

## Characterization of the Methionine Sulfoxide Reductase Activities of PILB, a Probable Virulence Factor from *Neisseria meningitidis*\*

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PILB has been described as being involved in the virulence of bacteria of *Neisseria* genus. The PILB protein is composed of three subdomains. In the present study, the central subdomain (PILB-MsrA), the C terminus subdomain (PILB-MsrB), and the fused subdomain (PILB-MsrA/MsrB) of *N. meningitidis* were produced as folded entities. The central subdomain shows a methionine sulfoxide reductase A (MsrA) activity, whereas PILB-MsrB displays a methionine sulfoxide reductase B (MsrB) activity. The catalytic mechanism of PILB-MsrB can be divided into two steps: 1) an attack of the Cys-494 on the sulfur atom of the sulfoxide substrate, leading to formation of a sulfenic acid intermediate and release of 1 mol of methionine/mol of enzyme and 2) a regeneration of Cys-494 via formation of an intradisulfide bond with Cys-439 followed by reduction with thioredoxin. The study also shows that 1) MsrA and MsrB display opposite stereoselectivities toward the sulfoxide function; 2) the active sites of both Msrs, particularly MsrB, are rather adapted for binding protein-bound MetSO more efficiently than free MetSO; 3) the carbon C $\alpha$  is not a determining factor for efficient binding to both Msrs; and 4) the presence of the sulfoxide function is a prerequisite for binding to Msrs. The fact that the two Msrs exhibit opposite stereoselectivities argues for a structure of the active site of MsrBs different from that of MsrAs. This is further supported by the absence of sequence homology between the two Msrs in particular around the cysteine that is involved in formation of the sulfenic acid derivative. The fact that the catalytic mechanism takes place through formation of a sulfenic acid intermediate for both Msrs supports the idea that sulfenic acid chemistry is a general feature in the reduction of sulfoxides by thiols.

Peptide methionine sulfoxide reductase (MsrA)<sup>1</sup> activity is described as being involved in the virulence of the pathogens

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<sup>1</sup> The abbreviations used are: Msr, MsrA, and MsrB, methionine sulfoxide reductase, methionine sulfoxide reductase A, and methionine sulfoxide reductase B, respectively; dimedone, 5,5-dimethyl-1,3-cyclohexanedione; DTT, dithiothreitol; DTNB, 5,5'-dithiobis(2-nitro)benzo-

*Escherichia coli*, *Streptococcus pneumoniae*, *Erwinia chrysanthemi*, *Mycoplasma genitalium*, and *Neisseria gonorrhoeae* (1–4). Inspection of the alignment of the corresponding protein sequences shows that all possess in common a sequence that displays an MsrA activity. This MsrA activity has now been well characterized at the structural level (5, 6) and the enzymatic level (7). In particular, a sulfenic acid intermediate has been shown to be formed on Cys-51 of *E. coli* MsrA during the reduction of the sulfoxide function of methionine sulfoxide (MetSO). The active site can be represented as an open basin in which Cys-51, located at the N terminus of an  $\alpha$ -helix, is accessible. Compared with the *E. coli* MsrA, the MsrAs from *S. pneumoniae* and from *N. meningitidis* or *N. gonorrhoeae* (called PILB) contain, in addition, an extension at the C terminus and at the C and N termini, respectively. This raised the question of the role of these extensions, in particular of the C-terminal extension. Sequence comparisons of the C-extension of PILB show amino acid identities with open reading frames of which no function has been assigned until recently. These sequences are detected in all kingdoms. Recently, the functions of the *E. coli* ortholog YeaA and an open reading frame downstream from the *msrA* gene from *Staphylococcus aureus*, which both have at least 50% amino acid identities with the C-subdomain of PILB, has been determined and shown to display a new Msr activity, called MsrB (8, 9).

The fact that the MsrB activity of YeaA is thioredoxin-dependent (8) indicates that at least a Cys residue is involved in the catalytic mechanism. Inspection of the amino acid sequences shows that two Cys are often conserved in putative MsrBs (see Fig. 1). One Cys, Cys-439, which is located in a CGWP(S/A)F motif is at least 50% conserved. The second one, Cys-494, which is included in an RYC(I/V/M)N motif is almost conserved.

In the present study, we show that in addition to an MsrA activity that is displayed by the central subdomain, called PILB-MsrA, the C terminus of PILB, called PILB-MsrB, possesses a thioredoxin-dependent MsrB activity. The catalytic mechanism of PILB-MsrB is shown to proceed via the sulfenic acid chemistry. The role of Cys-439 and Cys-494 has been demonstrated. The stereoselectivity in the reduction of the sulfoxide function and the catalytic parameters of the two subdomains have also been determined. The results are in favor of a structure of the active site of MsrBs different from that of the MsrAs.

ate; MetSO, methionine sulfoxide; Pht, phthalyl; TNB<sup>-</sup>, thionitrobenzoate (3-carboxy-4-nitrobenzenethiol); HPLC, high pressure liquid chromatography.

## EXPERIMENTAL PROCEDURES

**Plasmid Constructions, Site-directed Mutageneses, Production, and Purification of Wild-type and Mutant *N. meningitidis* PILB**—Plasmids pSKPILBmsrA, pSKPILBmsrB, and pSKPILBmsrAMsrB, designed for PILB-MsrA, PILB-MsrB, and PILB-MsrA/MsrB production, respectively, were obtained by cloning internal fragments 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, into the plasmid pDB125KSNN<sup>2</sup> between the *Nde*I and *Sac*I sites. Site-directed mutageneses were performed using the QuikChange site-directed mutagenesis kit (Stratagene).

The *E. coli* strain used for all Msr productions was HB101 (supE44, hsdS20 (r<sup>-</sup>, m<sup>-</sup>), recA13, ara-14, proA2, lacY1, galK2, rpsL20, xyl-5, mtl-1) transformed with the plasmid construction containing the coding sequence under the *lac* promoter.

