added to the assay, no DsbB reactivation was observed. This implies that deletion of the two major terminal oxidases in *E. coli* is sufficient to deplete cell membranes of DsbB-activating factors (Figure 4A). This provided genetic evidence that both terminal oxidases can reoxidize DsbB. In order to confirm that cytochrome *bo* oxidase is able to serve as an acceptor for electrons generated by disulfide bond formation, we purified this enzyme complex from an overexpressor strain of *E. coli* GO105/pJRHisA. This strain carries a plasmid that encodes the four subunits of cytochrome *bo* oxidase with subunit II fused to a histidine tag. Thus, a simple purification of the intact holoenzyme can be effected using nickel affinity chromatography (see Experimental Procedures). The genetic background of the strain that overexpresses cytochrome *bo* oxidase contains a deletion of the cytochrome *bd* gene *cyd*, ensuring that no cross-contamination with the *bd* oxidase occurs during the cytochrome *bo* purification procedure. Cytochrome *bo* oxidase was purified to >95% purity and tested for its ability to restore DsbB activity.

Reconstitution of the Disulfide Catalytic Machine with Purified Cytochrome *bo* and *bd* Oxidases: Relative Efficiencies of the Two Oxidases

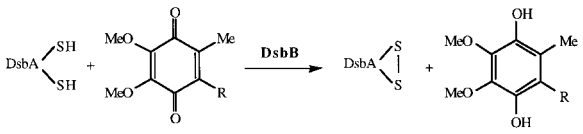
The purified cytochrome oxidases were quantified by the reported absorption coefficients for their heme groups (Miller and Gennis, 1983; Kita et al., 1984) and used to reconstitute the in vitro activity of DsbB. Figure 5 shows the data obtained from the fluorescence measurements. Cytochrome *bo* oxidase (6 pmol) was capable of reoxidizing 49 μmol DsbA per μmol DsbB per min (Figure 5, trace b). Cytochrome *bd* oxidase (6 pmol) could catalyze the reoxidation of 82 μmol DsbA per μmol DsbB per min (Figure 5, trace c). As a control, we also tested whether the two oxidases would reoxidize DsbA directly in the absence of DsbB, but no oxidation was observed (data not shown). This shows that electrons flow from DsbA via DsbB to either cytochrome *bd* or *bo* oxidase and then on to oxygen.

Cytochrome *bd* and *bo* oxidases are the major terminal oxidases under aerobic growth, catalyzing electron transport from the ubiquinone pool to molecular oxygen. Our observations established a clear link between oxidative protein folding and components of the electron transport system and strongly suggest that the protein folding process is driven by electron transport in vivo.

The catalytic efficiency of these cytochrome oxidases provides an explanation of how 97% pure DsbB could still be catalytically active. We calculate that cytochrome oxidases present as a 0.2% contaminant in our "photographically homogeneous" DsbB protein preparation would be sufficient to account for the observed DsbB activity. These results provide a stark reminder of the need to be certain of protein purity when characterizing enzymatic systems.

Ubiquinone Acts as an Electron Acceptor during DsbB Reoxidation

Cytochrome *bo* and *bd* terminal oxidases are structurally unrelated proteins that both catalyze the transfer of electrons from the ubiquinone pool to molecular oxygen (Miller and Gennis, 1983; Kita et al., 1984). We found it somewhat surprising that DsbB would interact directly with two unrelated proteins, so we decided to investigate the possibility that it uses ubiquinone as a common intermediate. The fact that either purified cytochrome *bo* or *bd* oxidase seems to be sufficient to reoxidize DsbB does not exclude the possibility that ubiquinone is involved, since these oxidases purified by conventional means contain tightly bound ubiquinone (Rumbley et al., 1997). It is also a possibility that DsbB binds ubiquinone. To test if ubiquinone is involved in disulfide catalysis, we attempted to reoxidize DsbB directly with ubiquinone-5 (coenzyme Q_1) and decyl-ubiquinone. These ubiquinone variants are commonly used as soluble analogs for ubiquinone-40 (coenzyme Q_8), which is present in the *E. coli* inner membrane. Ubiquinone-5 and decyl-ubiquinone proved to be spectacularly effective in reoxidizing DsbB—much more effective than small molecule reoxidants we tested such as ferricyanide or oxygen alone (Table 2). As a comparison, the turnover number for PDI-catalyzed disulfide isomerization is ~1 per min (Gilbert, 1997), making this eukaryotic enzyme about 400-fold slower than DsbB. It therefore appears that ubiquinone can serve as an effective electron acceptor in the reoxidation of DsbB. DsbB can thus be described as an enzyme catalyzing a two-substrate reaction between DsbA and ubiquinone.



R = isoprenyl residue $(C_5)_n$, n = 1–8.

Table 2. Quinones Act as Substrates for DsbB

Reoxidant	−DsbB	+DsbB
	μmol DsbA/min	μmol DsbA/μmol DsbB/min
Ubiquinone-5	<0.01	420
Decyl-ubiquinone	<0.01	372
Menadione	<0.01	67
Oxygen	<0.01	<0.01
Ferricyanide	<0.02	<0.02

Activities were measured by our fluorescence assay. The left column corresponds to the spontaneous oxidation of DsbA in the absence of DsbB, while the second column shows the rate in the presence of DsbB (1 nM). DsbA was at 10 μM, while the concentration of the reoxidants was 20 μM except oxygen, which was present at ~240 μM.

