The N-terminal Domain of PILB from Neisseria meningitidis Is a Disulfide Reductase That Can Recycle Methionine Sulfoxide Reductases*

Received for publication, January 11, 2005, and in revised form, January 21, 2005
Published, JBC Papers in Press, January 24, 2005, DOI 10.1074/jbc.M50385200

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The PILB protein of the Neisseria genus comprises three domains. Two forms have been recently reported to be produced in vivo. One form, containing the three domains, is secreted from the bacterial cytoplasm to the outer membrane, whereas the second form, which is cytoplasmic, only contains the central and the C-terminal domains. The secreted form was shown to be involved in survival under oxidative conditions. Although previous studies indicated that the central and the C-terminal domains display methionine sulfoxide reductase A and B activities, respectively, no function was described so far for the N-terminal domain. In the present study, the N-terminal domain of the PilB of Neisseria meningitidis was produced as a folded entity, and its biochemical and enzymatic properties have been determined. The data show that the N-terminal domain possesses a disulfide redox-active site with a redox potential in the range of that of thioredoxin. Moreover, the N-terminal domain, either as an isolated form or included in PilB, recycles the oxidized forms of the methionine sulfoxide reductases like thioredoxin. These results, which show that the N-terminal domain exhibits a disulfide reductase activity and probably has a thioredoxin-fold, are discussed in relation to its possible functional role in Neisseria.

The obligate human pathogens, Neisseria gonorrhoeae and Neisseria meningitidis are the only two pathogenic members of the Neisseria family of Gram-negative bacteria. N. gonorrhoeae, which colonizes mucosal epithelia of the genitourinary tract, is the causative agent of the disease gonorrhea, whereas N. meningitidis, which colonizes the nasopharynx, is the cause of two serious human diseases, pyogenic meningitis and meningococcal septicemia. Like many bacterial pathogens, the ability of these Neisseriae to infect their host is conditioned by successive steps of interactions with host cells and by the survival of the bacteria in the host environment (for review see Refs. 1 and 2). In particular, the bacteria have to resist the oxidative burst of the host, which generates a variety of reactive oxygen species. N. gonorrhoeae and N. meningitidis possess several antioxidant defense mechanisms among which the PilB protein was recently shown to play an essential role (3).

PilB is composed of three domains. The central and the C-terminal domains were shown to display methionine sulfoxide reductase (Msr) A and B activities, specific for the S and the R-isomers at the sulfur of MetSO, respectively (4–8). Although Msra and Msrb belong to two structurally unrelated classes of enzymes (9–13), they share a similar catalytic mechanism consisting of three steps, the third of which permits oxidized Msr under disulfide state to return back to reduced forms via reduction by thioredoxin (Trx) (8, 14, 15). The enzymology of the methionine sulfoxide reductase step and of the Trx-recycling process was recently well characterized (16, 17).

Different roles have been assigned to MsrA. One of the most important roles is to restore the function of proteins oxidized on their methionine residues (16, 19). In contrast, no role has been assigned so far to the N-terminal domain of PilB. Recently, two forms of PilB from N. gonorrhoeae have been characterized (3). One is secreted from the cytoplasm to the outer membrane as an entire polypeptide composed of the three domains. The second one is a truncated cytoplasmic form corresponding to amino acids 196–521 and therefore lacks the N-terminal domain. This form is produced from an internal AUG initiation codon corresponding to Met-195 (Ref. 3, see also “Results”). The fact that the extracytoplasmic localization of PilB was shown to be required for survival in the presence of oxidative damage raises the question of the function of the N-terminal domain in the periplasm and of its relationship with the Msra and Msrb activities.

Comparison of the primary structure of the N-terminal domain with those of the known proteins in the public data bases shows no significant identity with any protein excepted one from Fusobacterium nucleatum (see Fig. 1). The only peculiar feature is the presence of a CXXC signature.