For PILB-Msrs purification, cells were harvested by centrifugation, resuspended in a minimal volume of buffer A (50 mM Tris-HCl, 2 mM EDTA, pH 8) containing 20 mM dithiothreitol (DTT) and sonicated. The Msrs were then precipitated at 40, 50, and 60% ammonium sulfate ((NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>) saturation for PILB-MsrA/MsrB, PILB-MsrA, and PILB-MsrB, respectively. The contaminating proteins were removed by applying the enzymatic solutions onto exclusion size chromatography on ACA 54 resin at pH 8 (buffer A). Purified fractions were then pooled and applied onto a Q-Sepharose column equilibrated with buffer A, followed by a linear gradient of KCl (0–0.4 M) using a fast protein liquid chromatography system (Amersham Biosciences). The PILB-MsrA/MsrB was eluted at 100 mM KCl, whereas the PILB-MsrA and PILB-MsrB passed through. PILB-MsrA and PILB-MsrB were further purified on phenyl-Sepharose (Amersham Biosciences) equilibrated with buffer A, containing 1 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. PILB-Msrs were eluted with a linear gradient from 1 to 0 M in buffer A.

At this stage, wild-type PILB-MsrA/MsrB and wild-type and mutant PILB-MsrAs and PILB-MsrBs were pure as checked by electrophoresis on 12% SDS-polyacrylamide gel (10) followed by Coomassie Brilliant Blue R-250 staining and by electrospray mass spectrometry analyses.

Purified enzymes were stored at –20 °C in the presence of 50 mM DTT and 60% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. Under these conditions, the enzymes were stable for several weeks. Their molecular concentration was determined spectrophotometrically, using theoretical extinction coefficients at 280 nm deduced from the method of Scopes (11) (*i.e.* 26,200 M<sup>-1</sup>·cm<sup>-1</sup> for wild-type and mutant PILB-MsrAs, 17,330 M<sup>-1</sup>·cm<sup>-1</sup> for wild-type and mutant PILB-MsrBs, and 42,300 M<sup>-1</sup>·cm<sup>-1</sup> for wild-type PILB-MsrA/MsrB).

**Preparation of *L*-Met-*S*-SO, *L*-Met-*R*-SO, *D*-Met-*S*-SO, *D*-Met-*R*-SO, *L*-Met-*R,S*-SO-NHMe, *Ac-L*-Met-*R,S*-SO, and *Ac-L*-Met-*R,S*-SO-NHMe**—The *L*-Met-*S*-SO and *D*-Met-*R*-SO enantiomers were prepared according to Holland *et al.* (12). *L*- and *D*-methionine were treated with phthalic (Pht) anhydride using the method of Bose (13) to give Pht-*L*-Met-OH and Pht-*D*-Met-OH in 90–95% yields, respectively. Oxidation of Pht-*L*-Met-OH and Pht-*D*-Met-OH was carried out in methanol with H<sub>2</sub>O<sub>2</sub> and afforded the sulfoxide in a nearly equimolar (*S/R*)-composition as revealed by the split C<sup>1</sup>H<sub>3</sub> proton singlet in CDCl<sub>3</sub>/Me<sub>2</sub>SO-*d*<sub>6</sub> 4:1 (2.487 ppm for the heterochiral and 2.483 ppm for the homochiral diastereomers). Both Pht-*L*-Met-*S*-SO and Pht-*D*-Met-*R*-SO enantiomers were obtained by crystallization from methanol. The absolute configuration of the isomer *R* was confirmed by x-ray diffraction on single crystals.<sup>3</sup> The phthalic protecting group was eliminated with hydrazine hydrate in ethanol, and the *L*-Met-*S*-SO and *D*-Met-*R*-SO enantiomers were recovered by precipitation from water with acetone.

On the other hand, it was not possible by crystallization to obtain Pht-*L*-Met-*R*-SO and Pht-*D*-Met-*S*-SO enantiomers in a pure form. In both cases, the resulting sulfoxides were found to contain about 25% of the other isomer. Therefore, *L*-Met-*R*-SO and *D*-Met-*S*-SO were isolated from the *L*-Met-*R,S*-SO and *D*-Met-*R,S*-SO diastereomers by consuming the other isomer by enzymatic reduction with PILB-MsrA and PILB-MsrB, respectively. The experimental conditions were 100 mM DTT, 500 μM Msr, 50 mM Tris-HCl, 2 mM EDTA, pH 8, and the reaction mixtures were incubated overnight at 25 °C. Each MetSO isomer was then separated from Met on a 25-cm sephasil C18 reverse phase column on an ÄKTA explorer system (Amersham Biosciences) equilibrated with H<sub>2</sub>O/trifluoroacetic acid 0.1% buffer in the presence of 10% acetonitrile. Met was eluted isocratically after MetSO. The fractions corresponding to each sulfoxide isomer were pooled and concentrated in order to eliminate acetonitrile and trifluoroacetic acid.

*L*-Met-*R,S*-SO-NHMe and *Ac-L*-Met-*R,S*-SO-NHMe were classically prepared (14) from *N*-(*tert*-butoxycarbonyl)-*L*-Met-OH via the mixed anhydride with Me<sub>2</sub>CHCH<sub>2</sub>OCOC(NEt)(CHMe)<sub>2</sub> and MeNH<sub>2</sub>. Treatment of the resulting *N*-(*tert*-butoxycarbonyl)-*L*-Met-NHMe with 3 N HCl in EtOAc gave *L*-Met-NHMe, which was acetylated with AcCl/CHMe<sub>2</sub> into *Ac-L*-Met-NHMe. Oxidation of *L*-Met-NHMe and *Ac-L*-Met-NHMe with H<sub>2</sub>O<sub>2</sub> in methanol (12) resulted in *L*-Met-*R,S*-SO-NHMe and *Ac-L*-Met-*R,S*-SO-NHMe. *Ac-L*-Met-*R,S*-SO was purchased from Bachem.

**Quantification of the Free Cysteine Content with 5,5'-Dithiobis(2-nitro)benzoate (DTNB)**—Cysteine content was determined using DTNB under non-denaturing (buffer A) and denaturing conditions (final concentration of 1% SDS in buffer A), either in the absence or in the presence of 150 mM DL-Met-*R,S*-SO without the addition of any exogenous reducing system as previously described by Boschi-Muller *et al.* (7).

**Determination of Msr Activity in the Presence of DTT**—Msrs activities were determined with DL-Met-*R,S*-SO as a substrate at a concentration of 150 mM. The reaction mixture also contained 10 mM DTT and 5 μM wild-type PILB-MsrA/MsrB or wild-type or mutant PILB-MsrA or PILB-MsrB in buffer A.

