

The Thioredoxin Domain of *Neisseria gonorrhoeae* PilB Can Use Electrons from DsbD to Reduce Downstream Methionine Sulfoxide Reductases^{*§}

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The PilB protein from *Neisseria gonorrhoeae* is located in the periplasm and made up of three domains. The N-terminal, thioredoxin-like domain (NT domain) is fused to tandem methionine sulfoxide reductase A and B domains (MsrA/B). We show that the α domain of *Escherichia coli* DsbD is able to reduce the oxidized NT domain, which suggests that DsbD in *Neisseria* can transfer electrons from the cytoplasmic thioredoxin to the periplasm for the reduction of the MsrA/B domains. An analysis of the available complete genomes provides further evidence for this proposition in other bacteria where DsbD/CcdA, Trx, MsrA, and MsrB gene homologs are all located in a gene cluster with a common transcriptional direction. An examination of wild-type PilB and a panel of Cys to Ser mutants of the full-length protein and the individually expressed domains have also shown that the NT domain more efficiently reduces the MsrA/B domains when in the polyprotein context. Within this framework there does not appear to be a preference for the NT domain to reduce the proximal MsrA domain over MsrB domain. Finally, we report the 1.6 Å crystal structure of the NT domain. This structure confirms the presence of a surface loop that makes it different from other membrane-tethered, Trx-like molecules, including TlpA, CcmG, and ResA. Subtle differences are observed in this loop when compared with the *Neisseria meningitidis* NT domain structure. The data taken together supports the formation of specific NT domain interactions with the MsrA/B domains and its *in vivo* recycling partner, DsbD.

Although many examples of the fusion of the MsrA and MsrB domains into a single, multifunctional polyprotein exist (1, 2), the PilB proteins from *Neisseria gonorrhoeae*, *Neisseria meningitidis*, and *Fusobacterium nucleatum* are unique. The PilB proteins (also known as the MsrA/B polyprotein) are composed of three domains. The N-terminal (NT)³ domain functions as a thioredoxin-like disulfide reductase (3). The middle methionine sulfoxide reductase A (MsrA) and C-terminal methionine sulfoxide reductase B (MsrB) domains function with opposite substrate stereospecificity (2, 4, 5). MsrA reduces or repairs the S-form of methionine sulfoxide, whereas the MsrB domain is specific for the R-form. The polyprotein is thought to help *Neisseria* survive the burst of reactive oxygen species generated as a protection mechanism within the genitourinary tract of the host. The N terminus of PilB contains a signal sequence that is thought to target the protein to the outer membrane, thus orienting the protein in the periplasmic space (6). Removal of the targeting signal or production of the protein from an internal ribosome binding site results in cytoplasmic localization. In this location the antioxidant properties of the MsrA-MsrB domains appear to be lost when the organism is challenged with exogenous hydrogen peroxide (6).

The three-dimensional structures and catalytic mechanisms of individual MsrA and MsrB domains from several organisms have been characterized and recently reviewed (2, 5, 7–12). The last step of the Msr reaction requires the reduction of the disulfide bond within the Msr molecule to regenerate the active site for additional rounds of catalysis. When the Msr protein is present in the cytoplasm, the reduction is carried out by thioredoxin (Trx). Trx is then recycled by thioredoxin reductase (TrxR) at the expense of NADPH. For PilB, which is periplasmic, it appears that the reduction of the MsrA and MsrB domains is performed by the NT domain. The structure of the NT domain from *N. meningitidis* exhibits the Trx fold (13). Like many other proteins with a Trx fold, the NT domain presents a

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The atomic coordinates and structure factors (code 2H30) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (<http://www.rcsb.org/>).

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³ The abbreviations used are: NT, N-terminal domain of PilB; DTT, dithiothreitol; eTrxR, *E. coli* thioredoxin reductase; GST, glutathione S-transferase; MsrA, methionine sulfoxide reductase A; MsrB, methionine sulfoxide reductase B; Trx, thioredoxin; eTrx, *E. coli* Trx; TrxR, thioredoxin reductase; Mes, 4-morpholineethanesulfonic acid; r.m.s., root mean square; Met(O), methionine sulfoxide; MME, monomethyl ether.

Trp-Cys-Xaa-Xaa-Cys catalytic motif that undergoes oxidation-reduction cycling. The redox potential of this motif (-227 to -232 mV) from *N. meningitidis* PilB is slightly more oxidizing than the redox potential of *Escherichia coli* Trx (eTrx, -270 mV) (3).

The recombinant NT domain from *N. meningitidis* has been shown to reduce the individual, oxidized MsrA and MsrB domains similar to eTrx (3). A unique surface loop near the active site was identified (13). The observation that the NT domain cannot interact with the *E. coli* thioredoxin reductase (eTrxR) also suggested that this structural feature may be involved in specific interactions with NT-MsrA/B domains and its recycling partner. It is still unclear, however, if the NT domain of PilB can reduce the downstream MsrA and MsrB domains in the polyprotein context. Moreover, the source of electrons used by the NT domain to reduce the downstream Msr domains is unknown (10, 11). In an effort to address these questions, we have compared the efficiency of the methionine sulfoxide reduction process for PilB from *N. gonorrhoeae* with constructs of differing lengths and Cys to Ser mutations. We have also determined the 1.6-Å crystal structure of the NT domain from *N. gonorrhoeae*, identified the DsbD protein as a potential *in vivo* electron donor, and found that proteins homologous to DsbD, the NT domain, and the Msrs are next to each other in the genomes of some organisms, suggesting that they are part of the same methionine sulfoxide repair pathway.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—The full-length PilB protein from *N. gonorrhoeae* and domain variants were purified from *E. coli* using an N-terminal His tag within the pET-28b vector from Novagen as previously described (7). The constructs contained the following residues: full-length PilB, 23–522; NT domain, 23–182; MsrA domain, 183–362; MsrB domain, 375–522; and the MsrA-MsrB fusion, 183–522. Residue numbering is based on starting from the initiator methionine residue. For the constructs that contained the NT domain, the removal of the first 22 residues deleted the hydrophobic targeting signal. The proteins were treated with biotinylated thrombin to remove the His tag and purified via HiLoad Superdex75 size-exclusion and Resource Q columns. The desired fractions were pooled, dialyzed against 20 mM Hepes, pH 7.5, 100 mM NaCl, concentrated with a Centrprep YM10 concentrator (Amicon), aliquoted, flash frozen with liquid N₂, and stored at -80 °C. The Cys to Ser variants of each of the PilB domains and the Leu double mutant described below were generated using the QuikChange site-directed mutagenesis kit from Stratagene. The L38M/L41M double mutant of the NT domain of PilB was used to generate the protein containing selenomethionine for independent phase determination. Selenomethionine was incorporated into the Met double mutant by the growth of cells in minimal media and the repression of endogenous Met synthesis prior to induction (14).

