Laboratory Evolution of *Escherichia coli* Thioredoxin for Enhanced Catalysis of Protein Oxidation in the Periplasm Reveals a Phylogenetically Conserved Substrate Specificity Determinant*5

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Thioredoxin exported into the *Escherichia coli* periplasm catalyzes the oxidation of protein thiols in a DsbB-dependent function. However, the oxidative activity of periplasmic thioredoxin is insufficient to render *dsbA*− cells susceptible to infection by M13, a phenotype that is critically dependent on disulfide bond formation in the cell envelope. We sought to examine the molecular determinants that are required in order to convert thioredoxin from a reductant into an efficient periplasmic oxidant. A genetic screen for mutations in thioredoxin that render *dsbA*− cells sensitive to infection by M13 led to the isolation of a single amino acid substitution, G74S. In vivo the TrxA(G74S) mutant exhibited enhanced catalytic activity in the oxidation of alkaline phosphatase but was unable to oxidize FlgI and restore cell motility. In vitro studies revealed that the G74S substitution does not affect the redox potential of the thioredoxin-active site or its kinetics of oxidation by DsbB. Thus, the gain of function afforded by G74S stems in part from its altered substrate specificity, which also rendered the protein more resistant to reduction by DsbD/DsbC in the periplasm.

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Cells have evolved elaborate mechanisms for the spatial and temporal control of disulfide bond formation in proteins. Normally, protein oxidation occurs in exocyttoplasmic compartments (the endoplasmic reticulum or the bacterial periplasm), whereas in the cytoplasm disulfide bond formation is strongly disfavored (1, 2). Thiol oxidation, disulfide reduction, and isomerization are all accomplished by the action of multiple thiol-disulfide oxidoreductases. In the course of these reactions, the enzyme transfers electrons to or from substrate proteins and becomes oxidized or reduced in the process. To complete the catalytic cycle, the proper redox state of the thiol-disulfide oxidoreductase has to be restored by specialized enzymes that transfer electrons from non-thiol redox couples (such as NAD: NADH) or to the respiratory chain. For example, in the bacterial periplasmic space, disulfide bond formation is catalyzed by the highly efficient protein thiol oxidase DsbA (3). Upon transferring its disulfide to a substrate protein, DsbA becomes reduced and has to be recycled by the action of the membrane enzyme DsbB, which then transfers the electrons to the quinones. Similarly, in the cytoplasm, disulfide bond reduction is catalyzed by thioredoxin that is recycled by thioredoxin reductase (the product of the *trxB* gene), which in turn uses NADPH as electron donor (1). Interestingly, thiol-disulfide oxidoreductases with broad substrate specificity, such as DsaA, DsbC, thioredoxin, and glutaredoxin, all employ catalytic domains that belong to the thioredoxin superfamily. In contrast, the enzymes that recycle the general thiol-disulfide oxidoreductases (respectively DsbB, DsbD, thioredoxin reductase, and glutaredoxin reductase) are more structurally diverse.

Although DsaA and thioredoxin (TrxA) display a high degree of structural homology, in the cell they catalyze opposite reactions, namely thiol oxidation and disulfide reduction, respectively. At least two features have been shown to be responsible for the distinctly different functions of the two enzymes. First, the active site of DsaA is much more oxidizing than that of TrxA (4). The redox state of these and other thio-redox superfamily enzymes is critically dependent on the identity of the dipeptide sequence within the conserved Cys-X-X-Cys active site (5). Substitution of the PH sequence found in DsaA active site in thioredoxin, TrxA(G33P,P34H), imparts a more oxidizing redox potential $E'_0 = -204$ mV. By comparison, wild-type TrxA, which has a GP sequence in the active site, has an $E'_0 = -270$ mV (6). Second, in the periplasm DsaA is maintained in an oxidized state by DsbB, whereas in the cytoplasm TrxA is kept reduced by TrxB. Upon export of TrxA into the periplasm following fusion to an appropriate bacterial signal peptide, it becomes oxidized by DsbB and in turn serves as a general protein oxidant. Consequently, it can partially complement *dsbA*− strains, allowing, for example, the formation of small levels of active alkaline phosphatase (7, 8). The ability of periplasmic TrxA to catalyze the oxidative folding of alkaline phosphatase and restore motility is improved by replacing its...
active site dipeptide with that of DsbA, TrxA(G33P,P34H,G74S), and TrxA(G33P,P34H). pTrc99a-ssTorA-TrxA(G33P,P34H) with primers SalI-TrxA-F and R-trxA-Hind3 (5'--gatgccatatttcggcgcagtg-3') and R-Ovlp-TrxA(Aa) (5'--ctctccatgaaaaagatttggctggcgctggctggtttagttttagcgtttagcgcatcg-3'), as well as pTrc99a-ssBsA-TrxA(G74S) with primers Fat-dsbAss-TrxA-F (5'--ctttgcaattaacacctatgtattaatcggagagagtagatcgtgta-3') and R-trxA-Hind3. The two PCR fragments, N-terminal TrxA(G33P,P34H) that contains the mutation responsible for the G74S change was PCR-amplified from pTrc99a-ssTorA-TrxA(G33P,P34H), G74S was transferred into MC1000 F' dsbB::KanR by P1 transduction, and the CmR gene was deleted as described (10) resulting in LM108. All allele substitutions were confirmed by PCR.