In an attempt to identify the role of the N-terminal domain of PilB, a soluble form of the N. meningitidis domain of 143 amino acids, which only differs from its N. gonorrhoeae counterpart by four amino acids (see Fig. 1), has been overproduced and purified. Its biochemical and enzymatic properties have been determined. The results demonstrate that the N-terminal domain displays a disulfide reductase activity that is able to recycle...

* This research was supported by the Centre National de la Recherche Scientifique, the Ministère délégué à la Recherche (ACI BCMS047), the University Henry Poincaré Nancy I, the Association pour la Recherche sur le Cancer sur le Cancer Grant 5436), and the Institut National de Recherches 111 Bioingénierie. The University Henry Poincaré Nancy I, the Association pour la Recherche sur le Cancer sur le Cancer Grant 5436), the University Henry Poincaré Nancy I, the Association pour la Recherche sur le Cancer sur le Cancer Grant 5436), and the Institut National de Recherches 111 Bioingénierie.

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1 The abbreviations used are: Msr, methionine sulfoxide reductase; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; 2PDS, 2,2′-dipyridyl disulfide; Trx, thioredoxin; GSH, glutathione; GSSG, oxidized glutathione.

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Materials and Methods

Plasmid Constructions, Site-directed Mutageneses, Productions, and Purifications of the N-terminal Domain and of the Entire Form of PilB from N. meningitidis—Plasmid pETPlB was obtained by cloning the internal fragment of the pilB open reading frame synthesized by PCR (sequences of oligonucleotides not shown) using N. meningitidis Z2491 genomic DNA, kindly provided by Dr. M. K. Taha, between the NdeI and the SacI sites of the pET24c plasmid. To produce the N-terminal domain truncated after position 32, a deletion was done by NdeI digestion of the plasmid in which a second NdeI site has been introduced between the 100th and the 105th bp. Truncation at the C terminus after position 175 was obtained by site-directed mutagenesis of the Gly-176 position 175 was obtained by site-directed mutagenesis of the Gly-176 codon into a stop codon TTA. The resulting plasmid was named pETNterPlB. Site-directed mutageneses were performed using the QuickChange site-directed mutagenesis kit (Stratagene).

The strain used for N-terminal domain and entire PilB productions was BL21 (DE3) pLysS transformed with the pETPlB and pET195PlB plasmids, respectively. The overexpression of the N-terminal domain and of entire PilB was performed by addition of 1 mM isopropyl 1-thio-β-D-galactopyranoside in the culture medium at 0.6 A600. After 3 h of induction, cells were harvested by centrifugation, resuspended in minimal volume of buffer A (50 mM Tris-HCl, 2 mM EDTA, 0.3 M dithiothreitol (DTT), and sonicated. For the N-terminal domain and entire PilB, the supernatant was then precipitated at 70% ammonium sulfate saturation. The contaminating proteins were removed by exclusion size chromatography on ACA 54 gel (IBP) in buffer A. Purified fractions were then pooled and applied to a phenyl-Sepharose column equilibrated with buffer A, followed by a linear gradient of ammonium sulfate (0–1.0 M) connected to a fast protein liquid chromatography system (Amersham Biosciences). The two proteins were pure as checked by SDS-PAGE electrophoresis. The protein mass determined by electrospray mass spectrometry corresponded to that expected with no Met at the N terminus. The molecular concentrations were determined spectrophotometrically, using the extinction coefficient at 280 nm of 33.690 M−1 cm−1 for the N-terminal domain and 77.130 M−1 cm−1 for the entire PilB as deduced from the method of Scoopes (20).

Trxl from E. coli and MsrA and MsrB domains from N. meningitidis were prepared following experimental procedures already published (8, 21). The cysteine contents of the N-terminal domain and of E. coli Trxl were determined using 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) under non-denaturing conditions in buffer A as described previously (14).

Fluorescence Properties of the N-terminal Domain under Oxidized and Reduced Forms—The fluorescence spectra of the reduced and oxidized N-terminal domain (10 μM) in buffer A were recorded at maximum excitation (295 nm) and emission spectra were recorded at maximum excitation (295 nm).

