In Vivo and in Vitro Function of the Escherichia coli Periplasmic Cysteine Oxidoreductase DsbG*

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We have characterized in vivo and in vitro the recently identified DsbG from Escherichia coli. In addition to sharing sequence homology with the thiol disulfide exchange protein DsbC, DsbG likewise was shown to form a stable periplasmic dimer, and it displays an equilibrium constant with glutathione comparable with DsbA and DsbC. DsbG was found to be expressed at approximately 25% the level of DsbC. In contrast to earlier results (Andersen, C. L., Matthey-Dupraz, A., Missiakas, D., and Raina, S. (1997) Mol. Microbiol. 26, 121–132), we showed that dsbG is not essential for growth and that dsbG null mutants display no defect in folding of multiple disulfide-containing heterologous proteins. Overexpression of DsbG, however, was able to restore the ability of dsbC mutants to express heterologous multidisulfide proteins, namely bovine pancreatic trypsin inhibitor, a protein with three disulfides, and to a lesser extent, mouse urokinase (12 disulfides). As in DsbC, the putative active site thiol in DsbG are completely reduced in vivo in a dsbD-dependent fashion, as would be expected if DsbG is acting as a disulfide isomerase or reductase. However, the latter is not likely because DsbG could not catalyze insulin reduction in vitro. Overall, our results indicate that DsbG functions primarily as a periplasmic disulfide isomerase with a narrower substrate specificity than DsbC.

Disulfide bonds are essential for the correct folding and stability of many exocytoplasmic proteins (2). The oxidation of cysteine residues to form disulfide bonds can occur spontaneously in the presence of molecular oxygen. However, air oxidation is a slow, mechanistically complex reaction whose time scale is much longer than what is required for the folding of proteins under biosynthetic conditions, i.e. in the cell. As a result, both prokaryotic and eukaryotic cells have evolved elaborate enzymatic mechanisms for the catalysis of disulfide bond formation and for maintaining the proper thiol-disulfide redox balance in various cellular compartments.

In Gram-negative bacteria, disulfide bond formation normally occurs following export into the periplasmic space, which is topologically equivalent to the endoplasmic reticulum, albeit substantially more oxidizing (3, 4). Genetic and biochemical studies have unequivocally defined four proteins (DsbA, DsbB, DsbC, and DsbD) that are involved in the formation of disulfide bonds in secreted proteins (5). All the known Dsb proteins contain a Cys-X-X-Cys sequence that is characteristic of the thioredoxin superfamily (5). DsbA is a soluble periplasmic enzyme that serves as a potent catalyst of protein and peptide cysteine oxidation (6–8). It also exhibits some disulfide isomerization activity that may be important under some conditions in vivo (9–11). Once it has transferred its disulfide bond to a substrate, DsbA is rapidly reoxidized by the membrane protein DsbB, which in turn transfers its electrons either to molecular oxygen or to the quinone system (12, 13). The oxidation of protein cysteine by DsbA is very rapid but often results in the formation of incorrect disulfide bonds. The rearrangement of nonnative disulfides is catalyzed primarily by the dimeric periplasmic enzyme DsbC (8, 14, 15). For DsbC to be able to catalyze disulfide bond isomerization, its active site Cys-X-X-Cys sequence must be present in the dithiol form. Although the redox potentials of DsbA and DsbC are comparable, −89 mV (7) and −96 mV (calculated from Ref. 8), respectively, under steady state conditions in the periplasm DsbA is oxidized, whereas DsbC is almost exclusively reduced (10, 16, 17). Reduction of DsbC is mediated by cytoplasmic membrane protein DsbD and also depends on the cytoplasmic proteins thioredoxin (TrxA) and thioredoxin reductase (TrxB) (10, 17).

In addition to dsbA, dsbB, dsbC, and dsbD, Missiakas and Raina (18) have isolated additional genes that affect sensitivity to dithiothreitol (DTT). One of these genes was named dsbE and has been proposed to play a role in oxidative protein folding. However, dsbE is identical to ccmG, which has been shown to be an inner membrane protein involved in cytochrome biosynthesis in Escherichia coli and other bacteria (19–21). It is not yet known whether DsbE affects the oxidation state of proteins other than cytochrome c. Recently, Raina and coworkers (1) isolated a gene that functioned as a multicopy suppressor of DTT sensitivity in a dsbB− background. The same gene (dsbG) was also isolated in a search for mutants conferring increased sensitivity to DTT and an increase in σ8-dependent periplasmic heat shock response. Interestingly, Andersen et al. (1) reported that dsbG is required for growth unless the cells are provided with exogenous oxidants. This is surprising, since none of the other dsb genes, including dsbA, which encodes the main catalyst of protein oxidation in the periplasm, is essential. In addition, Andersen et al. (1) reported