Standard methods were used for all cloning steps (11). TrxA(G33P,P34H) was PCR-amplified from pFA7 (12) with primers SalI-TrxA-F (5'--ccttcgtgacagcaaatcggataattc-3') and R-trxA-Hind3 (5'--ctctccatgaaaaagatttggctggcgctggctggtttagttttagcgtttagcgcatcg-3'), as well as pTrc99a-ssBsA-TrxA(G74S) with primers Fat-dsbAss-TrxA-F (5'--ctttgcaattaacacctatgtattaatcggagagagtagatcgtgta-3') and R-trxA-Hind3 from pTrc99a-ssTorA-TrxA(G33P,P34H,G74S), digested with FatI and HindIII, and finally ligated into SalI- and HindIII-digested pTrc99a-ssTorA-TrxA resulting in pTrc99a-ssTorA-TrxA(G33P,P34H,G74S). The two PCR fragments, N-terminal TrxA(G33P,P34H) and C-terminal TrxA(G74S), were PCR-amplified from the core-
sponding pTrc99a-ssTorA plasmids with primers Sal1-TrxA-F and R-trxA-Hind3, digested with Sall and HindIII, and finally ligated into Sall- and HindIII-digested pTrc99a-ssDsbA-TrxA resulting in the constructs pTrc99a-ssDsbA-TrxA(G74S), pTrc99a-ssDsbA-TrxA(G33P,P34H), and pTrc99a-ssDsbA-TrxA(G33P,P34H,G74S). pET28a-TrxA(G74S) was constructed as follows. TrxA(G74S) was PCR-amplified with primers Fat1-trxA-F (5'–ccctctcatggatatattacctgac-3') and R-trxA-Hind3 from pTrc99a-ssTorA-TrxA(G74S), digested with FatI and HindIII, and finally ligated into Ncol- and HindIII-digested pET28a (Novagen).

TrxA Library Construction—Randomization of the trxA gene was performed by error-prone PCR (13) with primers Sall-TrxA-F and R-trxA-Hind3, then digested with Sall and HindIII, and finally ligated into Sall- and HindIII-digested pTrc99a-ssTorA-TrxA. Ligated plasmid DNA was first electroporated into E. coli Jude-1 resulting in a library size of 2 × 10⁶ clones. This library was then transferred by electroporation into LM106 resulting in 2 × 10⁹ transformants.

Minimal Media—MOPS low phosphate minimal medium was prepared as follows: MOPS minimal media (11) with 0.4% (w/v) glucose, 0.2% (w/v) casein enzymatic hydrolysate (C0626, Sigma), 0.05 mg/ml thiamine, and a total concentration of inorganic phosphates of 0.1 mM. M9 minimal medium was prepared as follows: MOPS minimal media (11) with 0.4% (w/v) glucose, 0.1% (w/v) casein enzymatic hydrolysate (C0626, Sigma), 2 mM MgSO₄, and 0.05 mg/ml thiamine.

M13 Phagemid Packaging—A single colony of MC1000 F⁻ cells harboring the plasmid pBAD18-CmR, which contains the M13 intragenic region, was inoculated in 6 ml of 2 × YT media (244020; BD Biosciences) with tetracycline (30 μg/ml) and chloramphenicol (30 μg/ml) and incubated at 37 °C with shaking. At an A₆₀₀ of 0.4–0.5, 1.0 ml of cells were infected with M13 KO7 (11) at a multiplicity of infection (m.o.i.) of 20 and incubated in a water bath at 37 °C for 30 min. Cells were then transferred to a flask containing 40 ml of 2 × YT with 0.2% (w/v) glucose with the antibiotics tetracycline (30 μg/ml) and chloramphenicol (30 μg/ml) and incubated at 37 °C with shaking. After 1 h, kanamycin (25 μg/ml) was added, and cells were grown overnight at 37 °C with shaking. The overnight culture was centrifuged for 30 min at 3500 rpm and at 4 °C. The supernatant was collected (~40 ml), and 8 ml of 20% (w/v) PEG-6000 with 2.5 M NaCl were added and then the mixture was kept on ice for 2 h and centrifuged again for 30 min at 3500 rpm and at 4 °C. The supernatant was discarded, and the packaged M13 phagemid pellet was resuspended in 2 ml of phosphate-buffered saline and stored at 4 °C.

