JOURNAL OF BACTERIOLOGY, Nov. 1997, p. 6602–6608 Vol. 179, No. 21
0021-9193/97/$04.00+0

Reduction of the Periplasmic Disulfide Bond Isomerase, DsbC, Occurs by Passage of Electrons from Cytoplasmic Thioredoxin

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Received 25 June 1997/Accepted 2 September 1997

The *Escherichia coli* periplasmic protein DsbC is active both in vivo and in vitro as a protein disulfide isomerase. For DsbC to attack incorrectly formed disulfide bonds in substrate proteins, its two active-site cysteines should be in the reduced form. Here we present evidence that, in wild-type cells, these two cysteines are reduced. Further, we show that a pathway involving the cytoplasmic proteins thioredoxin reductase and thioredoxin and the cytoplasmic membrane protein DsbD is responsible for the reduction of these cysteines. Thus, reducing potential is passed from cytoplasmic electron donors through the cytoplasmic membrane to DsbC. This pathway does not appear to utilize the cytoplasmic glutathione-glutaredoxin pathway. The redox state of the active-site cysteines of DsbC correlates quite closely with its ability to assist in the folding of proteins with multiple disulfide bonds. Analysis of the activity of mutant forms of DsbC in which either or both of these cysteines have been altered further supports the role of DsbC as a disulfide bond isomerase.

For the correct pattern of disulfide bonds to form in proteins, two hurdles must be overcome. First, the uncatalyzed rate at which the disulfide bonds are introduced in proteins in vitro is too slow to account for their rapid formation observed in vivo (1, 36). Second, in the case of proteins with more than two cysteines, oxidation of the inappropriate pairs of cysteines will result in the formation of nonnative disulfide bonds. To compensate for these potential limitations, cells have evolved an enzymatic machinery to catalyze the rapid and correct formation of disulfide bonds. First, the rate of disulfide bond formation in vivo is accelerated by protein catalysts such as bacterial DsbA (4, 20, 23). Second, incorrect disulfide bonds are "shuffled" by the action of disulfide bond isomerases, such as the eukaryotic enzyme protein disulfide isomerase (PDI) (7, 8).

In *Escherichia coli*, disulfide bond formation occurs in the periplasm. This process is catalyzed by DsbA, a 21-kDa protein with an active site displaying a thioredoxin-like fold, including a Cys-X-X-Cys motif (4, 20, 25). Oxidation of target proteins is achieved by thiol-disulfide exchange. The active-site cysteines in DsbA form a disulfide bond which is attacked by one of the cysteines in the target protein, resulting in a mixed disulfide. Resolution of the mixed disulfide leaves DsbA reduced and the target protein oxidized. DsbA is then reoxidized by DsbB, an integral cytoplasmic membrane protein (3, 14, 21, 26). How DsbB is, in turn, oxidized is unclear. Both *dsbA* and *dsbB* mutants display a severe pleiotropic defect in disulfide bond formation, resulting in, among other things, loss of motility, reduced alkaline phosphatase activity, and inability to assemble F pili (4, 20).

How incorrect disulfide bonds are isomerized in *E. coli* is less clear. Both in vivo and in vitro data suggest that DsbC acts as a disulfide bond isomerase (31, 38, 42). DsbC is a 23-kDa periplasmic protein which is presumed to assume a thioredoxin-like fold, based on the presence of conserved motifs in the primary sequence of the protein. Early studies on DsbC suggested that it might be involved in the formation of disulfide bonds. This conclusion was based on the finding that overexpression of DsbC could suppress the defect in disulfide bond formation observed in a *dsbA* mutant (27, 37). However, disulfide bond formation of proteins with single disulfide bonds such as OmpA or β-lactamase is unaffected by a *dsbC* null mutation (31; unpublished observation). We have presented evidence suggesting that, in wild-type cells, DsbC only plays a role in the folding of proteins that contain multiple disulfide bonds (31). Furthermore, the defects observed in the assembly of such proteins in a *dsbC* null mutant can be partially complemented by the addition of reduced but not oxidized dithiothreitol to the medium. These results are not consistent with a role in de novo disulfide bond formation, which is an oxidative reaction. Instead, these data suggest that the primary function of DsbC is actually isomerization of disulfide bonds. Alternatively, DsbC could act as a reductase, simply reducing incorrect disulfide bonds, which then are reoxidized by DsbA.

Another line of evidence for the proposed isomerase function of DsbC in vivo comes from the analysis of a cysteine mutant form of alkaline phosphatase, which ordinarily contains two disulfide bonds (38). A mutant form of alkaline phosphatase in which the first cysteine has been replaced with a serine residue causes about half of the newly synthesized molecules to form an incorrect disulfide bond. Isomerization of the disulfide bond to yield the correctly folded protein requires the presence of functional DsbC. Previous studies analyzing the effect of DsbC on the folding of bovine pancreatic trypsin inhibitor (BPTI) in vitro have shown that DsbC, unlike DsbA, is a good catalyst of disulfide bond isomerization. BPTI is a small protein with three disulfide bonds (42). The pathway for the formation of its disulfide bonds is known and includes an intermediate with nonnative disulfide bonds that require isomerization.

