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In Vivo Substrate Specificity of Periplasmic Disulfide Oxidoreductases*

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In *Escherichia coli*, a family of periplasmic disulfide oxidoreductases catalyzes correct disulfide bond formation in periplasmic and secreted proteins. Despite the importance of native disulfide bonds in the folding and function of many proteins, a systematic investigation of the *in vivo* substrates of *E. coli* periplasmic disulfide oxidoreductases, including the well characterized oxidase DsbA, has not yet been performed. We combined a modified osmotic shock periplasmic extract and two-dimensional gel electrophoresis to identify substrates of the periplasmic oxidoreductases DsbA, DsbC, and DsbG. We found 10 cysteine-containing periplasmic proteins that are substrates of the disulfide oxidase DsbA, including PhoA and FlgI, previously established DsbA substrates. This technique did not detect any *in vivo* substrates of DsbG, but did identify two substrates of DsbC, RNase I and MepA. We confirmed that RNase I is a substrate of DsbC both *in vivo* and *in vitro*. This is the first time that DsbC has been shown to affect the *in vivo* function of a native *E. coli* protein, and the results strongly suggest that DsbC acts as a disulfide isomerase *in vivo*. We also demonstrate that DsbC, but not DsbG, is critical for the *in vivo* activity of RNase I, indicating that DsbC and DsbG do not function identically *in vivo*. The absence of substrates for DsbG suggests either that the *in vivo* substrate specificity of DsbG is more limited than that of DsbC or that DsbG is not active under the growth conditions tested. Our work represents one of the first times the *in vivo* substrate specificity of a folding catalyst system has been systematically investigated. Because our methodology is based on the simple assumption that the absence of a folding catalyst should cause its substrates to be present at decreased steady-state levels, this technique should be useful in analyzing the substrate specificity of any folding catalyst or chaperone for which mutations are available.

The *Escherichia coli* periplasm contains a family of disulfide bond-forming (Dsb) proteins that catalyze disulfide bond formation and rearrangement. This family of thiol-disulfide oxidoreductases can be divided into two pathways: an oxidation pathway, consisting of the inner membrane protein DsbB and the periplasmic protein DsbA, and an isomerization pathway, composed of the inner membrane protein DsbD and the periplasmic proteins DsbC and DsbG. DsbA interacts with reduced substrates, catalyzing oxidation of their cysteine residues to disulfide bonds (1). After oxidizing substrate proteins, DsbA becomes reduced and is returned to the active oxidized form by donating its electrons to DsbB. DsbB shuttles these electrons to membrane-bound quinones, which transfer the electrons to molecular oxygen or anaerobic electron acceptors (2). DsbC and DsbG are the periplasmic components of the isomerization pathway. Although the periplasm is a highly oxidizing environment, the active sites of DsbC and DsbG are kept in the reduced form. This is accomplished by the inner membrane protein DsbD, which transfers electrons from the cytoplasmic protein thioredoxin to DsbC and DsbG (3).

DsbA, DsbC, and DsbG share a number of common features. All are members of the thioredoxin superfamily with an active site composed of 2 cysteine residues separated by 2 other amino acids in a C*XX*C motif, and all reside in the periplasm (4). How the oxidase DsbA and the isomerases DsbC and DsbG can function so differently with such similar structural features has been the focus of recent work. This research suggests that the oligomeric state of periplasmic oxidoreductases is an important determinant of function. DsbC and DsbG are dimers, whereas DsbA is a monomer. When DsbC is forced to become a monomer, it is readily oxidized by DsbB and functions as an oxidase (5). When DsbA is attached to the N-terminal dimerization domain of DsbC, the chimeric protein now functions as an isomerase (6). That the disulfide oxidation and isomerization pathways can so readily substitute for each other may indicate that substrate binding of these proteins is similar.

DsbA is the principal disulfide oxidant in the periplasm. A *dsbA*$^-$ strain exhibits numerous *in vivo* phenotypes such as loss of motility, attenuated virulence, and sensitivity to benzylpenicillin, all of which can be attributed to loss of disulfide bonds in proteins related to these processes (7, 8). *dsbA* null mutants also have a decreased growth rate in minimal medium compared with the wild-type strain and, when plated on minimal medium, show strain-dependent growth defects such as no growth or mucoid colonies (1). Although overexpression or modification (such as monomerizing DsbC or adding a signal sequence to thioredoxin) of other members of the thioredoxin family can complement the *dsbA*$^-$ phenotype (9), no Dsb protein has been found that, without manipulation, takes on the role of DsbA in its absence. DsbA seems to play a unique and central role in periplasmic protein folding. Despite this, studies of the effect of DsbA on substrate folding and function generally focus on a few well-characterized substrates such as alkaline phosphatase (10). The range of proteins whose *in vivo* folding is affected by DsbA remains unknown.

In vivo substrates of DsbC and DsbG are even less characterized than those of DsbA. In fact, although DsbC and DsbG are both believed to function as disulfide isomerases for *E. coli*

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proteins containing more than two disulfide bonds, the evidence for this is largely indirect. Both DsbC and DsbG have active sites that are maintained in reduced form, consistent with an isomerase activity (11, 12). Both have been shown to aid in the folding of eukaryotic proteins with multiple disulfides, and DsbC appears to act as an isomerase during the *in vitro* folding of bovine pancreatic trypsin inhibitor, a eukaryotic protein with three disulfides whose folding pathway has been very well studied (13). However, no effect of DsbC or DsbG on the *in vivo* function of a native *E. coli* protein has been demonstrated. Rietsch *et al.* (14) showed that the rate of accumulation of correctly folded alkaline phosphatase (two disulfides) is decreased very slightly (by ~15%) in a *dsbC* null mutant; however, the activity of alkaline phosphatase is unaffected by null mutations in *dsbC*. Because *in vivo* substrates of DsbC and DsbG have not been discovered, it is unclear whether DsbC and DsbG function on the same subset of periplasmic proteins or whether they have distinct substrate specificity.