E. coli MsrA was expressed in *E. coli* as a glutathione *S*-transferase fusion protein and purified on a glutathione-agarose column as previously described (15). The N-terminal glutathione *S*-transferase was removed by treatment with thrombin. *E. coli* MsrB (yeaA) was also produced as a poly-His fusion and puri-

fied in a similar manner to PilB proteins described above. In this case, however, the His tag was not removed (2). The α and γ domains of *E. coli* DsbD were overexpressed, purified, and quantified as in Collet *et al.* (16) using the following extinction coefficients (ϵ_{280} , in $\text{M}^{-1} \text{cm}^{-1}$): 20,340 for α and 8,370 for γ . The remaining protein concentrations were determined using the Bio-Rad protein assay kit using bovine serum albumin as the protein standard.

Met(O) Reductase Activity—The specific activity for the reduction of the Met(O) was determined as previously described using tritium-labeled *N*-acetyl-L-[³H]methionine sulfoxide, *N*-Ac-L-[³H]MetRSO, a racemic mixture of the R- and S-forms of methionine sulfoxide (17). Briefly, the 30- μl reactions contained 25 mM Tris, pH 7.4, 1.1–3.9 μM MsrA/B construct, 3.8 μM NT domain, 0.1–10 mM DTT, 200 μM *N*-Ac-L-[³H]MetRSO and were incubated at 37 °C for 10 min. For those reactions that contained the NADPH-eTrxR-eTrx reduction system the following concentrations were used: 1.7 mM NADPH, 2.9 μM eTrxR, and 6 μM eTrx. The reactions were quenched by the addition of 1 ml of 0.5 M HCl. The acidified solution was extracted with 3 ml of ethyl acetate, and the amount of product, *N*-Ac-L-[³H]Met, was determined by quantitating the amount of radioactivity in the ethyl acetate phase, corrected for the efficiency of extraction (50%). The reductase activities were converted to specific activity, moles of *N*-Ac-L-[³H]Met produced per mol of enzyme to account for the differences in protein size and amounts used in the assays. The NT domain alone exhibited no methionine sulfoxide reductase activity (data not shown). The experimental errors were within 10–15%.

Electron Flow from *E. coli* DsbD—The reduced forms of the α and γ domains of DsbD were made just prior to the experiment by incubating the proteins with 10 mM DTT at 37 °C for 30 min. The oxidized form of the NT domain was generated by incubating the protein with 20 mM oxidized glutathione at 37 °C for 20 min. For both of the treatments above, the excess DTT and oxidized glutathione were removed by passing the proteins through a PD-10 desalting column equilibrated with 25 mM sodium/potassium phosphate, pH 8, 0.5 mM EDTA. To test the electron flow pathway, stoichiometric amounts (1 μM) of the γ domain of DsbD in the reduced state and the NT domain of PilB in the oxidized state were incubated together at 37 °C. Then a catalytic amount of the α domain (100 nM) was added to the system. The fluorescence of the NT domain was monitored in a Hitachi F-4800 spectrofluorometer. The wavelength used for tryptophan excitation was 295 nm, and the emission was monitored at 330 nm.

Crystallization, Structure Determination, and Refinement—Crystals of the L38M/L41M mutant of the NT domain containing selenomethionine were obtained by the vapor diffusion method. Equal volumes of protein (30 mg ml^{-1} in 20 mM Hepes, pH 7.5, 100 mM NaCl) and well solutions (0.1 M Mes, pH 6.5, 0.2 M NH₄SO₄, 26% polyethylene glycol 2000 MME) were mixed and incubated at 4 °C. The crystals were transferred to a solution containing a cryoprotectant (0.1 M Hepes, pH 7.0, 0.2 M NH₄SO₄, 32% polyethylene glycol 2000 MME, 25% glycerol) prior to data collection at -170 °C. A three-wavelength multi-wavelength anomalous dispersion dataset (Table 1) was collected on beamline X25 at the National Synchrotron Light