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1 The abbreviations used are: DTT, dithiothreitol; PCR, polymerase chain reaction; Cm, chloramphenicol; CmR, Cm-resistant; AmpR, ampicillin-sensitive; KanR, kanamycin-resistant; StrpR, streptomycin-resistant; Tet, tetracycline; TetR, Tet-resistant; IPTG, isopropyl-β-D-thiogalactoside; BPTI, bovine pancreatic trypsin inhibitor; AMS, 4-acetamido-4-maleimidylstibine-2-2′-disulfonic acid; EGS, ethylene glycol-bis(succinimidylsuccinate); PDI, protein-disulfide isomerase; PAGE, polyacrylamide gel electrophoresis; kbp, kilobase pair(s).
that dsbG can catalyze insulin reduction in vitro and is partially responsible for the oxidation of alkaline phosphatase in the periplasm. We independently cloned and expressed DsbG and characterized its in vivo and in vitro function in detail. Contrary to the previous report, we show that dsbG is not an essential gene in E. coli; it is maintained by DsbD exclusively in reduced form, and it does not appear to affect protein oxidation in the periplasm. On the other hand, multicyclic expression of dsbG suppressed the effect of a dsbC− mutation on the folding of heterologous multidisulfide substrates. DsbG was found to have a very unstable disulfide much like DsbA and DsbC, but unlike these two proteins it appears to have narrow substrate specificity. Our results indicate that DsbG functions predominantly either as a reductant or as a catalyst for disulfide isomerization.

**EXPERIMENTAL PROCEDURES**

**Cloning and Expression**—The strains and plasmids used in this work are listed in Table I. The 5-kbp Eco52I/SalI fragment containing the E. coli dsbG gene from Kohara clone 166 (22) was isolated and cloned into Eco52I/SalI-digested pBR322, generating plasmid pPDsbG. The dsbG coding region was amplified from pPDsbG by polymerase chain reaction (PCR) using the primers 5′-AGGAATTCCAGGGTTCTCTCATGTTTAATAAGATTCTTAC-3′ and 5′-CCATCATGAGGATCCTTTTTCAAATGTATT-3′. The PCR product was digested with BsaI and BamHI and ligated into NcoI/BamHI-digested pTrc99A (Amer sham Pharmacia Biotech) or pET11d (Novagen, Madison, WI), generating plasmids pTrcdsbG and pETdsbG, respectively. Likewise, dsbG coding region was amplified from pPDsbG with the same forward primer and reverse primer 5′-CTAGAGGCTTCGATGTTATACCCCTATAATGAC-3′, digested with BsaI and XhoI, and ligated into pET-28a (Novagen, Madison, WI) that had been cut with NcoI and XhoI. The resulting plasmid, designated pETdsbG2his, contains the coding sequence for DsbG fused to a 6× histidine tag at its C terminus and a portion of the T7 promoter. Plasmid constructions were verified by automated DNA sequencing.

**Expression and Purification of DsbG**—Plasmid pETdsbG2his was transformed into strain BL21(DE3), and histidine-tagged DsbG was purified from the osmotic shock using nickel-chelate chromatography, following standard protocols (Qiagen, Santa Clarita, CA). The purity of DsbG in the nickel-Sepharose™ eluant was approximately 95% as judged by SDS-PAGE and Coomassie Brilliant Blue staining. This material was used to raise polyclonal antiserum in mice using standard protocols (23). Rabbit polyclonal antiserum against DsbA and DsbC was a gift of John Joly (Genentech, S. San Francisco, CA). Purified DsbA was purchased from Boehringer Mannheim. Purified DsbC was a kind gift of John Joly. All other chemicals were purchased from Sigma except as noted.

To purify DsbG overexpressed without a hexahistidine affinity tag, E. coli BL21(DE3) harboring the plasmid pTrcdsbG2 were grown in LB medium with 50 μg/ml ampicillin at 37 °C to midlog phase (A600 ~ 0.5). Cells were then transferred to a 25 °C water bath and induced with 0.5 mM IPTG for 8 h to maximize the concentration of soluble, mature protein in the periplasm. Immediately after induction, the cells were collected by centrifugation, and periplasmic fractions were obtained by the cold osmotic shock procedure as modified by Thorstenson et al. (24). The periplasmic fraction was dialyzed against 30 mM Tris-HCl, pH 8.5, 0.5 mM EDTA, and 50 mM NaCl and applied to a DEAE anion exchange column (Bio-Rad), equilibrated with the same buffer. The column was developed with a linear gradient of NaCl from 50 to 300 mM. Analysis of the collected fractions by SDS-PAGE showed that DsbG eluted between 100 and 150 mM NaCl. The peak fractions were then pooled, concentrated, and dialyzed against 10 mM Tris-HCl, pH 8.0, 0.5 mM EDTA buffer and applied to a Sephadex™-25 sizing column (Amersham Pharmacia Biotech) previously equilibrated with the same buffer. Densitometric analysis of a scanned SDS-PAGE gel revealed that following gel filtration the protein was at least 95% pure. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry was performed on a Voyager Biospectrometer (PerSeptive Systems Voyager Biospectro) with a mass resolution of greater than 95 and error of less than 0.07%.