To address these questions, we used two-dimensional gel electrophoresis to compare wild-type and *dsb*$^-$ strains. Comparison of wild-type and null strains by two-dimensional gel electrophoresis has been previously utilized to determine the *in vivo* role of various proteins such as the cysteine transporter CycDC (15). In the case of *dsb*$^-$ strains, we postulated that Dsb substrates would lack their correct disulfides, causing them to be unstable and aggregate or be degraded. These unstable proteins should therefore be less abundant in periplasmic extracts of the *dsb*$^-$ strain, allowing our two-dimensional gel system to identify those Dsb substrates whose disulfide bonds are important for their folding.

This method identified 10 disulfide-containing proteins that are likely substrates of DsbA as well as two *in vivo* substrates of DsbC: RNase I, a periplasmic RNase that contains four disulfides, and MepA, a periplasmic murein hydrolase that contains three presumptive disulfides. We confirm *in vivo* and *in vitro* that DsbC aids in the folding of RNase I, strongly suggesting that DsbC acts as a disulfide isomerase for some *E. coli* proteins with multiple disulfide bonds. Although we found no substrates for DsbG, we demonstrate that a *dsbG* null mutation has no effect on the *in vivo* function of RNase I, illustrating that DsbC and DsbG do not have identical *in vivo* substrate specificity.

EXPERIMENTAL PROCEDURES

Bacterial Strains—Strains containing null mutations in the *dsbA*, *dsbC*, and *dsbG* genes were the gift of Dr. George Georgiou (12). These mutations were introduced into wild-type MC1000 by P1 transduction to create isogenic strains containing the individual mutants *dsbA*::*kan*, *dsbC*::*cm*, and *dsbG*::*kan* and the double mutant *dsbC*::*cm*,*dsbG*::*kan*. As a control for periplasmic RNase I activity, the *rna*$^-$ strain D10 was used (16).

Culture Conditions and Periplasmic Extracts—Cells (350 ml) were grown aerobically at 37 °C in either Luria broth or M63 minimal medium (17) to A_{600} = 0.5 or 1.0. Periplasmic extraction was performed using a modified version of the cold osmotic shock method of Neu and Heppel (18). Briefly, cells (100 ml) were harvested by centrifugation at 5000 rpm for 20 min at 4 °C, and the pellet was gently resuspended in 1 ml of TSE buffer (0.2 M Tris (pH 8.0), 0.5 M sucrose, and 1 mM EDTA) using a wire loop. Cells were incubated in TSE buffer on ice for 30 min, transferred to a 2-ml microcentrifuge tube, and centrifuged at 16,000 rpm for 30 min at 4 °C. Supernatants were removed to a new microcentrifuge tube; this supernatant constituted the periplasmic extract.

Trichloroacetic Acid Precipitation and Two-dimensional Gel Sample Preparation—Trichloroacetic acid (100%) was added to these periplasmic extracts to a final concentration of 10%, followed by incubation on ice for 15 h at 4 °C. Samples were centrifuged at 16,000 rpm for 1 h at 4 °C, and the pellet was washed with 1.5 ml of ice-cold acetone, incubated on ice for 15 min, and centrifuged at 16,000 rpm for 1 h at 4 °C to remove residual trichloroacetic acid. This wash step was repeated; acetone was removed; and samples were air-dried.

Two-dimensional Gel Electrophoresis—First dimension sample buffer (550 μl; containing 7 M urea, 2 M thiourea, 1% (w/v) Serdolit MB-1, 1% (w/v) dithiothreitol, 4% (w/v) Chaps,[1] and 0.5% (v/v) Pharmalyte 3–10) was added to each sample, and the sample was shaken at 1000 rpm at 30 °C until fully dissolved (at least 2 h). Samples were centrifuged at 13,000 rpm for 1 h at 4 °C; 525 μl of supernatant was transferred to a new tube; and samples were centrifuged at 13,000 rpm for 1 h at 4 °C. For the isoelectric focusing first dimension, 450 μl of each sample was applied to a 24-cm IPG strip (Amersham Biosciences) containing an immobilized pH gradient (pH 3–10, nonlinear). Rehydration and first dimension separation were carried out on an IPGphor (Amersham Biosciences). IPG strips were equilibrated for second dimension using the standard protocol of Amersham Biosciences and then transferred to the second dimension, consisting of SDS-polyacrylamide (13% acrylamide) gels. Gels were stained with Coomassie Blue and destained with 10% acetic acid.

Two-dimensional Gel Analysis—Gels were scanned for analysis using an Epson TwainPro scanner. Gel scans were analyzed using the programs Delta 2D (Decodon) and PDQuest (Bio-Rad) according to the program instructions. Each analysis set consisted of at least three replicate gels for each mutant or wild-type strain. Each gel was run from a sample that was handled separately in all steps from cell growth to lysis in sample buffer to ensure reproducibility of results. If a protein spot was not present on all gels of the wild-type strain, it was excluded from analysis.

Mass Spectrometry—The protein spots of interest were excised from gels and subjected to trypsin digestion, followed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (Applied Biosystems) at the University of Michigan Proteomics Core Facility. Proteins were identified using both automated and manual peak identification. A MOWSE score of 10^4 (MS-Fit) or confidence interval of >95% (Applied Biosystems GPS Explorer), in addition to correct pI and molecular mass on our two-dimensional gels, was taken to be a correct identification.