Reduction of PilB by DsbD

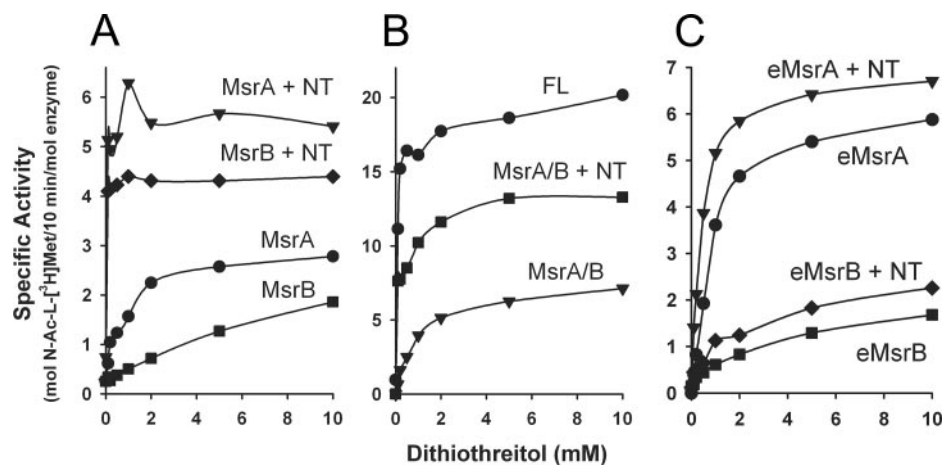


FIGURE 1. Met(O) reductase activity dependence on dithiothreitol and the NT domain. Each panel illustrates the data for MsrA/B constructs of different lengths or organismal sources. *A*, analysis of the addition of the NT domain to the individual *N. gonorrhoeae* MsrA and MsrB domains (MsrA and MsrB). *B*, comparison of the specific activity of the full-length (FL) MsrA/B protein from *N. gonorrhoeae* to the NT-truncated MsrA/B protein with and without the addition of the separately purified NT domain. *C*, analysis of the addition of the NT domain to the *E. coli* MsrA and MsrB proteins (eMsrA and eMsrB). The specific activity of each construct was determined as described under "Experimental Procedures."

Source, Upton, NY. The crystals exhibited $P2_12_12_1$ symmetry ($a = 43.3$, $b = 51.4$, $c = 80.3$) with one molecule in the asymmetric unit. Data were merged and scaled with d*Trek (Rigaku/MSK, The Woodlands, TX). Both engineered, selenium sites were found using SOLVE (occupancy of 0.74 and 0.83 with B values of 30.9 and 20.0, respectively) (18). The phases were improved by solvent flattening and maximum likelihood density modification with RESOLVE (18). The resulting 1.6-Å electron density maps were unambiguous, and the autobuild feature of RESOLVE was able to generate 94% (150/159 residues) of the starting model. A comparison of the experimental and composite omit electron density maps facilitated the modification of the model using O (19). The model was initially refined with CNS using alternating cycles of simulated-annealing, positional, and B -factor refinement (20). The last rounds of refinement were performed with REFMAC5 (21). The final model ($R_{\text{work}}/R_{\text{free}} = 18.8/20.9$, Table 1) contains residues 32–182 and 90 solvent molecules.

RESULTS

NT Domain More Efficiently Recycles the Downstream MsrA/B Domains When Present in the Polyprotein Context—Because the NT domain does not interact with eTrxR, one is not able to monitor the change in NADPH absorbance to measure the reduction of Met(O). Therefore, we chose to follow the Msr reaction by monitoring the reduction of tritiated *N*-acetyl-Met(O) (Fig. 1) (17). DTT, as has been known for some time, stimulates the MsrA and MsrB reactions at concentrations >1 mM by reducing the active site disulfide bond following the release of product. This finding is substantiated by the increase in activity of the individual MsrA and MsrB domains without the addition of the NT domain (Fig. 1A). The addition of the NT domain, however, enables a dramatic increase in reductase activity even at low DTT levels (0.1 mM, 20- to 50-fold molar excess to NT domain). We rationalize that this stimulation of activity is a consequence of the ability of the NT domain to

catalyze the reduction of MsrA or MsrB by DTT. Thus, we hypothesize that the electrons flow in a series of thiol-disulfide exchange reactions from DTT to the NT domain, and finally to the oxidized MsrA or MsrB domains. This idea is supported by an increase of Msr activity upon the addition of a 2-fold molar excess of the NT domain to the fused MsrA-MsrB domain construct (Fig. 1B). The further stimulation of the Msr activity when the NT domain is present in the full-length protein context suggests that the NT domain may reduce the downstream domains with greater efficacy. In contrast, the activity of the *E. coli* MsrA and MsrB (Fig. 1C) are not appreciably (~ 1.5 -fold) stimulated by the addition of the NT domain. This latter observation

implies that the NT domain has specific molecular interactions with its downstream MsrA and MsrB domains. Moreover, these interactions may not be made as effectively when the NT domain acts intermolecularly due in part to the loss of local concentration effects. One would expect, however, that by increasing the NT domain concentration >2 -fold, the Msr activity of the MsrA/B construct would asymptotically approach that of full-length PilB. The data presented support that the three domains of PilB function independently and that the NT domain appears not to be pre-associated with the MsrA/B domains. The presence of the NT domain in the polyprotein context greatly stimulates the reduction of the downstream Msr domains. As described in more detail below, the linker region between each domain appears to be of sufficient length to allow the free association of the domains during catalysis.

The NT Domain Reduces the MsrA and MsrB Domains Equivalently—The NT, MsrA, and MsrB domains each contain two Cys residues. These residues undergo disulfide bond formation during different stages of the catalytic cycle. To test the efficiency with which the NT domain reduces the downstream Msr domains, both Cys residues of each domain and of different domain combinations as indicated in Fig. 2, were mutated to Ser. The specific activity for each construct was monitored at a low DTT concentration (0.1 mM), a high DTT concentration (10 mM), and with the NADPH-eTrxR-eTrx reducing system (Fig. 2). The domains that are still active are indicated. When only the MsrA and MsrB domains are active (variants C68S/C71S/C440S/C495S and C68S/C71S/C207S/C349S, respectively), a similar stimulation in activity is observed with 10 mM DTT and the eTrx-based system. The presence of an active NT domain (variants C440S/C495S and C207S/C349S), however, results in a significant increase in specific activity particularly at 0.1 mM DTT, similar to data presented in Fig. 1. The further increase in MsrA and MsrB activity at 10 mM is most likely the result of the direct reduction of the MsrA/B domains by DTT