**Construction of dsbG− Mutant Strains**—Plasmid pPBdsbG was digested with AflII and Sse8387I; the large fragment was isolated, and its ends blunted with T4 polynuclease. This fragment was then ligated with either the 1.4-kbp BsaAI fragment of pACYC184 containing the chloramphenicol resistance gene to generate pPBdsbG-Cm or the 1.3-kbp kanamycin resistance cassette from pUC-4K to generate pPBdsbG-Kan. Plasmid pPBdsbG-Cm was linearized and transformed into the recD− strain D301 (25), and CmR, AmpR colonies were selected. Additionally, plasmid pPBdsbG-Kan was digested with Bpu36I and SpI1, and the fragment containing the KanR cassette was cloned into the E. coli chromosomal region flanking the dsbG gene from plasmid pBAD39, a vector containing a conditional IPTG-requiring replicon, an ampicillin resistance gene, and a wild type rpsL gene (Table I). MC4100 cells transformed with the resulting plasmid, pBADdsbG::Kan, were grown first in liquid medium without IPTG and then plated on LB agar containing kanamycin, and streptomycin for counterselection. Colonies that were KanR, AmpR, and SpI1 were selected, and correct constructs were confirmed both by immunoblotting to verify the absence of a DsbG band and by PCR using primers flanking the deletion region. The ΔdsbG:cm and ΔdsbG::Kan mutations were transduced into different strains using P2vir following standard protocols (26).

**Expression of BPTI and Urokinase**—Bovine pancreatic trypsin inhibitor (BPTI) expression was monitored by enzyme-linked immunosorbent assay as described previously (27). Urokinase activity was detected by indirect chromogenic assay as follows. Cultures were grown at 37 °C to A600 ~ 0.7, and protein synthesis was induced by adding IPTG to 1 mM. The cells were harvested 3 h later and lysed by French® pressure cell. Following centrifugation to remove insolubles, the total soluble protein was quantified by the Bradford assay (Bio-Rad), using bovine serum albumin as a standard. After further dilution to 50 μg of purified DsbA in Tris-CHI, pH 7.4, 0.01% Tween 80. In a microtitrate plate, 50 μl of whole cell lysate soluble fraction was mixed with 50 μl of human plasminogen (Calbiochem), 0.1 μg/μl in 50 mM Tris-CHI, pH 7.4, 0.01% Tween 80, 6 mM 6-aminohexanoic acid. The plasminogen substrate, 50 μl of 4 μM Spectrozyme® PL (American Diagnostica, Greenwich, CT), was added immediately, and the plate was incubated at room temperature for 60 min. The absorbance at 405 nm was measured, and after subtracting the background activity of a strain not expressing urokinase, compared with a standard curve prepared using human urokinase (Calbiochem).

**Redox Properties of DsbG**—The in vivo redox state of DsbG was assayed by derivatization of free thiols by 4-acetamido-4-maleimidyl-stilbene-2,2′-disulfonic acid (AMS) (Molecular Probes, Inc., Eugene, OR) unreacted or essentially unreacted for DsbC (10). The oxidized standard was purified DsbG without AMS added. The reduced standard was generated by reducing purified DsbG in 40 mM DTT for 20 min at room temperature followed by removing reducing agent using a gel filtration spin column (Bio-Rad) and AMS derivatization.

The redox equilibrium of DsbG with glutathione was assayed as described for DsbA (7). In this assay, the change in fluorescence intensity (excitation wavelength 280 nm) was measured at the wavelength of maximum emission (330 nm for DsbG and 324 nm for DsbA). Experiments were carried out in 100 mM sodium phosphate, pH 7.0, and 1.0 mM EDTA. Oxidized DsbA or DsbB (0.45 μM) was incubated at 25 °C in the presence of 0.1 mM GSSG and 0–2 mM GSH for 12 h before recording the fluorescence emission in an SLM-Amino Luminescence Spectrometer (series 2). The equilibrium concentrations of GSH and GSSG were calculated according to Equations 1–3:

$$\text{GSH} = \text{GSH}_{0} - 2R[Dsb]_{0} \quad (\text{Eq. 1})$$

$$\text{GSSG} = \text{GSSG}_{0} + R[Dsb]_{0} \quad (\text{Eq. 2})$$

$$R = \left( F - F_{\text{red}} / F_{\text{ox}} - F_{\text{red}} \right) \quad (\text{Eq. 3})$$

where $[\text{GSH}]_{0}$ and $[\text{GSSG}]_{0}$ are the initial concentrations of GSH and GSSG, $R$ is the relative amount of reduced protein at equilibrium, $[\text{Dsb}]_{0}$ is the initial concentration of DsbG or DsbA in the oxidized form, $F$ is the measured fluorescence intensity, and $F_{\text{red}}$ and $F_{\text{ox}}$ are the fluorescence intensities of completely oxidized and reduced protein. The equilibrium constant $K_{eq}$ was estimated from nonlinear regression analysis of the data according to Equation 4 (28).