In the absence of DsbB, almost no oxidation of DsbA occurred; this rate of oxidation was not accelerated by the addition of either ubiquinone or DsbB alone. These important controls show that ubiquinone cannot directly reoxidize DsbA. DsbB is required to catalyze the reoxidiation of DsbA in vitro as it is in vivo. The redox potential of ubiquinones (+0.11 V) seems to be perfectly suited to oxidize DsbA (−0.12 V) (Zapun et al., 1993).

To investigate if the cysteine residues necessary for DsbB activity in vivo are also necessary in our in vitro reconstitution system, we constructed a cysteine-less DsbB mutant and purified the resulting protein. Concentrations of this cysteine-free DsbB <100 nM were totally inactive in oxidizing DsbA in vitro, even in the presence of oxidized ubiquinone or cytochrome oxidases. Our assay is capable of detecting 0.1 nM wild-type DsbB. This means the cysteine-free DsbB is ≥1000-fold less active than wild-type DsbB. We also constructed and purified mutant proteins that contained either only the two active site cysteines present in the first periplasmic domain (Cys-41, Cys-44) or only the two active site cysteines present in the second domain (Cys-104, Cys-130). These mutant proteins were similarly inactive as the cysteine-free DsbB mutant protein. These results show that DsbB's cysteines are required in our in vitro reconstitution system as they are in vivo.

Components of the Disulfide Catalytic System

When this work was initiated, it was known that DsbA helps to catalyze oxidative protein folding reactions by providing a very reactive disulfide (Bardwell, 1994). DsbA is reoxidized in a reaction that requires DsbB and molecular oxygen (Bader et al., 1998). The direct reoxidant of DsbB was unknown. Now it appears that DsbB is directly reoxidized by oxidized ubiquinone, which is then reoxidized by cytochrome *bo* or *bd* terminal oxidases, which are then reoxidized by molecular oxygen (Figure 6). What then occurs under anaerobic conditions? Previous reports indicated that the DsbA–DsbB system does seem to function in vivo under anaerobic conditions. Disulfide bonds in acid phosphatase are formed efficiently anaerobically in vivo in a reaction that requires DsbA and DsbB (Belin et al., 1993).

Disulfide Oxidation under Anaerobic Conditions

Membranes prepared from a double null mutant in cytochrome *bd* and *bo* oxidases (DS253) are completely

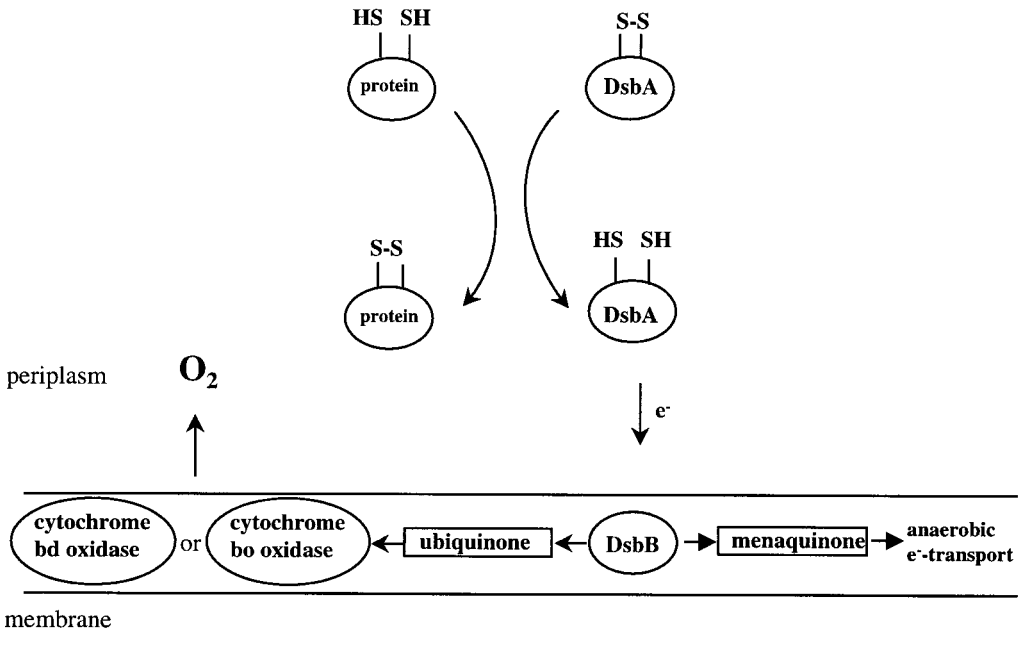


Figure 6. DsbB Links Disulfide Bond Formation to Electron Transport

The direction of electron flow is shown by straight arrows and that of disulfide flow by curved arrows.