RNase I Extraction—RNase I was isolated from the periplasm using the Tris/EDTA extract technique of Neu and Heppel (18). Briefly, cells were grown in Luria broth to A_{600} = 1, and 20 OD* ml was pelleted by centrifugation at 4000 rpm for 20 min at 4 °C. Cells were gently resuspended in 2 ml of ice-cold 0.2 M Tris (pH 8) and 1 mM EDTA and rotated at 4 °C for 30 min. This mixture was then centrifuged at 13,500 rpm for 30 min at 4 °C, and the supernatant was retained as the RNase I extract.

RNase I Activity Assay—RNase I activity was assayed using a Panvera RNase activity assay kit according to the directions supplied by the manufacturer, except that the RNase assay buffer was modified to contain 1 mM EDTA instead of 5 mM $MgSO_4$. This change allowed us to more specifically measure RNase I activity since it has been demonstrated that RNase I constitutes ~98% of the RNase activity of a crude cell extract in the presence of 1 mM EDTA (19). The RNase I extract (30 μl) was incubated at 37 °C for 15 h in modified RNase assay buffer (final concentrations of 25 mM Tris-HCl (pH 7.8) and 1 mM EDTA) in the presence or absence of fluorescein-labeled RNA (final concentration of 1 ng/μl) in a final volume of 50 μl. Positive and negative controls were always performed to ensure that no components of the assay were contaminated with exogenous RNases. These incubations were trichloroacetic acid-precipitated by addition of calf thymus DNA (final concentration of 0.8 mg/ml) and trichloroacetic acid (final concentration of 5%), incubation on ice for 10 min, and centrifugation at 16,000 rpm for 10 min at 4 °C. This supernatant (80 μl) was mixed with 500 μl of Beacon assay buffer (0.2 M Tris (pH 9.0), 0.5% SDS, and 0.02% sodium azide) and measured on a Hitachi F-4500 fluorescence spectrophotometer with an excitation wavelength of 488 nm and emission measured at 530 nm.

Reduction and Denaturation of RNase I—Plasmid expressing RNase I was the gift of Drs. Robert and Louis Lim. RNase I was expressed and purified to >95% purity as described (20). Reduction and denaturation of RNase I was carried out in 5 M guanidinium chloride, 30 mM Hepes (pH 7.5), and 5 mM dithiothreitol at 25 °C for 2 h. Buffer was exchanged into 4 M guanidinium chloride, 40 mM Hepes (pH 7.5), and 1 mM EDTA using a Nap5 column (Amersham Biosciences). Denatured and reduced RNase I was quantified using ϵ_{280} = 33,480 M^{-1}, whereas native RNase I was quantified using ϵ_{280} = 33,000 M^{-1} (Swiss Protein Database). The thiol content of denatured and reduced RNase I was measured with 5,5′-dithiobis(nitrobenzoic acid) as described (21), and denatured and reduced RNase I was found to be >95% reduced.

[1] The abbreviations used are: Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; HPLC, high pressure liquid chromatography.

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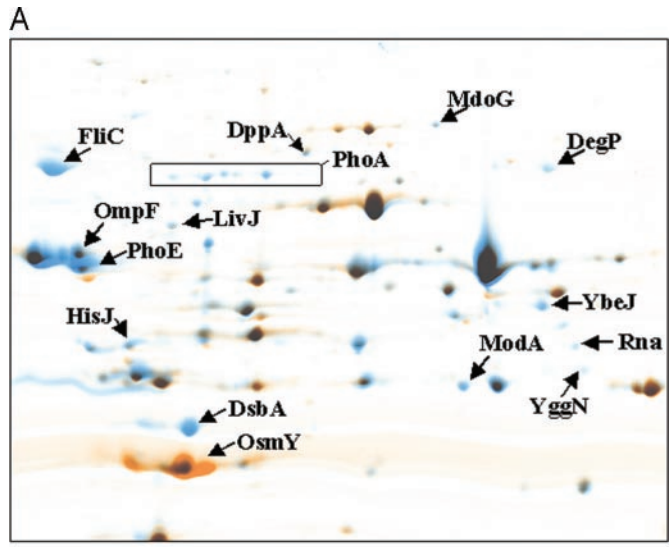
In Vitro RNase I Refolding—Refolding of denatured and reduced RNase I was initiated by dilution into redox buffer (40 mM Hepes (pH 7.5), 20 mM KCl, 1 mM EDTA, 6 mM oxidized glutathione, and 3 mM reduced glutathione) in the presence and absence of DsbC. DsbC was purified as described previously (22). Refolding was performed at 25 °C; and at various times, 100-μl aliquots were removed and quenched with 50 μl of 50% (v/v) acetonitrile and 50% (v/v) formic acid. Samples were centrifuged at 13,500 rpm for 10 min to remove aggregates and analyzed at 55 °C by reverse-phase HPLC (Waters 2695 separations module) on a C_{18} column (250 × 4.60 mm, Phenomenex Primesphere). The native and denatured/reduced forms were separated using a linear aqueous 34.4–36.4% acetonitrile gradient in 0.1% trifluoroacetic acid over 25 min at a flow rate of 0.5 ml/min.

RESULTS

Two-dimensional Gel Electrophoresis of the E. coli Periplasm—To identify possible substrates of Dsb proteins, we compared the proteome of *dsb*$^-$ strains with wild-type *E. coli*. The formation of disulfide bonds is vital to the proper folding of most of the proteins in which they are found. Dsb substrates should lack correct disulfide bonds, causing many of them to be unstable and to aggregate or undergo proteolysis. This should decrease the abundance of substrate proteins present in a periplasmic extract, which should be apparent on two-dimensional gels as the decreased amount of a protein spot in the *dsb*$^-$ strain relative to the wild-type strain. Because the Dsb proteins are located in the periplasm and should not catalyze disulfide bond formation in cytoplasmic proteins, we chose to examine the periplasmic cell fraction.