Initial rate measurements were carried out at 25 °C by following the appearance of free methionine measured by HPLC. To do so, aliquots of the reaction mixture were removed at different times of incubation up to 2.5 min, and the reaction was stopped by the addition of trifluoroacetic acid to a final concentration of 1%. In each aliquot, the quantity of Met formed was measured as previously described by Boschi-Muller *et al.* (7).

**Enzyme Kinetics in the Presence of Thioredoxin**—The ability of wild-type and mutant PILB-MsrAs and PILB-MsrBs and of wild-type PILB-MsrA/MsrB to reduce substrates was assayed in the presence of 1.28 μM *E. coli* thioredoxin reductase, 0.3 mM NADPH, and various concentrations of *E. coli* thioredoxin in buffer A.

Thioredoxin and thioredoxin reductase from *E. coli* were prepared following experimental procedures already published (15, 16).

Initial rate measurements were carried out at 25 °C on a Kontron Uvikon 933 spectrophotometer by following the decrease of the absorbance at 340 nm. The initial rate data were fitted to the Michaelis-Menten relationship using least squares analysis to determine *k*<sub>cat</sub> and *K*<sub>m</sub>. All *K*<sub>m</sub> values were determined at saturating concentrations of the other substrate.

**Stoichiometry of Met Formation in the Absence of a Regenerating System**—The reaction mixture, containing 150 mM DL-Met-*R,S*-SO and a 100–500 μM concentration of wild-type or mutant PILB-MsrA or PILB-MsrB or wild-type PILB-MsrA/MsrB, was incubated at 25 °C for 10 min in buffer A. Then the Met formed was quantified as previously described by Boschi-Muller *et al.* (7).

**Characterization of the Sulfenic Acid Intermediate**—The sulfenic acid intermediate was characterized spectrophotometrically by using thionitrobenzoate (TNB<sup>-</sup>) under non-denaturing conditions and by mass spectrometry analyses after modification (or no modification) with 5,5-dimethyl-1,3-cyclohexanedione (dimedone).

For spectrophotometric characterization, TNB<sup>-</sup> was prepared by reducing the corresponding disulfide using the procedure of Silver (17). Progress curves of TNB<sup>-</sup> disappearance for wild-type and mutant PILB-MsrAs and PILB-MsrBs were recorded at 412 nm in buffer A. Enzyme concentrations were 7.35 and 14.7 μM, and the TNB<sup>-</sup> concentration was 60 μM. The amount of TNB<sup>-</sup> consumed was calculated using an extinction coefficient at 412 nm of 13,600 M<sup>-1</sup>·cm<sup>-1</sup>.

For spectrometric characterization, analyses were performed for wild-type and mutant PILB-MsrAs and PILB-MsrBs, either after modification or not by DL-Met-*R,S*-SO and dimedone. All of the modification reactions were performed in buffer A in the presence of 20 μM enzyme. DL-Met-*R,S*-SO was added at a concentration of 150 mM, and the mixture was incubated 10 min at 25 °C. Then dimedone at a concentration of 20 mM was added, and the mixture was incubated overnight in the dark at room temperature. Mass spectrometric measurements were performed on a LCT electrospray time-of-flight mass spectrometer (Micromass, Manchester, UK). For mass analysis in denaturing conditions, Msr samples were diluted to 10 μM in a 1:1 water/acetonitrile mixture (v/v) containing 1% formic acid. Samples were continuously infused into the ion source at a flow rate of 5 μl/min. Spectra were recorded in the positive ion mode in the mass range 400–4000 *m/z*, after calibration of the instrument with a solution of horse heart myoglobin (Sigma) diluted to 2 μM in the 1:1 water/acetonitrile (1% formic acid) mixture.

<sup>2</sup> S. Marchal, personal communication.

<sup>3</sup> C. Didierjean, unpublished results.