The proposed function of DsbC as an isomerase poses a problem. The active site cysteines of an isomerase should be in the reduced state to be able to attack an incorrect disulfide bond in the target protein and catalyze its rearrangement, yet the environment of the periplasm is highly oxidizing. In particular, one might predict that DsbA would oxidize DsbC and render it inactive. We have previously proposed a pathway for

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TABLE 1. Strains used in this study

Name	Genotype[a]	Source or reference
MC1000	*araD139* Δ(*araABC-leu*)*7679 galU galK* Δ(*lac*)*X74 rpsL thi*	Laboratory collection
DHB4	MC1000 Δ*phoA*(*Pvu*II) *phoR* Δ*malF3*/F′ (*lacI*^{q}*ZYA*)	6
WP570	DHB4 Δ*trxA*	Laboratory collection
AD494	DHB4 *trxB*::Kan^r	10
WP758	*gshA*::Tn*10* Kan^r	Laboratory collection
WP812	*grxA*::Kan^r	Laboratory collection
WP840	*gor522*::mini-Tn*10*	Laboratory collection
RI89	MC1000 *phoR* Δ*ara-714 leu*$^+$	31
RI90	RI89 *dsbA*::Kan^r	31
RI179	RI89 Δ*dsbC*::Cam^r	31
A307	Δ*trxA307*	33
RI258	RI89 Δ*trxA307*	31
RI242	RI89 *dsbD*::mini-Tn*10* Cam^r	31
HMS157	Δ*trxA*::Kan^r	Stan Tabor
RI363	RI89 Δ*trxA*::Kan^r	This study
A304	*trxB*::Kan^r	33
RI319	RI89 *trxB*::Kan^r	This study
RI336	RI89 *gshA*::Tn*10* Kan^r	This study
STL117	*dsbC*::mini-Tn*10* Kan^r	24
RI393	RI89 dsbC::mini-Tn*10* Kan^r	This study
JCB752	MC1000 *phoR dsbB*::Kan^r	3
RI317	RI89 *dsbB*::Kan^r	This study
RI361	RI89 *dsbA*::Kan^r *dsbD*::mini-Tn*10* Cam^r	This study
RI318	RI89 *dsbB*::Kan^r *dsbD*::mini-Tn*10* Cam^r	This study
RI385	RI89 *dsbA*::Kan^r *zij*::Tn*10* *dsbB*::Kan^r *dsbD*::mini-Tn*10* Cam^r	This study

[a] For gene designations, see reference 2. Kan^r, kanamycin resistance; Cam^r, chloramphenicol resistance.

maintaining DsbC in the reduced state which involves the inner membrane protein DsbD (DipZ) and cytoplasmic thioredoxin. DsbD is a cytoplasmic membrane protein with a thioredoxin-like domain at its C terminus which is thought to be oriented towards the periplasm (28, 38a). DsbD (DipZ) was first discovered through its role in *c*-type cytochrome biogenesis and as a general reductant in the periplasm of *E. coli* (9, 28). Biogenesis of *c*-type cytochromes in a *dsbD* mutant is restored by addition of reducing agents (34), leading to the hypothesis that DsbD is involved in maintaining the cysteines in the heme binding site of the apo-cytochrome reduced, allowing the heme moiety to be attached. Thioredoxin is a highly abundant cytoplasmic protein which is involved in the reduction of certain cytoplasmic enzymes, including methionine sulfoxide reductase and ribonucleotide reductase (16, 32). Mutants lacking either thioredoxin or DsbD display certain phenotypes similar to those observed in a *dsbC* mutant, supporting the proposal that they are required for DsbC to function properly (31).

Here we present direct evidence that the active site of DsbC is present almost exclusively in the reduced state. We have analyzed the redox state of DsbC in vivo in both wild-type cells and mutants in the proposed reducing pathway. The redox state of DsbC correlates quite closely with the ability of the cell to correctly assemble multidisulfide bonded proteins. In addition, we present data showing that the thioredoxin reductase-thioredoxin system is the main source of reducing potential for this pathway. Analysis of the activity of mutant forms of DsbC lacking one or both active-site cysteines further supports its proposed role as a disulfide bond isomerase.

MATERIALS AND METHODS

Strains and media. The strains used in this study are listed in Table 1. For analysis of the redox state of DsbC, bacteria were grown in M63 minimal medium with glucose as the carbon source and supplemented with 18 amino acids (lacking cysteine and methionine). Cultures for urokinase zymograms were grown in NZ medium. For analysis of BPTI folding, cultures were grown at 37°C in M9 minimal salts supplemented with 0.2% glucose, 0.2% casein hydrolysate, and ampicillin or carbenicillin at 100 μg/ml, as required.