Identification of proteins affected by the absence of Dsb proteins required the specific isolation of periplasmic proteins and the exclusion of cytoplasmic proteins. Periplasmic proteins are estimated to constitute ~10–30% of the total cell protein; thus, only the most abundant periplasmic proteins are apparent on two-dimensional gels of whole cell extracts. To circumvent this problem, we devised a modified periplasmic extract that allowed us to cleanly separate periplasmic proteins from other cellular components. Each experiment was repeated at least three separate times in both Luria broth and minimal medium. Gene chip experiments have made it clear that the precise pattern of genomic expression is rather sensitive to the physiological state of the bacteria, including small batch-to-batch variations in minimal medium composition. We also observed this on our two-dimensional gels. To circumvent this problem, we simply excluded from analysis any proteins that were not present in very similar levels on all wild-type control gels. Mass spectrometry was performed on the most abundant spots and identified 123 spots representing 74 proteins that are confirmed or predicted periplasmic or outer membrane proteins.

dsbA$^-$ *Periplasmic Extracts Lack Many Disulfide-containing Proteins*—Consistent with previous observations (1), we noted that our *dsbA*$^-$ strain showed a decreased growth rate compared with the wild-type strain when grown in minimal medium, but not in rich medium. A comparison of the periplasmic proteins present on our wild-type and *dsbA*$^-$ gels revealed substantial differences between the two strains grown in minimal medium (Fig. 1*A*). Of particular interest are proteins with known disulfide bonds. Mass spectrometry analysis allowed us to identify 10 periplasmic proteins containing at least 2 cysteine residues whose abundance was decreased by at least 2-fold on our *dsbA*$^-$ gels (Table I). The structures of five of these proteins (PhoA, DppA, LivJ, HisJ, and RNase I) have been solved and show the presence of at least one disulfide bond (23–26). The effect of a *dsbA* null mutation on the folding of disulfide-containing proteins, as measured by our two-dimensional gel system, was much more pronounced in minimal medium than in rich medium. This is not surprising since cystine in rich medium has been shown to partially rescue



C

gene name	cysteine residues	confirmed disulfides	spot volume in *dsbC*$^-$ as % of wild type
rna	8	4	0% +/- 0 %
mepA	6		23% +/- 2%

FIG. 1. *A*, two-dimensional gel comparison of periplasmic proteins from the *dsbA*$^-$ and wild-type strains grown in minimal medium. A representative two-dimensional gel of a wild-type periplasmic extract (*blue spots*) is overlaid with a two-dimensional gel of a *dsbA*$^-$ periplasmic extract (*orange spots*). Proteins that were present in equal amounts in both strains appear as *black* or *brown spots*; proteins that were decreased in the *dsbA*$^-$ strain are *blue*; and proteins that were increased in the *dsbA*$^-$ strain are *orange*. For each strain, three independent periplasmic extracts of independent cultures were examined by two-dimensional gel electrophoresis. Spots that are identified here change in the *dsbA*$^-$ strain relative to the wild-type strain. As described under "Experimental Procedures," protein spots that were not present on all gels of the wild-type strain were excluded from analysis. *B*, MepA and RNase I protein levels are decreased in the *dsbC*$^-$ strain. Shown is a comparison of MepA and RNase I quantity in wild-type and various *dsb*$^-$ strains. Protein spots that were present in equal amounts in both strains are *black* or *brown*; proteins that were decreased in the *dsb*$^-$ strain are *blue*. *Arrows* indicate MepA and RNase I. A reference spot that did not decrease in the *dsbC*$^-$ strain is included for comparison, although this spot did decrease in the *dsbA*$^-$ strain. *C*, quantification of MepA and RNase I protein amounts in the *dsbC*$^-$ and wild-type strains.

dsbA$^-$ (27). All data presented for the *dsbA*$^-$ strain are therefore from experiments done in minimal medium, except in the case of two proteins, FlgI and MepA, which were not present on our wild-type minimal medium gels.

Of the candidate DsbA substrates identified, we found one well-studied DsbA substrate, periplasmic alkaline phosphatase (1). This protein contains 4 cysteine residues forming two disulfide bonds between Cys^{169} and Cys^{179} and between Cys^{287} and Cys^{337}. Alkaline phosphatase has been shown to be a substrate of DsbA: in the *dsbA*$^-$ strain, the protein lacks its disulfides and is therefore protease-sensitive (1). The abun-

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TABLE I
Cysteine-containing proteins are decreased in the dsbA⁻ strain

Likely substrates of DsbA are proteins containing at least 2 cysteine residues that are decreased by at least 2-fold in the *dsbA*⁻ strain. The percent decrease ± S.D. in *dsbA*⁻ spot volume relative to wild-type spot volume is shown. The number of cysteines in the proteins and disulfide bond content, if known, are also indicated.

Gene name	Cys residues	Confirmed disulfides	Spot volume in *dsbA*⁻
			% of wild-type
phoA	4	2	4 ± 4
ybeJ	2		13 ± 7
dppA	4	2	34 ± 2
degP	2	1	0 ± 0
hisJ	2	1	47 ± 8
yggN	2		0 ± 0
flgI	2		0 ± 0
rna	8	4	0 ± 0
mepA	6		29 ± 3
livJ	2	1	44 ± 13

TABLE II
Amounts of certain cysteine-less proteins are changed in the dsbA⁻ strain

The percent change ± S.D. in *dsbA*⁻ spot volume relative to wild-type spot volume is shown.

