In Vitro and *in Vivo* Redox States of the *Escherichia coli* Periplasmic Oxidoreductases DsbA and DsbC

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ABSTRACT: DsbC is a periplasmic protein of *Escherichia coli* that was previously identified by a genetic selection that rescued sensitivity to dithiothreitol in Tn10 mutagenized cells. The *Erwinia chrysanthemi dsbC* gene was identified in a previous genetic screen to restore motility in a *dsbA* null strain. In order to analyze the biochemical role of *E. coli* DsbC, the protein was overexpressed, purified, and compared with DsbA in terms of disulfide isomerization, thiol oxidation, and *in vivo* redox state. *In vitro,* DsbC and DsbA have an equivalent k_{cat} for disulfide isomerization with the model substrate, misfolded insulin-like growth factor-1. However, DsbA is a more effective oxidant than DsbC of protein dithiols. *In vivo,* DsbA is found exclusively in the oxidized state in wild-type strains grown in rich media. On the other hand, *in vivo* DsbC has one pair of cysteines oxidized and one pair reduced. *DsbD* is required to maintain this reduced pair of cysteines, confirming previous genetic results. A *dsbC* deletion strain showed decreases in the production of some, but not all, heterologous proteins containing multiple disulfide bonds. Notably, those proteins affected by the *dsbC* deletion do not have the cysteines paired consecutively.

In *Escherichia coli*, the formation of disulfides in secretory proteins occurs in the periplasm which is relatively oxidizing compared to the cytoplasm (*1*). These disulfide bonds are often required for protein function and the formation of protease-resistant conformations. The existence of periplasmic proteases induced during heat shock conditions or overexpression of outer membrane proteins suggests that there is quality control with respect to misfolded or unfolded proteins in the periplasm or outer membrane (*2*−*4*). To aid the formation of disulfides in the periplasm, proteins exist that promote rapid thiol oxidation (*5*−*8*). The genes for these proteins are termed *dsb* for disulfide bond formation. Four *dsb* genes have been identified by genetic screens and selections (*9*−*13*). Strains with mutations in the *dsb* genes, especially *dsbA* and *dsbB*, exhibit slower disulfide bond formation in periplasmic proteins when compared to wild-type strains.

The first *dsb* gene identified was *dsbA* (*9*, *14*). This protein contains only two cysteines which are part of a C-X-X-C motif in a thioredoxin-like domain (*15*). All disulfide oxidoreductases possess this C-X-X-C motif, and many contain domains similar to thioredoxin (*16*). In DsbA, the amino-terminal cysteine is more reactive and has the remarkable property of being reactive in acidic conditions (*17*, *18*). The pK_a of the reactive cysteine has been measured to be 3.4 (*19*, *20*). The protein has disulfide isomerase activity in addition to being a thiol oxidant (*21*). DsbA is reoxidized by DsbB, a cytoplasmic membrane protein (*10*, *22*). DsbB has six cysteine residues and is predicted to have four transmembrane sequences based on alkaline phosphatase fusions (*23*). Five cysteines are located in periplasmic loops including two cysteines which form a C-X-X-C motif. Since DsbB is a membrane protein, reoxidation of the protein may involve components of the respiratory electron transfer chain. *DsbB* mutants can be suppressed by the addition of low concentrations of cystine or the presence of either the *dsbA* or the *dsbC* genes on multicopy plasmids (*10*, *12*). *DsbA* mutants can also be suppressed by addition of cystine although a higher concentration is required than for *dsbB* mutants. Both *dsbA* and *dsbB* mutants show decreased alkaline phosphatase activity. Additionally, *dsbA* is required for the overproduction of heterologous proteins containing disulfides (*24*, *25*).

The *dsbC* gene was identified in several genetic selections (*12*, *26*). This gene can rescue sensitivity to dithiothreitol (DTT)[1] in mutagenized bacteria when present on a low-copy plasmid vector. Also when present on a multicopy vector, *dsbC* can rescue DTT and benzylpenicillin sensitivity in *dsbA*, *dsbB*, or *dsbAdsbB* strains. The protein is similar to DsbA in being a periplasmic protein containing a C-X-X-C motif which forms a reactive disulfide. Additionally, DsbC has a second set of cysteines that forms a disulfide bond (*27*). Since the two enzymes are found in the same cellular location and may be involved in forming disulfide bonds, we compared the two enzymes in terms of disulfide isomerase and thiol oxidant activities. Additionally, we examined the availability of the cysteines to thiol modifiers and determined the *in vivo* redox state. Our *in vivo* results confirm predictions based on genetic results that DsbC acts as a disulfide isomerase (*13*, *28*).