M13 Infectivity Assay—Cells were inoculated into 5.5 ml of fresh MOPS low phosphate minimal media and grown for ~14.5 h at 37 °C with shaking. Cell culture A₆₀₀ was adjusted to 1.0, and 1000 μl were transferred to 1.5-ml microcentrifuge tubes and mixed with 5 μl of pBAD18-CmR packaged M13 phagemid (titer of ~3 × 10⁹ colony-forming units/ml). The tubes were incubated in a 37 °C water bath for 30 min and then transferred to a 37 °C incubator with shaking for 1 h. Cells were plated on LB-Miller plates with chloramphenicol (30 μg/ml) and ampicillin (100 μg/ml) and grown at 37 °C overnight.

Alkaline Phosphatase Activity Assay—Cells were grown in MOPS low phosphate minimal media. The low phosphate concentration induces the synthesis of alkaline phosphatase (PhoA) from the chromosomal copy of the alkaline phosphatase gene (phoA). Cells were inoculated into 5.5 ml of fresh media and grown for ~14.5 h at 37 °C with shaking. Cell culture A₆₀₀ was adjusted to 1.0, and 20 μl were transferred to a 96-well plate and mixed with 30 μl of lysis buffer, a 2:1 mixture of B-PER bacterial protein extraction reagent (78248 Pierce), and a 0.4 M iodoacetamide solution. Lysis took place for 30 min with shaking and then 200 μl of 250 μg/ml p-nitrophenyl phosphate in 0.2 M Tris-HCl, pH 8, was added to each well. Hydrolysis of p-nitrophenyl phosphate was followed at A₄₀₅ on a plate reader (Beckman Instruments).

Cell Motility Assay—Cells were grown overnight in M9 minimal media with the appropriate antibiotic at 37 °C with shaking. The amount of cells was normalized to a final A₆₀₀ of 1.0, and 1.5 μl were spotted at the center of M9 minimal media motility plates (M9 minimal media with 0.3% (w/v) agar). Cells were grown at 37 °C for 29 h at which point the motility halos were determined.

Purification of TrxA(G74S)—BL21(DE3) (Stratagene) cells harboring the pET28a-TrxA(G74S) plasmid were grown in 2 liters of LB-Miller medium with kanamycin (50 μg/ml) at 30 °C until an A₆₀₀ of 0.7–0.8 was reached. Isopropyl β-thiogalactoside was then added to a final concentration of 0.1 mM, and shaking was continued for 5 h. Cultures were then centrifuged at 8000 rpm and 4 °C for 15 min (JA-10 rotor, Beckman Instruments). Cell pellets were resuspended and lysed in 35 ml of BugBuster protein extraction reagent (70584-4, Novagen) with 35 μl of Benzoase (25 units/μl) (Novagen) and 1 tablet of Complete EDTA-free protease inhibitor mixture (11-873-580-001, Roche Applied Science).

Bacterial extracts were then centrifuged for 40 min at 16,000 rpm at 4 °C, and the resulting supernatant was incubated at 60 °C for 10 min in a water bath, then cooled down to 4 °C on ice, and finally centrifuged for 40 min at 16,000 rpm at 4 °C. The supernatant was collected, filtered with a 0.22-μm filter (GeneMate, ISC BioExpress), and concentrated to a final total protein concentration of 20 mg/ml as determined with BCA protein assay kit (523225, Pierce). 2 ml of this solution was loaded into a HiPrep 16/10 DEAE FF ion exchange column (17-5090-01, Amersham Biosciences), and the presence of single colonies per plate. Plates were incubated at 37 °C overnight, and the number of single colonies was then determined.

M13 Infectivity Library Screening—75 μl of MC1000 F⁻ dsbA pTrc99a-ssTorA-TrxA EPPCR library frozen stock (~4 × 10⁹ cells) was inoculated in a flask with 400 ml of fresh MOPS low phosphate minimal media. Cells were grown at 37 °C with shaking for 7.5 h (final A₆₀₀ = 1.23) and then normalized to A₆₀₀ = 1.0, and 1.0 ml was transferred to a 1.5-ml microcentrifuge tube and infected with 20 μl of pBAD18-CmR packaged M13 phagemid (titer of ~3 × 10⁹ colony-forming units/ml). The tube was incubated in a 37 °C water bath for 30 min and then transferred to a 37 °C incubator with shaking for 1 h. All cells were plated in four 150 × 15-mm LB-Miller plates with chloramphenicol (30 μg/ml) and ampicillin (100 μg/ml) and grown at 37 °C overnight.
TrxA(G74S) in the eluate was confirmed by SDS-PAGE and Coomassie Brilliant Blue staining.