Gene name	Spot volume in *dsbA*⁻
	% of wild-type
fliC	4 ± 3
ompF	11 ± 9
ompE	12 ± 10
modA	20 ± 3
mdoG	32 ± 5
osmY	970 ± 280

dance of alkaline phosphatase was decreased by 25-fold on *dsbA*⁻ two-dimensional gels compared with wild-type gels. Other cysteine-containing proteins that were decreased by at least 2-fold include proteins involved in the transport of amino acids and peptides (DppA, HisJ, LivJ, and YbeJ), RNA degradation (RNase I), protein degradation (DegP), and murein degradation (MepA); a flagellar motor protein (FlgI); and a hypothetical protein of unknown function (YggN).

dsbA⁻ Affects Proteins without Cysteines—In addition to decreasing the quantity of proteins with disulfide bonds, the *dsbA*⁻ strain also affected the amount of some proteins with no cysteine residues (Table II). In minimal medium, the spot for flagellin (FliC) was decreased by 25-fold. Two outer membrane proteins, OmpF and PhoE, were decreased by ~10-fold. Two periplasmic proteins, glucans biosynthesis protein G (encoded by *mdoG*) and molybdate-binding periplasmic protein (encoded by *modA*), were also decreased in the *dsbA*⁻ strain (by 3- and 5-fold, respectively). Finally, the periplasmic protein osmotically inducible protein Y (encoded by *osmY*) was increased by ~10-fold in the *dsbA*⁻ strain grown in minimal medium. As described under "Discussion," these changes are likely due to pleiotropic effects of the *dsbA* null mutation on the regulation of these genes.

The dsbC⁻ Strain Exhibits Decreases in Proteins with Multiple Disulfides—The amounts of two periplasmic proteins, an abundant periplasmic RNase, RNase I, and the penicillin-insensitive murein endopeptidase, MepA, were dramatically decreased in the absence of DsbC (Fig. 1*B*). In the absence of DsbC, the protein spot for RNase I was missing, whereas the spot for MepA was decreased by 4-fold. RNase I and MepA both contain multiple cysteine residues. MepA contains 6 cysteines; all 6 are 100% conserved among the known or putative penicillin-insensitive murein endopeptidases, suggesting their structural importance to the protein. RNase I contains 8 cysteine residues. The crystal structure of this protein has been solved, and the disulfide connectivity is now known.[2] RNase I contains disulfides between Cys^{80} and Cys^{136}, Cys^{97} and Cys^{124}, and Cys^{197} and Cys^{234}, and a fourth disulfide bond between Cys^{25} and Cys^{39} is very likely to exist based on sequence homology to other T2 RNases whose crystal structures have been solved (28).

Neither MepA nor RNase I appears to rely on DsbG for proper folding. The effect of DsbG on MepA folding could not be determined with certainty: an adjacent protein that is increased in the *dsbG*⁻ strain obscured the effect of DsbG on MepA. The amount of RNase I in the *dsbG*⁻ strain was unchanged compared with the wild-type strain (Fig. 1, *B* and *C*), suggesting that RNase I folding relies only on DsbC and not on DsbG.

DsbC (but Not DsbG) Is Required for the in Vivo Function of RNase I—Our two-dimensional gels indicated that DsbC, but not DsbG, is required for the folding and function of RNase I. To address the *in vivo* effects of DsbC and DsbG on RNase I function, we examined RNase I activity in wild-type and *dsb*⁻ strains (Fig. 2). RNase I is the only known periplasmic RNase and accounts for ~98% of the *E. coli* RNase activity in crude extracts in the presence of 1 mM EDTA (19). We therefore used a Tris/EDTA extract technique specific for periplasmic RNase (18) and performed our assays in the presence of 1 mM EDTA. RNase I activity was determined by the ability of these Tris/EDTA extracts to degrade fluorescein-labeled RNA as measured by the release of soluble fluorescein-labeled RNA. RNase I activity in the *dsbC*⁻ strain was comparable with RNase I activity in the *rna* null strain D10. RNase I activity was ~12-fold higher in the *dsbG*⁻ strain than in the *dsbC*⁻ strain and was very similar to the activity in the wild-type strains. Finally, the *dsbC*⁻ *dsbG*⁻ double mutant strain showed base-line RNase activity similar to the *rna*⁻ strain.

DsbC Aids in the Refolding of RNase I in Vitro—DsbC has been shown to act as a disulfide isomerase for various eukaryotic proteins (29). RNase I contains four disulfide bonds, including one nonconsecutive disulfide, making it a logical substrate for a disulfide isomerase. However, it is also possible that the loss of RNase I activity in the *dsbC*⁻ strain occurs indirectly and is not the result of the interactions of DsbC with RNase I. To address this possibility, we examined the ability of DsbC to refold RNase I *in vitro*. Refolding of denatured and reduced RNase I was initiated by dilution of denatured and reduced RNase I into redox buffer in the presence and absence of DsbC. We used a redox buffer containing a mixture of GSH and GSSG to approximate the presence of the periplasmic reductant DsbD and the periplasmic oxidant DsbA *in vivo*.

RNase I refolding was analyzed using HPLC separation of folding intermediates. Native RNase I eluted from the column with a retention time of 21.5 min, whereas fully denatured and reduced RNase I eluted from the column with a retention time of 24.1 min. Not surprisingly, the GSH/GSSG redox buffer alone was able to refold RNase I to some extent. Addition of DsbC to redox buffer increased both the yield of native RNase I (Fig. 3, compare heights of peaks at 30 min with and without DsbC) and the rate of its refolding (compare locations of peaks at 10 min with and without DsbC). Refolding yield in the presence of DsbC corresponds to ~100% of the RNase I regaining native structure after only 10 min of incubation. Refolding in the absence of DsbC generated only ~50% recovery even after 30 min. These results suggest that the *in vivo* effects of DsbC on RNase I activity are due to direct interaction between the two proteins.

[2] L. W. Lim, personal communication.