Strain construction and plasmids. Standard molecular and genetic techniques were used for strain and plasmid construction (35). Cysteine mutant forms of DsbC were constructed by overlapping-strand exchange PCR in which codon residues were changed to serine codons by changing the first T of the target codon to A. The mutant alleles were cloned into vector pBAD33, a pACYC184-derived plasmid with a chloramphenicol resistance cassette (15). Expression of the mutant *dsbC* alleles is under the control of the arabinose P_{BAD} promoter.

Assay for the redox state of DsbC. Samples were taken from growing cultures at mid-log phase, and total protein was precipitated by adding an equal volume of 10% trichloroacetic acid. Pellets were washed with acetone, dried, and resuspended in 125 mM Tris · Cl (pH 8)–6 M guanidinium chloride–20 mM iodoacetic acid. After 10 min of incubation at room temperature, the iodoacetic acid was removed by gel filtration with a spin column packed with Sephadex G-25 resin equilibrated in 125 mM Tris · Cl (pH 8)–6 M guanidinium chloride. The samples were then reduced by addition of reduced dithiothreitol to a final concentration of 5 mM and incubation at room temperature for 15 min. Reduced cysteines were alkylated with iodoacetamide (final concentration, 50 mM; 5 min of incubation at room temperature). The alkylating reagent was again removed by gel filtration with a Sephadex G-25 spin column equilibrated in 58 mM Tris · PO_4 (pH 6.8)–8 M urea, and the samples were loaded on a urea gel. The separating gel was a 10% polyacrylamide gel with 8 M urea and 350 mM Tris (pH 8.9). The stacking gel was prepared by mixing 2 ml of 10% acrylamide–2.5% bisacrylamide, 1 ml of 0.47 M Tris · PO_4 (pH 6.9), 3.84 g of urea, 1 ml of riboflavin (0.04 mg/ml), and water to 8 ml; it was polymerized by exposure to UV light for 20 min. The running buffer contained 3.03 g of Tris base and 14.4 g of glycine dissolved in 1 liter of double-distilled H_2O. Gels were 1 mm thick and run at 4°C and 12 mA of constant current.

Urokinase assays. Urokinase assays were performed on strains transformed with plasmid pRDB8-A, which expresses mouse urokinase plasminogen activator constitutively (11). Whole-cell lysates of bacteria grown to exponential phase were separated on nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, and urokinase activity was demonstrated by assaying plasminogen activation by using plasminogen-casein agar as previously described (5).

Analysis of BPTI folding. BPTI folding was analyzed in strains harboring plasmid pTI103 containing the OmpA leader-BPTI fusion under control of the *lpp-lac* promoter (13). BPTI synthesis was induced by adding 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when the cultures reached an optical density at 600 nm of 0.3 to 0.35. Where indicated, glutathione was added at a final concentration of 5 mM at the time of induction. The cells were harvested 5 h after induction and lysed by French press (20,000 lb/in^2), and the soluble fraction was recovered by centrifugation (12,000 × *g*, 10 min, 4°C). The amount of native BPTI was determined by enzyme-linked immunosorbent assay (ELISA) using a polyclonal serum raised against native BPTI (29). Briefly, the amount of soluble protein in the clarified cell lysates was first quantified by the Bradford method (Bio-Rad, Hercules, Calif.) and diluted to 1.0 mg/ml in 100 mM carbonate buffer (pH 9.6). Subsequently, 100-μl aliquots were applied to a 96-well microtiter plate and incubated overnight at 4°C. Both the primary and secondary antibodies were used at a dilution of 1:1,000.

RESULTS

Redox state of DsbC in vivo. If the role of DsbC in vivo is that of a disulfide bond isomerase, then it should be maintained in the reduced state. We have tested this hypothesis by determining the redox state of the cysteines of DsbC in vivo.

DsbC has two disulfide bonds that have been assigned differing roles. Two cysteines (Cys_{98} and Cys_{101}) are arranged in a Cys-X-X-Cys motif typical for the active site of thioredoxin-like proteins. Cys_{98} is accessible to alkylating reagents in the native protein (42). The other two cysteines (Cys_{141} and Cys_{163}) form a structural disulfide bond that is buried in the native protein. For many proteins, one can observe differences in the oxidation state of the cysteines by differing mobilities on nonreducing SDS-PAGE. This was not possible with DsbC, since only reduction of the structural disulfide bond caused the mobility of the protein to change. Therefore, we analyzed the redox state of DsbC by alkylating the protein cysteines with iodoacetic acid and resolving the product on a urea gel without

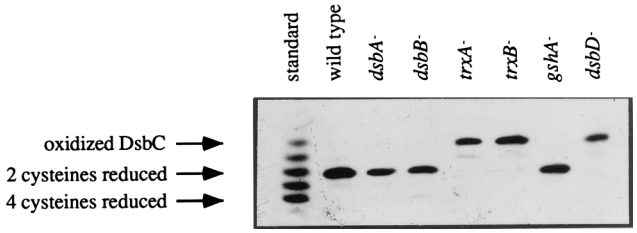

FIG. 1. Redox state of the active-site disulfide of DsbC in various mutant backgrounds. Total cellular protein was denatured and alkylated with iodoacetic acid and then separated by urea-PAGE. DsbC was detected by Western blotting with anti-DsbC antibody. The positions at which oxidized, active-site-reduced, and completely reduced DsbC migrate are indicated. The standard on the left is a mixture of DsbC alkylated with iodoacetic acid on none, one, two, three, or all four of the cysteines.