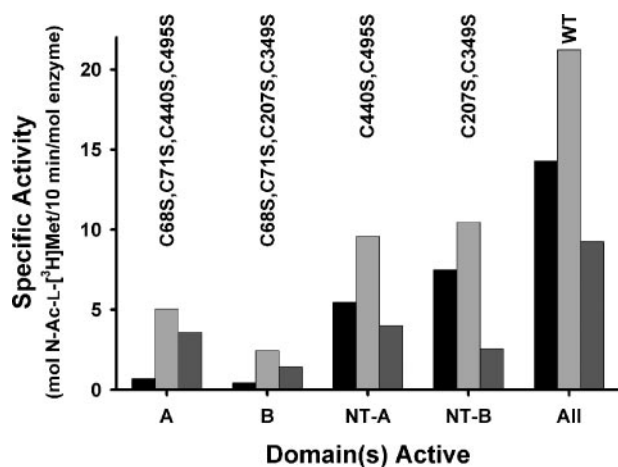


FIGURE 2. **Met(O) reductase activity for cysteine mutants of the full-length MsrA/B polyprotein from *N. gonorrhoeae*.** Each domain of the polyprotein contains two Cys residues. A panel of mutants was generated by systematically mutating the Cys residue pairs to Ser: NT domain, C68S and C71S; MsrA domain, C207S and C349S; MsrB domain, C440S and C495S. Mutation of these residues inactivates each domain. The specific activity for each construct is presented as the one or more domains that still contain the Cys residues and are therefore active: *black*, 0.1 mM DTT; *light gray*, 10 mM DTT; *dark gray*, NADPH-eTrxR-eTrx regeneration system. See "Experimental Procedures" for details.

(Fig. 1A). The lack of an increase in the eTrxR-stimulated activity supports the inability of the NT to interact with eTrxR. The activity of wild-type, full-length PilB (labeled "WT") at 0.1 mM DTT is essentially the sum of the activities for the Msr domains in the presence of the active NT domain. Therefore, it appears that the NT domain reduces the downstream Msr domains with the same relative efficiency, *i.e.* the NT domain does not preferentially reduce one Msr domain over another when both are present. The additive relationship for the Msr activity is reasonable given that the NT domain can only reduce one Msr domain at a time and must await reduction by DTT or its *in vivo* partner.

The α Domain of *E. coli* DsbD Is Able to Shuttle Electrons from DsbD- γ to the NT Domain—The PilB protein has been shown to be targeted to the periplasmic space in *N. gonorrhoeae* (6). This localization prevents the NT and Msr domains of the protein from interacting with the cytosolic NADPH-TrxR-Trx and other reducing systems. Therefore, a source of electrons for the reduction of the NT domain disulfide bond resulting from the reduction of the downstream Msr domains must be present in the periplasm. The periplasm is rich in proteins that exhibit thioredoxin folds and either catalyze disulfide bond formation (*e.g.* DsbA in *E. coli*) or disulfide bond isomerization (*e.g.* DsbC and DsbG) (22–25). The source of electrons for the latter proteins is the three domain protein DsbD (16, 26, 27). The membranous middle or β domain shuttles the electrons from the cytosolic NADPH-TrxR-Trx system to the Trx-like γ domain present in the periplasm. The electrons are then passed to the periplasmic, Ig-like α domain, which in turn reduces DsbC and DsbG. Therefore, the α domain of DsbD is a likely candidate for the electron donor to the NT domain of PilB.

To test the above hypothesis, we employed the *E. coli* α and γ domains of DsbD instead of the *N. gonorrhoeae* DsbD domains for the following reasons: (i) the individual DsbD domains from

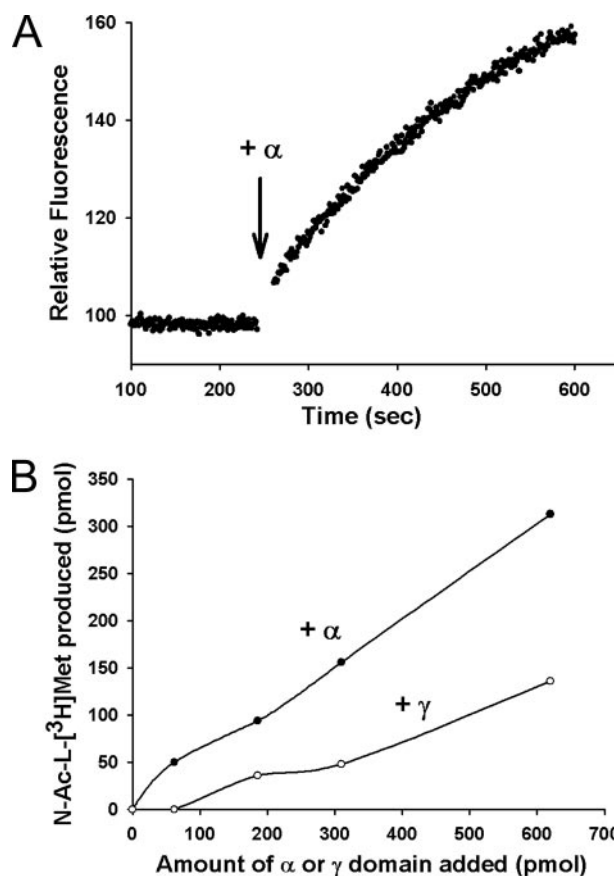


FIGURE 3. **Reduction of the NT domain by the α domain of *E. coli* DsbD.** A, the oxidized NT domain and the reduced γ domain of *E. coli* DsbD were mixed in equimolar concentrations (1 μ M), and the fluorescence was monitored at 330 nm. The α domain (+ α , 0.1 μ M) was added to the reaction after 250 s. B, reduction of *N*-acetyl-Met(O) facilitated by the α and γ domains of DsbD. The incubation was for 60 min at 37 °C.

