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# Kinetic Characterization of the Chemical Steps Involved in the Catalytic Mechanism of Methionine Sulfoxide Reductase A from *Neisseria meningitidis*\*

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**Oxidation of methionine into methionine sulfoxide is associated with many pathologies and is described to exert regulatory effects on protein functions. Two classes of methionine sulfoxide reductases, called MsrA and MsrB, have been described to reduce the S and the R isomers of the sulfoxide of methionine sulfoxide back to methionine, respectively. Although MsrAs and MsrBs display quite different x-ray structures, they share a similar, new catalytic mechanism that proceeds via the sulfenic acid chemistry and that includes at least three chemical steps with 1) the formation of a sulfenic acid intermediate and the concomitant release of methionine; 2) the formation of an intra-disulfide bond; and 3) the reduction of the disulfide bond by thioredoxin. In the present study, it is shown that for the *Neisseria meningitidis* MsrA, 1) the rate-limiting step is associated with the reduction of the Cys-51/Cys-198 disulfide MsrA bond by thioredoxin; 2) the formation of the sulfenic acid intermediate is very efficient, thus suggesting catalytic assistance via amino acids of the active site; 3) the rate-determining step in the formation of the Cys-51/Cys-198 disulfide bond is that leading to the formation of the sulfenic intermediate on Cys-51; and 4) the apparent affinity constant for methionine sulfoxide in the methionine sulfoxide reductase step is 80-fold higher than the  $K_m$  value determined under steady-state conditions.**

Methionine residues are easily oxidized to methionine sulfoxides (MetSO)<sup>1</sup> by reactive oxygen and nitrogen species produced either by aerobic metabolism or after exposure to various agents. This post-translation modification can provoke loss of protein function (1) and has been implicated in the aging process (for a review, see Ref. 2). Therefore, organisms have developed various defense strategies. One consists of restoring the biological function of the modified MetSO proteins. This is the role of methionine sulfoxide reductases (Msr), which reduce MetSO back to methionine (Met). There exist two classes of

MsrAs, called MsrA and MsrB. The physiological role of MsrAs has been particularly well illustrated. For instance, mice lacking MsrA show an atypical walking pattern and have a reduced life span (3), whereas in contrast, overexpression of the *msrA* gene in the nervous system markedly extends the life span of the fruit fly *Drosophila* (4).

MsrAs and MsrBs have been extensively characterized at both structural (5–9) and enzymatic levels (10–14). Although MsrAs from *Escherichia coli*, *Bos taurus*, and *Mycobacterium tuberculosis* and MsrB from *Neisseria gonorrhoeae* show quite different three-dimensional x-ray structures and are specific for the S and the R isomers of the sulfoxide of MetSO, respectively, both structural classes of Msrs present a similar, new catalytic mechanism that includes a minimum of three chemical steps (Scheme 1). This is the case for MsrA and MsrB from *Neisseria meningitidis* (13). In the first step, a Michaelis complex is formed between Msr and MetSO that precedes the nucleophilic attack of the catalytic cysteine on the sulfoxide of MetSO. Then, a 1,3-sigmatropic rearrangement of the tetrahedral transition state probably occurs, which leads to formation of a sulfenic acid intermediate with a concomitant release of 1 mol of Met/mol of Msr. In the second step, an intradisulfide bond is formed between the cysteine oxidized under sulfenic acid state and another cysteine. Finally, in the third step, a Michaelis complex is formed between the oxidized Msr and the reduced form of thioredoxin (Trx) that precedes the reduction of the disulfide bond of the Msr by Trx. Such a mechanism is in agreement with the ping-pong mechanism that was recently determined for the MsrA from *E. coli* (11) and for the MsrB from *N. meningitidis*.<sup>2</sup>

No information is presently available for any of the two classes of Msrs on the nature of the amino acids essential for the catalysis or on the substrate stereo specificities. Such studies require first determining the rates of all the steps implicated in the mechanism to interpret the effects of the amino acid substitutions introduced by site-directed mutagenesis. As mentioned above, a sulfenic acid intermediate is formed in the first step. However, no information is available on the nature of the transition state(s) that leads to the sulfenic acid intermediate and on the efficiency of the reduction of MetSO. Furthermore, we do not know how efficient the formation of the intradisulfide bond is. In addition, finally, no kinetic data are available for the Trx reduction process.

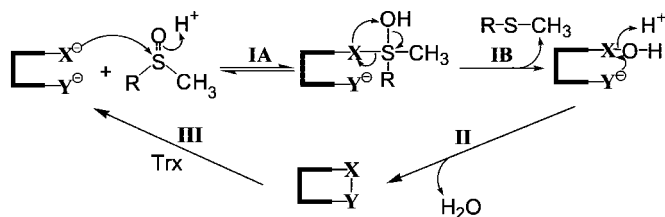
In the present study, the rates of the three steps have been investigated for the MsrA from *N. meningitidis*. To attain these rates, adapted experimental kinetics approaches were used. The data show that the step associated with the Trx reduction process is rate-limiting, whereas the rate of the sulfoxide re-

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<sup>1</sup> The abbreviations used are: MetSO, methionine sulfoxide; DTNB, 5,5'-dithiobis(2-nitro)benzoate; MES, 2(*N*-morpholino)ethanesulfonic acid; Msr, methionine sulfoxide reductase; MsrA, methionine sulfoxide reductase A; MsrB, methionine sulfoxide reductase B; Trx, thioredoxin.

<sup>2</sup> A. Olry, unpublished results.