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[1] Abbreviations: AMS, 4-acetamido-4′-maleimidostilbene-2,2′-disulfonate; DTT, dithiothreitol; DTNB, 5,5′-dithionitrobenzoic acid; HPLC, high-performance liquid chromatography; IPTG, isopropyl β-D-thiogalactoside; mis-IGF-I; misfolded IGF-I, cor-IGF-I, correctly folded IGF-I; GSH, reduced glutathione; GSSG, oxidized glutathione; SDS, sodium dodecyl sulfate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; PDI, protein disulfide isomerase; MGSA, melanocyte growth stimulating activity; hGH, human growth hormone; TCA, trichloroacetic acid.

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EXPERIMENTAL PROCEDURES

Materials. DsbA was purified as described previously (*21*). Oligonucleotides were synthesized by the Oligonucleotide Synthesis Group at Genentech. Q-Sepharose resin was obtained from Pharmacia. Ultrafiltration membranes were purchased from Amicon. Trifluoroacetic acid and 5,5′-dithionitrobenzoic acid (DTNB) were obtained from Pierce, and HPLC-grade solvents were obtained from Fisher. Pfu polymerase was purchased from Stratagene, and isopropyl thiogalactoside (IPTG) was from Boehringer Mannheim. All other reagents unless specified were purchased from Sigma. The strains used were all derived from 27C7 [ATCC 55244, W3110 *fhu*Δ phoAΔE15 Δ(argF-lac)169 ptrA3 degP::kanR ΔompT Δ(nmpC-fepE) ilvG2096] and are as follows: 37D6 (27C7 *ilvG2096 rbs*), 40C2 (37D6 *dsbA::kan*), 41F6 (37D6 *dsbB::kan*), 46C9 (37D6 Δ*dsbC*), 47H8 (37D6 Δ*dsbD*). Polyclonal antisera were generated in rabbits immunized with purified DsbA or DsbC by the Bioanalytical Technology Department at Genentech.

Expression and Isolation of dsbC. Two oligonucleotides were synthesized corresponding to the N- and C-termini of the DsbC gene with restriction endonuclease sites attached for ease of cloning. The N-terminal oligonucleotide was GCCTCTAGAGGGAAGAATTATGAAGAAAGGT, and the C-terminal oligonucleotide was CGAATCGATGCGAATTATTTACCGCTGGTC (restriction sites underlined). After PCR amplification using Pfu polymerase and W3110 chromosomal DNA, the PCR fragment was digested with *Xba*I and *Cla*I and ligated to (A) *Pst*I−*Xba*I 1.1 kb fragment encoding the tac promoter from the pBR322tacII plasmid (*29*) and (B) *Pst*I−*Cla*I fragment from pBR322. The plasmid construction contained *dsbC* behind the tac promoter on a pBR322 vector encoding both tetracycline and ampicillin resistance and was termed pJJ37. The plasmid was transformed into a lacI^q derivative of W3110 and grown in a 10 L fermenter. IPTG (1 mM) was added during mid log phase, and the cells were harvested 3 h after induction. Cell paste was stored at −20 °C. Wet cell paste (20 g) was resuspended in 200 mL of water and resuspended with the aid of a polytron tissue homogenizer. After being stirred for 2 h on ice, the cells were harvested, and the supernatant containing the periplasmic fraction was recovered. The supernatant was adjusted to 50 mM Mes, pH 6.0, with NaOH, 0.5 mM phenylmethanesulfonyl fluoride, and 1 mM EDTA. The solution was concentrated approximately 3-fold by ultrafiltration with an Amicon stirred cell and a YM10 membrane (Amicon). The extract was then passed over a 50 mL Q-Sepharose Fast Flow resin equilibrated in 50 mM Mes, pH 6.0, 1 mM EDTA. DsbC was found in the flow through fractions and pooled after analysis by SDS−PAGE. The protein was found to be 84% pure as analyzed by laser densitometry of Coomassie blue stained SDS−PAGE. This material was used for routine enzyme assays. A portion of the partially purified protein was further chromatographed by reversed phase HPLC and was analyzed by laser densitometry to be 91% pure. This material was used for antisera production and for some biochemical assays.