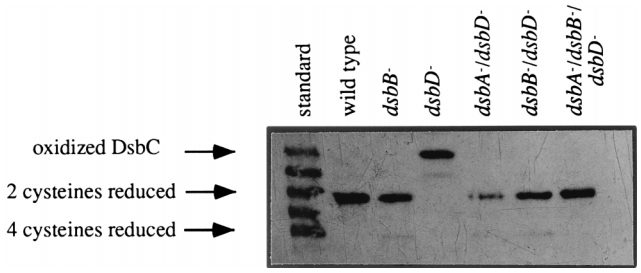

FIG. 2. Redox state of the active-site disulfide of DsbC in double mutants involving the reducing pathway (*dsbD*), as well as *dsbA* and/or *dsbB*. Total cellular protein was denatured and alkylated with iodoacetic acid and then separated by urea-PAGE. DsbC was detected by Western blotting with anti-DsbC antibody. The positions at which oxidized, active-site-reduced, and completely reduced DsbC migrate are indicated. The standard on the left is a mixture of DsbC alkylated with iodoacetic acid on none, one, two, three, or all four of the cysteines. w.t., wild type.

SDS. Under these conditions, the charge introduced by the acetic acid moiety causes a significant shift in the mobility of the protein, allowing easy analysis of the number of reduced cysteines. This technique was first used on purified DsbC by Zapun et al. in vitro to demonstrate that the structural disulfide bond in DsbC is accessible to alkylating reagents only if the protein is denatured (42).

When we analyzed the redox state of the cysteines in DsbC by alkylation with iodoacetic acid, we found that DsbC from wild-type cells migrated at the two-cysteines-reduced position, indicating that the protein is indeed maintained in a reduced state (Fig. 1). These experiments were performed under denaturing conditions, since the second cysteine of the active site is only poorly alkylated under nondenaturing conditions (42). We were able to alkylate DsbC under nondenaturing conditions as well (data not shown), indicating that we were analyzing the redox state of the active-site cysteines, since the structural disulfide bond is not accessible to alkylating reagents under these conditions (42). To test our proposal that thioredoxin and DsbD are required to maintain the active-site cysteines of DsbC reduced, we examined the state of these cysteines in *trxA* and *dsbD* backgrounds. In addition, since thioredoxin reductase is necessary to maintain thioredoxin in the reduced state, we also examined a *trxB* mutant. In all three mutant backgrounds, DsbC is present in the completely oxidized form (Fig. 1). Taken together, these results demonstrate that the active site of DsbC is reduced in a wild-type cell, consistent with its proposed role as a disulfide bond isomerase, and that the thioredoxin-DsbD pathway is required for DsbC to remain reduced.

Disruption of either *dsbA* or *dsbB*, the genes coding for the two known components of the disulfide bond-forming system in the periplasm, does not significantly affect the redox state of DsbC (Fig. 1); the active-site cysteines of DsbC are still in the reduced state. In addition, the structural disulfide bond of DsbC is still formed; only a small fraction of the protein is completely reduced in these backgrounds. The presence of the structural disulfide bond of this protein in the *dsbA* and *dsbB* mutants is not surprising; although these mutations reduce the rate of disulfide bond formation by at least 2 orders of magnitude, there is still a significant amount of background oxidation in these strains (4). Thus, one would expect, when looking at the form of the protein under steady-state conditions, most of DsbC to contain the structural disulfide bond. We did find, however, that the amount of the fully reduced form does vary somewhat with the growth phase of the cells.

We analyzed the effect of double mutant forms by combining *dsbA* and *dsbB* null mutations with a *dsbD* mutant. While a *dsbD* mutant results in the accumulation of DsbC in the oxidized form, combining the *dsbD* mutation with a *dsbA* or *dsbB* mutation results in the active site of DsbC being reduced again (Fig. 2). These results indicate that the DsbA-DsbB pathway is responsible for the accumulation of oxidized DsbC in a *dsbD* mutant. DsbC could be oxidized directly by DsbA, even though in vitro data show that oxidation of DsbC by DsbA is disfavored compared to the oxidation of a generic structural disulfide bond (42). Alternatively, oxidized DsbC could also accumulate if its interaction with a substrate protein led not to isomerization of the substrate protein disulfide bond but to reduction (39).