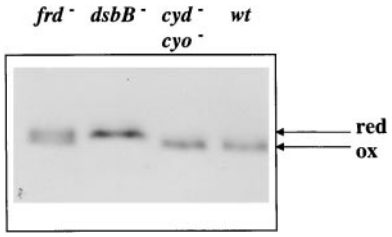


Figure 7. In Vivo Examination of Disulfide Bond Formation under Anaerobic Conditions

Western blot of total cell protein of *E. coli* strains either wild-type ZK126 (wt) or lacking either DsbB JCB819 (*dsbB*), the cytochrome oxidases DS253 (*cyd cyo*), or fumarate reductase MB21 (*frd*). The positions of the disulfide-bonded oxidized (ox) and the nondisulfide-bonded reduced (red) forms of β-lactamase that are shown were detected with antibody.

inactive in reoxidizing DsbB in vitro. The only final electron acceptor available in this in vitro system is oxygen. To test the possibility that DsbB can use alternate electron acceptors in vivo, we examined disulfide bond formation in the cell under anaerobic conditions. As an in vivo substrate, we examined the formation of disulfides in TEM β-lactamase. This substrate was chosen because it is stable in the absence of its disulfide and, once folded, does not oxidize (Bardwell et al., 1991). Thus, the ratio of oxidized to reduced β-lactamase present after isolation from bacteria provides a sensitive indicator of the effectiveness of the disulfide catalytic machinery present in vivo during the protein folding process. Since the affinity of cytochrome *bd* oxidase for oxygen is very high (K_m 20 nM–1 μM), we carried out our experiments in strains containing null mutations in both cytochrome *bd* and *bo* oxidases to avoid trace quantities of oxygen that would allow the flow of electrons through cytochrome *bd* oxidase. These strains were grown anaerobically and did not show any defect in disulfide bond formation, since β-lactamase migrates in the oxidized position (Figure 7). This strongly suggests that alternate electron acceptors can be used by the disulfide catalysts under anaerobic conditions in vivo. Menaquinone can substitute for ubiquinone as an electron shuttle under anaerobic conditions (Wallace and Young, 1977). Menaquinone acts to donate electrons to alternate final electron acceptors such as fumarate or nitrate. Thus, we tested if oxidized menaquinone could substitute for ubiquinone in our in vitro DsbA–DsbB system. It did so at about 16% of the rate of ubiquinone-5 (Table 2), suggesting that menaquinone can be used as an alternate electron acceptor for DsbB. This result predicts that a strain lacking both ubiquinone and menaquinone should show a strong defect in disulfide bond formation. Ito and coworkers have indeed observed that *ubiA menA* mutants, which make no functional ubiquinone or menaquinone, do have a strong defect in disulfide bond formation when starved for quinone precursors (Kobayashi et al., 1997). *UbiA* strains and *ubiD ubiX* double mutant strains, defective only in ubiquinone synthesis, were found to have a moderate but not complete disulfide defect (Kobayashi et al., 1997; Zeng et al., 1998). This suggests that enough electrons flow through menaquinone, even under aerobic conditions, to partially satisfy the disulfide bond formation needs of the cell. These results provide strong genetic support for a model that links disulfide catalysis to the electron transport system.

As an independent genetic test of our model, we reasoned that bacteria growing in a purely fermentative manner without electron transport should show a defect in disulfide bond formation. Thus, we tested if strains grown under conditions where very little electron transport occurs would be compromised in forming disulfides. We grew the fumarate reductase null mutant MB21 anaerobically on glucose-containing medium that lacks known alternate electron acceptors. This strain will derive almost its entire energy from the fermentation of glucose. Its use of oxygen was limited to the level of anaerobiosis achievable by the GasPak Anaerobic System (BBL Microbiology Systems). The strain cannot use any of the alternate electron acceptors because they are absent from the media. It will generate fumarate as part of the TCA cycle but cannot use it as an electron acceptor because of the fumarate reductase null mutation. Under these conditions, where very little electron transport occurs, the strain is indeed compromised in its ability to form disulfide bonds. At least half of the β-lactamase migrates in the reduced position, similar to the effect of a DsbA null mutation (see Figure 7 and Bardwell et al., 1991). This provides additional genetic support for our model.

In summary, DsbB links disulfide bond formation to electron transport (see Figure 6). Under aerobic conditions, DsbB donates its electrons to ubiquinone, which transfers its electrons to either cytochrome *bd* or *bo* oxidases; these are reoxidized by molecular oxygen. Under strictly anaerobic conditions, menaquinone can be used as an alternate electron acceptor. These results identify the sources of oxidative power used for protein folding and describe the path of electron flow following disulfide bond formation.

Discussion

Disulfide bond formation is catalyzed in the periplasm of prokaryotes by the DsbA–DsbB oxidative system. Although a lot is known about DsbA, it remained enigmatic how this important reaction in protein folding is linked to the metabolism of the cell. Genetic approaches established that DsbB is essential for the reoxidation of DsbA (Bardwell et al., 1993) but to date have been unable to resolve the complete pathway of disulfide bond formation. The direct reoxidant of DsbB was unclear, as was its final electron acceptor. The origin and flow of oxidants involved in eukaryotic protein folding is even more of a mystery (Bader et al., 1999; Cuozzo and Kaiser, 1999).