Determination of the Rate of Reduction of Oxidized N-terminal Domain by DTT—The rate was determined by recording the change in fluorescence intensity at 347 nm at an excitation wavelength of 295 nm. The change in fluorescence intensity was recorded for 3 min after the addition of DTT at various concentrations (5–40 μM) to oxidized N-terminal domain (2 μM) in degassed buffer B. The apparent second-order rate constant was calculated from the measured pseudo-first-order rate constants by dividing kobs value by the concentration of DTT.

Determination of the Redox Potential of the N-terminal Domain, the GSH/GSSG System—The change in fluorescence intensity (excitation wavelength 295 nm) was measured at the wavelength of maximum emission (347 nm). Experiments were carried out in degassed and H2O-purged buffer B, with 2 mM EDTA. Oxidized N-terminal domain (1 μM) was incubated at 30 °C in the presence of GSSG (0.1 mM) and varying concentrations of reduced glutathione (GSH) (0–180 mM) for 20 h before recording the fluorescence emission spectra. The equilibrium concentrations of GSH and GSSG were calculated according to Equations 1–3.

\[ [\text{GSH}] = [\text{GSH}_0] - 2R [\text{N-terminal domain}] \]  
\[ [\text{GSSG}] = [\text{GSSG}_0] + R [\text{N-terminal domain}] \]  
\[ R = \frac{(F - F_o)(F'_o - F_o)}{(F'_o - F)} \]  

where \([\text{GSH}]_0\) and \([\text{GSSG}]_0\) represent the initial concentrations of GSH and GSSG, respectively, \(R\) is the relative amount of reduced protein at equilibrium, \([\text{N-terminal domain}]_0\) is the initial concentration of the N-terminal domain under the oxidized state, \(F\) is the fluorescence intensity, and \(F'_o\) and \(F_o\) are the fluorescence intensities of the completely oxidized and reduced protein. The equilibrium constant \(K_{eq}\) was estimated according to Equation 4.

\[ \frac{[\text{GSH}]^2 [\text{GSSG}]}{[\text{GSH}]_0 + [\text{GSH}]^2 [\text{GSSG}]} = K_{eq} \]  

from a non-linear regression analysis of the data.

From the equilibrium constant and using the glutathione standard potential \(E^{0}_{\text{GSH}/\text{GSSG}} = -0.240\) V (22), the standard redox potential \(E^0\) was calculated with the Nernst equation (Equation 5).

\[ E^0 = E^{0}_{\text{GSH}/\text{GSSG}} - (RT ln F) \]  

in which \(F\) represents Faraday’s constant (23,040.612 cal mol−1 V−1), \(n\) is the number of electrons transferred (here \(n = 2\)), and \(RT\) is the product of the gas constant (1.987 cal K−1 mol−1) and the absolute temperature.

Redox Equilibrium between the N-terminal Domain and Trxl—Equilibrium reactions (250 μM) typically contained 15 μM each reduct-oxidative protein in degassed and H2O-purged solution of buffer B with 2 mM EDTA. The reduced form of each protein (1 μM) was prepared immediately before use by the incubation of protein for 1 h at room temperature in the presence of 50 mM DTT, followed by desalting on an Econo-Pac 10 DG column. Redox reactions between both proteins were initiated by mixing one protein in the reduced state and the other in the oxidized state. After 15 h of equilibration at 25 °C, each sample was quenched by the addition of 0.1% trifluoroacetic acid final. The oxidized and reduced forms of the proteins present in the samples were separated by reverse-phase high pressure liquid chromatography on a C8 column (4.6 mm × 100 mm) using a gradient from 30 to 80% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid in 25 min at a flow rate of 0.8 ml/min at room temperature. Column effluent was monitored at 215 and 280 nm. The amounts of oxidized and reduced forms of each protein in the quenched equilibrium mixture were determined from peak areas after integration of the chromatograms. Essentially identical results were obtained from analysis of chromatograms recorded at 215 and 280 nm, and the former was used in a subsequent analysis. The mechanism of thiol-disulfide exchange is described as a two-step reaction proceeding through a mixed disulfide intermediate (Equation 6). It is assumed that the mixed disulfide intermediate is not populated under standard conditions.