Since DsbA and DsbC are thought to have similar structural motifs, the thioredoxin-like fold, it seemed possible that DsbB might be able to oxidize DsbC. A *dsbA-dsbD* double mutant has the same effect on the redox state of DsbC as a *dsbA-dsbB-dsbD* triple mutant, with no detectable oxidized DsbC. This finding argues that DsbB cannot oxidize DsbC, since one would otherwise expect a portion of the DsbC molecules to be fully oxidized in the *dsbA-dsbD* double mutant. It is possible that the reason why no oxidized DsbC is detectable in a *dsbA-dsbD* double mutant is that it is used up to form disulfide bonds in proteins. Disruption of *dsbD* is known to suppress the defect in disulfide bond formation of a *dsbA* mutant. Suppression can be easily visualized by assaying the motility of the strain; a *dsbA* mutant is nonmotile, since it cannot assemble flagella. This suppression is dependent on DsbC, which argues that oxidized DsbC is responsible for disulfide bond formation in a *dsbA-dsbD* double mutant. If DsbB can oxidize DsbC, then one would expect suppression of the *dsbA* null mutation by disruption of *dsbD* to be at least partially dependent on DsbB. The *dsbD* mutant, therefore, would be unable to suppress a *dsbA-dsbB* double mutant or at least to do so only to a lesser extent. This is not the case; the *dsbD* null mutant can suppress a *dsbA-dsbB* double mutant, as judged by its restoration of motility (data not shown).

Our results suggest that thioredoxin and thioredoxin reductase are involved in maintaining DsbC reduced and active. However, in addition to the thioredoxin-thioredoxin reductase pathway, there is a second major reducing pathway in the cytoplasm, the glutathione-glutaredoxin pathway (17, 18). Glutathione reductase reduces glutathione, which, in turn, reduces glutaredoxins, a family of disulfide bond oxidoreductases having a thioredoxin-like fold. We wondered whether the glutathione-glutaredoxin pathway might also be involved in the maintenance of DsbC in the reduced state. However, we have found that a null mutation in *gshA*, the gene encoding one of

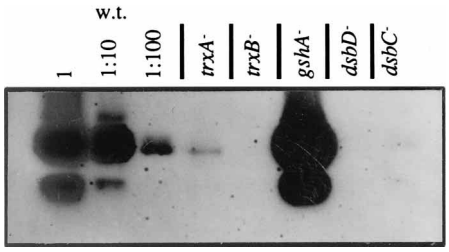

FIG. 3. Urokinase activities in *trxA*, *trxB*, *gshA*, *dsbD*, and *dsbC* mutants strains. Strains expressing periplasmic urokinase constitutively were grown to mid-log phase and harvested. Total cellular protein was separated by nonreducing SDS-PAGE, and urokinase activity was demonstrated by its ability to activate plasminogen on casein-plasminogen agar. Relative activities of various mutant strains were compared to dilutions of an extract prepared from the wild-type parental strain.

the enzymes required for the biosynthesis of glutathione, does not affect the redox state of DsbC (Fig. 1).

Effect of thioredoxin reductase and *gshA* mutations on urokinase activity and folding of BPTI. We have shown that mutations in *dsbC*, as well as mutations (*trxA* and *dsbD*) affecting the reductants thioredoxin and DsbD, significantly interfere with the assembly of proteins with more than one disulfide bond (31). We wished to directly correlate the DsbC-dependent assembly of such proteins with the redox state of DsbC. To this end, we assayed the assembly of active mouse urokinase and of fully folded BPTI secreted into the periplasm of *E. coli* in various mutant backgrounds. Urokinase is a eukaryotic plasma protein with a total of 12 disulfide bonds, 6 of which are located in the C-terminal protease domain. Protease activity can be easily observed by using a zymogram, assaying the ability of urokinase to activate plasminogen. BPTI is a small protein with three disulfide bonds in its native conformation. The disulfide bond formation pathway of BPTI has been studied extensively in vitro. BPTI can fold properly in *E. coli* if exported to the periplasm (30). The oxidation of its disulfide bonds depends on the DsbA-DsbB system (30).

As we had previously found that urokinase activity is dramatically reduced in a *trxA* mutant, it was not surprising that we found that elimination of thioredoxin reductase activity (*trxB* mutation) also results in a decrease in urokinase activity similar to that seen in a *dsbC* mutant (Fig. 3). In contrast, a *gshA* null mutation, which has little or no effect on the redox state of DsbC, has no discernible effect on urokinase activity (Fig. 3). Collectively, these results indicate a strong correlation between the redox state of DsbC and the assembly of multiply disulfide-bonded proteins. Moreover, they support the proposition that reduced DsbC is the functional form in wild-type cells.