$$R = \left( [\text{GSH}]_{0}^{2} [\text{GSSG}]_{0} / [\text{GSH}]_{0}^{2} [\text{GSSG}]_{0} \right) \quad (\text{Eq. 4})$$

**Chemical-cross-linking**—Chemical cross-linking was used to determine the oligomeric state of DsbG. The reactions were performed by adding 0.1 volumes of MeSO containing various concentrations (0–5 mM) of the amine cross-linker ethylene glycol-bis(succinimidylsuccinate) (EGS) (Pierce) to protein solutions containing 250 μg/ml of purified DsbG or DsbA. Each reaction was incubated at 4 °C for 30 min and then quenched by the addition of glycine to 75 mM. The samples were resolved by SDS-PAGE (10–20% resolving gels), and the cross-linked products were visualized by Coomassie Brilliant Blue staining.
TABLE I

| Strain | Relevant genotype | Source or reference |
|--------|------------------|---------------------|
| BL21(DE3) | F− strain (DE3) | Laboratory collection |
| MC4100 | F− araD139Δ (argF lac) U169 | Laboratory collection |
| SF110 | KS272 degP41 Δ (ompT entF) | 43 |
| RI89 | MC1000 phiR Δara714 leu + | 14 |
| RI90 | RI89 dsbA::Kan | 14 |
| RI177 | RI89 ΔdsbC::Cm | 14 |
| RI242 | RI89 ΔdsbD::mini-Tn10 Cm | 14 |
| SR3324 | MC4100 Δsdc::Kan | S. Raina |
| D301 | RP451 recD | 25 |
| PB301 | D501 Δsdc::Cm | This study |
| PB303 | RI89 ΔdsbG::Cm | This study |
| PB306 | MC4100 Δsdc::Cm | This study |
| PB343 | MC4100 Δsdc::Kan | This study |
| PB349 | RI89 ΔdsbG::Kan | This study |

Plasmid Relevant features Source or reference
pACYC184 | General cloning vector, p15A ori, chloramphenicol acetyltransferase (Cm\(^{3}\)) gene | New England Biolabs (Beverly, MA) |
pACYC177 | General cloning vector, p15A ori | New England Biolabs |
plBS22 | General cloning vector, colE1 ori | New England Biolabs |
plUC-4K | Aminoglycoside Phosphotransferase (Kan\(^{3}\)) cassette | Pharmacia |
pT799A | Tc promoter expression vector | Novagen |
pET-11d | T7 expression vector | Novagen |
pET-28a | T7 expression vector, C-terminal 6\(\times\) histidine tag | Novagen |
prDDB-A | Constitutive secreted expression of mouse urokinase-type plasminogen activator | 44 |
pIT103 | BPTI fused to the OmpA leader peptide under the control of the lpp-lac promoter | 45 |
pBAD39 | IPTG-requiring conditional replicon, rpsL (Strep\(^{3}\)) gene | L. M. Guzman (Millennium Pharmaceuticals) |
puPA184 | Urokinase gene from pRDB-A cloned into pACYC184 | This study |
pBBPIT | OmpA-BPTI gene from pIT103 cloned into pACYC177 | This study |
pSidB | 5-kbp chromosomal region containing dsbG cloned into pBR322 | This study |
pTredB | Minimal dsbG coding region cloned into pT99a under tcr control | This study |
pETdBG2 | Minimal dsbG coding region cloned into pET-11d behind T7 promoter | This study |
pETdBG2his | dsbG gene without its stop codon cloned into pET-28a with C-terminal 6\(\times\) histidine fusion | This study |
pPBdBG2::Cm | pPBdBG with deletion spanning the first 187 codons of dsbG and insertion of Cm\(^{3}\) from pACYC184 | This study |
pPBdBG2::Kan | pPBdBG with deletion spanning the first 187 codons of dsbG and insertion of Kan\(^{3}\) from pUC-4K | This study |
pBADdBG2::Kan | 2.8-kbp fragment from pPBdBG2::Kan containing Kan\(^{3}\) and dsbG flanking regions cloned into pBAD39 | This study |