The apparent concentration equilibrium constant, \(K_{eq}\), was calculated according to Equation 7.

\[ \text{Trxl}^{\text{SS}} + \text{Nter}^{\text{SS}} \rightleftharpoons \text{Trxl}^{\text{SS}} \text{Nter} = \text{Trxl}^{\text{SS}} + \text{Nter}^{\text{SS}} \]  

and the redox potential of the N-terminal domain \(E^0\) was then calculated using the Nernst equation (Equation 8), using the Trxl standard potential \(E^{0}_{\text{Trxl}}\) of −0.270 V at pH 7 and 25 °C (23), in which \(n\) is the number of electrons transferred in the reaction (here \(n = 2\)), \(F\) is Faraday’s constant (23,040.612 cal mol−1 V−1), and \(R\) is the gas constant (1.987 cal K−1 mol−1).

Redox Titration of Met Reaction—In the case of MsrA/B domains alone, the reaction mixture containing Met-(R)-SO (100 mM) and MsrA/B (at two concentrations of 50 and 100 μM), in the absence or presence of stoichiometric concentration of N-terminal domain (i.e. 50 and 100 μM), was incubated at 25 °C for 60 min in buffer A. Then the fluorescence was measured at maximum excitation (347 nm) at an emission wavelength of 450 nm. Fluorescence was recorded for 3 min after the addition of DTT at various concentrations (5–40 μM) to oxidized N-terminal domain (2 μM) in degassed buffer B. The apparent second-order rate constant was calculated from the measured pseudo-first-order rate constants by dividing kobs value by the concentration of DTT.
mixture was injected onto a Sephasil C18 column for Met quantification as already described (14).

Determination of the Kinetic Parameters—Msr activities were determined with Ac-L-Met-SO (2 μM) or Ac-L-Met-NHMe, which is a better substrate than Met-(R)-SO (8), at a saturating concentration of 200 mM. The reaction mixture also contained MsrA (2 μM) or MsrB (1 μM), and the pH was kept at 7.4 by the presence of Tris-HCl 10 mM. Initial rate measurements were carried out at 25 °C by following the appearance of the peptide using a reverse phase chromatography as described previously (17). The concentration of Ac-L-Met-SO formed was plotted against the time, and the data were fit to a linear model to attain initial rates. The initial rate data were fit to the Michaelis-Menten relationship using least squares analysis to determine kinetic parameters. The pseudo-first-order rate constants were determined at each pH by fitting the absorbance at 343 nm to Equation 9,

\[ k_{\text{obs}} = k_{\text{cat}} + k_0 \]  

(Eq. 9)

where \( k_0 \) is the burst magnitude, and \( c \) represents the value of the ordinate intercept.

The second-order kinetic constants \( k_2 \) were calculated by dividing the \( k_{\text{obs}} \) value by the concentration of 2PDS and then fitting to Equation 10,

\[ k_2 = k_{\text{min}} + \frac{k'}{10^{\text{pK}_a - \text{pH} + 1}} \]  

(Eq. 10)

in which \( k_{\text{min}} \) represents the second-order kinetic constant at pH 6, and \( k' \) represents the second-rate constant for the thiolate form. In the case of C67S protein, \( k_{\text{min}} \) = 0.