Sulfhydryl Reactivity. The Dsb proteins were reduced with DTT at room temperature in 20 mM Tris-HCl, pH 8.0, for 30 min. The reduced protein was then gel-filtered into 10 mM HCl using a NAP-5 (Pharmacia) column. Protein concentration was determined by A_{280} using extinction coefficients calculated by the method of Gill and von Hippel (*30*). 4-Acetamido-4′-maleimidostilbene-2,2′-disulfonate (AMS) (Molecular Probes) in 50 mM sodium phosphate, pH 7.0, was used at a final concentration of 10 mM for 10 min at room temperature. Derivatization was done in 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 1 mM EDTA, and the protein concentration used was 1 mg/mL. To terminate the reaction, the pH of the solution was lowered with 0.1 volume of 3 M HCl, and the proteins were then analyzed by nonreducing SDS−PAGE on 16% Tris−glycine gels. For mass spectrometry analysis, the derivatized protein was gel-filtered a second time into 10 mM HCl. Ellman's reagent (DTNB) was added to quantitate free sulfhydryl content by the procedure of Riddles et al. (*31*). Additionally, 0.5 mM coumarin maleimide (Molecular Probes, Eugene, OR) in dimethylformamide was used to quantitate free sulfhydryl content by absorption spectroscopy ($\lambda = 385$ nm, $\epsilon = 33\,000$ M^{-1} cm^{-1}) after removal of the reducing agent.

Disulfide Isomerase Activity. DsbC or DsbA was freshly reduced, gel-filtered into 10 mM HCl, and added at a concentration of 1 μM to misfolded IGF-I (mis-IGF-I) (*32*) in 100 mM Tris-HCl, pH 7.5, 100 mM KCl, and 1 mM EDTA. Aliquots were quenched by pipetting into 0.1 volume of 3 N HCl chilled on ice in glass vials. The samples were analyzed for conversion to correctly folded IGF-I (cor-IGF-I) by reversed phase HPLC as described previously (*21*). Whenever a glutathione redox buffer was included, the solutions were made fresh, and the isomerization measured in the absence of enzyme was subtracted from the isomerization measured in the presence of enzyme plus redox buffer.

Thiol Oxidant Activity. DsbC was added to reduced and denatured IGF-I (45 μM). Oxidation of IGF-I by DsbC was compared with DsbA- or GSSG-stimulated oxidations at pH 7.0, 7.5, and 8.0 in 0.1 M potassium phosphate buffer. Quantitation of cor-IGF-I and mis-IGF-I was performed by reversed phase HPLC after incubating for 4 h at 37 °C.

Determination of in Vivo Redox State. Fresh overnight cultures were diluted to 0.05 OD_{550} unit in 25 mL of LB media plus the appropriate antibiotic in 125 mL baffled flasks and incubated at 37 °C, at 225−240 rpm. After 2−2.5 h, the OD_{550} was measured, and 0.5 OD_{550} unit was precipitated with 10% trichloroacetic acid (TCA) and incubated on ice according to the procedure of Kishigami et al. (*33*). The precipitates were centrifuged for 15 min at 16 000*g*, 4 °C, and washed with cold acetone. The samples were centrifuged for 10 min and the acetone supernatants discarded. The pellets were air-dried and resuspended in 100 μL of 10 mM AMS in 50 mM Tris-HCl, pH 8.0, 1% SDS, and 1 mM EDTA (freshly made). The samples were vortexed 30 min at room temperature, heated 5 min at 37 °C, and reprecipitated with the addition of 1 mL of cold acetone. The samples were centrifuged, supernatants were discarded, and the pellets were resolubilized in 100 μL of Laemmli 2× sample buffer without reducing agents. After SDS−PAGE, the proteins were transferred to nitrocellulose and probed with rabbit polyclonal antisera generated against purified proteins. Antibodies were detected by the ECL detection system (Amersham).

dsbC and dsbD Knockout Construction. To delete the *dsbC* gene from the chromosome, the procedure of Metcalf et al. (*34*) as modified by Bass et al. (*35*) was employed. The upstream and downstream regions of the *dsbC* gene were amplified by PCR and ligated together in a vector allowing positive (ampicillin resistance) and negative selection (su-