**SCHEME 1. Proposed catalytic mechanism of MsrA and MsrB from *N. meningitidis*.** A Michaelis complex is formed between Msr and MetSO, which precedes the nucleophilic attack of Cys-X on the sulfur atom of the sulfoxide function of MetSO (step IA). Then, a 1,3-sigmatropic rearrangement of the tetrahedral transition state probably occurs, which led to formation of a sulfenic acid intermediate with a concomitant release of 1 mol of Met/mol of enzyme (step IB). Steps IA and IB are favored by acid-assisted catalysis. In step II, nucleophilic attack of Cys-Y on the sulfur atom of the sulfenic acid leads to formation of a Cys-X/Cys-Y disulfide bond and a release of a water molecule, which is also favored by acid-assisted catalysis. In step III, return of the active site to a fully reduced state proceeds via reduction of the Msr disulfide bond by reduced Trx.

duction is very rapid and is rate-determining in the process leading to the formation of the intradisulfide bond. These results are discussed in relation with the three-dimensional structures of MsrAs described so far.

#### EXPERIMENTAL PROCEDURES

**Site-directed Mutagenesis, Production, and Purification of Wild-type and Mutant *N. meningitidis* MsrAs**—The *E. coli* strain used for all *N. meningitidis* MsrA productions was BE002 (MG1655 msrA::spec $\Omega$ , msrB:: $\alpha$  3kana), transformed with the plasmidic construction pSKPILBmsrA containing the coding sequence of *msrA* under the *lac* promoter (13). The BE002 strain was kindly provided by Dr F. Barras. Its use prevented expression of endogenous wild-type MsrA and MsrB from *E. coli* and thus avoided any contamination of the activity of the *N. meningitidis* MsrA by the Msrs from *E. coli*. Site-directed mutageneses were performed using the QuikChange site-directed mutagenesis kit (Stratagene).

Purifications were realized as described previously (13) with minor modifications. A Q-Sepharose column was done at 4 °C, and wild-type and mutant proteins were eluted at 80 mM KCl. At this stage, wild-type and mutant MsrAs were pure, as checked by electrophoresis on 12.5% SDS-PAGE gel followed by Coomassie Brilliant Blue R-250 staining and by electrospray mass spectrometry analyses.

Storage of the enzymes was done as described previously (13). The molecular concentration was determined spectrophotometrically, using extinction coefficient at 280 nm of 26,200 M<sup>-1</sup> cm<sup>-1</sup> for wild-type and C198S MsrA (13) and 20,480 M<sup>-1</sup> cm<sup>-1</sup> for W53F MsrA. In this report, *N. meningitidis* MsrA amino acid numbering is based on *E. coli* MsrA.

**Quantification of the Free Cysteine Content with 5,5'-Dithiobis(2-nitro)benzoate (DTNB)**—Cysteine content of MsrA and Trx was determined using DTNB under nonreducing conditions in buffer A (50 mM Tris-HCl, 2 mM EDTA, pH 8) as described previously (10).

**Preparation of MsrA and Trx, under Oxidized Disulfide State**—MsrA oxidation was achieved by mixing 100  $\mu$ M MsrA with 100 mM MetSO in buffer A. The MetSO used was DL-Met-R,S-SO, of which only the S isomer is a substrate for MsrA. For Trx, oxidation was achieved by mixing 500  $\mu$ M Trx with 1 mM DTNB in buffer A. After 10 min of incubation at room temperature, oxidized proteins were passed through an Econo-Pac 10 DG desalting column (Bio-Rad) equilibrated with buffer A. Oxidation of both proteins in the disulfide state was checked by titration with DTNB.

**Fluorescence Properties of Wild-type and Mutated MsrAs and Trx**—The fluorescence characteristics of 1) the wild-type MsrA in its reduced form and Cys-51/Cys-198 disulfide state, 2) the C198S MsrA in its reduced form and sulfenic acid state, 3) the W53F MsrA in its reduced form and Cys-51/Cys-198 disulfide state, and 4) the Trx in its reduced and disulfide state were recorded on an flx spectrofluorometer (SAFAS) thermostated at 25 °C, in buffer A with 10  $\mu$ M of each protein (see the legends of Figs. 1 and 2 for more details).

**Determination of the Rate of Met Formation and of Thiol Loss by Single Turnover Quenched-flow Experiments**—Quenched-flow measurements were carried out at 25 °C on a SX18MV-R stopped-flow apparatus (Applied PhotoPhysics) fitted for the double-mixing mode and adapted to recover the quenched samples. The apparatus worked in a

pulsed mode. Under the conditions used, a minimum aging time of about 25–40 ms was determined. Equal volumes (57.5  $\mu$ l) of a solution containing 550  $\mu$ M MsrA in 50 mM MES, pH 5.5, or buffer A and a solution containing 200 mM MetSO in 50 mM MES, pH 5.5, or buffer A were mixed in the aging loop. The mixture was then allowed to react for 40–500 ms before being mixed with 115  $\mu$ l of a quenching aqueous solution containing 2% of trifluoroacetic acid. Quenched samples were then collected in a 200- $\mu$ l loop. For each aging time, four shots were done, and the four corresponding quenched samples were pooled in a volume of 700  $\mu$ l and then analyzed.

To precipitate the protein, 50  $\mu$ l of 100% trifluoroacetic acid were added to aliquots of 300  $\mu$ l of the quenched samples. Samples were centrifuged at 12,000  $\times$  g for 30 min at room temperature. Met quantification in the resulting supernatant was carried out by reverse-phase chromatography as described previously in Ref. 10, with slight modifications: 100  $\mu$ l were injected onto a 4.6  $\times$  250 mm Atlantis dC18 reverse-phase column (Waters) on an AKTA explorer system (Amersham Biosciences), equilibrated with H<sub>2</sub>O, 7.5% acetonitrile, 0.1% trifluoroacetic acid. Met was eluted isocratically after MetSO.