The buffer of the collected fractions (≈21 ml) was exchanged to 25 mM HEPES, pH 7.5, and the solution was concentrated to a final volume of 1.5 ml. 0.75 ml of this solution was loaded into a Superdex 200 HR 10/30 gel filtration column (17-1088-01, Amersham Biosciences). The buffer of the collected fractions that contained TrxA(G74S) (≈3 ml) was exchanged to 25 mM HEPES, pH 7.5, and the solution was concentrated to a final volume of 0.25 ml with a final protein concentration of 39 mg/ml. The purity of the preparation was examined by SDS-PAGE followed by Coomassie Brilliant Blue staining and was found to be >99%.

**Determination of Protein Concentrations**—Protein concentrations for the in vitro assays were determined using their extinction coefficients at 280 nm and were calculated as described (14). Absorbance at 280 nm for the corresponding proteins was measured in 6 × guanidine hydrochloride, 20 mM sodium phosphate buffer, pH 6.5.

**Kinetics of Oxidation by DsbB in Vitro**—Stopped flow absorbance measurements were performed on a Hi-Tech Scientific SF61 instrument (1.0-cm path length) in single-mixing mode. The typical reaction contained around 100 μM of thioredoxin variants, 10 μM DsbB, and 200 μM ubiquinone-1 (coenzyme Q1; Sigma). Thioredoxin variants and a DsbB/Q1 mix were incubated in PND buffer (50 mM sodium phosphate, 300 mM NaCl, 0.04% dodecyl maltoside, pH 8.0) at 10 °C before mixing. Thioredoxin variants were freshly reduced as described above. The fractions of reduced protein were determined by Ellman’s assay, and the values were more than 95%. The absorbance after mixing was recorded at 510 nm. One data set contained three to four successive shots, and data for each trace was obtained three to four successive shots, and data for each trace was taken from a single-mixing instrument in single-mixing mode. The typical reaction contained around 100 mM sodium phosphate buffer, pH 6.5.

**Kinetics of Hirudin Isomerization in Vitro**—21 μM scrambled hirudin and 21 μM freshly reduced protein (wild-type TrxA and TrxA(G74S)) were incubated in the isomerization buffer (20 mM sodium phosphate, 130 mM sodium chloride, 0.13% polyethylene glycol 8000) at 25 °C. Aliquots of 120 μl were removed after different reaction times and quenched with 15 μl of formic acid and 15 μl of acetonitrile. Hirudin folding intermediates were separated by reverse-phase HPLC on a C18 column at 55 °C in a 19–25% acetonitrile gradient in 0.1% (v/v) trifluoroacetic acid. The absorbance was recorded at 220 nm.

**Distribution of Gly74 and Ser74 among Bacterial Thioredoxins**—Protein BLAST from NCBI was used to identify which amino acids are present at the −2 position with respect to the highly conserved Pro76 of thioredoxin cis-Pro loop. Specifically, the nonredundant data base was searched with the *E. coli* K12 TrxA protein sequence (GenBank accession number AAC76786) using the PSI-BLAST algorithm with a threshold of 0.005 and four rounds of iterations. The first 500 Blast hits with the exception of three-dimensional structures from the Protein Data Bank were used to determine the corresponding amino acids at the −2 position with respect to Pro76 of thioredoxin cis-Pro loop. Taxonomy of the different species was obtained from the NCBI Taxonomy web page. See the Supplemental Material for a detailed list of the results of this search.

**RESULTS**

**A Screen for Periplasmic Protein Oxidation Using M13 Infection**—One of the phenotypes of *dsbA*− mutant strains is resistance to the single-stranded filamentous phage M13 (16). Infection of *E. coli* by M13 is initiated by the binding of the pilI protein of the phage onto the conjugative sex pili encoded by the F factor (17). The F pili are extracellular protein structures that participate in bacterial conjugation (18). *dsbA*− null mutant strains seem to be resistant to M13 infection due to their inability to properly assemble the F pilus (16).

We took advantage of the dependence of M13 infection on disulfide bond formation in the host cell to develop an efficient screen for protein oxidation in the periplasm. Briefly, *E. coli* is infected with filamentous phage that packages the phagemid pBAD18-CmR, a plasmid containing a filamentous phage packaging sequence as well as a chloramphenicol resistance gene cassette (19). Upon infection by M13, F+ cells receive the CmR gene cassette and are able to form colonies on antibiotic-containing plates. As expected, infection of *dsbA*− mutant cells resulted in a dramatic reduction in the number of chloramphenicol-resistant colonies because they lack functional F pili and are therefore resistant to infection by the M13 phagemid (Table 2). In addition, no colonies could be obtained in a *dsbA*− *dsbB*− background where periplasmic protein oxidation is completely abolished.