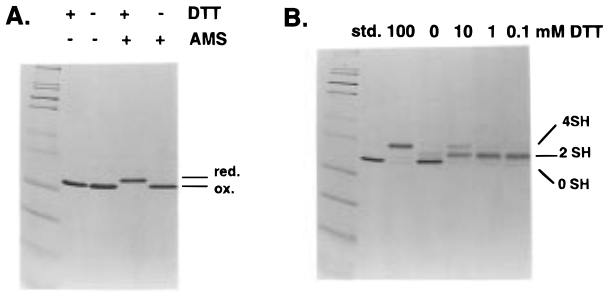

FIGURE 1: Electrophoresis of AMS-derivatized proteins. Both panels are Coomassie blue stained nonreducing SDS−PAGE (16% Tris−glycine) using purified Dsb proteins. In each panel, the left-hand lane depicts molecular mass standards (from the top, 200, 116, 97, 66, 55, 36, 31, 21, 14, 6, and 3.5 kDa). Panel A shows DsbA with and without reduction by DTT followed by AMS conjugation (lanes marked +). Panel B shows DsbC reduced with varying concentrations of DTT followed by reaction with AMS (except for the lane marked std.). std. is a DsbC that has not been reduced or modified with AMS.

crose resistance). Knockouts were confirmed by PCR as lacking the *dsbC* gene and also were confirmed by lack of the DsbC protein in immunoblots of whole cell extracts. The *dsbD* gene knockout was similarly done except knockouts were confirmed only by PCR.

Overexpression of Heterologous Proteins. Expression of heterologous proteins was performed with the *phoA* promoter where induction was done using low phosphate concentration in the media (*36*). All plasmids were pBR322-based. Overnight cultures grown in rich media were diluted approximately 50-fold into low-phosphate media and shaken at 37 °C for 24 h. Protein expression was monitored by Coomassie blue staining of SDS−PAGE and quantitated by laser densitometry. The band of interest was quantitated as a percent of the total protein in the entire gel lane. For the quantitation of human Fab produced, Western blotting followed nonreducing SDS−PAGE, and the blots were probed with a mouse anti-human κ chain antibody (Zymed Laboratories) and detected with a horseradish peroxidase-conjugated antibody and the ECL system (Amersham). The films were quantitated by laser densitomery.

RESULTS

Biochemical Properties of DsbC. In order to study the oxidoreductase activities of DsbC, the *dsbC* gene was amplified from W3110 chromosomal DNA and cloned into an overexpression vector. After purification, there was no detectable disulfide isomerization activity. To determine if the protein possessed disulfide isomerase activity, varying concentrations of DTT were added and incubated for 30 min at room temperature. The reducing agent was removed by a small gel filtration column and the protein checked for disulfide isomerase activity as well as accessibility of cysteines to modifying reagents. The reduced protein did have disulfide isomerase activity as assayed by the conversion of misfolded IGF-I to correctly folded IGF-I (see below). Figure 1B shows that the different redox states of DsbC can be detected by nonreducing SDS−PAGE after trapping with 4-acetamido-4′-maleimidostilbene-2,2′-disulfonate (AMS). Each conjugated AMS moiety increases the molecular mass by 490 daltons. The highest concentration of DTT (100 mM) used in Figure 1B produced completely reduced DsbC. When 10 mM DTT was used, some completely reduced DsbC was observed as well as a band which migrated between completely oxidized and reduced DsbC. When either 1 or 0.1 mM DTT was used, the intermediate band was the major isoform. Mass spectrometry determined that this derivative had two AMS groups added and therefore is likely to be singly reduced DsbC. The major species in each lane in Figure 1 were confirmed by mass spectrometry (data not shown). Proteolytic digestion followed by reverse phase HPLC and mass spectrometry confirmed that both cysteines in the C-X-X-C site were derivatized by AMS and that the other two cysteines were linked by a disulfide bond (data not shown). Based on these results, we conclude that mild reduction under native conditions yields two free cysteines and that the cysteines in the active site are accessible to maleimide reagents.