The other part of the quenched samples that were not treated with 100% of trifluoroacetic acid was used to 1) determine the protein concentration from the absorbance at 280 nm and 2) quantify the free cysteine content, using 2,2'-dipyridyldisulfide as a thiol probe, in the presence of urea to avoid precipitation of the protein in the cuvette. Progress curves of pyridine-2-thione production were recorded at 343 nm in 1.1 M urea, buffer A. Enzyme concentration was 6.19  $\mu$ M, and 2,2'-dipyridyldisulfide concentration was 665  $\mu$ M. The amount of pyridine-2-thione formed was calculated using an extinction coefficient at 343 nm of 8,080 M<sup>-1</sup> cm<sup>-1</sup>.

Data were plotted as mol of Met formed per mol of MsrA and as free remaining thiols per mol of MsrA, both as a function of time. The rate of Met formation was determined by fitting the curve to the monoexponential equation (Equation 1) in which  $a$  represents the fraction of Met formed per mol of MsrA and  $k_{\text{obs}}$  represents the rate constant.

$$y = a(1 - e^{-k_{\text{obs}}t}) \quad (\text{Eq. 1})$$

The rate of loss in free thiols was determined by fitting the curve to the monoexponential equation (Equation 2) in which  $y_0$  represents the number of free remaining thiols,  $a$  represents the number of oxidized thiols, and  $k_{\text{obs}}$  represents the rate constant.

$$y = y_0 + a e^{-k_{\text{obs}}t} \quad (\text{Eq. 2})$$

**Kinetics of the Formation of the Cys-51/Cys-198 MsrA Disulfide Bond in the Absence of Trx by Single Turnover Stopped-flow Experiment**—Kinetics of the MsrA fluorescence increase associated with the formation of the Cys-51/Cys-198 disulfide bond were measured at 25 °C on a SX18MV-R stopped-flow apparatus (Applied PhotoPhysics) fitted for fluorescence measurements. The excitation wavelength was set at 284 nm, and the emitted light was collected using a 320-nm cutoff filter. One syringe contained MsrA in buffer A (10  $\mu$ M, final concentration after mixing), and the other one contained MetSO at various concentrations in buffer A (25 mM to 1 M, final concentration). An average of six runs was recorded for each MetSO concentration. Rate constants,  $k_{\text{obs}}$ , were obtained by fitting fluorescence traces with the monoexponential equation (Equation 3) in which  $c$  represents the end point,  $a$  represents the amplitude of the fluorescence increase (<0), and  $k_{\text{obs}}$  represents the rate constant.

$$y = a e^{-k_{\text{obs}}t} + c \quad (\text{Eq. 3})$$

Data were fit to Equation 4 using least squares analysis to determine  $k_{\text{obs max}}$  and  $K$  for MetSO.  $S$  represents the MetSO concentration, and  $K$  represents the apparent affinity constant.

$$k_{\text{obs}} = k_{\text{obs max}} S/K + S \quad (\text{Eq. 4})$$

**Kinetics of the Reduction of the Cys-51/Cys-198 MsrA Disulfide Bond by Trx by Single Turnover Stopped-flow Experiments**—Kinetics measurements of the Trx fluorescence quenching associated with the formation of the Cys-32/Cys-35 disulfide bond upon the reduction of the oxidized MsrA were carried out as described above. The excitation wavelength was set at 310 nm, and the emitted light was collected using a 320-nm cutoff filter. One syringe contained the oxidized MsrA in buffer A (25  $\mu$ M, final concentration), and the other one contained the reduced Trx at various concentrations in buffer A (from 20 to 800  $\mu$ M, final concentration). An average of six runs was recorded for each concentration of Trx. Rate constants,  $k_{\text{obs}}$ , were obtained by fitting fluorescence traces with the monoexponential Equation 3 with  $a > 0$ .

**Determination of  $k_{cat}$  and  $K_m$  for Trx under Steady-state Conditions—**Kinetic parameters were determined in steady-state conditions with the Trx reductase recycling system (*E. coli* Trx reductase (4.8  $\mu\text{M}$ ), NADPH (1.2 mM), saturating concentration of MetSO (100 mM)) and by varying the concentrations of Trx. Initial rate measurements were carried out at 25 °C in buffer A on a Kontron Uvikon 933 spectrophotometer by following the decrease of the absorbance at 340 nm due to the oxidation of NADPH. Initial rate data were fitted to the Michaelis-Menten relationship using least squares analysis to determine  $k_{cat}$  and  $K_m$  for Trx. *E. coli* Trx1 and Trx reductase were prepared following experimental procedures already published (15, 16).

## RESULTS AND DISCUSSION

MsrA from *N. meningitidis* possesses two cysteines at position 51 and 198 (13). As mentioned in the Introduction, the catalytic mechanism of the MsrA from *N. meningitidis* involves three chemical steps: a) the reduction of MetSO, which leads to the formation of a sulfenic acid intermediate on Cys-51 and a concomitant release of 1 mol of Met/mol of enzyme; b) the formation of an intradisulfide bond between Cys-51 and Cys-198; and c) the reduction of the disulfide bond by Trx, which finally liberates MsrA and Trx under reduced and oxidized forms, respectively.

To investigate the rates of all these steps thus implied setting up experimental approaches that selectively apprehended each rate. For the formation of the sulfenic acid intermediate, the rate was determined by following that of the Met formation under single turnover conditions, *i.e.* in the absence of Trx. For the formation of the Cys-51/Cys-198 disulfide bond, two methods were used, also under single turnover conditions. The first one took advantage of the observation that the fluorescence emission intensity of MsrA increased upon going from the reduced to the oxidized disulfide forms. The second one used a specific thiol reagent to follow the rate of the oxidation of Cys-51 and Cys-198, which was shown to occur concomitantly. The third step was apprehended by following the rate of the reduction of the Cys-51/Cys-198 disulfide bond by Trx under single turnover conditions, using as a probe the quenching of the Trx fluorescence intensity upon going from reduced to oxidized forms. Finally, the catalytic constants of MsrA were determined for the MetSO reductase step and the Trx-reducing step as well, under conditions in which the concentrations of the substrates were nearly saturating.