In Vitro Oxidoreductase Activity Assays—The ability of DsbA, DsbC, and DsbG to catalyze the reduction of human insulin (Sigma catalog no. I-5523) in the presence of DTT was tested as described previously by Holmgren (29). A stock solution of 5 mM insulin was freshly prepared in 0.1 M potassium phosphate buffer, pH 7.0, and 2 mM EDTA before each assay. The reaction mixtures were prepared directly in cuvettes using 0.1 M potassium phosphate buffer, pH 8.0; 4.5 mM cCMP; GSH and GSSG (at predetermined concentrations to provide a redox buffer); 0.1 M potassium phosphate buffer, pH 7.0, and 2 mM EDTA before each assay. The reaction mixtures consisted of 50 mM Tris acetate buffer, pH 8.0; 4.5 mM cCMP; GSH and GSSG (at predetermined concentrations to provide a redox buffer); pure DsbG or DsbC at concentrations ranging from 1 to 20 μM or 1.5 μM bovine PDI as a positive control. After equilibrating the reaction at 25 °C, the assay was initiated by the addition of reduced, denatured RNase A to a final concentration of 8 μM. Hydrolysis of cCMP by the reduced RNase was monitored every 30 s for 1 h as an increase in the absorbance at 296 nm. As a negative control, the uncatalyzed reaction was recorded in parallel under identical redox conditions. The concentration of active RNase at any time in each assay was calculated as described by Lyles and Gilbert (30).

RESULTS

Cloning and Expression of dsbG—A BLAST (31) search of the E. coli genome indicated the presence of an open reading frame,\(^2\) having 49% similarity and 30% identity to 220 residues of the E. coli DsbC protein. Because of the high degree of similarity between DsbC and the identified hypothetical protein, we reasoned that it may have a role in the formation of disulfide bonds in the periplasmic space. While this work was in progress, Raina and co-workers (1) identified the same open reading frame in a genetic screen for resistance to dithiothreitol and named it dsbG (GenBankTM accession no. AF000956). We cloned the complete 248-amino acid coding region of dsbG into a T7 expression vector fused to a 6\(\times\) histidine tag and purified the expressed protein by immobilized metal affinity chromatography. The N-terminal sequence of the mature purified protein was verified by automated Edman degradation, and cleavage of the signal peptide was shown to occur after Ala\(^{17}\). Antibodies were raised against the resulting material and used to probe a Western blot of whole cell extracts of E. coli not carrying any plasmids. A band of the expected mobility could be detected in lysates of exponential phase cells growing

\(^2\) Found in GenBankTM accession no. AE000166 as b6064 (PID: g1786821) (42). The actual dsbG start codon is 20 amino acids downstream of that identified here.
Characterization of E. coli DsbG

A significantly lower number of KanR colonies relative to the number of transduced. If the introduction of a DsbG mutation resulted in the accumulation of second site suppressor mutations, as suggested by Andersen et al. (1), the dsbG::ΩTet mutation could be transduced onto the chromosome only when the cells were grown in the presence of low molecular weight oxidants such as cystine or oxidized DTT. Despite finding that a dsbG::ΩKan mutation could be transduced without supplementation of oxidants, they nevertheless hypothesized that this observation resulted from the accumulation of second site suppressor mutations. On the basis of these results, they concluded that mutations in dsbG are conditionally lethal. To evaluate this hypothesis, we first constructed a large deletion in dsbG comprising 187 codons and marked it by the insertion of fragments of either 1.4 or 1.3 kbp containing, respectively, a CmR or a KanR gene. First, the CmR-marked dsbG deletion was integrated into the chromosome by homologous recombination in a recD strain. Hundreds of CmR, AmpS colonies were obtained, as expected for a gene that is not essential for viability. Colonies were picked at random and proven to contain ΔdsbG::Cm by PCR using the appropriate primers. Subsequently, the allele was transferred to different strains by P1 transduction.

In a second approach, the ΔdsbG::Kan mutation was inserted into the suicide vector pBAD39 that can only replicate in cells lacking IPTG. KanR, StrepR, and AmpS colonies were tested by sensitivity to ampicillin on plates that also contained IPTG, streptomycin and sensitivity to ampicillin on plates that also contained IPTG. KanR, StrepR, and AmpS colonies were tested by PCR and were all found to carry the expected deletion in dsbG. In order to test whether suppressor mutations had arisen in our dsbG− strains, we transduced the ΔdsbG::Kan mutation to either a wild type strain or the same strain carrying dsbG+ on a multicopy plasmid (pTrcdsbG2). For comparison, the same procedure was repeated in order to recombine the degP41 mutation, a nonlethal KanR-marked deletion in the gene encoding the periplasmic protease DegP (32), into the chromosome of the same two acceptor strains. The ratio of KanR transductants in RI89 to RI89 (pTrcdsbG2) was the same (3:2) regardless of whether ΔdsbG::Kan or the unrelated degP41 mutation was transduced. If the introduction of a dsbG gene disruption in the haploid strain was lethal, and growth could only occur via the accumulation of second site suppressor mutations, as suggested by Andersen et al. (1), then we would expect that transduction of the ΔdsbG::Kan allele into RI89 to result in a significantly lower number of KanR colonies relative to the number of colonies obtained by transducing the unrelated degP41 allele. This, however, was not the case. In summary, the above results clearly show that dsbG is not necessary for growth in RI89. Also, the frequency with which dsbG− transductants could be obtained in MC4100, RI90, and SR3324 strains suggests that dsbG is not essential in these genetic backgrounds either. The dsbG mutation was found to have no effect on alkaline phosphatase activity, growth at 42 °C, or the growth rate in rich or minimal media (data not shown).