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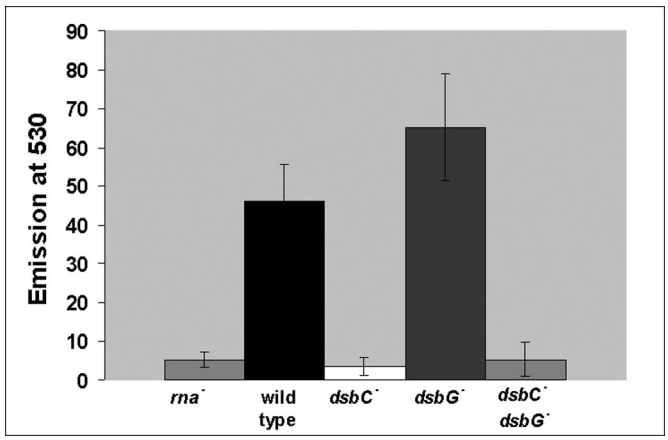

FIG. 2. **RNase I activity is decreased in the *dsbC*⁻ (but not *dsbG*⁻) strain.** RNase I was isolated by Tris/EDTA extraction from wild-type and *dsb*⁻ strains, and its activity was quantified by measuring the degradation of fluorescein-labeled RNA. Fluorescein-labeled RNA was incubated with Tris/EDTA extracts at 37 °C for 16 h, and insoluble RNA was precipitated by addition of calf thymus DNA and 5% trichloroacetic acid. The fluorescence of soluble fluorescein-labeled RNA (representing the degraded fraction) was measured by excitation at 488 nm and emission at 530 nm.

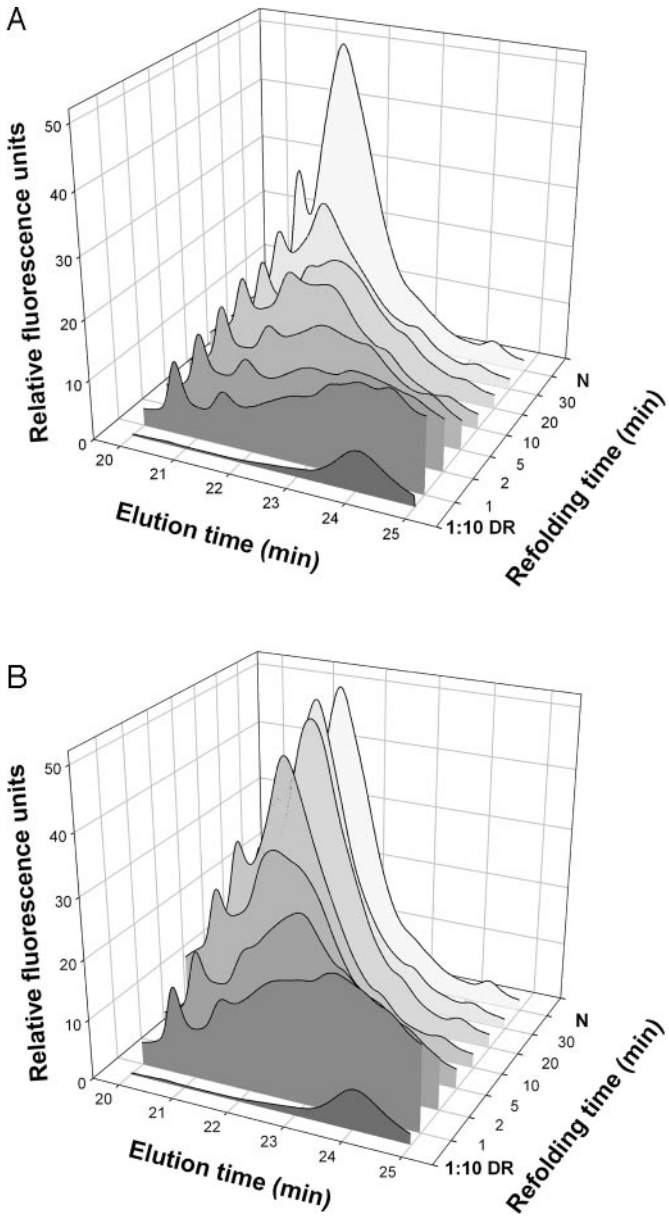

FIG. 3. **DsbC aids in the refolding of denatured and reduced RNase I *in vitro*.** Denatured and reduced RNase I was diluted (final concentration of 4 μM) into redox buffer and allowed to refold at 25 °C for various amounts of time. Samples were acid-trapped, and folding intermediates were separated by reverse-phase HPLC. Refolding was performed in the absence (*A*) and presence (*B*) of 4 μM DsbC. Native RNase I (*N*) eluted at 21.5 min; fully denatured and reduced RNase I eluted at ~24.1 min. To avoid obscuring the right-hand portion of these plots, the elution profile of a 10-fold dilution of denatured and reduced RNase I is shown and is indicated by *1:10 DR*.

DISCUSSION

We used two-dimensional gel electrophoresis of *dsb*⁻ strains to examine the role of Dsb proteins in the *in vivo* folding of *E. coli* proteins. The well-studied oxidase DsbA is generally believed to catalyze disulfide bond formation in most or all disulfide-containing periplasmic and outer membrane proteins. A bioinformatics search performed in our laboratory revealed that, of the ~700 periplasmic and membrane proteins listed in the Swiss Protein Database, more than half have 2 or more cysteine residues, suggesting that DsbA may have >300 *in vivo* substrates. It is not altogether surprising that DsbA may have a large number of *in vivo* substrates given the wide range of disulfide-containing heterologous proteins that can be expressed in active form in the *E. coli* periplasm (30). Despite the abundance of possible *in vivo* DsbA substrates, only a small number have been identified. These substrates generally fall into two categories: 1) certain *E. coli* proteins such as alkaline phosphatase that are used as model substrates and are thus particularly well characterized (31); and 2) those proteins whose structural instability in the *dsbA*⁻ strain causes notable phenotypic changes such as loss of motility and decreased virulence (32). However, structural instability of other proteins in the *dsbA*⁻ strain may cause no noticeable phenotypic changes under laboratory conditions, causing us to overlook many proteins that rely on correct disulfide formation for proper functioning.