**Characterization of the Fluorescence Properties of the MsrA and Trx under Reduced and Disulfide Oxidized Forms—**MsrA, under reduced form, presented a maximum excitation wavelength at 284 nm and a fluorescence emission maximum at 302 nm with a shoulder around 340 nm (Fig. 1). This suggested a contribution of both tyrosine and tryptophan residues to the fluorescence signal when excitation was set at 284 nm. In MsrA from *N. meningitidis*, there are 16 tyrosines and 1 tryptophan at position 53. When excitation was done at 295 nm, a fluorescence emission maximum was observed at 340 nm (Fig. 1). This suggested the contribution of only Trp-53 at this excitation wavelength. This was confirmed by the fact that W53F MsrA did not exhibit any fluorescence emission at 340 nm when the excitation was done at 295 nm (spectrum not shown). When excited at 295 nm, oxidized MsrA in the Cys-51/Cys-198 disulfide state also showed a fluorescence emission maximum at 340 nm but with a fluorescence increase of 85% (integrated from 320 to 450 nm) when compared with that of the reduced form (Fig. 1). Inspection of the x-ray structure shows that Trp-53 is situated in an  $\alpha$  helix near the catalytic Cys-51 (5). Therefore, the formation of the Cys-51/Cys-198 disulfide bond, which is located near Trp-53, is likely responsible for the increase of the fluorescence emission intensity at 340 nm.

As already shown by others, Trx1 from *E. coli* exhibited a fluorescence emission maximum at 342 nm when excited at 280

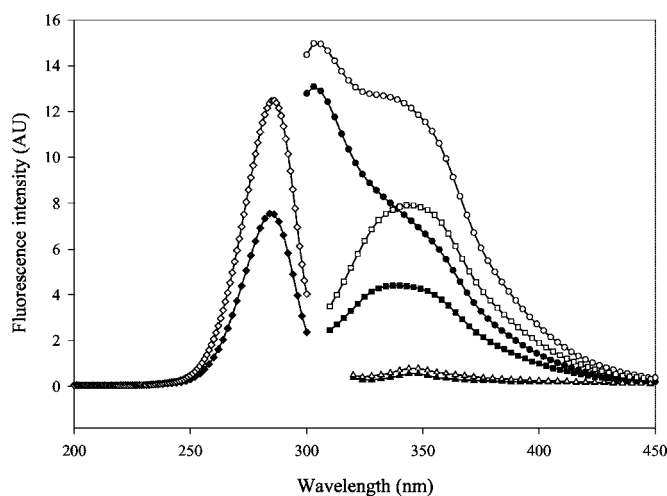
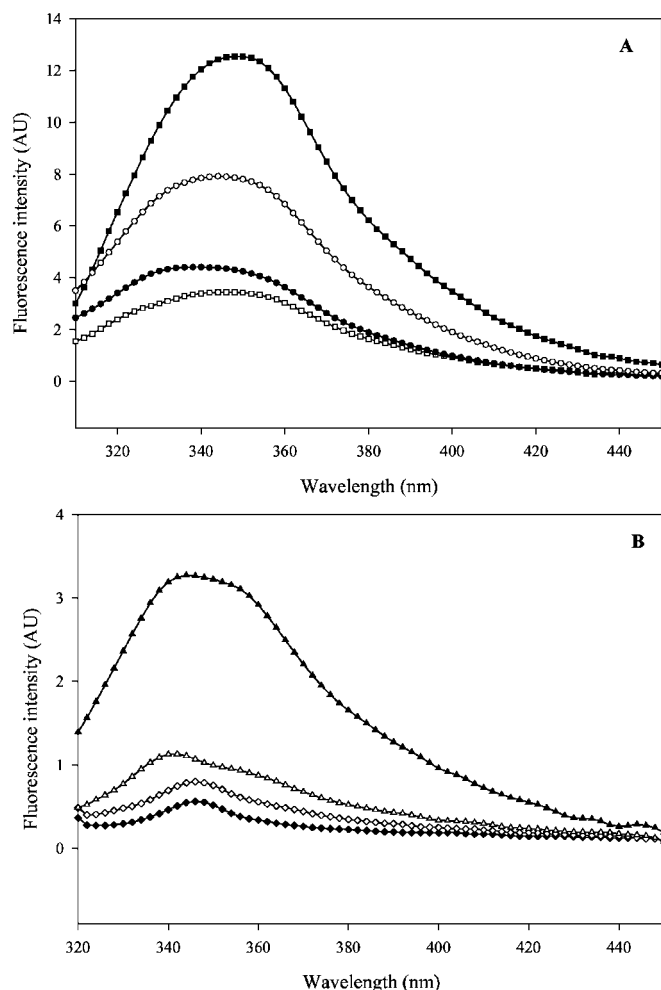


FIG. 1. **Fluorescence spectra of *N. meningitidis* MsrA under reduced and disulfide oxidized state.** Excitation spectra of reduced ( $\blacklozenge$ ) and oxidized ( $\diamond$ ) MsrA (emission followed at 340 nm), emission spectra of reduced ( $\bullet$ ) and oxidized ( $\circ$ ) MsrA (excitation at 284 nm), reduced ( $\blacksquare$ ) and oxidized ( $\square$ ) MsrA (excitation at 295 nm), and reduced ( $\blacktriangle$ ) and oxidized ( $\triangle$ ) MsrA (excitation at 310 nm) were recorded with 10  $\mu\text{M}$  protein at 25 °C in buffer A.

nm (17). Two tryptophans are present in the *E. coli* enzyme at positions 28 and 31, and it was shown that Trp-28 is mainly responsible for the strong quenching of the fluorescence emission intensity observed when Trx is oxidized in the Cys-32/Cys-35 disulfide state (17–19). Therefore, following the rate associated with the formation of the Cys-32/Cys-35 Trx disulfide bond on a stopped-flow apparatus equipped for fluorescence measurements can give information on the nature of the rate-limiting step in the Trx-reducing process provided that the fluorescence signal of the Msr does not interfere. As mentioned above, the reduction of the Cys-51/Cys-198 MsrA bond also leads to a quenching of the fluorescence emission intensity at 340 nm. However, as shown in Fig. 2, when the excitation was done at 295 nm, the contribution of MsrA to the quenching of the fluorescence signal is small when compared with that of Trx. It is even insignificant when the excitation was done at 310 nm and when considering the ratio of the fluorescence emission intensity of both partners, integrated from 320 to 450 nm (Fig. 2).