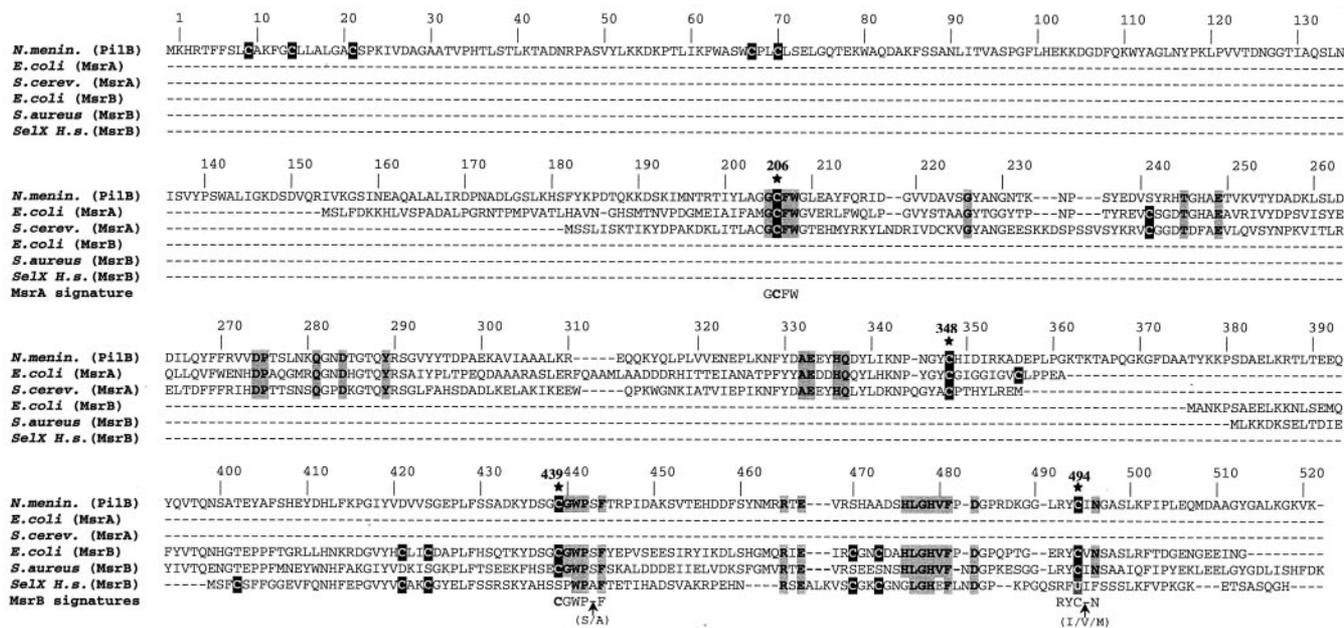


FIG. 1. Sequence alignment of *N. meningitidis* PILB, *E. coli*, and *S. cerevisiae* MsrAs and *E. coli*, *S. aureus*, and *Homo sapiens* MsrBs. The sequences of PILB from *N. meningitidis* (*N. meningitidis*), of MsrAs from *E. coli* and *S. cerevisiae* (*S. cerev.*), and of the MsrBs from *E. coli*, *S. aureus*, and *H. sapiens* (*selX H.s.*) were aligned with Bioedit software. The numbering of amino acid residues indicated is based on the numbering of the *N. meningitidis* PILB sequence. The cysteine residues are shown in white on black boxes, and conserved residues in all putative Msr sequences ( $\geq 90\%$  in 35 MsrA sequences and in 64 MsrB sequences) are shown in gray boxes. Black stars indicate the positions of the four catalytic Cys residues of *N. meningitidis* MsrA (Cys-205 and Cys-348) and MsrB (Cys-439 and Cys-494). Sequence signatures around Cys-205 (at least 90% invariant), around Cys-439 (at least 50% invariant), and around Cys-494 (at least 90% invariant) are also indicated below the alignment.

## RESULTS AND DISCUSSION

### Justification of the Truncations on PILB

PILB contains 522 amino acids and is composed of three subdomains. The N-subdomain is suggested to encode a disulfide oxidoreductase. The central subdomain is an ortholog to *E. coli* and *Saccharomyces cerevisiae* MsrAs, whereas the C-subdomain displays high sequence similarities to *E. coli* and *S. aureus* MsrBs (Fig. 1).

**Truncation after the N-subdomain**—Sequence comparisons show that at least two MsrAs from *S. pneumoniae* and *Bacillus subtilis* (Pedant code gi\_14972133 and gi\_2634588) and one recently produced by truncation of the N terminus of *E. coli* MsrA (18) have an N terminus starting from the position corresponding to amino acid 196 of PILB. All three MsrAs were shown to be active (1, 18, 19). Thus, truncation was done after position 194 of PILB. The PILB-MsrA/MsrB protein (amino acids 195–521) was produced in a soluble form.

**Truncations between MsrA and MsrB Subdomains on PILB**  
**Truncated at the N-subdomain**—Two plasmidic constructs were used to produce soluble MsrA and MsrB subdomains. In the first construct, the truncation was done after position 388 of PILB which corresponds to the N terminus of putative MsrB from *Klebsiella pneumoniae* (Pedant code b\_kpn.contig523orf8). In this case, the PILB-MsrA (amino acids 195–388) was produced in a soluble form, whereas the truncated form corresponding to the MsrB subdomain (amino acids 390–521) was not soluble. Thus, to obtain a soluble form of the MsrB subdomain, another construct was used, where truncation was done after position 375 of PILB, which corresponds to the N terminus of the putative *Sinorhizobium meliloti* MsrB (NCBI accession number AE007290.1) and of the *E. coli* MsrB (8). In this case, PILB-MsrB was produced in a soluble form (amino acids 376–521). Such a result suggests that both Msrs fold independently within PILB. This is also consistent with the fact that the two genes are often located at different positions on chromosomal DNAs.

### Biochemical Properties of MsrA, MsrB, and MsrA/MsrB Subdomains of PILB

Truncated PILB variants and their mutants were overexpressed in an *E. coli* strain using the corresponding DNA sequences under the *lac* promoter. All forms of PILB variants were obtained pure as judged by SDS-PAGE gels and mass spectrometry analyses.

DTNB reagent revealed four Cys for PILB-MsrA/MsrB and two Cys for both PILB-MsrA and PILB-MsrB under denaturing conditions (Table I). These results are in agreement with the PILB DNA sequence that indicates four Cys at positions 206 and 348 in PILB-MsrA and at positions 439 and 494 in PILB-MsrB. Under native conditions, all of the Cys were also reactive regardless of the subdomains. This shows that 1) both Cys of the MsrA subdomain are easily accessible, similar to what is described for *E. coli* MsrA; 2) both Cys of the MsrB subdomain are also accessible; and 3) the accessibility in PILB-MsrA/MsrB of each couple of Cys within each domain is not significantly altered by the presence of the second subdomain. This again supports an independent folding of each subdomain.

### Stoichiometry of Methionine Formation and Thioredoxin Recycling Activity

**The PILB-MsrA Subdomain**—Stoichiometry of methionine formation was determined in the absence of reductant (Table II). One mol of methionine was formed with a loss of two thiols, which is in agreement with formation of a disulfide bond between Cys-206 and Cys-348. One mol of methionine was also formed with mutant C348S with a loss of one thiol, whereas no methionine was formed with mutant C206S. In the presence of thioredoxin, a recycling activity was observed with PILB-MsrA wild type but not with the mutants (Table II). Together, these results are in agreement with formation of a sulfenic acid on Cys-206 and regeneration of Cys-206 via formation of an internal disulfide bond with Cys-348 followed by its reduction by

TABLE I

Free sulfhydryl content of wild-type and Cys to Ser mutants of *N. meningitidis* PILB-MsrA and PILB-MsrB and PILB-MsrA/MsrB wild type

The values indicated represent the average of two independent measurements of at least two enzyme concentrations (S.D. range 10%). Cysteine content was determined spectrophotometrically using DTNB under non-denaturing and denaturing conditions (10% SDS), in the absence or in the presence of 150 mM DL-Met-R,S-SO without any regenerating system (see "Experimental Procedures"). Data presented in the table are those obtained under non-denaturing conditions. Data obtained under denaturing conditions are identical but are not presented for reasons of clarity of the table.