Table 1: Kinetic Constants for Disulfide Isomerization of mis-IGF-I[a]

	K_M (μM)	k_{cat} (min^{-1})
DsbA	43	0.2
DsbC	10	0.2
PDI*	36	3.4

[a] The K_M and k_{cat} values were determined by Michaelis−Menten kinetic analysis of double reciprocal plots with the largest standard deviations being 20%. The parameters for DsbA were remeasured and were identical to the previously published report (*21*). For bovine PDI, a redox buffer of 100 μM GSH and 25 μM GSSG was used to maintain the linearity of product formation with time.

AMS derivatization followed by nonreducing SDS−PAGE as well as mass spectrometry was used to examine the availability of the two cysteines in DsbA. As can be seen in Figure 1A, there is very little difference in electrophoretic mobility between reduced and oxidized DsbA without any further modifications. After AMS conjugation, there is a noticeable decrease in mobility for reduced DsbA. Mass spectrometry revealed that a mixture of singly and doubly derivatized DsbA was formed. Approximately two-thirds of the material was doubly derivatized and one-third of the protein was singly modified. Our gel system cannot resolve the difference between the two species, resulting in the appearance of a single band rather than two bands. The carboxyl-terminal cysteine, C33, in DsbA has been shown to be less reactive and more difficult to derivatize than the amino-terminal cysteine C30 (*18*).

Quantitation of thiol content by absorption spectroscopy yielded misleading results. After reduction of DsbC with 100 mM DTT followed by gel filtration, both Ellman's reagent and coumarin maleimide yielded approximately 2 mol of accessible cysteine/mol of protein. Ellman's reagent indicated 2.1 moles of cysteine per mol of DsbC, and coumarin maleimide yielded 1.9 mol of cysteine/mol of protein. The reaction with Ellman's reagent was complete within 1 min at room temperature. Subsequent mass spectroscopy analysis of the coumarin maleimide reaction indicated four maleimide groups, confirming the protein was completely reduced.

Disulfide Isomerase Activity of dsbC. DsbC reduced with 1 mM DTT exhibited disulfide isomerase activity after removal of the reducing agent. This enzyme activity was quantitated and compared with the isomerase activities of DsbA and bovine protein disulfide isomerase (PDI) (see Table 1). Although DsbC had a higher affinity for the mis-IGF-I substrate, the k_{cat} values for DsbC and DsbA were equivalent and significantly less than for PDI. It should be noted that PDI requires the presence of a redox buffer for

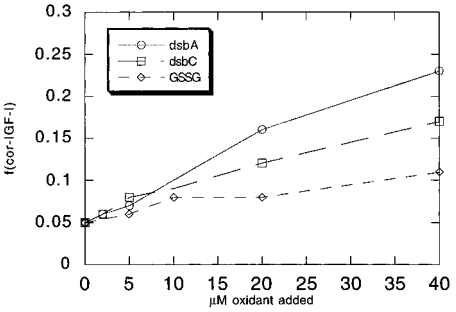

FIGURE 2: Oxidation of reduced and denatured IGF-I. IGF-I (45 μM) was oxidized at pH 7.5 by either DsbA (circles), DsbC (squares), or GSSG (diamonds). The *y*-axis corresponds to the fraction of IGF-I that has folded to the cor-IGF-I isomer.

maximum isomerase activity and loses this activity rapidly after removal of low molecular weight thiols (data not shown). Both DsbA and DsbC can isomerize disulfides independent of a redox buffer, which may be important considering the periplasmic location of these enzymes and the uncertain availability of low molecular weight thiols depending on the growth media and pH.

Oxidation of Reduced and Denatured IGF-I. To assay the ability of DsbC to oxidize reduced proteins, reduced and denatured IGF-I was prepared and added to oxidized DsbC. The levels of cor-IGF-I and mis-IGF-I were measured and compared to the levels when DsbA was added instead of DsbC. For comparison, refolding where GSSG was the primary oxidant was also performed. The resulting ratio between cor-IGF-I and mis-IGF-I formed was consistently 2:1 (*21*). The refolding reactions were conducted at three different pH values (7.0, 7.5, 8.0). As shown in Figure 2, DsbA was a better oxidant than DsbC and GSSG at all pH values. Only pH 7.5 is shown for clarity. DsbC was not much better than GSSG at pH 7.0 but was better at pH 7.5 and 8.0. The amount of product formed can be easily manipulated in these experiments by adjusting the IGF-I to oxidant ratio. Decreasing this ratio increases the yield of cor-IGF-I produced. These experiments were originally designed to detect catalytic refolding so the amount of substrate relative to the starting oxidant concentration was high. The starting concentration of IGF-I was 45 μM, which means that the concentration of disulfides formed would be 135 μM if all the IGF-I folded to the three-disulfide form. It should be noted that the refolding reaction described here is stoichiometric and not catalytic unlike the isomerase reaction where catalysis does occur. There has been only one example to date of either DsbA or DsbC acting as a catalyst in protein thiol oxidation *in vitro* (*17*). The ranking of DsbA > DsbC > GSSG as oxidants is consistent with the published redox potentials and equilibrium constants (*18*, *27*, *37*).