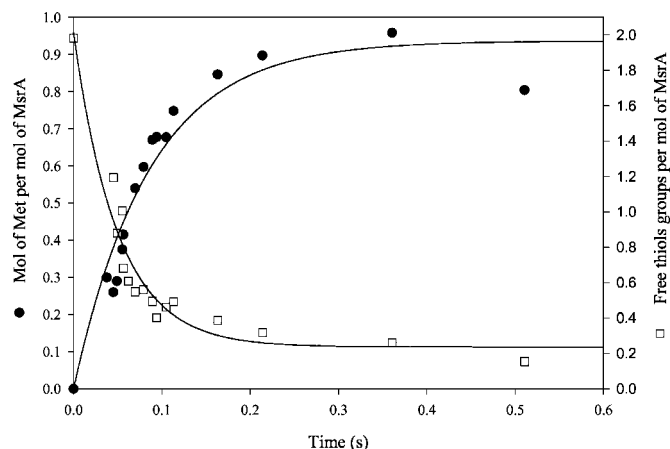
**Rate of the Met Formation—**A means to determine the rate of the formation of the sulfenic acid intermediate was to attain that of the Met, which is formed concomitantly. To do so, the stopped-flow apparatus was adapted to be used in a quenched-flow mode. At various times of incubation of MsrA with MetSO, in the absence of Trx, the reaction mixture was quenched by mixing it with trifluoroacetic acid. Met was then quantified by reverse-phase chromatography analysis (see “Experimental Procedures” for more details). The study was done at 100 mM MetSO, a concentration that is known to be saturating under steady-state conditions (13). At pH 8, which is the optimum pH determined under steady-state conditions (data not shown), a Met burst was observed with a stoichiometry of 0.9 mol of Met/mol of MsrA. However, the rate of the Met formation was too fast, at least 100  $\text{s}^{-1}$ , to be determined with the apparatus adapted for quenched-flow experiments, which gave a minimum aging time around 30 ms. In contrast, at pH 5.5, the curve profile fitted to the monoexponential Equation 1 with a  $k_{obs}$  value of  $12 \pm 1 \text{ s}^{-1}$  and a stoichiometry of  $0.9 \pm 0.1 \text{ mol of Met/mol of MsrA}$  (Fig. 3). Under steady-state conditions, at 200  $\mu\text{M}$  Trx and 100 mM MetSO,  $k_{cat}$  values were 7 and 0.6  $\text{s}^{-1}$  at pH 8 and 5.5, respectively. Therefore, the data demonstrated that the rate-limiting step in MsrA occurs after the formation of the



**FIG. 2. Fluorescence emission spectra of *E. coli* thioredoxin under reduced and oxidized state and comparison with those of *N. meningitidis* MsrA under reduced and oxidized state.** In A, fluorescence spectra of 10  $\mu\text{M}$  reduced (■) and oxidized (□) Trx and reduced (●) and oxidized (○) MsrA in buffer A were recorded at 25 °C on excitation at 295 nm. In B, fluorescence spectra of 10  $\mu\text{M}$  reduced (▲) and oxidized (△) Trx and reduced (◆) and oxidized (◇) MsrA in buffer A were recorded at 25 °C on excitation at 310 nm.

sulfenic acid intermediate and is associated with either the Cys-51/Cys-198 disulfide bond formation or the Trx reduction process.

**Rate of the Formation of Cys-51/Cys-198 MsrA Disulfide Bond**—First, the rate of formation of the Cys-51/Cys-198 disulfide bond was determined at 100 mM MetSO in the absence of Trx by following the increase of the MsrA fluorescence emission intensity due to the formation of the Cys-51/Cys-198 disulfide bond. Two pH conditions were investigated. At pH 8 and 5.5, a fast increase of the fluorescence signal was observed. The curves fitted to the monoexponential Equation 3 with  $k_{\text{obs}}$  values of  $144 \pm 1 \text{ s}^{-1}$  and  $11 \pm 1 \text{ s}^{-1}$ , respectively (Fig. 4). The  $k_{\text{obs}}$  value at pH 5.5 is similar to that of the Met formation at the same pH. Therefore, this strongly suggested that the step leading to the formation of Met, and thus of the sulfenic acid intermediate, is rate-determining in the formation of the Cys-51/Cys-198 disulfide bond. In other words, the rate of the nucleophilic attack of Cys-198 on the Cys-51 sulfenic intermediate is limited by that determining the formation of the sulfenic acid intermediate, which as a consequence does not accumulate. Therefore, even if a change in the fluorescence emission intensity of the MsrA occurs upon the formation of the sulfenic acid, this should not interfere with the increase of the fluorescence