E. coli are well characterized biochemically and structurally (22–27), (ii) the α and γ domains from *E. coli* show no change in fluorescence upon oxidation-reduction (16), (iii) there is high sequence identity (32%) for the entire DsbD protein between these organisms, and (iv) the *N. gonorrhoeae* DsbD domains have not been cloned or characterized in any way. Thus, the use of the readily available *E. coli* DsbD domains allows an evaluation of the ability of the homologous α domain to reduce the NT domain of PilB.

The oxidized form of the separate NT domain was generated by incubation with 20 mM oxidized glutathione (see "Experimental Procedures"). As has been previously shown for the NT domain from *N. meningitidis* and other Trx-like molecules, the NT domain of PilB exhibits a marked increase in fluorescence upon reduction of the disulfide bond (3). This property was used to monitor the reduction of the disulfide bond of the *N. gonorrhoeae* NT domain (excitation, 295 nm; emission, 330 nm; \sim 1.9-fold increase in fluorescence, data not shown). The oxidized NT domain (1 μ M) was pre-mixed with an equivalent amount of the reduced form of the γ domain of DsbD and monitored for 250 s (Fig. 3A). The flat baseline that was observed is consistent with the inability of the γ domain to donate electrons to the NT domain. The addition of a catalytic amount (0.1 μ M) of the reduced α domain, however, resulted in

TABLE 1
Crystallographic data, phasing, and refinement statistics

Crystallographic data			
Wavelength (Å)	0.9790	0.9791	0.9500
Resolution range (Å)	33.1-1.6	33.1-1.6	33.1-1.6
Observed reflections	313,298	320,508	306,862
Unique reflections	45,498	45,439	45,501
$R_{\text{merge}}^{a,b}$ (%)	4.5 (27.0)	5.9 (29.7)	4.2 (18.4)
Completeness (%)	99.6 (99.2)	99.4 (98.6)	99.3 (98.7)
$\langle I \rangle / \langle \sigma(I) \rangle^c$	20.9 (5.8)	15.8 (5.2)	25.0 (7.9)
Phasing (figure of merit)			
SOLVE			0.60 (0.36)
RESOLVE			0.71 (0.40)
Refinement			
R_{work}^d (%)			18.8 (19.3)
R_{free}^e (%)			20.9 (22.0)
r.m.s. deviations			
Bond lengths (Å)			0.02
Bond angles (°)			1.85
Average B-factor (Å ²)			
Protein			20.9
Solvent			20.1

^a Numbers in parentheses refer to the highest resolution shell (1.60–1.66 Å).

^b $R_{\text{merge}} = \sum |I - \langle I \rangle| / \sum I$, where I is the observed intensity and $\langle I \rangle$ is the average intensity of multiple symmetry-related observations of that reflection.

^c $\langle I \rangle / \langle \sigma(I) \rangle$, the r.m.s. value of the intensity measurements divided by their estimated standard deviation.

^d $R_{\text{work}} = \sum_{\text{hkl}} \|F_o - |F_c|\| / \sum_{\text{hkl}} |F_o|$, where F_o and F_c are the observed and calculated structure factors, respectively, for the 95% of the data used in refinement.

^e R_{free} is calculated as for R_{work} for 5% of the data excluded from refinement.

a marked increase in fluorescence from 250 to 600 s. The α and γ domains do not show a change in fluorescence with a change in oxidation state (16). Thus, the small amount of the α domain was able to accept the electrons from the γ domain to reduce the NT domain of PilB. The reduction, however, was not complete (~50%), *i.e.* the fluorescence did not plateau. This observation is most likely due to the similarity in the reduction potentials for all three proteins: -227 to -232 mV for the NT domain from *N. meningitidis*, -229 mV for the α domain of *E. coli* DsbD, and -241 mV for the γ domain (3, 16). The addition of $0.5 \mu\text{M}$ more of the γ domain displaced the equilibrium and did result in complete reduction of the NT domain (data not shown). Moreover, the addition of the reduced α domain resulted in the formation of *N*-acetyl-Met, the product of the reaction (Fig. 3B). Similar to the ability of the NT domain and eTrx to reduce individual Msr domains, the Trx-like γ domain appears to be able to directly reduce the MsrA and MsrB domains of PilB resulting in a low level of Met(O) reduction.

NT Domain Is Different from Other Membrane-tethered Thioredoxins—The 1.6 Å structure of the NT domain from *N. gonorrhoeae* (residues 32–182 visible) was determined using the multiwavelength anomalous dispersion method via selenomethionine incorporation (Table 1). Because the NT domain, however, does not naturally contain any Met residues, except for the N terminus, Leu³⁸ and Leu⁴¹ were mutated to Met, an approach often used for structure solution (14, 28). The Cys⁶⁸-Pro⁶⁹-Leu⁷⁰-Cys⁷¹ motif was found to be in the reduced state (Fig. 4A) and located near Trp⁶⁷, Tyr¹⁴⁰, and Pro¹⁴¹ within the canonical Trx fold (Fig. 4B). In contrast to the *N. meningitidis* structure (13), the orientation of the Leu⁷⁰ side chain as defined by the electron density was unambiguous. The proximity of Cys⁶⁸ to Trp⁶⁷ is consistent with the observed increase in fluorescence for the reduced state of the NT domains from *N. meningitidis* (3) and *N. gonorrhoeae* (data not shown).