The dsbG deletion mutants were also tested for their effect on the folding of heterologous proteins containing multiple disulfide bonds, such as bovine pancreatic trypsin inhibitor (BPTI) and mouse urokinase. BPTI is a 6.5-kDa protein with three disulfides, whereas mouse urokinase is a 48-kDa protein containing 12 disulfides. The folding of both proteins had been shown to be strongly dependent on the presence of functional dsbA, dsbB, dsbC, and dsbD genes (14, 17, 33, 34). However, with the levels of folded BPTI and active urokinase in the dsbG− strain were identical to their wild type counterparts (data not shown).

We furthermore examined the effect of overexpressed DsbG on dsbG folding in heterologous proteins. While overexpression of DsbG from the trc promoter in a wild type strain could not improve the yield of BPTI or urokinase, it could complement the folding defect in a dsbC− strain. In the case of BPTI, the yield of native protein was restored to wild type levels in the dsbC− strain; dsbG overexpression could not, however, complement a null mutation in dsbD (Fig. 1A). With urokinase, the activity was only restored to approximately 15% of the wild type level (Fig. 1B).

In Vivo Redox State—Unlike DsbC, which contains a struc-
natural disulfide in addition to its active site disulfide, mature DsbG has a single pair of cysteine residues. We examined the in vivo redox status of the active site disulfide in DsbG under normal growth conditions in wild type cells as well as in null mutants of dsb family genes. Briefly, exponentially grown cells were lysed in the presence of 10% trichloroacetic acid to denature DsbG while preventing the rearrangement of free thiols. Subsequently, free thiols were blocked with AMS, and the AMS-conjugated (reduced) and unconjugated (oxidized) proteins were resolved by SDS-PAGE under nonreducing conditions (10, 12, 35). We found that at steady state DsbG is present exclusively in reduced form in wild type cells and in dsbA\(^{-}\), dsbB\(^{-}\), and dsbC\(^{-}\) mutants. In contrast, in dsbD\(^{-}\) cells the protein was completely oxidized (Fig. 2A). Furthermore, there was no significant change in the expression level of DsbG in any of the dsb mutants. Upon introduction of a plasmid overexpressing DsbG from an inducible promoter, a small amount of DsbG is found to be oxidized. The reduced state, however, is overwhelmingly favored in all strains except, of course, the dsbD null (Fig. 2B).

In Vitro Characterization of DsbG—DsbG was purified from the periplasmic fraction of a culture harboring a plasmid containing the dsbG gene under the control of the T7 promoter. The overexpressed periplasmic protein was extracted by cold osmotic shock (Fig. 3A, lane 3) and partially purified by DEAE anion exchange chromatography, yielding a preparation that contained more than 90% pure DsbG protein (Fig. 3A, lane 4).

To further remove other protein contaminants, peak fractions were loaded onto a Sephadex™-75 size exclusion column. Fractions containing DsbG eluted slightly ahead of the ovalbumin (44 kDa) marker and yielded a preparation that was over 95% pure (Fig. 3A, lane 5). The molecular weight of the purified protein was determined by matrix-assisted laser desorption/ionization time-of-flight spectrometry and shown to agree with the calculated molecular weight within the error of measurement.

Upon gel filtration, DsbG eluted as a 45–50-kDa protein, raising the possibility that DsbG (subunit molecular mass 25.7 kDa) may form a homodimer as has been shown for DsbC (8). As an independent means to establish the quaternary structure of DsbG, purified protein was subjected to cross-linking with EGS, and the migration of the complex was analyzed by SDS-PAGE and Coomassie Brilliant Blue staining (Fig. 3B). These data revealed that, indeed, with increasing concentration of EGS, the electrophoretic mobility of DsbG shifted to approximately twice the expected 25.7-kDa mass of the monomer (Fig. 3B, lanes 3–5). As a negative control, purified DsbA, known to be a monomer, did not shift in mobility when subjected to the same concentrations of cross-linking agent (data not shown). These results suggest that DsbG exists predominantly in the homodimer form in the periplasmic space of E. coli, which is consistent with its estimated molecular weight from gel filtration analysis.