Enzyme	No. of Cys	No. of Cys measured			
		Without DL-Met-R,S-SO	With DL-Met-R,S-SO	Decrease in free thiols <sup>a</sup>	
		<i>mol Cys/mol enzyme</i>			
				Calculated	Theoretical
PILB-MsrA	2	1.9	0.1	1.8	2
C206S PILB-MsrA	1	1.0	1.0	0	0
C348S PILB-MsrA	1	0.9	0	0.9	1
PILB-MsrB	2	2.0	0.1	1.9	2
C439S PILB-MsrB	1	1.0	0.1	0.9	1
C494S PILB-MsrB	1	1.0	1.0	0	0
PILB-MsrA/MsrB	4	4.0	0.4	3.6	4

<sup>a</sup> The difference in the number of free cysteine thiols upon treatment with DL-Met-R,S-SO versus no treatment.

TABLE II

Stoichiometry of Met formed in the absence of regenerating system and enzymatic activity with DTT or thioredoxin as reductant

The values indicated represent the average of three independent measurements of at least two enzyme concentrations (S.D. range 10%). The quantity of Met formed for stoichiometry determination was determined by HPLC, after reaction with 150 mM DL-Met-R,S-SO without any regenerating system as described under "Experimental Procedures." The activities of wild-type and mutant enzymes were assayed with 150 mM DL-Met-R,S-SO, 10 mM DTT, and 5  $\mu$ M enzyme or with 150 mM DL-Met-R,S-SO, 200  $\mu$ M thioredoxin, 1.28  $\mu$ M thioredoxin reductase, and 0.5–50  $\mu$ M enzyme as described under "Experimental Procedures." NA, no activity ( $<6 \cdot 10^{-2}$   $\mu$ mol/min/ $\mu$ mol).

Enzyme	Stoichiometry	Activity with DTT	Activity with thioredoxin
	<i>mol Met formed/mol enzyme</i>	$\mu$ mol Met formed/min/ $\mu$ mol enzyme	$\mu$ mol Met formed/min/ $\mu$ mol enzyme
PILB-MsrA	0.9	3	220
C206S PILB-MsrA	0	NA	NA
C348S PILB-MsrA	0.9	37	NA
PILB-MsrB	1.0	3	12
C439S PILB-MsrB	1.0	36	NA
C494S PILB-MsrB	0	NA	NA
PILB-MsrA/MsrB	1.8	4.2	170

thioredoxin. This is a situation similar to that described for *E. coli* MsrA except that the Cys-206–Cys-348 bond (equivalent to the Cys-51–Cys-198 bond in *E. coli* MsrA) is reducible by thioredoxin in PILB-MsrA, whereas only the Cys-198–Cys-206 bond is reducible in *E. coli* MsrA. Such a difference remains to be explained. Further evidence of the formation of a sulfenic acid comes from the use of TNB<sup>-</sup> and dimedone, which are specific reagents for sulfenic acid. In the case of mutant C348S, in which no disulfide bond can be formed, a decrease of the absorbance at 410 nm by 1 eq of TNB<sup>-</sup>/mol was observed when the mutant was first incubated with MetSO and then treated with an excess of TNB<sup>-</sup> (Table III). Incubation of mutant C348S with MetSO and a subsequent addition of dimedone led to an increased mass of 138 Da. This increase is that expected if a covalent adduct is formed with dimedone.

**The PILB-MsrB and MsrA/MsrB Subdomains**—One mol of methionine per mol of PILB-MsrB was formed in the absence of reductant. Moreover, the loss of thiols was in agreement with formation of a disulfide bond between Cys-439 and Cys-494 (Table I). To investigate the role of Cys-439 and Cys-494, mutations of these residues into Ser were done. Substituting Ser for Cys-494 abolished any activity, whereas the mutant C439S showed a reductase activity with a stoichiometry of 1 mol of methionine in the absence of DTT (Table II). When the mutant C439S was incubated with MetSO in the absence of DTT and then treated with TNB<sup>-</sup>, a decrease of the absorbance at 410 nm equivalent to that of 1 mol of TNB<sup>-</sup> was observed. Moreover, when the mutant was incubated under the same conditions and then subsequently treated with dimedone, a mass increase of 154 Da was observed instead of the expected value of 138 Da. This difference of 16 Da remains to be explained. A possibility is that, under the experimental conditions used, the

sulfide that is formed on MsrB can be easily oxidized into sulfoxide (Table III). In the presence of thioredoxin, a recycling activity was observed with wild-type PILB-MsrB but not with the mutants (Table II). Altogether, these results show that 1) the C-terminal subdomain of PILB displays an Msr activity; 2) the mechanism involves formation of a sulfenic acid intermediate; 3) the essential Cys involved in reduction of MetSO and in formation of the sulfenic acid derivative is Cys-494; and 4) the regeneration of Cys-494 is done via formation of a disulfide bond with Cys-439 followed by its reduction by thioredoxin. This mechanism is reminiscent of that described for MsrA from *E. coli* except that only one intradisulfide bond is formed in MsrB of PILB for regenerating the active Cys-494. The fact that no thioredoxin-recycling process was observed in mutant C439S indicates that, similar to MsrA and more generally to all mechanisms where a sulfenic acid is formed except for the BCP protein and Prx1p (20, 21), the sulfenic acid intermediate formed in MsrB cannot be reduced via a double displacement mechanism involving formation of a disulfide bridge between Cys-494 and Cys-32 of thioredoxin followed by formation of a disulfide bond between Cys-32 and Cys-35 of thioredoxin and release of Cys-494. The fact that an efficient recycling activity was observed on mutant C439S with DTT as a reductant (Table II) indicates that DTT can easily attack the sulfenic acid intermediate on Cys-494 in contrast to thioredoxin. Thus, this supports a nonaccessibility of the sulfenic acid within the active site of PILB-MsrB to thioredoxin. In this context, knowledge of the three-dimensional structure of MsrB under the sulfenic acid intermediate state will be informative.