In Vivo Redox State. Since both DsbA and DsbC can form and shuffle disulfides *in vitro*, we wanted to determine the redox state of each *in vivo*. To do this, we employed the procedure of Kishigami et al. (*33*) since we had also noted a problem in determining the *in vivo* redox of DsbA. This procedure utilizes TCA precipitation to inhibit an activity capable of reducing DsbA during sample preparation. Live cells are TCA-precipitated and then resuspended in a denaturing solution containing AMS to block free thiols. Since AMS is a highly soluble reagent in aqueous solutions, it is ideal for thiol trapping in concentrated protein solutions. The electrophoretic mobilities of the oxidized and reduced proteins were then compared with *in vitro* generated

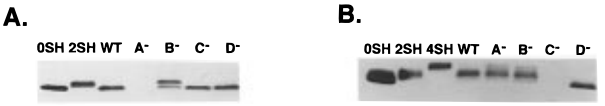

FIGURE 3: Oxidation state of DsbC and DsbA *in vivo*. Immunoblots probed with anti-dsb polyclonal sera of whole cell lysates from wild-type and mutant *dsb* strains. Each lane contains 0.025 OD equivalents of lysates. Panel A was probed with anti-DsbA sera. The first two lanes refer to *in vitro* generated standards where DsbA was derivatized with AMS without (0SH) and with (2SH) prior reduction by DTT. Each standard corresponds to 5 ng of DsbA. Panel B was probed with anti-DsbC sera. The first three lanes (0SH, 2SH, 4SH) are *in vitro* generated standards corresponding to reduction with 0, 1, and 100 mM DTT prior to reaction with AMS. Each standard is 5 ng.

standards. There is a very clear separation between oxidized and reduced proteins as can be seen in Figure 3. In *E. coli* strains with wild-type *dsb* genes, DsbA was found in the oxidized state exclusively in agreement with Kishigami et al. (*33*). On the other hand, DsbC was found exclusively in the singly reduced form. The presence of *dsbA* or *dsbB* mutations resulted in some completely reduced DsbC. The strain background used in these experiments has three protease mutations which may increase the half-life of completely reduced DsbC *in vivo*. The presence of a functional *dsbD* gene is needed to maintain the singly reduced form of DsbC as a *dsbD* mutant yields only oxidized DsbC.

dsbC Knockout Strain. To gauge the importance of DsbC in heterologous protein secretion, we disrupted the chromosomal copy of the gene as described under Experimental Procedures. The mutant strain was then transformed with plasmids for overexpression and secretion of heterologous proteins containing varying numbers of disulfide bonds. Similarly, these plasmids were transformed into *dsbA* null hosts for comparison. All heterologous protein secretion is severely impaired in *dsbA* null hosts which is consistent with a previous report concerning antibody fragment production in a *dsbA* knockout strain (*24*). In our experiments, no heterologous protein accumulated in the *dsbA*-disrupted strain to the point where it was detected by Coomassie blue staining. This was the case even when the secretory protein did not contain any cysteine residues. The *dsbC* knockout strains exhibited much milder phenotypes than the *dsbA* knockouts. The *dsbC* phenotype varied from protein to protein. Some proteins like human growth hormone (hGH) and a human Fab (anti-CD18) showed little defect in the overall yield of soluble protein (see Figure 4A). Both of these examples have multiple disulfide bonds. A secreted heterologous protein (lymphotoxin) without any disulfide bonds shows no defect in overall yield. Some proteins do have defects in accumulation such as human IGF-I and melanocyte growth stimulating activity (MGSA). Both of these examples were found to be decreased by 50% in the total yield.