**RESULTS**

**Production and Purification of the N-terminal Domain and of the Entire Form of PilB**—The fact that PilB was shown to be secreted from the cytoplasm to the outer membrane of N. gonorrhoeae supported the presence of at least an N-terminal signal peptide. Therefore, the strategy to produce a soluble form of the N-terminal domain and of entire PilB should take into account this putative peptide. As indicated in Fig. 1, the N-terminal 31 amino acids of PilB were predicted to constitute a signal peptide using the SignalP software. Using the Network Protein Sequence Analysis web interface, an α-helix between amino acids 4 and 22 was also predicted. Production of the N-terminal domain also required us to define where the truncation should be introduced between the C terminus of the N-terminal domain and the N terminus of the MsrA domain. For that purpose, the position of the truncation was based on the fact that 1) the MsrA domain was previously produced in a soluble form from a DNA construct in which a stop codon was introduced at a position corresponding to amino acid 195 (8), and 2) no secondary structural element is predicted after amino acid 171.

Production of a soluble form of PilB in good yield required not only to remove the peptide signal but also to change the AUG codon corresponding to Met-195 into an Ala one. Indeed, when overexpressed in E. coli two forms of PilB were shown to be produced in the cytoplasm of E. coli i.e. one form corresponding to amino acids 33–521 and the other, produced in a higher yield, corresponding to amino acids 196–521 (data and SDS-PAGE, not shown). This result strongly suggested that the AUG codon of Met-195 can be used as an internal initiation codon as already postulated for N. gonorrhoeae PilB (8). This is indeed the case. When an Ala codon was substituted for the Met codon as already postulated for N. gonorrhoeae PilB, no amino acid between positions 33 and 521 was produced and in a higher yield.

For N-terminal domain production, two plasmidic constructs under T7 promoter were therefore built that took in account all the information described above and then were tested for protein production in E. coli. Only one construct corresponding to truncations at the N terminus after position 32 and at the C terminus after position 175 gave a high overexpression of the N-terminal domain. The reason why truncation done at position 194 gave a small production of protein remains unknown. Therefore, only the plasmidic construct that coded the N-terminal domain from amino acids 33 to 175 was used to produce the domain. Purification was achieved by sequential ammonium sulfate precipitation, chromatography on ACA54, Q-Sepharose and phenyl-Sepharose. At the end of the purifica-
N-terminal Domain of PILB from N. meningitidis

Excitation spectra of the reduced (●) and oxidized (○) N-terminal domains (excitation at 295 nm) and the emission spectra of reduced (▲) and oxidized (▼) N-terminal domains (excitation at 295 nm) were recorded with protein (10 μM) at 25 °C in buffer A.

Characterization of the Redox Properties of the N-terminal Domain—The two cysteines in the N-terminal domain are separated by only two amino acids (Fig. 1). Therefore, the fact that they are included in a signature found in disulfide oxidoreductases suggested that these cysteines were redox sensitive. The addition of GSSG or DTNB led to the loss of the two cysteines as proved by DTNB titration. Addition of DTT, then followed by gel filtration restored the thiol titration. These results indicated that the Cys residues, Cys-67 and Cys-70 are oxidized by GSSG or DTNB and form an intramolecular disulfide bond that is sensitive to reduction by DTT.

It is known that proteins of the thiol-disulfide oxidoreductase family such as Trx show an increase in fluorescence emission intensity upon reduction of their active site disulfide bond. In the case of the Trx1 from E. coli, a 2.5-fold increase is observed, which was attributed essentially to the tryptophan located directly before the CXXC signature (24). In the N-terminal domain (amino acids 33–175), there exist five tryptophans of which one is situated at position 66 also before the CXXC motif (Fig. 1). Therefore, it was expected that the N-terminal domain behaved like Trx1. As shown in Fig. 2, when the oxidized N-terminal domain was excited at 295 nm for the selective excitation of tryptophan, an addition of 1 mM DTT led to a 2.0-fold increase in the fluorescence emission intensity at 347 nm. This result suggested that, as observed for E. coli Trx1, the fluorescence emission of Trp-66 of the N-terminal domain is quenched by formation of the disulfide bond.