When PILB-MsrA/MsrB was tested in the absence of reductant, 2 mol of methionine per mol of subdomain were formed with a loss of four thiols (Tables I and II). These results indicate

TABLE III

Characterization of the sulfenic acid intermediates by electrospray mass spectrometry analyses and spectrophotometric titration with TNB<sup>-</sup>

The values indicated for spectrophotometric titration with TNB<sup>-</sup> represent the average of two independent measurements of at least two enzyme concentrations (S.D. range 10%). Sulfenic acid content was determined spectrophotometrically using TNB<sup>-</sup> under denaturing conditions (0.1% SDS), after reaction with 150 mM DL-Met-*R,S*-SO in the absence of any regenerating system as previously described (see "Experimental Procedures"). Molecular masses of wild-type and mutant PILB-MsrA and PILB-MsrB were determined by electrospray mass analysis without any modification or after reaction with 150 mM DL-Met-*R,S*-SO and/or 20 mM dimedone as described under "Experimental Procedures." Molecular mass and difference in mass are expressed in daltons.

Enzyme	TNB <sup>-</sup>		Electrospray mass spectrometry analyses			
	Native	After MetSO treatment	Before modification		After modification with MetSO and dimedone	
			Theoretical mass	Measured mass	Measured mass	Mass difference <sup>a</sup>
	<i>mol TNB<sup>-</sup> / mol enzyme</i>					
Wild-type PILB-MsrA	0	0	21,896	21,898 ± 1	21,897 ± 1	-1 ± 2
C206S PILB-MsrA	0	0	21,880	21,881 ± 1	21,880 ± 1	-1 ± 2
C348S PILB-MsrA	0	1	21,880	21,880 ± 1	22,018 ± 1	+138 ± 2
Wild-type PILB-MsrB	0	0	16,374	16,374 ± 1	16,373 ± 1	-1 ± 2
C439S PILB-MsrB	0	1	16,358	16,358 ± 1	16,512 ± 1 <sup>b</sup>	+154 ± 2
C494S PILB-MsrB	0	0	16,358	16,358 ± 1	16,358 ± 1	+0 ± 2

<sup>a</sup> Mass differences measured by electrospray ionization-mass spectrometry correspond to the differences between the mass of the protein before and after treatment.

<sup>b</sup> A second peak, representing about 50% of the enzyme population, and with a mass of 16,390 ± 1 Da was observed. This corresponds to a mass increase of 32 Da compared with native C439S PILB-MsrB. This peak can correspond to a population of sulfenic acid derivative that would be oxidized into sulfonic acid under the experimental conditions used.

that both Msrs are active within PILB-MsrA/MsrB and thus that the presence of one subdomain does not prevent the reductase activity of the other subdomain.

#### Catalytic Constants and Stereoselectivity in MetSO Reduction

The catalytic constants of PILB-MsrA and PILB-MsrB were first determined using the mixture of four diastereomers DL-Met-*R,S*-SO as a substrate under conditions where thioredoxin concentrations were not limiting (Table IV).  $K_m$  for MetSO and thioredoxin and  $k_{cat}$  values of PILB-MsrA and PILB-MsrB were 9 mM, 75 μM, and 3.7 s<sup>-1</sup> and 56 mM, 34 μM, and 0.2 s<sup>-1</sup>, respectively. The catalytic values of PILB-MsrA are in the range of those recently determined for *E. coli* MsrA (18), but the  $K_m$  and  $k_{cat}$  values for MetSO are significantly different from those recently described by Grimaud *et al.* (8) for *E. coli* MsrA and *E. coli* MsrB ( $K_m$  values for MetSO are 170 μM and 6.7 mM, and  $k_{cat}$  values are 20 and 0.6 min<sup>-1</sup>, respectively). This is because the concentration of 5 μM of thioredoxin used in their experiments was not saturating. In the case of MsrB, the observed discrepancy could be due to structural differences between MsrBs from *N. meningitidis* and *E. coli*. In fact, a control experiment done on *E. coli* MsrB showed that a saturating concentration of thioredoxin is also only attained at 75 μM (data not shown). In this context, it is interesting to note that  $K_m$  values of *E. coli* MsrB and of *E. coli* MsrA for *E. coli* thioredoxin are 3–5-fold better than  $K_m$  for *Chlamydomonas reinhardtii* thioredoxin (data not shown). This therefore suggests that thioredoxin affinity is in part species-dependent. Therefore,  $K_m$  values of *N. meningitidis* MsrA and MsrB for *N. meningitidis* thioredoxin are probably lower than those reported for *E. coli* thioredoxin.

DL-Met-*R,S*-SO was shown to be quantitatively reduced by PILB-MsrA/MsrB. In contrast, only 50% of the mixture of diastereomers of MetSO was reduced by either PILB-MsrA or PILB-MsrB. Moreover, only L-Met-*S*-SO and D-Met-*S*-SO were quantitatively reduced by PILB-MsrA, whereas D-Met-*R*-SO and L-Met-*R*-SO were only reduced by PILB-MsrB. These results support the data reported by Grimaud *et al.* (8) on oxidized calmodulin, suggesting that MsrB is stereospecific for the *R* isomer of the sulfoxide of MetSO, whereas, as has already been shown, MsrA is stereospecific for the *S* isomer (22, 23).

The catalytic constants were then determined for the isomers

that are substrates (Table IV). The  $K_m$  value of PILB-MsrA for L-Met-*S*-SO is 3-fold lower than for D-Met-*S*-SO, whereas the  $K_m$  value of PILB-MsrB for L-Met-*R*-SO is decreased 6-fold compared with that for D-Met-*R*-SO. This suggests that the configuration at the carbon C $_{\alpha}$  is not a determining factor for efficient binding to both Msrs. Compared with the  $k_{cat}/K_{MetSO}$  value of 1,200 M<sup>-1</sup>·s<sup>-1</sup> for PILB-MsrA with L-Met-*S*-SO, the catalytic efficiency of PILB-MsrB is at least 60-fold lower with L-Met-*R*-SO. The low catalytic efficiency of PILB-MsrB raised the question of whether protein-bound MetSO is a better substrate than free MetSO. Therefore, the catalytic constants were determined for Ac-L-Met-*R,S*-SO-NHMe. In this case, the amino and carboxyl groups of MetSO are engaged in amide bonds, and thus no charge is present. As shown in Table IV, the  $K_m$  value of MsrB for Ac-L-Met-*R,S*-SO-NHMe was decreased by a factor of 16 compared with that of L-Met-*R*-SO. Taking into account the fact that Ac-L-Met-*S*-SO-NHMe is neither a substrate nor an inhibitor (see below), the  $K_m$  value is in fact 32-fold decreased. A similar effect was observed with MsrB from *E. coli*.<sup>4</sup> For PILB-MsrA,  $K_m$  was decreased by a factor of 7 compared with that for L-Met-*S*-SO, taking also into account the fact that Ac-L-Met-*R*-SONHMe is not a substrate. On the other hand, the  $K_m$  value of PILB-MsrB for Ac-L-Met-*R,S*-SO was similar to that for Ac-L-Met-*R,S*-SO-NHMe, whereas a 3.7-fold increase in the  $K_m$  value was observed for L-Met-*R,S*-SO-NHMe. PILB-MsrA behaves similarly to PILB-MsrB except that a 13-fold increase in the  $K_m$  value was observed for Ac-L-Met-*R,S*-SO. Anyway, the highest  $k_{cat}/K_{MetSO}$  values of both Msrs are observed for Ac-L-Met-*R,S*-SO-NHMe (Table IV). Thus, this suggests that the active sites of both Msrs are adapted for binding protein-bound L-MetSO more efficiently than free L-MetSO. Finally, it is important to note that no inhibition of the two PILB-Msrs was observed in the presence of methionine and of the L-Met-SO isomer, which is not a substrate, at a concentration as high as 100 mM, with N-Ac-L-Met-*R,S*-SO-NHMe as a substrate.