**FIG. 3. Time-resolved appearance of Met and disappearance of free thiols of *N. meningitidis* MsrA under single turnover kinetics.** A quenched-flow experiment was carried out at 25 °C in 50 mM MES, pH 5.5. Briefly, reduced MsrA (275  $\mu\text{M}$ , final concentration) was allowed to react with MetSO (100 mM, final concentration) in the aging loop of the stopped-flow apparatus before being mixed with a 2% trifluoroacetic acid aqueous solution (1% final concentration). After recovery, quenched samples were used for Met quantification and free thiols titration as well (see “Experimental Procedures” for more details). Symbols represent experimental points (● for Met, □ for free thiols). Data were fitted to Equations 1 and 2, respectively, and gave rate constants of  $11 \pm 1$  and  $20 \pm 2 \text{ s}^{-1}$ , respectively.

signal observed upon the formation of the Cys-51/Cys-198 disulfide bond. To confirm this interpretation, C198S MsrA was produced. In this mutated protein, the Cys-51 sulfenic acid intermediate accumulates (13). Its formation led to a quenching of the fluorescence signal of Trp-53 (data not shown) in contrast to the increase observed when following the formation of the Cys-51/Cys-198 disulfide bond in wild-type MsrA. Thus, the fact that only an increase of the fluorescence signal was observed in the wild-type enzyme again supports the nonaccumulation of the sulfenic acid intermediate in the wild-type enzyme. The fact that the  $k_{\text{obs}}$  value at pH 8 is  $144 \text{ s}^{-1}$  confirms that the  $k_{\text{obs}}$  value of the Met formation could not be attainable in the previous quenched-flow experiment with the stopped-flow apparatus.

A second approach for determining the rate of the formation of the Cys-51/Cys-198 disulfide bond was to follow the loss of the two free thiol groups. This analysis was done with the samples obtained from the quenched-flow experiments described above. As suggested above, as soon as Cys-51 is oxidized into sulfenic acid form, Cys-198 forms a disulfide bond with Cys-51. Consequently, the enzyme should pass directly from the reduced state form with two free thiols to the oxidized Cys-51/Cys-198 disulfide form with no more free thiols. At pH 5.5 and using 100 mM MetSO, the curve profile fitted to the monoexponential Equation 2, with the loss of two cysteines and a  $k_{\text{obs}}$  value of  $20 \pm 2 \text{ s}^{-1}$  (Fig. 3). The fact that the  $k_{\text{obs}}$  value is in the range of that found for the Met formation again supports a rate-determining step associated with the formation of the sulfenic acid intermediate and the nonaccumulation of the sulfenic acid intermediate.

**Rate of the Reduction of Cys-51/Cys-198 MsrA Bond by Trx**—As already mentioned, the rate of reduction of the Cys-51/Cys-198 MsrA disulfide bond was determined on a stopped-flow apparatus by following selectively the quenching of the fluorescence emission intensity of Trx upon going from the reduced to an oxidized form, *i.e.* Cys-32/Cys-35 intradisulfide state or MsrA Cys-198/Trx Cys-32 interdisulfide form. Under the experimental conditions used, at pH 8, the concentration of the Cys-51/Cys-198 disulfide MsrA was 25  $\mu\text{M}$ , and the concentration of the reduced Trx was varied from 75  $\mu\text{M}$  to up 800  $\mu\text{M}$ .