In earlier studies it had been observed that post-translational export of TrxA using the PhoA signal sequence is inefficient because the rapid folding of thioredoxin in the cytoplasm interferes with its translocation through the Sec pore (20). In contrast, thioredoxin can be exported with high efficiency by using a signal peptide that engages the co-translational SRP-depend-

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4 The abbreviations used are: HPLC, high pressure liquid chromatography; m.o.i., multiplicity of infection; Tat, twin arginine transporter; MOPS, 4-morpholinepropanesulfonic acid.
ent pathway (20). For this study, we reasoned that since TrxA folds rapidly in the cytoplasm, it should be compatible with export via the twin arginine transporter (Tat) pathway, which is responsible for the membrane translocation of proteins that have completed their folding prior to export (21). The gene for thioredoxin was therefore fused to the Tat-specific signal peptide of the E. coli trimethylamine N-oxide reductase (TorA) (22) downstream from the trc promoter in the plasmid pTrc99a.

Expression of the ssTorA-TrxA fusion partially restored alkaline phosphatase activity in dsbA− cells to a level ~40% of that observed in the wild-type parental strain (Table 3). However, no antibiotic-resistant colonies were obtained upon infection with M13 containing the pBAD18-CmR phagemid (Table 2) indicating that the localization of TrxA to the periplasm was unable to restore the defect in F pilus assembly caused by the absence of DsbA. Interestingly, the more oxidizing thioredoxin was therefore fused to the Tat-specific signal peptide downstream from the trc promoter in the plasmid pTrc99a.

Isolation of TrxA Mutants Conferring Increased M13 Infectivity—The trxA gene in pTrc99a-ssTorA-TrxA was subjected to random mutagenesis by error-prone PCR resulting in a library of 2 × 10⁶ transformants of MC1000 dsbA F (21). Sequencing of eight clones selected at random revealed an average of 2.4 nucleotide substitutions per gene. Cells were grown in MOPS minimal media for 7.5 h and then infected with M13 phagemid to the PhoA activity of the wild-type parental strain (Table 3). How-ever, no antibiotic-resistant colonies were obtained upon infection with M13 containing the pBAD18-CmR phagemid (Table 2) indicating that the localization of TrxA to the periplasm was unable to restore the defect in F pilus assembly caused by the absence of DsbA. Interestingly, the more oxidizing thioredoxin variant, TrxA(G33P,P34H), did not allow M13 infectivity, yet it was fully able to restore the formation of active PhoA in the absence of DsbA. Interestingly, the more oxidizing thioredoxin was therefore fused to the Tat-specific signal peptide downstream from the trc promoter in the plasmid pTrc99a.
ity was determined in MC1000 dsbA cells grown in MOPS minimal media (Table 3). Interestingly, ssDsbA-TrxA(G74S) resulted in a lower level of PhoA activity compared with export via the ssTorA signal peptide, and the same trend was observed for wild-type TrxA. Consistent with these results, Western blot analysis revealed that both ssDsbA-TrxA(G74S) and ssDsbA-TrxA are expressed at a much lower level than the corresponding constructs with a ssTorA signal sequence (Fig. 1).

In order for an enzyme to be able to serve as a protein oxidant, it has to be maintained in a predominantly oxidized state by DsbB and to avoid reduction by the DsbD-DsbC pathway. Kinetic factors prevent the reduction of DsbA by DsbC or by DsbD (25). TrxA however is prone to reduction in the periplasm. Expression of ssTorA-TrxA in dsbA− dsbD− cells results in substantially higher PhoA activity to a level equal to that observed in MC1000 (Table 3) and Ref. 8. Because ssTorA-TrxA alone gives maximal PhoA activity in this strain background, we examined instead the effect of the G74S mutation in cells expressing thioredoxin via the DsbA signal sequence. ssDsbA-TrxA(G74S) resulted in statistically indistinguishable PhoA activities in cells with or without DsbD (Table 3). This result suggests that unlike the wild-type thioredoxin, TrxA(G74S) is significantly less susceptible to reduction by the DsbD-DsbC reduction pathway in vivo.