The development of a specific assay for DsbB enabled us to reconstitute the DsbA/DsbB system in vitro and show that oxygen can serve as the final electron acceptor (Bader et al., 1998). By fractionating and purifying proteins from *dsbB* null membranes that restore DsbB activity, we now demonstrate that either cytochrome *bd* or *bo* oxidase can function to reoxidize the DsbA–DsbB system. These oxidases are the two major terminal oxidases of the electron transport chain in *E. coli* (Miller and Gennis, 1983). Their role in electron transport is to

Table 3. Bacteria and Plasmids

Strain/Plasmid	Genotype	Reference/Source
WM76	DH5α pWM76 placI^q-tet	this work
JCB819	MC1000 *dsbB*::kan5 *phoR malF-lac*Z102 *zih*::Tn*10*	lab collection
ZK126	W3110 Δ*lac*U169 *tna*-2	D. Siegele, Texas A + M
DS187	W3110 Δ*lac*U169 *tna-2* Δ*cyd*::cam	D. Siegele, Texas A + M
DS253	ZK126 Δ*cyd*::cam Δ*cyo*::kan	D. Siegele, Texas A + M
GL101	Δ*cyo*::kan	R. Gennis, U of Illinois
GO105/pTK1	Δ(*cyd*AB′)455, *z bg* − 2200::Km^R*cyo*, *recA*, *srl*-300::Tn10 *mcrA*, F′ [*lacI*^{q}Δ(*lac*Z)M15, *proAB*$^+$]	R. Gennis, U of Illinois
GO103/pJRhisA	Δ*cyd*::kan	R. Gennis, U of Illinois
MB21	ZK126 zjd::Tn10 Δ(*frdABCD*)18	this work
pWM76	*dsbB* C8A, C49V	this work
pTK1	*cydA*$^+$, *cydB*$^+$	R. Gennis, U of Illinois
pJRhisA	*cyoA*$^+$, *cyoB-histag*$^+$, *cyoC*$^+$, *cyoD*$^+$	R. Gennis, U of Illinois

shuttle electrons from ubiquinone to oxygen (Kita et al., 1984; Puustinen et al., 1991). Our work shows that oxidized ubiquinone can effectively drive the DsbA–DsbB system, suggesting that ubiquinone is an intermediate in this pathway. Under anaerobic conditions, menaquinone was found to be able to function as an alternate electron acceptor (see Figure 6). This branching of the disulfide catalytic pathway at DsbB ensures that disulfide bond formation will proceed whether the cell grows under aerobic or anaerobic conditions. This redundancy provides testimony to how important it is to the cell that the disulfide bond formation reaction in the protein folding pathway take place.

Cytochrome *bo* and *bd* oxidases differ in their expression pattern and affinity for oxygen. Cytochrome *bo* oxidase is abundant under aerobic conditions but is repressed up to 140-fold during anaerobic growth (Cotter et al., 1990). Cytochrome *bd* oxidase is maximally expressed in response to limiting oxygen concentrations (≤7% air saturation) (Cotter et al., 1990; Tseng et al., 1996). The low apparent K_m for oxygen (20 nM–1 μM) of cytochrome *bd* oxidase agrees with its function being the major oxidase under low aeration (Jünemann et al., 1995). The apparent very high affinity of cytochrome *bd* oxidase for oxygen, reflected by its extremely low K_m, may allow it to scavenge enough oxygen to allow disulfide bond formation to proceed efficiently even under conditions many would consider anaerobic.

DsbB's choice of quinones as electron acceptors allows various pathways for electron flow to support disulfide bond formation. The route should go via cytochrome *bo* oxidase under aerobic conditions. The cell will switch over to using cytochrome *bd* oxidase under conditions of low oxygen concentration. Under truly anaerobic conditions, flow can occur via menaquinone to alternate final electron acceptors such as fumarate, nitrate, or DMSO. The multiple branching of the electron transport pathway at DsbB also helps to explain why selections for mutations severely defective in disulfide bond formation yielded only *dsbA* and *dsbB* (Bardwell et al., 1993). Individual mutations in either cytochrome *bo* or *bd* oxidase do not show a severe defect in the ability to maintain DsbB's activity because these oxidases can substitute for each other. Even a double mutant that eliminates both oxidases will not show a defect in disulfide bond formation, because DsbB can use the alternate electron acceptor menaquinone. We would predict that to stop disulfide bond formation one would need to remove both menaquinone and ubiquinone from the cell. Unfortunately, a *ubiA menA* double mutant is inviable unless supplied with quinone analogs. Ito and colleagues washed a *ubiA menA* double mutant free from quinone analogs and found that a defect in disulfide bond formation develops in the dying cells (Kobayashi et al., 1997; Kobayashi and Ito, 1999). These in vivo experiments are very strongly suggestive of a relationship between disulfide bond formation and electron transport.

Our results show how disulfide bond formation is linked to electron transport and imply that oxidative protein folding is driven by electron transport. They also help resolve a long-standing mystery: the source of oxidative power used for protein folding and how these oxidizing equivalents flow within the cell. It remains to be seen if a similar connection can be made in the eukaryotic oxidative folding system.

Experimental Procedures

Construction of a DsbB Mutant Lacking the Nonessential Cysteines

A *dsbB* mutant that lacks the two nonessential cysteines *dsbB* Cys8Ala, Cys49Val was constructed by PCR mutagenesis using the following primers: CB8 (5′-CCCCCGCATGCTGCGATTTTTGA ACC-3′), CB9 (5′-GGAAGATCTGCGACCGAACAGATCACG-3′), WM4 5′-GCCGAATAACGCCGACGCGTTCATAATTA-3′), and WM3 (5′-TGA ACGCGTCGGGTTATTCGGCGTTCTG-3′). This mutation was cloned into the expression plasmid pQE70 (Qiagen). The entire sequence of the *dsbB* coding region was verified. We changed these cysteines because this change prevents the disulfide-linked aggregation behavior that the wild-type DsbB protein is susceptible to. These cysteines are not conserved, and substituting them by mutation has no deleterious effect on function either in vivo or in vitro. The Cys8Ala, Cys49Val DsbB protein is referred to in this paper simply as DsbB. Construction of expression plasmids for DsbB mutants lacking various essential cysteines was performed by cloning mutations previously constructed by Jander et al. (1994) into pQE70. Additional mutagenesis was performed on these plasmids using primer WM7 (5′AGGAGAAATTAAGCATGCTGCGATTTTTGAACCAATGTTCACA AGGCCG-3′) and the primers described above to generate three mutant *dsbB* genes: one that contained no cysteines whatsoever, one that contained only Cys-40 and Cys-44, and one that contained only Cys-104 and Cys-130.