It has been previously shown that the fluorescence emission spectra of thioredoxin and DsbA are strongly dependent on the redox states of the enzymes (7, 36–38). The fluorescence intensity at 324 nm of reduced DsbA at pH 7.0 is 3-fold higher than that of the oxidized protein. Reduced DsbG likewise exhibits increased fluorescence emission at 330 nm at pH 7.0, compared with the oxidized form (Fig. 4A), but the difference is smaller.
than that of DsbA. In contrast, there is no difference in the spectroscopic properties of active site reduced and oxidized DsbC (8).

The observed differences in fluorescence intensities of reduced or oxidized DsbA and DsbG were used to measure the equilibrium concentrations of the oxidized and reduced forms in the presence of different ratios of oxidized (GSSG) and reduced (GSH) glutathione. The DsbG/glutathione redox equilibrium and its equilibrium constant are given by Equations 5 and 6.

\[
\text{DsbG}_{\text{red}} + \text{GSSG} \rightleftharpoons \text{DsbG}_{\text{ox}} + 2\text{GSH} \quad \text{(Eq. 5)}
\]

\[
K_{eq} = [\text{DsbG}_{\text{ox}}][\text{GSH}]^2/[\text{DsbG}_{\text{red}}][\text{GSSG}] \quad \text{(Eq. 6)}
\]

DsbA or DsbG was incubated in the presence of 100 μM GSSG and increasing concentrations of GSH (0–2 mM), and the relative amount of reduced enzyme at equilibrium \( R \) was measured over the range from fully oxidized to fully reduced protein (Fig. 4B). Nonlinear regression was used to fit the data to Equation 4. The equilibrium constant \( K_{eq} \) for the reduction of DsbA by glutathione was determined to be 130 μM. This result agrees well with the values of 120 and 80 μM reported by Wunderlich and Glockshuber (7) and Zapun et al. (6), respectively, for DsbA. Under identical conditions, the equilibrium constant for DsbG was estimated to be 140 μM. Thus, the DsbG disulfide bond is nearly as unstable as that of DsbA and slightly less so than that of DsbC \( (K_{eq} = 200 \mu M) \) (8). The general behavior of the DsbG titration by glutathione indicates that its redox potential is comparable with those of DsbA and DsbC.

The ability of DsbG to catalyze the reduction of insulin in the presence of DTT was evaluated. Reduction of insulin leads to cleavage of the two interchain disulfide bonds, causing the β-chain of insulin, which is insoluble, to aggregate and form a precipitate whose formation can be recorded by measuring the turbidity in the sample at 650 nm. Assays were performed exactly as described by Holmgren and co-workers (29). As expected, the addition of 5.0 μM DsbC or DsbA resulted in the rapid precipitation of insulin, which could be detected after 6 min for DsbC and 18 min for DsbA (Fig. 5A). At lower concentrations of either protein (2.5 μM), the time at which the turbidity could initially be detected was increased, and the precipitation rate was slower (data not shown). Andersen et al. (1) had reported that DsbG has insulin reduction activity comparable with that of DsbA. However, in our hands DsbG was not able to reduce insulin above the background levels observed in...
the control reaction with DTT alone (Fig. 5A). No activity was observed with different preparations of DsbG expressed with or without a hexahistidine tail at 25 or 37 °C (Fig. 5A) or with high concentrations of DsbG (up to 20 μM). We also failed to see any activity with protein that had been prereduced with 15 mM DTT (data not shown). On the basis of these results, we concluded that DsbG is unable to catalyze the reduction of insulin, at least under the standard assay conditions.

We also examined whether DsbG could catalyze the oxidative renaturation of reduced, denatured RNase (30). The addition of reduced, denatured RNase to a glutathione redox buffer containing a catalytic protein and the RNase substrate cCMP results in an increase of RNase activity, which is monitored as an increase in the rate of cCMP hydrolysis measured at 296 nm. The concentration of refolded RNase was determined by measuring the absorbance change at 296 nm produced by RNase-catalyzed hydrolysis of cCMP (initial concentration 4.5 mM) at pH 8.0 in 50 mM Tris acetate buffer (see “Experimental Procedures”) in the presence of redox buffer (0.2 mM GSSG, 1.0 mM GSH). Concentrations of PDI, DsbC, DsbG, and DsbG-His are indicated in the key. Reactions were started with the addition of 8 μM reduced, denatured RNase.

**Fig. 5. Oxidoreductase activity of DsbG compared with other disulfide catalysts.** A, the enzymatic reduction of insulin in the presence of DTT was assayed by turbidimetric assay as described previously (29). Reactions were performed in a final volume of 800 μl containing 0.1 M potassium phosphate buffer, pH 7; 2 mM EDTA; 0.35 mM DTT; 0.13 mM insulin; and purified DsbA, DsbC, or DsbG at 5 μM. The background reduction by DTT without any enzyme present was used as a control. B, analysis of the kinetics of oxidative refolding of RNase. The concentration of refolded RNase was determined by measuring the absorbance change at 296 nm produced by RNase-catalyzed hydrolysis of cCMP (initial concentration 4.5 mM) at pH 8.0 in 50 mM Tris acetate buffer (see “Experimental Procedures”) in the presence of redox buffer (0.2 mM GSSG, 1.0 mM GSH). Concentrations of PDI, DsbC, DsbG, and DsbG-His are indicated in the key. Reactions were started with the addition of 8 μM reduced, denatured RNase.

substantial gain in RNase activity with catalytic concentrations of enzyme in the absence of glutathione redox buffer, suggesting that oxygen-dependent activation of RNase does not provide a significant source of oxidizing equivalents under these conditions.