By taking advantage of the fluorescence intensity change between oxidized and reduced N-terminal domains, the redox equilibrium constant $K_{eq}$ of the domain was determined based on the variation of the fluorescence message at various GSH/GSSG ratios. Thus, the oxidized N-terminal domain was incubated in the presence of 100 μM GSSG and increasing concentrations of GSH from 0 to 180 mM. The fraction of reduced N-terminal domain at equilibrium was measured by the intrinsic N-terminal domain fluorescence assuming no significant equilibrium concentration of N-terminal domain/glutathione mixed disulfide. After fitting the data by nonlinear regression according to Equation 4 (see "Materials and Methods") (Fig. 3), an equilibrium constant for the N-terminal domain/glutathione system of 0.54 ± 0.09 m was determined. A standard redox potential of −0.232 ± 0.004 V for the active site cysteines of the N-terminal domain at 30 °C and pH 7.0 ($E'_{\text{on}}$) was calculated from the Nernst equation (Equation 5) using a value of −0.240 V (22) for the glutathione standard redox potential ($E'_{\text{GSSG}}$).

To confirm the result, we used another method based on the analysis of the direct protein–protein redox equilibrium between the N-terminal domain and the E. coli Trx1, which has a well established redox potential $E'_{\text{Trx1}}$ of −0.270 V (23). To ensure that the redox equilibrium was indeed attained after incubation for 15 h, we checked with each protein pair that identical equilibrium constants were obtained irrespective of the redox state of the initial mixture. In both cases, the redox potential of the N-terminal domain ($E'_{\text{on}}$) was determined to be −0.227 ± 0.005 V at pH 7. This is in good agreement with the value determined using the GSH/GSSG system as a reference.

Determination of the $pK_{\text{app}}$ Values of the Cys Residues of the Redox Center of the N-terminal Domain—The $pK_a$ values of the Cys were determined using 2PDS over a pH range of 6–10.5 under conditions where the wild-type, C67S, and C70S N-terminal domains are stable. The reaction of 2PDS with the N-terminal wild-type domain followed pseudo-first-order kinetics with formation of 2 mol of pyridine-2-thione/mol of the domain, as determined from the absorbance change at 343 nm (data not shown). The pH-$k_a$ curve between pH 6 and 10.5 fitted to a monosigmoidal profile with a $pK_{\text{app}}$ value of 9.3 ± 0.2 and a $k$ value of $1.6 \times 10^4$ M$^{-1}$s$^{-1}$ (Fig. 4A).

As expected, the kinetics of 2PDS with the C67S and C70S
proteins followed pseudo-first-order but with formation of only 1 mol of pyridine-2-thione/mol of N-terminal domain (data not shown). The pH-\(k_2\) curve with the C67S protein passed through the origin and fitted to a monosigmoidal profile with a \(pK_{app}\) value of 9.5 ± 0.2 and a \(k_2\) value of \((1.8 \times 10^4 \text{ M}^{-1} \text{s}^{-1})\), respectively, for the wild-type, a \(pK_{app}\) of 9.1 ± 0.1 with a \(k_2\) value of \((1.4 \times 10^4 \text{ M}^{-1} \text{s}^{-1})\), respectively, for the C70S N-terminal domains. In the C70S protein, the Cys-67 reacted rapidly even at pH 6, with a \(k_2\) constant of \(1.4 \times 10^4 \text{ M}^{-1} \text{s}^{-1}\), indicating that the \(pK_{app}\) value of Cys-67 was below 6 as expected (Fig. 4C). The fact that the \(k_2\) value is 3-fold lower than that of the wild type remains to be explained. Another \(pK_{app}\) value of 9.1 ± 0.1 with a \(k_2\) value of \(1.3 \times 10^4 \text{ M}^{-1} \text{s}^{-1}\) was also determined from the pH-\(k_2\) curve, suggesting the presence of an amino acid not yet determined.
identified near Cys-67 whose deprotonation increases the reactivity of the thiolate of Cys-67 which is likely involved in decreasing the $pK_a$ of Cys-67.