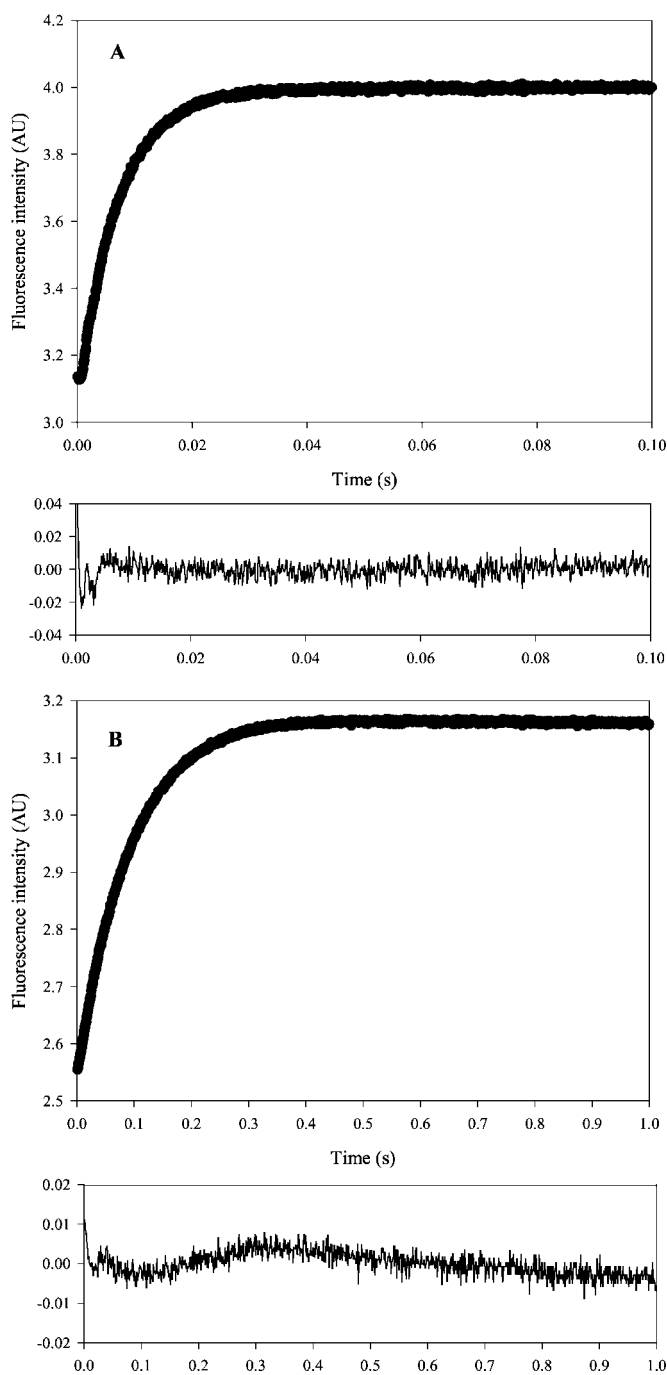


FIG. 4. Rate of formation of the Cys-51/Cys-198 MsrA disulfide bond measured by fluorescence stopped-flow experiment under single turnover kinetics. The MsrA fluorescence increase associated with the disulfide bond formation was recorded on a stopped-flow apparatus at 25 °C in buffer A (A) or in 50 mM MES, pH 5.5 (B). In both experiments, MsrA and MetSO final concentrations were 10  $\mu\text{M}$  and 100 mM, respectively. Excitation wavelength was set at 284 nm, and emitted light was collected via a 320 nm cutoff filter. The upper frames show the recorded fluorescence trace, and the lower frames show the residual values of the best monoexponential fit. Rate constants were  $144 \pm 1$  and  $11 \pm 1 \text{ s}^{-1}$  in buffer A and in MES buffer, pH 5.5, respectively.

For each concentration of Trx, a  $k_{\text{obs}}$  value was determined. At a concentration of 200  $\mu\text{M}$  Trx, a  $k_{\text{obs}}$  value of  $9.5 \text{ s}^{-1}$  was obtained (Fig. 5). This value is in the range of the rate value of  $7 \text{ s}^{-1}$  found under steady-state conditions, *i.e.* 200  $\mu\text{M}$  Trx and 100 mM MetSO, and under conditions in which the process of Trx reduction by thioredoxin reductase and NADPH was shown not to be rate-limiting. These results demonstrated that

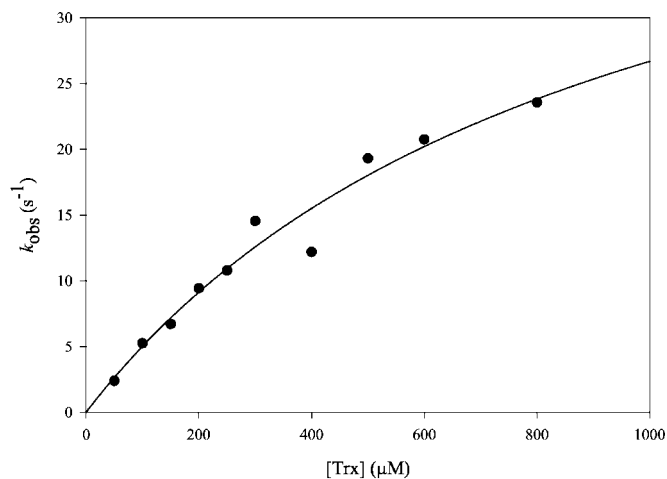


FIG. 5. Rate of reduction of the Cys-51/Cys-198 *N. meningitidis* MsrA disulfide bond by *E. coli* Trx measured by fluorescence stopped-flow experiment. The Trx fluorescence quenching associated with Trx disulfide bond formation was recorded on a stopped-flow apparatus at 25 °C in buffer A by mixing reduced Trx and oxidized MsrA. MsrA final concentration was 25  $\mu\text{M}$ . Excitation wavelength was set at 310 nm, and emitted light was collected via a 320 nm cutoff filter. Data were then fitted to the Michaelis-Menten relationship by least squares regression, which gave  $k_{\text{obs max}}$  and  $K_m$  values of  $50 \pm 10 \text{ s}^{-1}$  and  $930 \pm 300 \mu\text{M}$ , respectively. Symbols (●) represent experimental points.

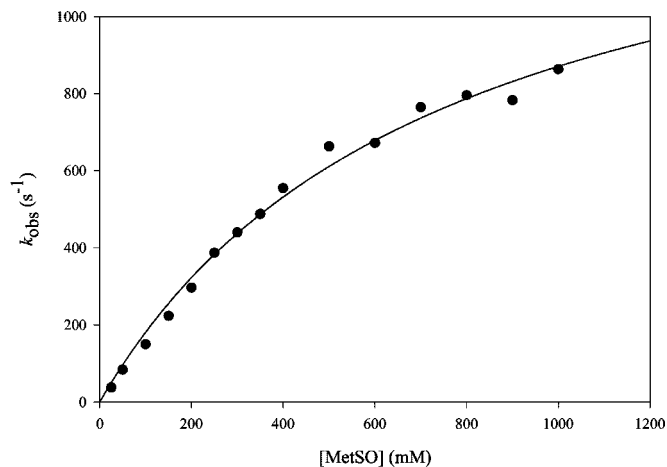


FIG. 6. Determination of the catalytic parameters of the MetSO reductase step of the *N. meningitidis* MsrA. The MsrA fluorescence increase associated with the Cys-51/Cys-198 disulfide bond formation was recorded on a stopped-flow apparatus at 25 °C in buffer A. MsrA final concentration was 10  $\mu\text{M}$ . Excitation wavelength was set at 284 nm, and emitted light was collected via a 320-nm cutoff filter. Data were then fitted to Equation 4 by least squares regression which gave  $k_{\text{obs max}}$  and apparent affinity constant values of  $1510 \pm 90 \text{ s}^{-1}$  and  $740 \pm 80 \text{ mM}$ , respectively. Symbols (●) represent experimental points.

the rate-limiting step of MsrA is associated with the Trx reduction process. Furthermore, we could conclude that the rate-limiting step is prior to the dissociation of oxidized Trx from reduced MsrA, a step that occurs after the chemical process. In this context, the rate-limiting step can be associated either with the formation of the Michaelis complex between the oxidized MsrA and the reduced Trx or with the chemical processes associated with either the formation of the interdisulfide bond between Cys-198 of the MsrA and Cys-32 of Trx or the nucleophilic attack of Cys-35 of Trx on Cys-32 engaged in an interdisulfide bond with the Cys-198 of MsrA. Although additional experiments are needed, it is probable that the formation of the Cys-32/Cys-35 Trx intradisulfide bond is efficient, similar to what is observed for the formation of the Cys-51/Cys-198 MsrA

intradisulfide bond, and therefore is not rate-limiting.