Analysis of BPTI folding in the periplasm of various redox mutants mirrors the result obtained with urokinase. Mutations in *dsbC*, *trxA*, and *trxB* severely decrease the yield of correctly folded BPTI, whereas mutations in the genes for glutathione reductase (*gor*) and glutaredoxin I (*grxA*) and in *gshA* have, at most, only a slight effect (Fig. 4A). Interestingly, addition of reduced glutathione to the medium was able to rescue the defect in BPTI folding in the case of the *trxA* and *dsbD* mutants, but not in the case of *dsbC* or *trxB* (Fig. 4B). Reduced glutathione added to the medium can therefore substitute for components of the reduction pathway but not alleviate the *dsbC* defect. The *trxB* mutant does not fit this model; pulse-chase analysis, however, revealed that a large portion of the BPTI protein did not have its signal sequence cleaved, indicating that it was, in fact, retained in the cytoplasm (data not shown). The accumulation of this species of BPTI may be due to the introduction of disulfide bonds prior to export. Disruption of *trxB* does allow disulfide bond formation to occur in the cytoplasm (10).

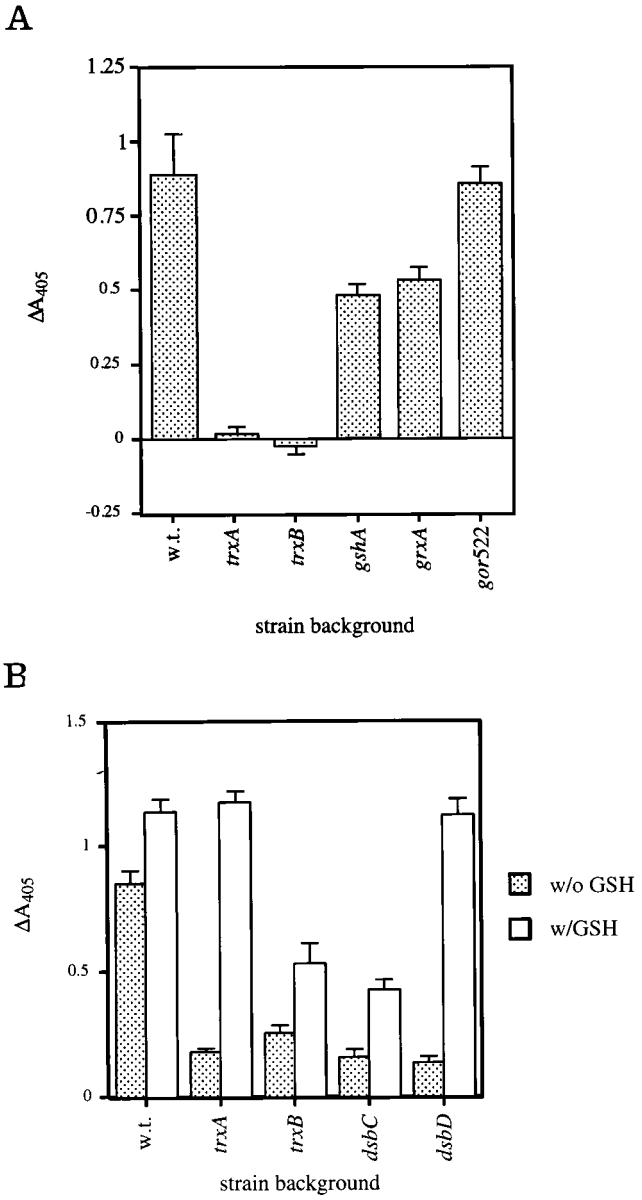

FIG. 4. Yield of native BPTI in *trxA*, *trxB*, *gshA*, *gor*, *dsbD*, and *dsbC* mutant strains. Strains expressing BPTI were grown to mid-log phase and harvested. The level of native BPTI in cell lysates was determined by ELISA with an antiserum specific for native BPTI. (A) BPTI ELISA measuring the level of native BPTI in cell lysates of a wild-type (w.t.) strain and *trxA*, *trxB*, *gshA*, *gor*, *dsbD*, and *dsbC* mutant strains. (B) Complementation by exogenously added 5 mM reduced glutathione (GSH). Cells were grown with or without 5 mM reduced glutathione. The level of native BPTI in cell lysates was determined by ELISA.

A single cysteine mutant of DsbC can partially complement a urokinase defect. In yeast, a mutant form of PDI in which the second cysteines of the two active sites had been changed to serine residues was able to complement a ΔPDI mutant for growth (22). This mutant form of PDI, while displaying no oxidizing or reducing activity, can still serve to isomerize disulfide bonds in vitro (22). The authors of reference 22 con-