Several results presented in this study argue for a structure of the active site of PILB-MsrB different from that of PILB-MsrA, which can probably be extended to all MsrBs and Msrs. In particular, the two Msrs exhibit opposite stereoselectivities toward the sulfoxide function, and the sulfoxide isomer, which

<sup>4</sup> A. Olry, S. Boschi-Muller, and G. Branlant, unpublished results.

TABLE IV

Kinetic parameters for the methionine sulfoxide reductase activity of wild-type PILB-MsrA, PILB-MsrB, and PILB-MsrA/MsrB

Reactions were carried out in 50 mM Tris-HCl, 2 mM EDTA buffer, pH 8, at 25 °C as described under "Experimental Procedures." Enzyme concentration was 0.5  $\mu$ M for wild-type PILB-MsrA and PILB-MsrA/MsrB, and 2–6  $\mu$ M for wild-type PILB-MsrB. Data presented were obtained by fitting the experimental data to the Michaelis-Menten equation. NA, no activity (*i.e.*  $k_{\text{cat}} \leq 3 \cdot 10^{-3} \text{ s}^{-1}$ ); ND, not determined.

Enzyme	Substrate	$k_{\text{cat}}$ $\text{s}^{-1}$	$K_{\text{sulfoxide}}$ $\text{mM}$	$k_{\text{cat}}/K_{\text{sulfoxide}}$ $\text{M}^{-1} \cdot \text{s}^{-1}$	$K_{\text{Trx}}$ $\mu\text{M}$
PILB-MsrA	DL-Met- <i>R,S</i> -SO	3.7 $\pm$ 0.4	9 $\pm$ 4	410 $\pm$ 200	75 $\pm$ 15
	L-Met- <i>S</i> -SO	2.5 $\pm$ 0.1	2.1 $\pm$ 0.2	1200 $\pm$ 150	ND
	D-Met- <i>S</i> -SO	2.5 $\pm$ 0.1	6.0 $\pm$ 0.2	400 $\pm$ 100	ND
	L-Met- <i>R</i> -SO	NA			
	D-Met- <i>R</i> -SO	NA			
	Ac-L-Met- <i>R,S</i> -SO-NHMe <sup>a,b</sup>	3.4 $\pm$ 0.2	0.6 $\pm$ 0.2 <sup>c</sup>	5600 $\pm$ 200 <sup>c</sup>	ND
	Ac-L-Met- <i>R,S</i> -SO	2.1 $\pm$ 0.2	8 $\pm$ 2 <sup>c</sup>	250 $\pm$ 40 <sup>c</sup>	ND
	L-Met- <i>R,S</i> -SO-NHMe	2.0 $\pm$ 0.1	1.7 $\pm$ 0.3 <sup>c</sup>	1200 $\pm$ 200 <sup>c</sup>	ND
PILB-MsrB	DL-Met- <i>R,S</i> -SO	0.2 $\pm$ 0.1	56 $\pm$ 7	4 $\pm$ 2	34 $\pm$ 6
	L-Met- <i>S</i> -SO	NA			
	D-Met- <i>S</i> -SO	NA			
	L-Met- <i>R</i> -SO	0.36 $\pm$ 0.01	19 $\pm$ 2	19 $\pm$ 6	ND
	D-Met- <i>R</i> -SO	0.30 $\pm$ 0.03	122 $\pm$ 13	2 $\pm$ 1	ND
	Ac-L-Met- <i>R,S</i> -SO-NHMe <sup>a,d</sup>	0.46 $\pm$ 0.04	1.2 $\pm$ 0.4 <sup>c</sup>	380 $\pm$ 60 <sup>c</sup>	ND
	Ac-L-Met- <i>R,S</i> -SO	0.28 $\pm$ 0.01	1.6 $\pm$ 0.2 <sup>c</sup>	175 $\pm$ 50 <sup>c</sup>	ND
	L-Met- <i>R,S</i> -SO-NHMe	0.17 $\pm$ 0.01	4.5 $\pm$ 0.5 <sup>c</sup>	40 $\pm$ 20 <sup>c</sup>	ND
PILB-MsrA/MsrB	DL-Met- <i>R,S</i> -SO	2.8 $\pm$ 0.2	9 $\pm$ 2	300 $\pm$ 100	114 $\pm$ 31
	L-Met- <i>S</i> -SO	2.5 $\pm$ 0.2	1.5 $\pm$ 0.3	1600 $\pm$ 300	ND
	L-Met- <i>R</i> -SO	0.08 $\pm$ 0.01	3 $\pm$ 1	25 $\pm$ 8	ND

<sup>a</sup> No inhibition was observed in the presence of up to 100 mM Met.

<sup>b</sup> No inhibition was observed in the presence of up to 100 mM L-Met-*R*-SO.

<sup>c</sup>  $K_{\text{sulfoxide}}$  and  $k_{\text{cat}}/K_{\text{sulfoxide}}$  should be divided and multiplied by 2, respectively, considering the fact that only one stereomer is a substrate and that the second one is not an inhibitor.