One example of this is the finding that the periplasmic protease DegP is absent on our *dsbA*⁻ gels. DegP is a periplasmic protease that is essential under heat shock conditions and has been proposed to transition between a protease and a chaperone depending on cellular conditions (33). The crystal structure of this protein has been solved; however, one region, residues 55–79, is too flexible to be crystallized (34). This region contains 2 conserved cysteine residues and is one of the main areas of sequence dissimilarity between DegP and the highly homologous (75% sequence homology) nonessential protein DegQ (35). Recently, Skorko-Glonek *et al.* (36) showed that, under reducing conditions, DegP undergoes autocleavage, causing it to dissociate from a stable hexamer into catalytically active monomers. This is consistent with our finding that DegP is absent on our gels of the *dsbA*⁻ strain, likely because reduced DegP is not stable and either degrades itself or becomes vulnerable to other periplasmic proteases.

We found nine other cysteine-containing proteins that were decreased by at least 2-fold in the *dsbA*⁻ strain compared with the wild-type strain. Six of these proteins (PhoA, DppA, RNase I, FlgI, HisJ, and LivJ) contain at least one known structural disulfide bond. This suggests that these proteins are true substrates of DsbA and represents a substantial increase in the number of known DsbA substrates. Four of these proteins are involved in the periplasmic transport of various substances: YbeJ is a periplasmic binding protein, likely specific for aspartate and glutamate; HisJ is a periplasmic histidine-binding protein; LivJ is a periplasmic branched-chain amino acid transporter; and DppA is periplasmic dipeptide transporter (24, 25,

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37, 38). This technique identified the predicted protein YggN as a DsbA substrate. PSORT predicts this protein to be localized to the periplasm (39), and its sequence aligns with a putative periplasmic protein in *Salmonella typhimurium*. Our results suggest that this protein is indeed a periplasmic *E. coli* protein. We also found two DsbA substrates that contain multiple disulfides and are also substrates of DsbC: RNase I (four disulfides) and MepA (6 conserved cysteines). These are discussed in more detail below. Finally, *in vitro* folding and disulfide bond formation of one *in vivo* DsbA substrate (PhoA) has been previously examined, and the role of DsbA in *in vitro* PhoA folding is well characterized. Similar to the *in vitro* oxidant GSSG (oxidized glutathione), purified DsbA dramatically increases disulfide bond formation in both *in vitro* synthesized and reduced, denatured purified PhoA (31, 40). DsbA can also catalyze *in vitro* disulfide bond formation in a large number of eukaryotic proteins (40, 41); therefore, DsbA is very likely to catalyze *in vitro* disulfide bond formation in the remainder of its *in vivo* substrates.

The absence of DsbA affected six proteins containing no cysteines. One of these can be explained by a known relationship to a disulfide-containing protein. The cysteine-less flagellin protein FliC was absent in the absence of DsbA. This finding is consistent with previous work that demonstrates that flagellar synthesis is a highly regulated process in which transcription of late genes such as *fliC* is dependent upon synthesis of earlier genes (42). The flagellar P-ring motor protein FlgI contains 2 cysteine residues that form an intramolecular disulfide bond in a DsbA- and DsbB-dependent manner (43). On our gels, FlgI was absent in the *dsbA*$^-$ strain.

The cysteine-less outer membrane porin proteins OmpF and PhoE were both decreased to ~10% of the wild-type levels in the *dsbA*$^-$ strain grown in minimal medium. The effect of a *dsbA*$^-$ mutation on the expression of OmpF has been previously studied and shown to cause a 10-fold reduction in the amount of OmpF in the outer membrane (44). Furthermore, the effect of *dsbA*$^-$ on OmpF is due to decreased transcription rather than changes in translation or protein stability (44). The effect of a *dsbA*$^-$ strain on PhoE has not been previously demonstrated; however, OmpF and PhoE share a number of common features. Both are general diffusion porins, allowing passage of small hydrophilic molecules through the outer membrane (45). The crystal structures of OmpF and PhoE have been solved: both are trimers, with each monomer composed of 16 β-sheets (46). OmpF and PhoE are both regulated by osmolarity (44, 47). The two-component regulatory system EnvZ-OmpR induces the expression of the *ompF* gene under low osmolarity and decreases the expression of *ompF* under high osmolarity (48). PhoE expression also decreases in response to high osmolarity; however, the EnvZ-OmpR system has been shown to be uninvolved (47). Based on these similarities, we hypothesize that *dsbA*$^-$ may affect PhoE as it does OmpF (at a transcriptional rather than post-transcriptional level), although this remains to be determined.

Two other cysteine-less proteins whose levels change in the *dsbA*$^-$ strain compared with the wild-type strain are also regulated by osmolarity. The periplasmic protein OsmY is induced by hyperosmotic stress. The *osmY* gene has been shown to be induced by 8–10-fold in high osmolarity medium (49). On our *dsbA*$^-$ gels, OsmY was increased by ~9-fold compared with the wild-type gels. The periplasmic protein MdoG is involved in the synthesis of osmoregulated periplasmic glucans (50). Osmoregulated periplasmic glucans are a group of periplasmic oligosaccharides that are increased in medium of low osmolarity (51, 52). MdoG was decreased by ~5-fold on our *dsbA*$^-$ gels. The direction of change in these four proteins suggests that the *dsbA*$^-$ strain is either subject to or incorrectly sensing an increase in osmolarity that does not affect the wild-type strain. There are a few models that could account for this observation. First, it is possible that the *dsbA*$^-$ strain does not form a disulfide in a protein involved in osmoregulation, causing the cell to incorrectly sense an increase in osmolarity. Although the inner membrane-bound sensor protein EnvZ contains 1 cysteine residue, it is unlikely that this protein is affected by the absence of DsbA since the cysteine is likely located on the cytoplasmic side of the inner membrane (53). Second, the *dsbA*$^-$ strain could be experiencing increased osmolarity due to a change in cell wall permeability caused by lack of proper disulfide bond formation in outer membrane porins such as OmpA, which contains one disulfide.