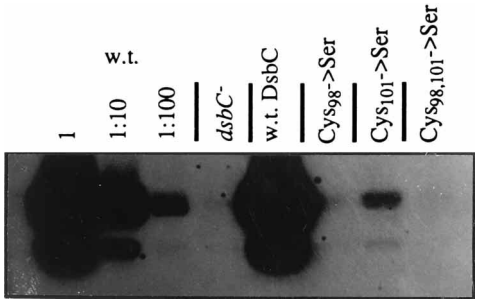

FIG. 5. A mutant form of DsbC in which the second cysteine of the active site has been replaced can partially complement the defect in urokinase activity of a *dsbC* null mutant strain. Strains expressing periplasmic urokinase constitutively were grown to mid-log phase and harvested. Plasmid-based expression of wild type (w.t.) and mutant forms of *dsbC* was induced by the addition of arabinose to the medium to a final concentration of 0.05%. Total cellular protein was separated by nonreducing SDS-PAGE, and urokinase activity was demonstrated by its ability to activate plasminogen on casein-plasminogen agar. Relative activities of various mutant forms of DsbC were compared to those of dilutions of an extract prepared from the wild-type parental strain. The strains assayed were RI89 (wild type), RI393 (RI89 *dsbC*::mini-Tn*10* Kan^r), RI393/pDSBC, RI393/pDSBC$_{Cys\text{-}98\rightarrow Ser}$, RI393/pDSBC$_{Cys\text{-}101\rightarrow Ser}$, and RI393/pDSBC$_{Cys\text{-}98,\ \text{-}101\rightarrow Ser}$.

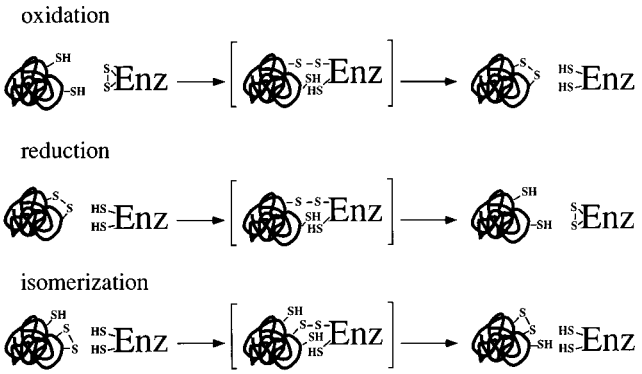

FIG. 6. Catalysis of disulfide bond formation, reduction, and isomerization by thiol-disulfide exchange. Oxidation, reduction, and isomerization of disulfide bonds are thought to occur via a mixed-disulfide intermediate between the enzyme (Enz) and the target protein. Unlike disulfide bond oxidation or reduction, the redox state of the enzyme does not change in the case of disulfide bond isomerization; only the first cysteine of the active site is involved in the reaction (7, 8, 12).

cluded that the mutant protein complemented an isomerization defect in the PDI null mutant and that this is the essential function of PDI in vivo.

We have used active-site mutant forms of DsbC to determine whether it behaves like PDI. The wild-type *dsbC* gene and mutant *dsbC* genes in which either one or both active-site cysteines had been changed to serine residues were expressed at approximately wild-type levels in strains producing periplasmic urokinase (as determined by Western blot analysis [data not shown]). Mutants lacking both active-site cysteines or the first cysteine of the active site are not able to complement a *dsbC* null mutation (Fig. 5). However, the mutant in which the reactive cysteine is intact but the second cysteine of the active site has been changed to a serine can partially complement the *dsbC* null mutation (Fig. 5). Since there can be no disulfide bond at the active site of this mutant protein, the protein cannot act as an oxidase. Furthermore, the protein is unlikely to act as a reductase without the ability to be oxidized itself to generate a disulfide bond. Thus, the finding that the single-cysteine mutant protein can partially complement the *dsbC* null mutation is best explained if DsbC acts as a protein disulfide isomerase.

DISCUSSION

We have demonstrated that the function of DsbC correlates with its redox state. In wild-type strains where significant amounts of urokinase and BPTI assemble, the Cys-X-X-Cys active cysteine pair is fully reduced. In mutants defective in the reducing pathway composed of thioredoxin reductase, thioredoxin, and DsbD, DsbC is found with its active-site cysteines fully oxidized. At the same time, such mutant strains (and *dsbC* mutants) are severely defective in the assembly of urokinase and BPTI. In contrast, the mutation *gshA*, which eliminates the second major reducing pathway in the cytoplasm, does not affect the redox state of DsbC, nor does it affect the folding of urokinase and BPTI.

These results strongly suggest that in the functional form of DsbC in wild-type cells the active-site cysteines are reduced. This finding, along with our previous results (31), indicates that DsbC plays no significant role in the de novo formation of disulfide bonds in a wild-type background. Rather, the correlation of activity with the reduced state of DsbC is consistent with its proposed role as a disulfide bond isomerase. The most accepted model for protein disulfide bond isomerization requires that a reduced, reactive cysteine in the protein catalyst attacks an incorrect disulfide bond in a target protein, resulting in a mixed disulfide between the two proteins. Resolution of the mixed disulfide leads to isomerization of the original disulfide bond (8, 19, 39, 40). Perhaps the isomerization reaction will then be driven to yield the native or correct disulfide bond because it allows the protein to take on an energetically favorable conformation.