The N-terminal Domain Is Capable of Recycling Methionine Sulfoxide Reductase Activities—With the objective to get more information on the role of the N-terminal domain included in PilB, we first tested the ability of the N-terminal domain alone to reduce the oxidized Msra and MsrB domains, produced independently as recently described (8). For that purpose, the stoichiometry of Met formation/mole of Msra or MsrB was determined in the presence and absence of the N-terminal domain. Two mol of Met were formed/mole of Msra or MsrB when an equimolar concentration of the N-terminal domain was added, whereas in its absence, only 1 mol of Met/mole of Msra or MsrB was formed (Table I). Two mol of Met were also formed/mole of entire PilB in the presence of either L-Met-(S)-SO, which is selectively reduced by Msra, or L-Met-(R)-SO which selectively reduced by MsrB (8). Altogether, these results clearly showed that the N-terminal domain, either as an isolated form or included in PilB, is able to reduce the disulfide bond in Msra and in MsrB but did not give any information on the efficiency of the recycling process. Such data required the determination of the kinetic parameters, i.e., the $k_{cat}$ value and the $K_m$ for the N-terminal domain. This was done in the presence of saturating concentrations of Ac-L-Met-SO-NHMe (Table I). The rate was determined by following the formation of Ac-L-Met-NHMe. In the case of Msra, a $k_{cat}$ value of 4.1 $s^{-1}$ and a $K_m$ of 280 $\mu$M for the N-terminal domain were determined, which are in the range as those determined for Trx1 from E. coli (15) and from N. meningitidis.2 In contrast, within the concentration range of 50–800 $\mu$M of the N-terminal domain tested, no saturating effect was observed for Msra. At 800 $\mu$M, a $k_{obs}$ value of 4.10$^{-2}$ $s^{-1}$ was determined that is 80-fold lower than the $k_{cat}$ value determined with Trx1 from E. coli (8) and from N. meningitidis.2

**DISCUSSION**

PilB of N. meningitidis is composed of three domains. Although the central and the C-terminal domains display Msra and MsrB activities, respectively, the function of the N-terminal domain is unknown. In this study, we have characterized the biochemical and enzymatic properties of a soluble form of the N-terminal domain.

The only two Cys of the N-terminal domain are located in a WCPLC motif that has been shown to form a disulfide redox-active site. The percentage of a helix and of $B$ sheets predicted from CD spectra of the N-terminal domain and of the Trx1 from E. coli are similar (data and CD spectra not shown). Altogether, these results suggested that the redox-active site is located in a Trx-like fold. Trx-like proteins are widely distributed and implicated in the control of the redox environment of subcellular compartments. The disulfide oxidoreductase activity of CXXC-containing proteins depends on various factors, among which the redox potential and the $pK_a$ of the thiol group of the N-terminal Cys in the motif. The redox potential ($E_m$ = −230 mV) of the N-terminal domain is similar to those of different thiol reductants such as E. coli Trx (−270 to −267 mV) (23), E. coli Grx (−233 to −198 mV), and GSH (−240 mV) (22). This suggested that the N-terminal domain probably acts as a reductant. However, the $pK_a$ value of the N-terminal Cys in the WCPLC motif, which is below 6 corresponds to a value usually observed for an oxidant and not for a disulfide reductant. For instance, the $pK_a$ of the corresponding catalytic Cys in E. coli Trx1 is 7.5, whereas it is of 3.5 in the periplasmic oxidase DsbA (25, 26). The fact that the apparent second-order rate constant of reduction of the oxidized N-terminal domain by DTT (5.10$^3$ $\mu$M$^{-1}$s$^{-1}$) is in the same range as that measured on oxidized Trx1 from E. coli (10$^3$ $\mu$M$^{-1}$s$^{-1}$) and 3 orders of magnitude slower compared with that measured on oxidized DsbA (10$^6$ $\mu$M$^{-1}$s$^{-1}$) (27, 28) is another piece of data that supports a function of the N-terminal domain as a reductant.