Growth of Strains

The strains used are shown in Table 3. The DsbB-overproducing strain WM76 was grown to OD_{600} = 1.0 in 1 l of Luria broth (LB) containing 100 μg/ml ampicillin in 5 l baffled shaker flasks, induced with 15 μM IPTG, and harvested 4 hr after induction. JCB819 and GL101 were grown in LB and harvested after cells had reached stationary phase. DS187 was grown in LB into midlog phase. DS253, which lacks both terminal oxidases, was grown under anaerobic conditions. LB (500 ml) was inoculated from a single colony and grown for at least 24 hr in sealed containers containing an atmosphere of H_2 and CO_2 (GasPak Anaerobic System) without shaking at 30°C.

GO103/pJRhisA and GO105/pTK1 were generous gifts from Dr. Robert Gennis, University of Illinois. These strains overexpress cytochrome *bo* and cytochrome *bd* terminal oxidases, respectively, and were grown essentially as described before (Kaysser et al., 1995; Rumbley et al., 1997).

Preparation of Membranes

All steps were carried out at 4°C. Cells were centrifuged for 20 min at 5000 × g and resuspended in 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 1 mM PMSF. The suspension was passed through a French pressure cell twice at 16,000 psi and centrifuged for 20 min at 10,000 × g to remove cell debris. Membranes were prepared from the supernatant by ultracentrifugation at 100,000 × g for 1.5 hr in a Beckman type 50.2Ti rotor. The membrane pellet was resuspended in 50 mM sodium phosphate (pH 8.0), 300 mM NaCl unless otherwise noted.

Purification of Proteins

DsbB Protein

DsbB was purified over a Ni-NTA column (Qiagen) from an overexpressing strain as described before (Bader et al., 1998). Minor contaminating proteins were removed either by precipitating the protein at pH 4.5 or by hydroxyapatite chromatography. The pH 4.5 precipitation step involved simple dialysis overnight against 50 mM sodium citrate (pH 4.5), 300 mM NaCl followed by centrifugation at 40,000 × g. The DsbB remained soluble at this pH, but contaminates were insoluble. The hydroxyapatite chromatographic step was performed as follows. DsbB-containing fractions from the Ni-NTA column were pooled and loaded directly onto a 10 ml hydroxyapatite (Biorad) column equilibrated with 50 mM sodium phosphate (pH 8.0), 300 mM NaCl, 0.02% n-Dodecyl-β-D-maltoside (Anatrace). The column was then washed with 50 mM sodium phosphate (pH 6.2), 100 mM NaCl, 0.02% n-Dodecyl-β-D-maltoside, and DsbB eluted with a linear 120 ml gradient ranging from 50 mM to 500 mM sodium phosphate (pH 6.2), 0.02% n-Dodecyl-β-D-maltoside. Contaminate-free fractions were pooled, concentrated, and dialyzed against 10 mM HEPES (pH 7.5), 300 mM NaCl. DsbB was stored at −70°C.

DsbB-Activating Factor #1

The first DsbB-activating factor was purified from membranes as follows. Membranes isolated from JCB819 (*dsbB*::kan5) were resuspended and solubilized in 50 mM sodium phosphate (pH 8.0), 100 mM NaCl, 1 mM PMSF, 0.5% n-Dodecyl-β-D-maltoside for 1.5 hr at a protein concentration of 5–10 mg/ml. The sample was then centrifuged at 100,000 × g. Solid ammonium sulfate was added to 60% saturation, stirred for 10 min, and centrifuged at 13,000 × g. The supernatant was collected and dialyzed overnight versus 50 mM sodium phosphate (pH 8.0), 100 mM NaCl, 1 mM EDTA. The sample was loaded onto a 25 ml Q Sepharose column (Pharmacia) that had been equilibrated with the dialysis buffer containing 0.02% n-Dodecyl-β-D-maltoside. The column was eluted with a 150 ml gradient from 100–250 mM NaCl. Fractions were tested for their ability to reactivate DsbB, pooled, and loaded onto a 10 ml hydroxyapatite. This column was equilibrated with 50 mM sodium phosphate (pH 8.0), 100 mM NaCl, 0.02% n-Dodecyl-β-D-maltoside. After loading of the sample, the column was washed with 200 mM sodium phosphate (pH 6.8), 0.02% n-Dodecyl-β-D-maltoside. The DsbB-activating component was eluted with 500 mM sodium phosphate (pH 6.8), 0.02% n-Dodecyl-β-D-maltoside and dialyzed overnight versus 25 mM Bis-Tris (pH 6.4), 20 mM NaCl, 1 mM EDTA. After dialysis, it was further purified over a 10 ml PBE-94 chromatofocusing column (Pharmacia) equilibrated with the same buffer plus 0.02% n-Dodecyl-β-D-maltoside. The column was washed with two column volumes of equilibration buffer and eluted with Polybuffer 74-HCl (1:8) (pH 4.0). Fractions were tested in the fluorescence assay, and those who could reactivate DsbB were pooled, concentrated, and dialyzed versus 25 mM sodium phosphate (pH 7.0), 100 mM NaCl. The sample was further analyzed by SDS-PAGE and UV/Vis spectroscopy. To obtain spectra of a reduced sample, a few grains of dithionite were added to the cuvette and the spectra recorded. N-terminal sequencing was carried out at the University of Michigan protein sequencing core.