We have analyzed the ability of cysteine mutant forms of DsbC to complement the defect in urokinase folding of a *dsbC* null mutant. For thioredoxin-like proteins to catalyze disulfide bond formation or reduction by thiol disulfide exchange (Fig. 6), both cysteines of the active site have to be intact. This is not the case for disulfide bond isomerization, in which, theoretically, only the reactive cysteine of the active site should be sufficient to catalyze the rearrangement of disulfide bonds in a target protein (Fig. 6). The requirement of only a single cysteine for function has been shown for PDI (22). Here we show that mutated DsbC in which only the second cysteine in the active site is replaced is able to partially complement the *dsbC* null mutant for urokinase activity. This finding supports the proposed function of DsbC as an isomerase in vivo. While a single-cysteine mutant form of DsbA can still form a mixed disulfide with a small redox molecule such as glutathione and this mixed disulfide form can then act as an oxidant (41), the periplasm of *E. coli* is thought to be devoid of small, thiol-containing compounds. Thus, the single-cysteine mutant form of DsbC could not form mixed disulfides and then go on to oxidize disulfide bonds. While the second cysteine in the active site of a disulfide bond isomerase is not required for full function, it may be required to rescue the protein from an unproductive interaction with a substrate protein (39, 40). By forming a disulfide bond in the active site of the isomerase, the mixed disulfide bond between the enzyme and the substrate is broken, thereby allowing the enzyme to escape from the unproductive interaction. The inability of the single-cysteine mutant form of DsbC to perform this type of "escape" reaction could explain the failure of the mutant protein to fully complement the *dsbC* null mutant.

DsbC was first detected in *E. coli* when it was found that

multicopy plasmids carrying the gene could partially suppress a *dsbA* null mutation. That is, at high-level expression, DsbC can act as an oxidant. This finding suggests that overexpressed DsbC exceeds the reductive capacity of DsbD, thus allowing some oxidized DsbC to accumulate. The oxidation of DsbC in this case is due to either oxygen or oxidants in the medium used. We have also found that reductive-pathway mutants (*trxA* and *dsbD*) can suppress a *dsbA* null mutation and that this suppression is dependent on the presence of DsbC (31). Presumably, when the reductive pathway is disabled, a portion of DsbC accumulates in the oxidized form and is able to catalyze the formation of disulfide bonds, thereby suppressing the defect in the *dsbA* null mutant. While we have not observed any DsbC with its active-site cysteines in the oxidized form in the *dsbA-dsbD* double mutant, the restoration of disulfide bond formation is so weak in this background (31) that the amount of oxidized DsbC may be undetectable.

The thioredoxin-DsbD pathway for the reduction of DsbC appears to be necessary because in the absence of this pathway, DsbA maintains the active-site cysteines of DsbC disulfide bonded. Our results show that the accumulation of fully oxidized DsbC in reductive-pathway mutant strains can be reversed by eliminating DsbA or DsbB. DsbC could be oxidized directly by DsbA or become oxidized as a result of escaping from a nonproductive interaction with a substrate protein that has been oxidized by DsbA.

Several cytoplasmic enzymes, ribonucleotide reductase, adenosine-3′-phosphate-5′-phosphosulfate reductase, and methionine sulfoxide reductase, require the reduction of active-site disulfide bonds as part of their catalytic cycle. Both thioredoxins and glutaredoxins are thought to be able to provide reducing potential for the first two of these enzymes. However, methionine sulfoxide reductase appears to be reduced only via the thioredoxin pathway. Our finding that mutations in *trxA* and *trxB* caused DsbC to be completely oxidized while mutations in *gshA* did not affect the redox state of DsbC provides a second example of a thioredoxin-specific pathway. It is unclear whether the apparent specificity for the thioredoxin-thioredoxin reductase system is due to the particular redox potential of thioredoxin or to a specific interaction of thioredoxin with other components of the reducing pathway.

The nature of the reductive pathway, i.e., the transfer of electrons from the cytoplasmic thioredoxin through the cytoplasmic membrane to DsbD and thence to DsbC, presents some intriguing questions. While there may be other intermediate components that facilitate this transfer, we have no evidence of any such components. If thioredoxin is the direct electron donor to DsbD, a number of different possible mechanisms can be imagined for this process. DsbD has a number of cysteines in addition to the two in the active site of the thioredoxin-like domain that are conserved between the *E. coli* and *Haemophilus influenzae* homologs. Passage of the electrons may be achieved by a series of intramolecular disulfide bonds or through transfer via other amino acid components. Other, more unusual mechanisms, including flipping of portions of DsbD within the membrane, can be postulated. Understanding of this process awaits more detailed structural and structure-function studies on DsbD.

ACKNOWLEDGMENTS

We thank Susan Lovett for providing strain SL117 and plasmid pJC763, as well as Stan Tabor for strain HMS157 and John Joly for the anti-DsbC serum. We gratefully acknowledge members of the Beckwith laboratory for helpful discussions. We also thank Terri Luna for medium preparation and Ann McIntosh for administrative assistance.

This work was supported by the National Science Foundation (grant MCB9406275 to J.B.) and the National Institute of General Medical Sciences (grant GM41883 to J.B. and grant GM47520 to G.G.). J.B. is an American Cancer Society Research Professor.

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