A superposition of the *N. gonorrhoeae* NT domain with *E. coli* Trx (eTrx) (Fig. 4C) reveals that the NT domain contains an additional α -helix and β -strand ($\alpha 4$ – $\beta 4$). This insert is found in other membrane-tethered, Trx-like proteins, including TlpA (Fig. 4D), CcmG, and ResA (29–31). These added features are particularly evident when a sequence alignment is made on a structural basis (supplemental Fig. S1). Moreover, the *N. gonorrhoeae* NT domain contains an additional eight-residue insertion, Phe¹⁰⁰–Gly¹⁰⁷, similar to the NT domain from *N. meningitidis* (Fig. 4E) (13). The conformation of this loop sequence is, however, subtly different in the structures. For example, Leu¹⁰¹ and His¹⁰² at the apex of the loop are further away from Cys⁶⁸ and Tyr⁶⁷ in the *N. gonorrhoeae* structure. The side chain of Lys¹⁰⁵ is also present in a completely different rotamer position. In addition to these differences, the *N. gonorrhoeae* structure also delineated the positions and geometries of additional residues relative to the *N. meningitidis* structure: residues 32–33 and 177–182. The lack of significant molecular interactions from these residues to the rest of the domain suggests that the core of the NT domain contains residues 23–170 (Fig. 4B). The remaining residues, 171–182, most likely function as a flexible linker to the adjacent MsrA domain.

DISCUSSION

In most organisms the Msr proteins are expressed as separate domains and are reduced by the cytoplasmic NADPH-TrxR-Trx reduction system. In contrast, the Msr domains of PilB are fused in tandem to an N-terminal, Trx-like domain and located in the periplasm (6). This gene arrangement suggests that the role of the NT domain is to reduce the downstream Msr domains in the polyprotein context. Moreover, this proposition suggests that there is a specific electron donor within the periplasm to the NT domain.

The crystal structures of the NT domain from *N. gonorrhoeae* and *N. meningitidis* (Fig. 4E) reveal a unique loop insertion that differentiates it from other membrane-tethered Trx-like proteins, including TlpA, CcmG, and ResA (13, 29–31). The presence of this loop and undoubtedly other surface feature differences to *E. coli* Trx support the inability of the NT domain to interact with *E. coli* TrxR and to activate eMsrA and eMsrB (Fig. 1C). These observations also suggest that the structure of the NT domain may enable specific protein-protein interactions with the downstream Msr domains.

The observed higher specific activity of full-length PilB with the addition of a low concentration of DTT than when the NADPH-eTrxR-eTrx reducing system is present (Figs. 1 and 2) further supports this notion. This idea of restrictive protein partners (*i.e.* substrate specificity) is reminiscent of the specific interactions between CcmG and CcmH in the cytochrome *c* maturation process and other periplasmic protein-protein interactions (23, 24, 30, 32).

In order for the NT domain to reduce the downstream Msr domains there must be sufficient flexibility in the linker regions between the domains. The crystal structures of the NT, MsrA, and MsrB domains were examined to estimate the length of the linker between each of the domains. For example, the NT domain core is made up of residues 32–170 (Fig. 4 and supplemental Fig. S1) based on the comparison to eTrx. The addi-