DISCUSSION

The periplasmic oxidoreductases DsbA and DsbC have dual activities *in vitro* depending on their redox states. When oxidized, these proteins can donate their reactive disulfide bond to reduced protein substrates readily. When reduced, they can act as disulfide isomerases and shuffle mispaired disulfides. Both functions are desirable, yet Gram-negative bacteria seem to have at least two different proteins to perform these reactions in the periplasm. To understand

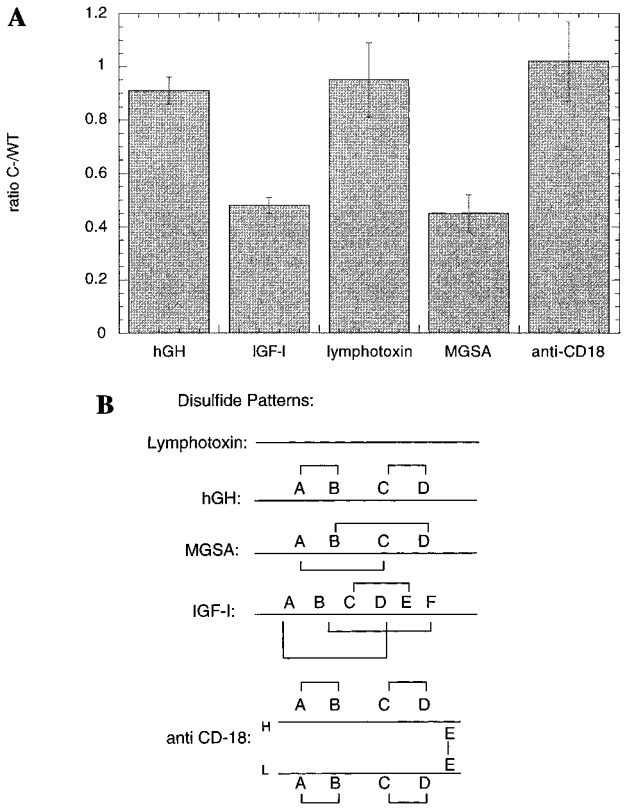

FIGURE 4: Yields of recombinant proteins produced in either wild-type or dsbC mutant strains. (A) Cell lysates were prepared and separated by SDS−PAGE. After staining with Coomassie blue, the protein yields were quantitated by laser densitometry and expressed as the yield of protein in the mutant strain divided by the yield in the wild-type strain ($n = 3-6$). Error bars represent one standard deviation. Panel B depicts the cysteine pairings of the proteins tested. H and L refer to the heavy and light chain derived polypeptides of the anti-CD18 Fab fragment. E denotes the two cysteines involved in the interchain disulfide.

which protein performs oxidation and/or isomerization *in vivo* and to complement previous genetic investigations, we investigated the *in vivo* redox state of each. DsbA is found in the oxidized form unless the strain has a mutant DsbB. DsbC is found in the singly reduced form unless the strain is deficient in DsbD function. These results are consistent with genetic experiments showing that *in vivo* DsbA is a protein thiol oxidant and DsbC is a disulfide isomerase (*10*, *13*, *28*).

While our *in vivo* redox determinations are qualitatively similar to those of Missiakas et al. (*13*), we see only oxidized DsbA and primarily singly reduced DsbC in wild-type strains whereas the previously published results show mixtures of oxidized and reduced proteins. We attribute these differences to two possible reasons. The first factor is the trapping procedure involved. Missiakas et al. (*13*) used iodoacetamide to trap the cysteines while we use a maleimide reagent. Maleimides tend to be more cysteine-specific and faster reacting with cysteines than the haloacetates (*38*−*41*) although microenvironmental factors can greatly influence thiol reactivity and specificity (*20*, *42*). Additionally, the three redox states of DsbC cannot be differentiated with iodoacetamide trapping followed by SDS−PAGE. Only two forms can be observed (data not shown and *13*). The use of the maleimide derivative that adds 490 daltons per covalent modification resolves the different redox forms of DsbC by SDS−PAGE. The second difference between the two procedures involves media growth conditions. Missiakas et al. (*13*) used minimal media in order to radiolabel the cultures whereas we used rich media.