This raises the question of the role of the N-terminal domain of PilB in the periplasm. As indicated in the Introduction, the fact that it is fused with Msra and MsrB domains suggested a function of the N-terminal domain associated with the Mar activities. This is indeed the case. The N-terminal domain is able to reduce the oxidized form of Msra from N. meningitidis with a catalytic efficiency, $k_{cat}/K_m$ of 0.015 $\mu$M$^{-1}$s$^{-1}$, similar to that observed with Trx1 from E. coli and N. meningitidis. Therefore, the N-terminal domain can act as a disulfide reductase and is probably folded as a Trx. However, the fact that no saturating concentration effect for the N. meningitidis Msra is observed supports subtle three-dimensional structural differences between the N-terminal domain and Trx1 from E. coli and N. meningitidis. This is confirmed by the fact that the E. coli Trx reductase is not able to reduce the oxidized N-terminal domain (data not shown) in contrast to that observed with oxidized Trx1. In that context, the knowledge of the x-ray structure of the N-terminal domain, the resolution of which is under progress, will be very informative.

The kinetic parameters obtained in the present study have been determined with separated and soluble domains. In vivo, PilB is localized in the periplasm on the outer membrane. Therefore, the question arises of whether the N-terminal domain in PilB is operative in recycling the Msra and MsrB activities in the in vivo context. No data are presently avail-

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**TABLE I**

Stoichiometry of Met formation/mol of Msrs and kinetic parameters of Msrs in the presence of the N-terminal domain

| Stoichiometry (mol Met/mol Msr) | With the N-terminal domain | With Trx 1 |
|-------------------------------|---------------------------|-----------|
|                               | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ |
| Msra                          |            |       |               |            |       |               |
| Without N-terminal domain     | 1          | 2     | ND            | 3.4 ± 0.2$^a$ | 75 ± 15$^a$ | 0.045       |
| With 1 eq of N-terminal domain| 4.1 ± 0.2  | 280 ± 30 | 0.015         | 1.1 ± 0.2$^b$ | 58 ± 12$^b$ | 0.019       |

$^a$ From Ref. 8.

$^b$ From Ref. 15.
able. However, what is known from the present study done in vitro is that the disulfide bonds formed within PilB MsrA and MsrB domains are accessible and reduced by the N-terminal domain included in PilB. But this result does give any indication of whether the recycling process by the N-terminal domain is intra or intermolecular. This question is of importance in the context of the localization of PilB in vivo. What can be concluded, however, is that whatever the mechanism, i.e. intra- or intermolecular, 1 mol of PilB is sufficient to reduce 1 mol of a mixture of a protein-Met-(R,S)-SO and to recycle the fractions of MsrA and MsrB domains in PilB, which have been oxidized under the disulfide state. Another point that has to be addressed is the nature of the proteins that are repaired in the periplasm by PilB and the possible relationship with the pathogenic character of *Neisseria*. Finally, the fact that the N-terminal domain displays a disulfide reductase activity suggests that in the periplasm of *N. meningitidis* and *N. gonorrhoeae* a disulfide oxidoreductase is present to recycle the N-terminal domain from the oxidized to the reduced form. Its nature remains to be identified.

The PilB organization is a specific human pathogen bacteria from the *Neisseria* genus. A similar organization only exists in *F. nucleatum*, a Gram-negative anaerobe, which is a human opportunistic pathogen (29). Therefore, the N-terminal domain which has 1) no homologue in other bacteria, 2) an outer membrane localization, and 3) a fold likely similar to Trx1 but with subtle structural differences, could be a good candidate as a drug target against pathogenic *Neisseria*.

Acknowledgments—We thank Dr. M. K. Taha for the gift of the *N. meningitidis* DNA and Dr. A. Van Dorsselear and G. Chevreux for mass determination. We also thank Dr. S. Azza and C. Gauthier for their efficient technical help, and A. Ory for helpful discussion.

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