The single disulfide bond in FlgI is essential for motility. E. coli cells deficient in periplasmic disulfide bond formation exhibit lack of motility (26, 27). The motility of dsbA− cells expressing the different thioredoxin variants was determined (Fig. 2). Neither ssTorA-TrxA nor ssTorA-TrxA(G74S) were able to restore motility in dsbA− cells (Fig. 2, B and C). In contrast, expression of the TrxA variant with the DsbA active site, ssTorA-TrxA(G33P,P34H), conferred partial motility. Interestingly, introduction of the G74S mutation into ssTorA-TrxA(G33P,P34H) reproductively resulted in lower motility relative to cells expressing ssTorA(G33P,P34H) alone (Fig. 2, E and F). These results are to be contrasted with the effect of the “CPHC” active site (G33P,P34H substitutions) and the G74S mutation on M13 infectivity. As discussed above, change to the CPHC active site did not restore infectivity, whereas the G74S amino acid substitution resulted in the formation of 4 × 10^3 (±1 × 10^3) cm^3 colonies. Thioredoxin containing both the CPHC active site and G74S substitutions, TrxA(G33P,P34H,G74S), conferred a slight but statistically significant increase in the number of colony-forming units obtained following M13 infection. Collectively, these results suggest that the G74S mutation exerts a differential effect on the oxidation of various periplasmic substrates.

**Biochemical Characterization of the G74S Variant**—Structural modeling of the G74S change indicated that it resides close to the active site (Fig. 3). Changes close to the active site can affect the redox potential of the reactive cysteine (Cys^32) and in turn may be responsible for the increased activity of the mutant thioredoxin. To establish if the G74S change affected the redox potential of thioredoxin, the TrxA(G74S) variant was expressed in a preparative scale and purified to homogeneity, and its redox potential was determined by direct protein-protein redox equilibria with the γ domain of DsbD (28). These measurements show that there is no significant difference between the redox potential of wild-type thioredoxin, E′_0 = −270 mV (28), and the TrxA(G74S) variant E′_0 = −269 (±1) mV. This finding is in agreement with the in vivo infectivity results with the TrxA(G33P,P34H) variant and confirmed that the improved oxidative properties of TrxA(G74S) are not due to a change to a more oxidizing redox potential.

The in vivo results in strains with and without DsbD cannot establish if avoidance of reduction by the DsbD-DsbC pathway is the only reason for the improved oxidase activity of the G74S change. The more efficient oxidation by DsbB could also be a contributing factor. In order to address this issue, the kinetic parameters for the oxidation of wild-type TrxA and TrxA(G74S) by DsbB were determined in vitro by using enzyme-monitored turnover described for glucose oxidase by Gibson et al. (29). This method allows the measurement of k_cat and apparent K_m values in a single experiment by following the time course of the decay of an enzyme intermediate upon reaction with an excess amount of substrate. During the oxidation of DsbA by DsbB, cysteine 44 of DsbB forms a purple charge-transfer intermediate with the DsbB-bound quinone cofactor (30). This intermediate is character-
ized by a strong absorbance peak at 510 nm that allows the study of the kinetic cycle of DsbB in detail by using stopped flow methods (31). We have shown that wild-type thioredoxin and several active site variants also form intermediates that induce an absorbance peak at 510 nm and that this method can be used to measure the corresponding kinetic parameters for the in vitro oxidation by DsbB.9 A $K_m$ of 11 $\pm$ 1.2 $\mu M$ and $V_{max}$ of 29 $\pm$ 0.6 min$^{-1}$ were obtained for wild-type TrxA, and a $K_m$ of 8.5 $\pm$ 0.4 $\mu M$ and $V_{max}$ of 25 $\pm$ 0.6 min$^{-1}$ were obtained for TrxA(G74S). Both wild-type TrxA and TrxA(G74S) display almost identical kinetic parameters, $V_{max}/K_m$ value of 2.6 $\pm$ 0.3 min$^{-1}$ $\mu M^{-1}$ for TrxA and 2.9 $\pm$ 0.2 min$^{-1}$ $\mu M^{-1}$ for TrxA(G74S), indicating that the kinetics of oxidation of both thioredoxins by DsbB are very similar.

There are a large number of cysteines in the pili proteins that are required for M13 infectivity. Therefore, another possible explanation for our in vivo data was that TrxA(G74S) could allow M13 infectivity because it possesses enhanced isomerase activity. To test the isomerase activity of the G74S thioredoxin variant, we measured its ability to isomerize disulfides in scrambled hirudin to generate the native form of the protein as described by Quan et al.5 Both wild-type thioredoxin and TrxA(G74S) had identical hirudin isomerization ability (Fig. 4) making this possible explanation for the G74S variant less likely.

To further establish the oxidative properties of TrxA(G74S), we measured the oxidation rate of reduced hirudin by oxidized thioredoxin in vitro. Hirudin is a 65-amino acid protein with three disulfide bonds in its native state that has been used as a model protein to characterize protein oxidation in vitro (33–35). Using stopped flow fluorescence measurements, we determined the second-order rate constant of hirudin oxidation. TrxA(G74S) displayed a slightly slower rate of oxidation, with a second-order rate constant of 6.2 $\times$ 10$^4$ M$^{-1}$ s$^{-1}$, compared with wild-type thioredoxin that showed a second order rate constant of 8.0 $\times$ 10$^4$ M$^{-1}$ s$^{-1}$. Overall, these results support the view that the main effect of the G74S substitution is an alteration of thioredoxin specificity, which prevents the reduction of thioredoxin by the DsbD/DsbC isomerization pathway in conjunction with a change of the reactivity toward certain substrate proteins that receive the disulfide bonds.