DsbB-Activating Factor #2

The second DsbB-activating factor was purified from n-Dodecyl-β-D-maltoside extracted membranes (DS187). The sample was loaded onto a DEAE column equilibrated with 20 mM Tris-HCl (pH 8.0), 20 mM NaCl, 0.02% n-Dodecyl-β-D-maltoside. The second DsbB-activating factor eluted >200 mM NaCl and was loaded directly onto a hydroxyapatite column equilibrated with 10 mM sodium phosphate (pH 7.4), 20 mM NaCl, 0.02% n-Dodecyl-β-D-maltoside. The column was then washed with a 100 ml gradient from 10 to 400 mM sodium phosphate (pH 7.4), 0.02% n-Dodecyl-β-D-maltoside. The DsbB-activating factor was eluted with 700 mM sodium phosphate (pH 7.4), 0.02% n-Dodecyl-β-D-maltoside.

Cytochrome Oxidases

The cytochrome *bo* terminal oxidase was purified from GO103/pJRhisA over Ni-NTA according to a published procedure (Rumbley et al., 1997). For the purification of cytochrome *bd* oxidase from GO105/PTK1, we followed the protocol described by Kaysser et al. (1995) except that n-Dodecyl-β-D-maltoside was used as a detergent.

DsbA

DsbA was purified essentially as described by Wunderlich and Glockshuber (1993).

All proteins purified from overexpression strains were >95% pure, as judged by SDS-PAGE.

K_m Determination for Oxygen

To determine the K_m value for oxygen as a substrate of the disulfide catalytic system, rapid kinetic measurements under anaerobic and partially aerobic conditions were performed on a Hi Tech Scientific Model SF-61 stopped flow flourospectrophotometer scrubbed to remove any trace of oxygen (Bull and Ballou, 1981). To achieve different concentrations of oxygen as substrate, two tonometers were used. One tonometer contained purified DsbB and the other contained DsbA. Initially, each tonometer was rendered completely anaerobic by equilibration with oxygen-scrubbed argon exchanged 12 times over 25 min. The lack of oxygen was verified by lack of DsbB-catalyzed DsbA oxidation. Next, the tonometer containing DsbA was equilibrated to various concentrations of oxygen ranging from 0.1% to 20% O_2 at 22.5°C. It was assumed that the resulting O_2 concentration in each fast flow mixed reaction would be one-half the amount equilibrated into the single tonometer. Initial trials revealed virtually no change in the rate of the initial reaction until a concentration of less than 40 μM oxygen was present. Thus, we estimated the K_m value for oxygen to be <40 μM. To obtain various low concentrations of oxygen ranging between 0 and 50 μM, the DsbA tonometer was equilibrated with 5% oxygen and then mixed with known concentrations of protocatechuate (PCA) and protocatechuate dioxygenase (PCD) to deplete a specific amount of the oxygen out of the tonometer before being used in the DsbB/DsbA reaction. When reactions were carried out at substoichiometric levels of O_2, the point in the reaction curve where oxygen was depleted was denoted by a sharp termination in the progress curve, which in itself also indicates a small K_m value for oxygen. In later trials, it was discovered that PCA/PCD was not necessary to scrub the oxygen in the tonometer to a known value. When 5% or less O_2 is introduced into an extensively degassed tonometer, the measurable oxygen concentration was observed to slowly decline. This is probably because the Teflon washers in the prefire chamber are extracting oxygen out of the solution. Teflon is known to have a high affinity for oxygen. We exploited this phenomenon to rapidly generate initial velocity measurements at a large number of low oxygen tensions. By calculating the extent of DsbA oxidized via its fluorescence quench in the completed reaction, we were able to determine the

oxygen concentration actually present in the reaction. From a plot of the initial reaction rate versus the observed oxygen concentration, we calculated a K_m value for oxygen (Figure 1).

Activity Assay

The activity of DsbB can be followed by monitoring the redox state of its in vivo substrate DsbA using fluorescence spectroscopy (Bader et al., 1998). DsbA was reduced by incubation with 10 mM DTT. DTT was removed by gel filtration using a PD-10 column and stored in distilled water containing 0.1 mM EDTA at −70°C. The reoxidation of DsbA in the presence of DsbB in 50 mM sodium phosphate (pH 6.0), 300 mM NaCl, 0.5 mM EDTA at 30°C was followed by fluorescence change using an excitation wavelength of 295 nm and an emission wavelength of 330 nm. The assay volume was 600 μl. The quinone reaction used the same buffer plus 0.1% n-Dodecyl-β-D-maltoside. The rates expressed in terms of μmol DsbA per μmol DsbB min^{-1} were derived from the initial linear fluorescence decrease by using a ratio of fluorescence of reduced DsbA/oxidized DsbA = 3.4 at pH 6.0. Spontaneous air oxidation of DsbA is negligible for this period of time.