Consistent with previous studies, bovine PDI was shown to catalyze the oxidative refolding of RNase (Fig. 5B). Similarly, DsbC was also efficient in catalyzing the reactivation of RNase. The $k_{cat}$ and $K_m$ values for this reaction were 0.24 ± 0.01 min$^{-1}$ and 16 ± 3 μM, which are 3-fold slower and 2-fold higher, respectively, than those reported for PDI (39). The presence of DsbG (5 μM) resulted in a rate of RNase reactivation that was reproducibly slightly above background. Identical results were obtained with either native DsbG or DsbG-His-tagged protein (Fig. 5B). These results indicate that DsbG is a poor catalyst of oxidative protein refolding, at least using RNase as the substrate. Furthermore, DsbG (5 μM) could not act synergistically with PDI or DsbC, failing to give a reactivation rate higher than PDI or DsbC alone.
We first identified DsbG based on its homology with E. coli DsbC. A data base search also revealed that DsbG is highly similar to an unidentified open reading frame from *Pseudomonas aeruginosa* (47% identity, 70% similarity), allowing for conservative substitutions, distinct from the putative DsbC (GenBank accession no. AF057031) of *P. aeruginosa*, with the two exhibiting similarity (25% identity, 43% similarity) comparable with that between the *E. coli* DsbC and DsbG.

The *E. coli dsbG* gene was shown to encode a dimeric periplasmic protein, which in exponentially growing cells is expressed at a level about 25% that of DsbC. The two proteins share a number of similarities; they are both dimeric, form an unstable disulfide bond, and are maintained in the reduced state in the periplasm via the action of DsbD (10, 17). High level expression of DsbG could fully complement the defect in the folding of BPTI and partially complemented the formation of active urokinase in a *dsbC* mutant background. In *vitro*, the folding pathway of BPTI has been shown to involve the formation and subsequent rearrangement of intermediates with non-native disulfide bonds. There is evidence that DsbC catalyzes kinetically important disulfide bond isomerization steps in the folding of BPTI in the periplasm and most likely has a similar effect on urokinase (17, 34). The ability of DsbG to fully restore the expression of BPTI and partially restore that of urokinase suggests that DsbG also is able to effect disulfide bond isomerization. Remaining to be determined is whether DsbG is a bona fide catalyst of disulfide isomerization or whether its overexpression acts indirectly, for example by altering the redox state of the periplasm.

DsbG contains a Cys-X-X-Cys sequence that is found in the active site of proteins belonging to the thioredoxin superfamily. This is consistent with DsbG’s being a redox active protein that may play a role in disulfide bond isomerization. The Cys-Pro-Tyr-Cys sequence in DsbG forms an unstable disulfide bond that is readily reduced by glutathione. Interestingly, DsbA, DsbC, and DsbG all exhibit equilibrium constants with glutathione in the 80–200 μM range (6–8), considerably lower than that of thioredoxin (10 μM) (40). Despite the highly oxidizing nature of the periplasmic space, DsbG is found exclusively in the reduced state in wild type cells. Only under conditions of overexpression did we observe a small amount of oxidized protein (Fig. 2B). On the other hand, DsbG was completely oxidized in a *dsbD*− background, suggesting that, in the absence of a membrane protein reductant, DsbG becomes completely oxidized.

While this work was in progress, Andersen *et al.* isolated *dsbG* genetically and examined its function in *vitro* (1). We have found several discrepancies between our results and those of Andersen *et al.* First of all, they reported that a *dsbG* null linked to TetR could not be crossed onto the chromosome unless the cells were supplemented with high concentrations of low molecular weight oxidants. Although a *dsbG*::ΩKan null allele could be transduced without supplementation by oxidants, they attributed the appearance of transductants to suppressor mutations and went on to conclude that *dsbG* is essential. In contrast, we found the following: 1) a KanR-, or a KmR-marked null allele could be transduced from a *recD*− strain into a variety of recipient strains at a normal frequency; 2) a null allele could be readily recombined into the chromosome from a suicide plasmid, again at a high frequency; and 3) finally, confirming the absence of second site suppressors, we found

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2 Found in Contig 204 of the unfinished *Pseudomonas* Genome Project, available on the World Wide Web at http://www.pseudomonas.com/.

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