Distribution of Gly74 and Ser74 among Bacterial Thioredoxins—In order to establish if there are any wild-type bacterial thioredoxins with a serine at position 74, a protein homology search with E. coli TrxA sequence was performed. Taking as reference proline 76, which is highly conserved among members of the thioredoxin family (36), we looked for amino acid residues that are present at the position — 2 with respect to Pro76 based on an overall alignment. This preliminary analysis should give a general overview of how conserved are glycine and serine at this position of the thioredoxin fold but is not meant to be exhaustive. Surprisingly, out of the over 400 bacterial thioredoxins analyzed, 47% contain a serine instead of a glycine at that position (see Supplemental Material). Asparagine and alanine are also found at this position but at much lower frequencies, 5 and 2%, respectively. Glycine is mostly found among members of the proteobacteria group (more than 90%), whereas serine seems to be distributed among a wider range of prokaryotic groups. Additionally, serine is also present in some Archaea and eukaryotic thioredoxins.

DISCUSSION

Thioredoxin exported into the periplasm of dsbA− mutant strains serves as a catalyst of thiol oxidation, a function that constitutes a complete reversal of its physiological role, namely the reduction of protein disulfide bonds (7, 8). In the periplasm, thioredoxin becomes oxidized by DsbB and then transfers its disulfide bond onto substrate proteins. Earlier studies suggested that an important factor affecting the efficiency of disulfide bond formation by periplasmic thioredoxin is the redox potential of the active site of the enzyme (7, 12). The latter is in turn modulated by the sequence of the dipeptide separating the two Cys residues in the active site (5).

The purpose of this study was to investigate the molecular determinants that are required for TrxA, the prototypical member of the thioredoxin family to function as an efficient catalyst of disulfide bond formation in the periplasmic space. We examined the effect of TrxA on the formation of functional F pili, which are required for infection by M13 (16). The F-specific proteins TraN, -U, -H, and -W and TrbC have 22, 11, 6, 1, and 2 conserved cysteines, respectively (37).

The large number of cysteines in F proteins would be expected to place a much greater burden onto the cellular machinery for protein oxidation and isomerization compared with Flgl or PhoA. Indeed, infectivity by M13 is reduced by more than 5 orders of magnitude in a dsbA− null mutant. We found that TrxA exported into the periplasm using a Tat-specific signal peptide was unable to restore M13 infectivity. Importantly, the TrxA variant with the CPHC active site, which has a significantly higher redox potential, also failed to complement the loss of infectivity observed in dsbA− mutants. A genetic screen for mutations in TrxA that could partially restore M13 infectivity led to the isolation of several different alleles that overwhelmingly contained the amino acid substitution G74S. Expression of ssTorA-TrxA(G74S) in dsbA− cells resulted in a significant increase in the number of M13-infected cells. In addition, this mutation completely restored PhoA

5 Quan, S., Schneider, I., Pan, J., Von Hacht, A., and Bardwell, J. C. A. (2007) J. Biol. Chem. 282, 28823–28833.
activity of MC1000 *dsbA* cells to the same level as in the wild-type strain. However, cells expressing ssTorA-TrxA(G74S) were found to be non-motile, indicating that the mutant thioredoxin was unable to oxidize FlgI. It is interesting to compare the phenotypes conferred by TrxA(G74S) with those of the TrxA(G33P,P34H) mutant. While the latter enzyme could not restore infectivity by M13, it was nonetheless able to confer wild-type level PhoA activity as well as partial motility. A TrxA(G33P,P34H,G74S) triple mutant conferred a statistically reproducible increase in M13 infectivity yet exhibited lower motility than the TrxA(G33P,P34H) variant alone. Overall, our data indicate the following: 1) the G74S mutation enhances disulfide bond formation *in vivo* for certain substrates but not for others, and 2) in combination with the CPHC active site, G74S can exhibit either positive or negative effect, depending on the phenotype assayed.