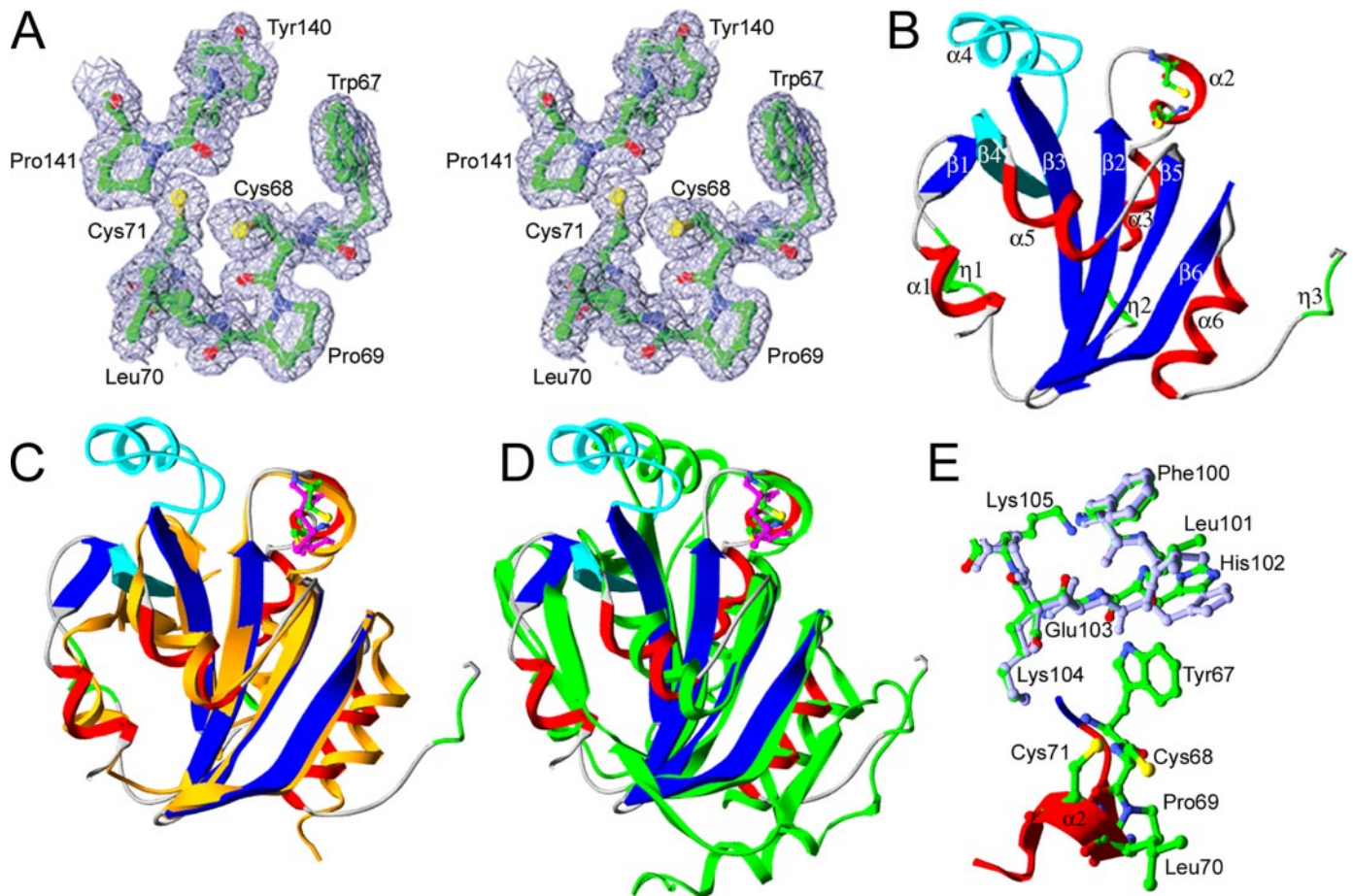


FIGURE 4. Structure of the NT domain from *N. gonorrhoeae* and its similarity to eTrx and periplasmic Trx homologs. *A*, stereo view of the multiwavelength anomalous dispersion-phased, experimental electron density map contoured at 1σ . The Cys-X-X-Cys motif of the NT domain, Cys⁶⁸-Pro⁶⁹-Leu⁷⁰-Cys⁷¹, was found to be in the reduced state and in close proximity to Tyr¹⁴⁰ and Pro¹⁴¹. Atom coloring scheme: red, oxygen; blue, nitrogen; green, carbon; and yellow, sulfur. *B*, overall fold of the NT domain. Coloring scheme for secondary structural elements: red, α -helices (α 1– α 5); blue, β -strands (β 1– β 6); and green, 3_{10} helices (η 1– η 3). The inserts unique to the membrane-anchored Trx homologs (α 4 and β 4) are colored cyan (also see supplemental Fig. S1). Cys⁶⁸ and Cys⁷¹ are shown in ball and stick representation. *C*, superposition of the NT domain onto eTrx (gold, PDB code 2TRX (43); 1.39 Å r.m.s. displacement with 256 backbone atoms superimposed). The oxidized Cys³²-X-X-Cys³⁵ motif of eTrx is shown in magenta. *D*, superposition of the NT domain onto TlpA (green, PDB code 1JFU (29); 1.77 Å r.m.s. displacement with 308 backbone atoms superimposed). The oxidized Cys⁷²-X-X-Cys⁷⁵ motif of TlpA is shown in magenta. TlpA contains an additional N-terminal, 70-residue extension shown on the lower, right-hand side. *E*, close-up view of the unique NT domain loop from *N. gonorrhoeae* (green) superimposed onto the NT domain from *N. meningitidis* (light blue, PDB code 2FY6 (13); 0.56 Å r.m.s. displacement with 572 atoms superimposed). *A*–*E* were generated with Swiss PDB Viewer and POV-RAY (44).

tional residues present in the structure (residues 171–182) exhibit coil and short helical structures that do not directly contact the rest of the protein. Based on the hydrogen-bonding patterns of the N termini of the *E. coli* and bovine MsrA proteins, the core of the MsrA domain consists of residues 35–228 (corresponds to residues 197–360 for *N. gonorrhoeae* PilB) (8, 9). This core assignment is in accord with the near wild-type activity of the eMsrA protein when residues 1–41 have been truncated (33). For the *N. gonorrhoeae* MsrB domain, the core structure contains residues 405–506 with residues 377–404 also present in loosely associated coil and α -helical structures (2). Based on these considerations the linker between the NT and MsrA domains and the MsrA and MsrB domains could range from 15–27 and 14–44 residues, respectively. Therefore, it appears that the linker segments between the domains are long enough to impart considerable flexibility to the full-length PilB protein so that the NT domain is not hindered from accessing the Msr domains.

The PilB protein from *N. gonorrhoeae* has been localized to

the outer membrane using diagnostic markers for this compartment (6). Because anchoring to the membrane would restrict the movement of the NT domain, the MsrA and MsrB domains would have to “bend back” to the NT domain to be reduced. The long linker lengths between the domains should make these movements possible. Thus, membrane attachment of PilB could be functionally important as has been observed for CcmG and CcmE (34). It is not clear at this time, however, whether an intermolecular interaction between two PilB proteins predominates in the physiological environment. In this situation the NT domain of one protein may reduce the Msr domains of an adjacent PilB protein. The efficient *in vitro* recycling of the MsrA and MsrB domains by the NT domain, particularly in the full-length protein context (Figs. 1 and 2), supports the reduction process occurring in an intramolecular manner.

The periplasm is an oxidizing environment in which most cysteine residues are disulfide-bonded. However, electrons are transported from the cytoplasmic NADPH pool to the