Determination of the Periplasmic Redox State In Vivo

The isogenic strains ZK126, DS253, and MB21 were transformed with pUC18 in order to introduce a source of β-lactamase. JCB819 *dsbB*::kan5 transformed with the same plasmid served as a disulfide-defective control strain. An overnight culture grown in LB glucose was used to dilute cells 1:500 into minimal media supplemented with 0.4% glucose and 0.1% casamino acids. Anaerobic growth was carried out in sealed jars (GasPak Anaerobic System) for 24 hr. Disulfide exchange was prevented by trapping with 0.1 M iodoacetamide in 50 mM Tris (pH 8.5) for 10 min at 37°C. Protein was precipitated with 8% TCA and resuspended in 0.9 M Tris (pH 8.5), 1% SDS, 0.5 mM EDTA, 0.1 M iodacetamide. The oxidized form of β-lactamase was separated from its reduced form on 14% SDS-PAGE, blotted onto nitrocellulose, and detected with anti-β-lactamase antibody (5-prime-3-prime Inc.).

Protein Determination

Total membrane protein concentration was determined by the Sigma protein kit, which is based on the method of Lowry. The concentration of DsbB in membranes was determined as described (Bader et al., 1998). An absorption coefficient of 47.9 $mM^{-1}cm^{-1}$ at 280 nm was used to calculate the concentration of DsbB. The concentration of cytochrome *bo* and *bd* oxidases was determined as described before (Miller and Gennis, 1983; Kita et al., 1984).

Acknowledgments

We thank Ursula Jakob for her encouragement and many helpful discussions and comments on the manuscript. We gratefully acknowledge Robert B. Gennis and Blanca Barquera (University of Illinois) for providing the cytochrome *bo* and *bd* overexpression strains and for many helpful suggestions. We would like to thank Gary Cecchini (University of California, San Francisco) for the fumarate reductase knockout strain. We thank Charlie Yocum and Bob Bender for comments on the manuscript. This work was supported by the National Institutes of Health grants (J. C. A. B. and D. P. B.), a BMBF grant (J. C. A. B.), and a scholarship from the PEW charitable trusts (J. C. A. B.). W. M. was supported by a joint Parke-Davis/University of Michigan postdoctoral fellowship.

Received May 13, 1999; revised June 16, 1999.

References

Andersen, C.L., Matthey-Dupraz, A., Missiakas, D., and Raina, S. (1997). A new *Escherichia coli* gene, dsbG, encodes a periplasmic protein involved in disulphide bond formation, required for recycling DsbA/DsbB and DsbC redox proteins. Mol. Microbiol. *26*, 121–132.

Bader, M., Muse, W., Zander, T., and Bardwell, J. (1998). Reconstitution of a protein disulfide catalytic system. J. Biol. Chem. *273*, 10302–10307.

Bader, M., Winther, J.R., and Bardwell, J.C.A. (1999). Protein oxidation: prime suspect found "not guilty." Nat. Cell Biol. *1*, E56–E58.

Bardwell, J.C. (1994). Building bridges: disulphide bond formation in the cell. Mol. Microbiol. *14*, 199–205.

Bardwell, J.C., McGovern, K., and Beckwith, J. (1991). Identification of a protein required for disulfide bond formation in vivo. Cell *67*, 581–589.

Bardwell, J.C., Lee, J.O., Jander, G., Martin, N., Belin, D., and Beckwith, J. (1993). A pathway for disulfide bond formation *in vivo*. Proc. Natl. Acad. Sci. USA. *90*, 1038–1042.

Belin, P., Quemeneur, E., and Bouquet, P.L. (1993). A pleiotropic acid phosphatase-deficient mutant of *E. coli* shows premature termination in the *dsbA* gene. Mol. Gen. Genet. *242*, 23–32.

Bessette, P.H., Cotto, J.J., Gilbert, H.F., and Georgiou, G. (1999). *In vivo* and *in vitro* function of the Escherichia coli periplasmic cysteine oxidoreductase DsbG. J. Biol. Chem. *274*, 7784–7792.

Bull, C., and Ballou, D.P. (1981). Purification and properties of protocatechuate 3,4 dioxygenase from Pseudomonas putida. J. Biol. Chem. *256*, 12673–12680.

Cotter, P.A., Chepuri, V., Gennis, R.B., and Gunsalus, R.P. (1990). Cytochrome o (cyoABCDE) and d (cydAB) oxidase gene expression in *Escherichia coli* is regulated by oxygen, pH, and the fnr gene product. J. Bacteriol. *172*, 6333–6338.

Cuozzo, J.W., and Kaiser, C.A. (1999). Competition between glutathione and protein thiols for disulphide-bond formation. Nat. Cell Biol. *1*, 130–135.

Fabianek, R.A., Hennecke, H., and Thony-Meyer, L. (1998). The active-site cysteines of the periplasmic thioredoxin-like protein CcmG of *Escherichia coli* are important but not essential for cytochrome c maturation *in vivo*. J. Bacteriol. *180*, 1947–1950.

Frand, A.R., and Kaiser, C.A. (1998). The ERO1 gene of yeast is required for oxidation of protein dithiols in the endoplasmic reticulum. Mol. Cell *1*, 161–170.

Gilbert, H.F. (1997). Protein disulfide isomerase and assisted protein folding. J. Biol. Chem. *272*, 29399–29492.