The DsbA−B system is the major oxidant of secretory proteins. When these proteins are inactivated, suppressors can be found either in multicopy or in chromosomal dosages. Increased DsbC expression can suppress the lack of DsbA or DsbB function (*12*). Alternatively, mutations in *dsbD* can arise that are loss of function mutations that disrupt the redox state of DsbC (*13*, *28*). In *dsbD* mutants, all of the DsbC protein is found in the oxidized form where it now can function as an oxidant. The presence of the *dsbC* gene is required for suppression of these *dsbA* and *dsbB* mutants. Therefore, selections have uncovered a role for the DsbC−D system that is normally not the primary function of this pathway. Mutations in cytoplasmic redox effectors can also suppress the *dsbA* null strains, illustrating that the DsbC−D pathway interacts with the thioredoxin system (*28*).

The *in vivo* redox states of DsbA and DsbC coupled with *in vitro* observations of DsbC oxidation by DsbA (*27*) suggest an interesting dilemma, a futile redox cycle. Singly reduced DsbC can be oxidized by DsbA *in vitro*, albeit slower than the oxidation of other protein thiols (*27*). To the extent that oxidized DsbA reacts with reduced DsbC in the periplasm, thioredoxin-driven reduction of DsbD will be required to maintain DsbC in a reduced state. The thioredoxin-driven reduction of DsbD results ultimately in the consumption of NADPH. Additionally, reoxidation of DsbB with subsequent oxidation of DsbA will be required.

The investigation of the accessibility of the active site cysteines of DsbC *in vitro* has led to some discrepancies with previously published results (*27*). We consistently find that two cysteinyl residues are available for thiol modifiers while Zapun et al. (*27*) found only one cysteinyl residue modified in the absence of denaturant with iodoacetate, iodoacetamide, or GSSG. In our experiments, the extent of cysteine derivatization varies with the class of modifier. AMS consistently reacted with two free cysteines without any evidence of singly modified protein. Haloacetates do give mixtures of singly and doubly modified protein, with approximately a 4:1 ratio of doubly versus singly modified protein. The differences between these observations and those published previously may be due to the buffer conditions employed. We included sodium chloride and used a pH similar to those conditions in which bacteria are typically cultured. The previous study used a pH of 8.7 to maximize thiol reactivity. It is possible that the increased pH changed the local protein structure such that the second cysteine was actually less reactive. Another possibility is that the inclusion of sodium chloride increased the reactivity of the second cysteine even at the lower pH employed in our study.

The reactivity of the two cysteines in DsbA did not vary depending on the type of reagent used. A 2:1 ratio of doubly versus singly modified DsbA was observed whether AMS or iodoacetamide was used. A word of caution is justified here as we noticed that absorption spectroscopy could not reliably determine the number of cysteines derivatized and that mass spectrometry was a better method. This was true for both DTNB and the fluorescent maleimide derivatives.

The fact that overproduction of different heterologous proteins is differentially affected in the absence of DsbC is an interesting finding. When the cysteine pairings were consecutive rather than nonconsecutive (Figure 4B), the yield of overexpressed protein was not significantly affected.

However, when the cysteine pairings were nonconsecutive, the yield of overexpressed protein was decreased by 50%. This decrease in yield may be due to increased proteolysis although both strains have a kanamycin resistance gene inserted into the *degP* locus, a major periplasmic protease. *DsbC* null strains have an increased sigma E response (*43*) and may turn on periplasmic proteases other than just *degP*. Mispaired disulfide isomers are probably more sensitive to proteases and may be degraded as part of the quality control mechanisms in the periplasm. Cysteine pairing may occur as the secretory protein is still translocating so that the first two cysteines in the primary sequence form a disulfide. If this pairing is correct, as in growth hormone, alkaline phosphatase, or OmpA (*28*), then the absence of DsbC is not critical for obtaining the proper disulfide conformation and increasing the stability of the protein. If this pairing is incorrect, as in MGSA, IGF-I, or a mutant alkaline phosphatse (*44*), the absence of DsbC is significant and affects the yield of overexpressed protein. Consistent with these results is the dependence of Fab′ accumulation on increased PDI expression but only when the cysteine pairings were nonconsecutive (*45*). The yield of a similar Fab′ with consecutive cysteine pairings was not affected by increased PDI expression. The reason that the *dsbC* knockout shows such mild phenotypes relative to *dsbA* knockouts may be that very little disulfide isomerization is required for growth in standard media. Very few periplasmic and outer membrane proteins have more than two disulfides per monomer.

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