Reduction of PilB by DsbD

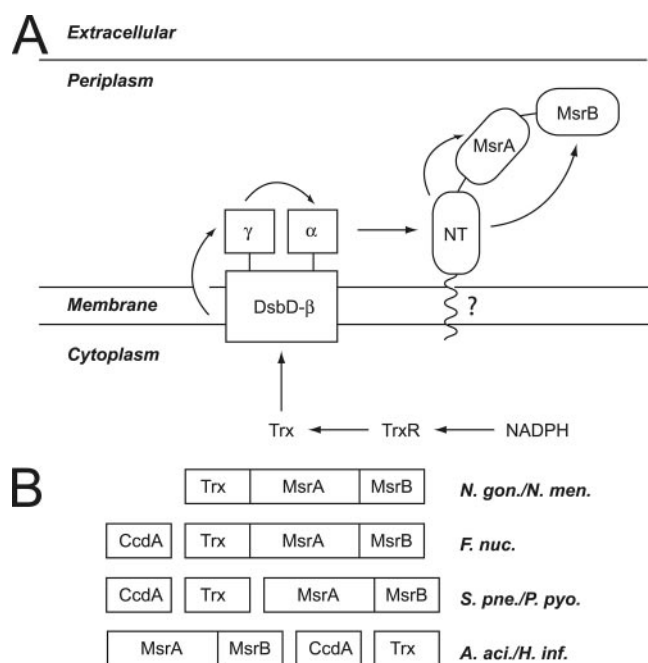


FIGURE 5. *A*, proposed electron flow pathway for the reduction of Met(O) within the periplasm. *B*, representative gene cluster arrangements observed in bacteria. *N. gonorrhoeae* and *N. meningitidis* (*N. gon.* and *N. men.*) both contain the N-terminal, Trx-like domain directly fused to the downstream tandem MsrA and MsrB domains. These organisms contain a gene for a DsbD homolog distant from the Trx-MsrA-MsrB polyprotein gene. In *F. nucleatum* (*F. nuc.*) a CcdA homolog is found adjacent to the polyprotein. In *S. pneumoniae* and *S. pyogenes* (*S. pne.* and *S. pyo.*), the Trx-like gene is no longer fused to the MsrA/B domains, but does have a CcdA gene homolog just upstream. In contrast, the MsrA/B gene fusions of *A. actinomycetemcomitans* and *H. influenzae* (*A. aci.* and *H. inf.*) are located upstream of the CcdA and Trx homologs. The direction of transcription for all genes is from left to right.

periplasm to keep some periplasmic proteins in a reduced state for type I and type II cytochrome *c* maturation and other disulfide bond shuffling processes (24, 25, 31, 35). The key protein of this electron transport system is DsbD, a membrane protein with one integral and two periplasmic domains (Fig. 5A). Some bacteria possess a stripped-down version of DsbD, called CcdA, which only has the trans-membrane domain. For DsbD the electrons from the cytoplasmic Trx are shuttled between the three domains in the following progression: the central, trans-membrane β domain; the C-terminal, periplasmic γ domain, which contains a Trx fold; and the N-terminal α domain, which exhibits an immunoglobulin-like fold (16, 32, 36–39). Each of the domains contains two Cys thiols. The α domain can transfer its electrons through the reduction of disulfide bonds to DsbC, DsbG, and CcmG. CcdA is able to reduce other proteins such as ResA and HelX, even though it does not contain an α or γ domain (31, 40). These observations prompted us to test whether the well characterized α domain of *E. coli* DsbD could reduce the oxidized form of the NT domain of PilB.

As shown in Fig. 3A, the γ domain of *E. coli* DsbD is not able to reduce the oxidized NT domain. Only upon the addition of a catalytic amount of the α domain does reduction occur as illustrated by the increase in tryptophan fluorescence in the active site. It is not surprising that the reduction was not 100% complete, because the reduction potentials of all three proteins are quite similar. Moreover, the addition of the α domain enables the reduction of *N*-acetyl-Met(O) (Fig. 3B). The low level of

activity observed with the addition of the Trx-like γ domain is most likely a result of direct reduction of the Msr domains, similar to the ability of the NT domain and eTrx. Thus by inference, the DsbD homolog from *N. gonorrhoeae* (32% sequence identity) should be able to provide reducing equivalents to the NT domain for the reduction of the downstream Msr domains (Fig. 5A). This finding is not consistent with the conclusions from the superposition of the *N. meningitidis* NT domain onto the crystal structure of *E. coli* CcmG/DsbE complexed with the α -domain of DsbD (13, 19). Because DsbD and its homologs are the only known source of electrons to the periplasm and all homologs of the NT domain seem to be anchored to the inner membrane, the outer membrane localization of the *N. gonorrhoeae* PilB remains a conundrum.

It is important to note that future studies will be necessary to verify that the α and γ domains of the *N. gonorrhoeae* DsbD protein function in a similar manner to *E. coli* DsbD. In particular, it will be interesting to know whether the *N. gonorrhoeae* DsbD domains couple better to the NT domain and to assess the specificity of the DsbD-NT domain interaction by comparison to other known targets of DsbD reduction in *E. coli*. Currently nothing is known about the *Neisseria* periplasmic reduction system, including DsbD. Further investigation is clearly warranted. It is tempting to speculate that components of the periplasmic reduction system and PilB in *N. gonorrhoeae* may be attractive targets for new antimicrobials (13).

These observations encouraged us to further investigate the genomic relationships of Trx, DsbD, MsrA, and MsrB homologs in the available genome databases. Gene cluster analysis was performed using the SEED server from the Fellowship for Interpretation of Genomes (www.theseed.org) (41). The four common cluster motifs are shown in Fig. 5B. As mentioned above, the *N. gonorrhoeae* and *N. meningitidis* PilB proteins contain all functional domains fused into one polyprotein. The DsbD gene for these bacteria is not near the PilB gene. In contrast, the *F. nucleatum* PilB gene is downstream of a CcdA homolog. A similar gene arrangement is seen in *Streptococcus pneumoniae* and *Streptococcus pyogenes* except that the Trx-like domain is not fused to the MsrA/B domains. Another variation in this latter theme is the transposition of the MsrA/B fusion protein in front of the CcdA- and Trx-like genes in *Actinobacillus actinomycetemcomitans* and *Haemophilus influenzae*. These gene clusters support the functional linkage between the transfer of electrons from a DsbD/CcdA homolog to a Trx-like protein followed by reduction of MsrA and MsrB domains within the periplasm of each representative organism (Fig. 5A). Moreover, all the Trx-like proteins and the MsrA/MsrB fusions mentioned above contain an N-terminal targeting signal for the periplasm as judged by the SignalP server (42).

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