Grauschopf, U., Winther, J.R., Korber, P., Zander, T., Dallinger, P., and Bardwell, J.C. (1995). Why is DsbA such an oxidizing disulfide catalyst? Cell *83*, 947–955.

Guilhot, C., Jander, G., Martin, N.L., and Beckwith, J. (1995). Evidence that the pathway of disulfide bond formation in *Escherichia coli* involves interactions between the cysteines of DsbB and DsbA. Proc. Natl. Acad. Sci. USA *92*, 9895–9899.

Hennecke, J., Spleiss, C., and Glockshuber, R. (1997). Influence of acidic residues and the kink in the active-site helix on the properties of the disulfide oxidoreductase DsbA. J. Biol. Chem. *272*, 189–195.

Hwang, C., Sinskey, A.J., and Lodish, H.F. (1992). Oxidized redox state of glutathione in the endoplasmic reticulum. Science *257*, 1496–1502.

Jander, G., Martin, N.L., and Beckwith, J. (1994). Two cysteines in each periplasmic domain of the membrane protein DsbB are required for its function in protein disulfide bond formation. EMBO J. *13*, 5121–5127.

Joly, J.C., and Swartz, J.R. (1997). *In vitro* and *in vivo* redox states of the *Escherichia coli* periplasmic oxidoreductases DsbA and DsbC. Biochemistry *36*, 10067–10072.

Jünemann, S. (1997). Cytochrome *bd* terminal oxidase. Biochim. Biophys. Acta *1321*, 107–127.

Jünemann, S., Butterworth, P.J., and Wrigglesworth, J.M. (1995). A suggested mechanism for the catalytic cycle of cytochrome *bd* terminal oxidase based on kinetic analysis. Biochemistry *34*, 14861–14867.

Kaysser, T.M., Ghaim, J.B., Georgiou, C., and Gennis, R.B. (1995). Methionine-393 is an axial ligand of the heme b558 component of the cytochrome *bd* ubiquinol oxidase from *Escherichia coli*. Biochemistry *34*, 13491–134501.

Kita, K., Konishi, K., and Anraku, Y. (1984). Terminal oxidases of *Escherichia coli* aerobic respiratory chain. I. Purification and properties of cytochrome b562-o complex from cells in the early exponential phase of aerobic growth. J. Biol. Chem. *259*, 3368–3374.

Kobayashi, T., and Ito, K. (1999). Respiratory chain strongly oxidizes the CXXC motif of DsbB in the Escherichia coli disulfide bond formation pathway EMBO J. *18*, 1192–1198.

Kobayashi, T., Kishigami, S., Sone, M., Inokuchi, H., Mogi, T., and Ito, K. (1997). Respiratory chain is required to maintain oxidized states of the DsbA-DsbB disulfide bond formation system in aerobically growing *Escherichia coli* cells. Proc. Natl. Acad. Sci. USA *94*, 11857–11862.

Miller, M.J., and Gennis, R.B. (1983). The purification and characterization of the cytochrome d terminal oxidase complex of the *Escherichia coli* aerobic respiratory chain. J. Biol. Chem. *258*, 9159–9165.

Missiakas, D., Schwager, F., and Raina, S. (1995). Identification and characterization of a new disulfide isomerase-like protein (DsbD) in *Escherichia coli*. EMBO J. *14*, 3415–3424.

Pollard, M.G., Travers, K.J., and Weissman, J.S. (1998). Ero1p: a novel and ubiquitous protein with an essential role in oxidative protein folding in the endoplasmic reticulum. Mol. Cell *1*, 171–182.

Puustinen, A., Finel, M., Haltia, T., Gennis, R.B., and Wikstrom, M. (1991). Properties of the two terminal oxidases of *Escherichia coli*. Biochemistry *30*, 3936–3942.

Rietsch, A., Belin, D., Martin, N., and Beckwith, J. (1996). An *in vivo* pathway for disulfide bond isomerization in *Escherichia coli*. Proc. Natl. Acad. Sci. USA *93*, 13048–13053.

Rumbley, J.N., Furlong Nickels, E., and Gennis, R.B. (1997). One-step purification of histidine-tagged cytochrome bo3 from *Escherichia coli* and demonstration that associated quinone is not required for the structural integrity of the oxidase. Biochim. Biophys. Acta *1340*, 131–142.

Tseng, C.P., Albrecht, J., and Gunsalus, R.P. (1996). Effect of microaerophilic cell growth conditions on expression of the aerobic (cyoABCDE and cydAB) and anaerobic (narGHJI, frdABCD, and dmsABC) respiratory pathway genes in *Escherichia coli*. J. Bacteriol. *178*, 1094–1098.

Wallace, B.J., and Young, I.G. (1977). Role of quinones in electron transport to oxygen and nitrate in *E. coli*. Biochim. Biophys. Acta *461*, 84–100.

Wunderlich, M., and Glockshuber, R. (1993). Redox properties of protein disulfide isomerase (DsbA) from *Escherichia coli*. Protein Sci. *2*, 717–726.

Zapun, A., Bardwell, J.C., and Creighton, T.E. (1993). The reactive and destabilizing disulfide bond of DsbA, a protein required for protein disulfide bond formation *in vivo*. Biochemistry *32*, 5083–5092.

Zeng, H., Snavely, I., Zamorano, P., and Javor, G.T. (1998). Low ubiquinone content in *Escherichia coli* causes thiol hypersensitivity. J. Bacteriol. *180*, 3681–3685.