<sup>d</sup> No inhibition was observed in the presence of up to 100 mM L-Met-*S*-SO.

is not a substrate, does not bind. Moreover, no sequence identity is detectable. In particular, the conserved sequence of MsrA around Cys-51 (*i.e.* GCFW, which is located at the N terminus of an  $\alpha$ -helix) is different from the conserved region around Cys-494 of PILB-MsrB (*i.e.* RYC(I/V/M)N). However, the following common properties are shared by MsrA and MsrB. 1) Their chemical mechanisms are similar, with sulfenic acid formation. This supports the idea that sulfenic acid chemistry is a general feature in the reduction of sulfoxides by thiols and thus excludes another chemical mechanism proposed by Wallace and Mahon (24). 2) The recycling process is probably dependent on thioredoxin *in vivo*. 3) The presence of the sulfoxide function is a prerequisite for binding to Msrs as supported by the absence of inhibition in the presence of a large excess of methionine.

The fact that MsrA and MsrB subdomains are fused in *N. meningitidis* PILB and also in *S. pneumoniae* (1) in which the first subdomain is not present also raised the question of whether the fusion of MsrA and MsrB subdomains had an inhibitory, an additive, or a synergetic effect on the efficiency of MetSO reduction. Therefore, the catalytic constants were also determined on wild-type PILB-MsrA/MsrB. As shown in Table IV, the catalytic efficiency determined with DL-Met-*R,S*-SO was in the range of that of PILB-MsrA. This suggests that the presence of PILB-MsrB does not change significantly the catalytic efficiency of PILB-MsrA. This is confirmed by the fact that  $k_{\text{cat}}$  and  $K_m$  values for L-Met-*S*-SO of PILB-MsrA/MsrB were similar to those of PILB-MsrA. The fact that the catalytic efficiency,  $k_{\text{cat}}/K_m$ , for L-Met-*R*-SO of PILB-MsrA/MsrB was similar to that of PILB-MsrB also suggests that the presence of PILB-MsrA does not change significantly the catalytic efficiency of PILB-MsrB. However,  $k_{\text{cat}}$  and  $K_m$  values of PILB-MsrB were 4.5- and 6-fold decreased, respectively. These differences remain to be explained.

Sequence comparisons of putative MsrBs show that Cys equivalent to Cys-494 of PILB-MsrB is replaced by a selenocysteine in human (25) and mouse ortholog SelXs. Taking into account the fact that selenocysteine is chemically more reactive than a Cys, one can hypothesize that substituting selenocys-

teine for Cys will favor the efficiency of the reduction of the sulfoxide function at least of the nucleophilic attack on the sulfoxide function. Inspection of the primary structure of SelXs shows that the Cys equivalent to Cys-439 of PILB-MsrB located in the signature CGWP(S/A)F is replaced by a Ser, whereas there exist four Cys at positions equivalent to 421, 424, 470, and 473, which are conserved in at least 40% of the MsrB putative sequences. The fact that these Cys have been recently shown to be involved in binding of metal in *E. coli* MsrB<sup>4</sup> excludes their participation in the thioredoxin-recycling reductase activity. Therefore, if SelXs are expressed and are catalytically active, this means that either an alternative recycling thiol system other than that involving thioredoxin is operative or Cys at another position plays a similar role as Cys-439 of PILB-MsrB. In this context, it would be informative to test the activity of the putative MsrBs that have no Cys at position 439.

Bacterial virulence relies on the adhesins and cytoadherence-related proteins of the pathogenic bacteria to bind to host cells. It also depends on the ability of the pathogenic bacteria to survive oxidative damages caused by reactive oxygen or nitrogen species. In this context, it was suggested that PILB from *N. gonorrhoeae* contributed to the production or the maintenance of the functional properties of adhesins by mechanisms not clearly elucidated (1). More recently, convincing experiments showed that MsrA is required for full virulence of the plant pathogen *E. chrysanthemi* (2) and of the human pathogen *M. genitalium* (3). The role of the bacterial MsrA would be at least to protect the protein structures of such bacteria, in particular methionine, from oxidative damage or through alternate virulence-related pathways. The fact that MsrA is specific for the *S* isomer of MetSO while MsrB is *R*-specific and that PILB expresses both Msr activities suggests that MsrB is also involved in bacterial virulence.

#### CONCLUSION

We have shown that the MsrB subdomain of PILB reduces MetSO via a two-step catalytic mechanism involving sulfenic acid chemistry. The first step leads to reduction of MetSO into Met with a concomitant formation of a sulfenic acid on the

catalytic Cys-494. The second step consists in the regeneration of Cys-494 via formation of a disulfide bridge between Cys-494 and Cys-439 followed by reduction of the disulfide bridge by thioredoxin. Cys-494 is almost conserved, whereas Cys-439 is only conserved in 50% of the MsrB putative sequences. This argues for the generality of the sulfenic acid mechanism described in the present study but at the same time raises the question of whether alternative mechanisms in Cys-494 regeneration are functioning.

PILB-MsrB recognizes only the *R* isomer of the sulfoxide function, whereas PILB-MsrA recognizes the *S* isomer. These differences in stereospecificities are of particular importance *in vivo*, since they allow the complete reduction of Met-*R,S*-SO back to methionine. It is also shown that 1) the presence of the sulfoxide function is a prerequisite for binding to both Msrs and 2) the catalytic efficiency of PILB-MsrB is significantly lower than that of PILB-MsrA. The results also suggest that protein-bound L-Met-SO is a better substrate than free L-MetSO, in particular for MsrB. Several results from the present study argue for a structure of the active site of MsrBs different from that of MsrAs. In this context, the Cys-494 that is situated in the signature RYC(I/V/M)N and is oxidized into a sulfenic acid during the catalytic event is probably not located in an environment similar to that of Cys-51 of *E. coli* MsrA. Thus, knowledge of the three-dimensional structure of a MsrB combined with site-directed mutageneses will be useful for characterizing its active site, in particular the amino acids involved in the stereospecificity and catalysis.

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**Characterization of the Methionine Sulfoxide Reductase Activities of PILB, a Probable Virulence Factor from *Neisseria meningitidis***  
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