There are at least two possible explanations for these results. G74S may exert its effect on the catalysis of disulfide bond formation either by influencing the redox potential of thioredoxin or, alternatively, by changing the substrate specificity of the enzyme. In the crystal structure, glycine 74 is located within 7 Å of the catalytic thiol for reduced TrxA (Protein Data Bank code 1XOB). Therefore, mutations at that location might potentially affect the redox potential of the catalytic cysteine. However, *in vitro* determination of the redox potential of TrxA(G74S) by direct protein-protein redox equilibria with the γ domain of DsbD established that this was not the case. Also, TrxA(G74S) is oxidized by DsbB with kinetic parameters almost identical to those of the wild-type enzyme. Thus, we favor the second explanation, namely that the ability of TrxA(G74S) to complement *dsbA* mutants is due to a change in its substrate specificity. Several lines of reasoning, including the results of independent genetic studies, support this interpretation. First, as was mentioned above, *in vivo* TrxA(G74S) catalyzes the oxidation of certain cell envelope proteins (PhoA, F pilus proteins) but not others (FlgI). *In vitro*, TrxA(G74S) displays a slightly slower oxidation rate of hirudin than wild-type TrxA. Similarly, the G74S change appears to render the protein resistant to reduction by the DsbC-DsbD pathway *in vivo* and thus allowing it to be maintained in an oxidized state as required for disulfide bond formation (Table 3). On the other hand, it should be noted that the G74S mutation did not affect the *in vitro* kinetics of oxidation of thioredoxin by DsbB. Second, glycine 74 is part of the so-called cis-Pro loop, which contains a highly conserved proline in the less common cis-conformation and is composed the following amino acids: Arg<sup>73</sup>–Gly<sup>74</sup>–Ile<sup>75</sup>–Pro<sup>76</sup> (36). This loop is located at the end of the third α-helix (α3) and the beginning of the fourth β-sheet (β4) of the thioredoxin structure in front of the CXXC motif of the active site of TrxA that is positioned at the beginning of the second α-helix (α2) (Fig. 3). Gly<sup>74</sup> is in close proximity to the putative substrate binding surface of TrxA that encompasses residues 33–34, 75–76, and 91–93 (38). In fact, the solved crystal structures of *E. coli* thioredoxin complex with T7 DNA polymerase (39) and *E. coli* thioredoxin reductase (TrxB) (40) highlight the importance of the cis-Pro loop in substrate-binding interactions. Third, earlier biochemical and genetic studies point to the significance of Gly<sup>74</sup> in terms of determining the substrate specificity of thioredoxin.

Thioredoxin is required for the function of T7 DNA polymerase and for the growth of the phage in *E. coli*. Thioredoxin Y from *Rhodobacter sphaeroides*, which exhibits a 47% amino acid identity with the *E. coli* enzyme but contains the same active site motif (CGPC), supports T7 replication. However, a G74S substitution in thioredoxin Y abrogated its ability to form a catalytically active complex with T7 DNA polymerase (41). Similarly, Himawan and Richardson (42) identified a G74D substitution in *E. coli* thioredoxin that does not allow T7 growth in *E. coli*. Finally, a genetic screen for mutations that prevent the formation of a complex between cytoplasmic thioredoxin and filamentous phage proteins that are essential for phage assembly also led to the isolation of a G74D substitution (43). An analysis of the phylogenetic distribution of the amino acid occupancy of position 74 shows that serine is widely distributed among bacterial thioredoxins. Our results and those of others strongly suggest that the substitution between glycine and serine at position 74 causes a change in the substrate specificity of thioredoxin. Together these results indicate that this position in the thioredoxin structure is a physiologically conserved substrate specificity determinant. This may help explain why some prokaryotes like *E. coli* contain more than one thioredoxin (TrxA and TrxC). As mentioned before, TrxA has a Gly at position 74, whereas TrxC has a Ser at the equivalent position of the cis-Pro loop, thus suggesting that one possible explanation for this redundancy is a different subset of specificities that may allow these two thioredoxins to perform different physiological roles.

In summary, a genetic screen for mutations in thioredoxin expressed in the periplasm of *dsbA*<sup>−</sup> cells that partially restored infectivity by M13 led to the isolation of an amino acid substitution that changed the substrate specificity of the enzyme. Such change resulted in an altered reactivity with substrate proteins that receive the disulfide bonds and allowed thioredoxin to avoid reduction by the DsbC-DsbD pathway. Interestingly, this amino acid substitution did not change the highly reducing redox potential of the protein thus highlighting the key role of substrate specificity in the conversion of thioredoxin from a cytoplasmic reductant into an efficient periplasmic oxidant. Earlier studies demonstrated the significance of active sites with a more oxidizing redox potential on the ability of thioredoxin to serve as a periplasmic oxidant. However, mutations in the active site that affect the redox potential can also alter the substrate specificity of the enzyme. Bessette et al. (44) and more recently Quan et al. (45) noticed that active site mutations that improve oxidation *in vivo* do so not just because they enhance the redox potential of the protein. Decoupling the effect of mutations on the catalytic rate of disulfide exchange reactions from the effects of substrate specificity (*K<sub>m</sub>* effects) requires careful kinetic analysis of protein oxidation. In the absence of such data, the relationship between active site mutations and changes in the redox potential must be interpreted with care.

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