The periplasmic molybdate-binding protein ModA has not been shown to be regulated by osmolarity and was decreased by ~5-fold on our *dsbA*$^-$ gels, though it contains no cysteines. The decrease in ModA in the *dsbA*$^-$ strain is probably due to a pleiotropic effect of the *dsbA* null mutation on *modA* transcription since an insertion in the *dsbA* gene of *S. typhimurium* causes decreased *modA* transcription (54). Further experimentation will be needed to confirm the effect of a *dsbA* null mutation on these genes and to probe the mechanism by which the *dsbA* gene affects proteins lacking cysteine residues.

Of the many cysteine-containing proteins affected by the *dsbA*$^-$ strain, we were especially interested in those DsbA substrates that contain more than one disulfide bond since they are possible substrates for the putative periplasmic isomerases DsbC and DsbG. We found four proteins affected by the *dsbA*$^-$ strain that are likely to contain two or more disulfide bonds. These proteins are the periplasmic alkaline phosphatase PhoA (two disulfides), dipeptide-binding protein DppA (two disulfides), periplasmic RNase I (four disulfides), and penicillin-insensitive murein endopeptidase MepA (6 cysteines, each 100% conserved). The *dsbC*$^-$, *dsbG*$^-$, and *dsbC*$^-$ *dsbG*$^-$ strains did not strongly affect the amount of PhoA and DppA compared with the wild-type strain. It has been shown that the *dsbC*$^-$ strain exhibits a slightly decreased rate of alkaline phosphatase folding *in vivo*, although it shows no change in alkaline phosphatase activity (14). The quantity of RNase I and MepA was dramatically decreased in the *dsbC*$^-$ and *dsbC*$^-$ *dsbG*$^-$ strains. The amounts of RNase I and MepA were unaffected by the absence of DsbG, suggesting that DsbG does not aid in the folding of these two proteins *in vivo*. We confirmed this to be the case for RNase I, whose *in vivo* function we demonstrated to be DsbC but not DsbG-dependent.

The number of disulfides present in RNase I makes it likely that the ability of DsbC to isomerize disulfide bonds is the activity that allows proper functioning of RNase I. Our *in vitro* results suggest that this is the case. *In vitro*, DsbC increased both the rate of RNase I refolding and the yield of native RNase I compared with redox buffer alone. These data indicate that DsbC can directly interact with RNase I to catalyze its folding, likely through disulfide bond isomerization. The increased yield of native RNase I in the presence of DsbC may also indicate that the chaperone activity of DsbC prevents aggregation of unfolded RNase I, allowing productive folding to occur.

That DsbG does not affect the *in vivo* function of RNase I demonstrates that DsbC and DsbG do not have identical *in vivo* roles. However, our method failed to detect any *in vivo* substrates for DsbG. This could be for a few reasons. First, it is possible that DsbG has a limited substrate specificity and that this set of proteins was not apparent on our two-dimensional gels, on which we could visualize and identify just over 10% of the ~700 proteins that the Swiss Protein Database annotates as periplasmic and outer membrane proteins. Second, it is

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possible that DsbG has an *in vivo* function that is not related to its ability to act on disulfide bonds and thus does not affect the amount of disulfide-containing proteins that we examined. However, the absence of DsbG did not cause a noticeable decrease in any of the other proteins visible on our gels. Finally, the effects of DsbG, which is the least abundant of the periplasmic Dsb proteins (12), may be too subtle to be detected by our methods. The *in vivo* role of DsbG remains to be determined.

Finally, we found it interesting that the *dsbC*$^-$ strain so dramatically affected the levels of two proteins with multiple disulfide bonds (RNase I and MepA) while leaving unchanged the levels of two other DsbA-dependent proteins (PhoA and DppA). There are two models to explain the roles of DsbC and DsbA *in vivo*. First, it has been suggested that DsbA acts immediately on newly translocated proteins as they are entering the periplasm (10). This would cause formation of disulfides between cysteine residues that are nearest to each other in the primary sequence of the protein (consecutive). This model suggests that DsbC is utilized mainly in the folding of proteins in which at least one native disulfide is nonconsecutive, whereas DsbA is important for disulfide formation in any disulfide-containing protein expressed to the periplasm. A significant amount of evidence supports this model based on *in vivo* folding of heterologous proteins: a *dsbC*$^-$ strain is unable to fold heterologous proteins containing nonconsecutive disulfides, such as insulin-like growth factor-1, but can fold proteins with multiple consecutive disulfides, such as human growth hormone (11). Second, it is possible that DsbA cannot act immediately on all newly translocated proteins and that incorrect disulfide formation occurs in both proteins that contain consecutive disulfides and those that contain nonconsecutive disulfides. The disulfide connectivity of MepA remains unknown; however, the disulfide connectivity of PhoA, DppA, and RNase I has been determined. PhoA and DppA each contain only consecutive disulfide bonds, whereas RNase I contains one nonconsecutive disulfide in addition to three consecutive disulfides. In our system, RNase I expression was decreased by the absence of DsbC, whereas PhoA and DppA expression was unaffected. This result adds support to the idea that DsbC is more important in the folding of proteins with nonconsecutive disulfides.

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