The  $K_m$  Trx and  $k_{\text{obs max}}$  values cannot be determined with accuracy (Fig. 5). This is because when Trx is in a large excess when compared with MsrA, the fluorescence quenching message measured upon the formation of the oxidized Trx becomes small as compared with the fluorescence message of the remaining reduced Trx and, as a consequence, the quenching signal cannot be measured with great precision. Therefore, only an estimation of the  $K_m$  Trx and of the  $k_{\text{obs max}}$  values was made, *i.e.* 500–900  $\mu\text{M}$  and at least 30  $\text{s}^{-1}$ , respectively. The values of  $K_m$  for Trx and  $k_{\text{obs max}}$  are much higher than those described so far by our group and other groups, which were determined under steady-state conditions with the Trx reductase recycling system. This apparent discrepancy is probably due to the fact that under the steady-state conditions used, the rate of the Trx reductase process becomes rate-limiting at high concentrations of Trx.

**Rate of the Met Formation under Saturating MetSO Concentration**—The fact that the Trx reduction step is rate-limiting suggested that the  $K_m$  value for MetSO determined under steady-state conditions was much lower than the apparent affinity constant in the MetSO reductase step. Indeed, having a slow step following a fast catalytic one always lowers the  $K_m$  value for the substrate. Thus, the concentration of MetSO, *i.e.* 100 mM, which was used for determining the  $k_{\text{obs}}$  of the reductase activity, was probably not saturating. This is indeed the case. At pH 8, using the fluorescence signal increase observed upon the formation of the Cys-51/Cys-198 bond, in the absence of Trx, a  $k_{\text{obs}}$  value of 795  $\text{s}^{-1}$  was observed at 800 mM MetSO. From the curve, an apparent affinity constant value of 740 mM for MetSO with a  $k_{\text{obs max}}$  value of 1510  $\text{s}^{-1}$  can be estimated (Fig. 6). These values are 80- and 40-fold higher than those determined under steady-state conditions at 200  $\mu\text{M}$  Trx, respectively. As shown above, the rate of the formation of the sulfenic acid intermediate is rate-determining in the process leading to the formation of the Cys-51/Cys-198 disulfide bond. Therefore, the  $k_{\text{obs}}$  and affinity constant values correspond to those of the MetSO reductase step. In this context, it should be noted that the affinity constant values for the D and the L isomers of MetSO are similar (data not shown) and have to be divided by 2, taking into account the fact that the R isomer of the sulfoxide function of MetSO is neither a substrate nor an inhibitor of MsrA (13).

**Conclusion**—We have shown that 1) the rate-limiting step of the *N. meningitidis* MsrA reaction is associated with the thio-redoxin reduction process and 2) the formation of the sulfenic acid intermediate is rate-determining within the two-step processes leading to the formation of the intradisulfide Cys-51/Cys-198 intermediate. As already mentioned (11), three subclasses of MsrAs exist. The *N. meningitidis* enzyme, which is the prototype of the first subclass, contains two essential cysteines at positions 51 and 198. The second one, which is represented by *E. coli* and *B. taurus* MsrAs, possesses three essential cysteines at positions 51, 198, and 206. In this case, only the Cys-198/Cys-206 disulfide bond was shown to be reduced by Trx (10). In fact, the reduction of the Cys-51/Cys-198 bond probably does not occur because of its nonaccumulation. As soon as the Cys-51/Cys-198 disulfide intermediate is formed, Cys-206, which is located in a flexible region, attacks it and forms the Cys-198/Cys-206 disulfide bond, which is then reduced by Trx. The fact that in C206S *E. coli* MsrA, the Cys-51/Cys-198 disulfide bond can be reduced by Trx (data not shown) supports our interpretation. The third subclass, which is represented by the *Bacillus subtilis* MsrA, contains two essential cysteines at positions 51 and 54. Also, in this case, the rate-limiting step is associated with reduction by Trx (data not shown).

Under saturating concentrations of MetSO, at pH 8, the rates of formation of the sulfenic acid intermediate and of the Cys-51/Cys-198 disulfide bond are 1510  $\text{s}^{-1}$  and at least 1510  $\text{s}^{-1}$ , respectively. Thus, the chemical process that leads to the formation of the sulfenic acid is efficient. Cys-51 is probably activated by the active site, and the electrophilic character of the sulfur atom of the substrate sulfoxide and the rate of the 1,3-sigmatropic rearrangement of the tetrahedral transition state are likely increased via an acid catalyst belonging to the active site, which has been recently shown to be Glu-94 (data not shown). Both activations should favor the efficiency of the reductase process. Also, the rate of formation of the Cys-51/Cys-198 disulfide bond is fast. Therefore, the nucleophilic attack of Cys-198 on sulfenic acid Cys-51 is also efficient. Inspection of the three-dimensional structures of MsrAs solved so far and comparison of the primary structures of MsrAs show that Cys-198 is located in a flexible region with no secondary structural elements (5). The fact that 1) a sulfenic acid is chemically unstable; 2) the Cys-198, as part of a flexible region, can easily approach position 51; and 3) an acidic amino acid likely intervenes to favor the release of the water molecule from the Cys-51 oxidized in the sulfenic acid state can explain why the formation of the Cys-51/Cys-198 disulfide bond is so efficient. Studies are presently underway to characterize all the amino acids of the MsrA from *N. meningitidis*, which are involved not only in the chemical activation of the three steps but also in the substrate specificities.

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**Kinetic Characterization of the Chemical Steps Involved in the Catalytic Mechanism of Methionine Sulfoxide Reductase A from *Neisseria meningitidis***

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