Characterization of the Amino Acids from *Neisseria meningitidis* MsrA Involved in the Chemical Catalysis of the Methionine Sulfoxide Reduction Step*

Mathias Antoine¹, Adeline Gand¹, Sandrine Boschi-Muller, and Guy Branlant²

From the Maturation des ARN et Enzymologie Moléculaire, Unité Mixte de Recherche, CNRS-UHP 7567, Nancy Université, Faculté des Sciences et Techniques, Bld des Aiguillettes, BP 239, 54506 Vandoeuvre-les-Nancy, France

Methionine sulfoxide reductases (Msrs) are ubiquitous enzymes that reduce protein-bound methionine sulfoxide back to Met in the presence of thioredoxin. *In vivo*, the role of the Msrs is described as essential in protecting cells against oxidative damages and as playing a role in infection of cells by pathogenic bacteria. There exist two structurally unrelated classes of Msrs, called MsrA and MsrB, specific for the S and the R epimer of the sulfoxide function of methionine sulfoxide, respectively. Both Msrs present a similar catalytic mechanism, which implies, as a first step, a reductase step that leads to the formation of a sulfenic acid on the catalytic cysteine and a concomitant release of a mole of Met. The reductase step has been previously shown to be efficient and not rate-limiting. In the present study, the amino acids involved in the catalysis of the reductase step of the *Neisseria meningitidis* MsrA have been characterized. The invariant Glu-94 and to a lesser extent Tyr-82 and Tyr-134 are shown to play a major role in the stabilization of the sulfurate transition state and indirectly in the decrease of the pK_app of the catalytic Cys-51. A scenario of the reductase step is proposed in which the substrate binds to the active site with its sulfoxide function largely polarized via interactions with Glu-94, Tyr-82, and Tyr-134 and participates via the positive or partially positive charge borne by the sulfur of the sulfoxide in the stabilization of the catalytic Cys.

Methionine sulfoxide reductases (Msr)³ are enzymes that catalyze the reduction of free and protein-bound methionine sulfoxide (MetSO) back to Met. Two structurally unrelated classes of MsrS have been described so far. MsrAs are stereo specific toward the S isomer on the sulfoxide of the sulfoxide function, whereas MsrBs are specific toward the R isomer. Both classes share a similar three-step catalytic mechanism (Scheme 1). First, the reductase step leads to formation of a sulfenic acid intermediate on the catalytic cysteine concomitantly with the release of one mole of Met/mole of Msr. Then, an intra-disulfide bond is formed via the attack of a second Cys (called the recycling Cys) on the sulfenic acid intermediate accompanied by release of a water molecule. Finally, the disulfide bond is reduced by thioredoxin (Trx) in the last step. Recently, the kinetics of the three steps have been investigated for MsrA and MsrB domains of the PilB protein of *Neisseria meningitidis* (1, 2). For both classes of Msrs, the rate-limiting step is associated with the Trx recycling process, whereas the rate of formation of the intra-disulfide bond is governed by that of formation of the sulfenic acid intermediate, the rate of which is fast.

The three-dimensional structures of the MsrA from *Escherichia coli*, *Bos taurus*, and *Mycobacterium tuberculosis* have been recently solved by x-ray crystallography (3–5). The active site can be represented as an opened basin readily accessible to the MetSO substrate in which the catalytic Cys-51 is located at the entrance of the α helix. In all the structures, the active site is occupied by a molecule that is covalently or non-covalently bound to the catalytic cysteine. In the case of *E. coli* MsrA, a dimethyl arsenate molecule is covalently bound, whereas it is a dithiothreitol molecule in *B. taurus* enzyme. In the case of *M. tuberculosis* MsrA, a methionine residue from a neighboring monomer occupies the active site. In all three structures, a water molecule is present, the position of which can mimic the oxygen atom of the sulfoxide function of MetSO. This water molecule is tightly H-bonded to three invariant amino acid residues, *i.e.* Tyr-82, Glu-94, and Tyr-134. All the three structures also support the involvement of invariant Phe-52 and Trp-53 in the substrate recognition via the formation of a hydrophobic pocket in which the ε methyl group of MetSO can bind.

Study of the reduction mechanism of dimethyl sulfoxide (Me₂SO) by methanethiol in Me₂SO solution has recently been investigated by quantum chemistry calculations (6). It was shown that 1) a sulfurate species is formed prior to formation of either a sulfenic acid intermediate or a disulfide species and 2) the rate-limiting step is governed by proton transfer between the thiol and the sulfamide functions prior to sulfurate formation. Although these conclusions are derived from studies based on a model in solution, they provide a framework for the study of the chemical reductase step occurring within the MsrA active site.

In the present study, the role of Glu-94, Tyr-82, and Tyr-134 residues and how the catalytic Cys-51 is stabilized in the reductase step of the MsrA from *N. meningitidis* have been investi-
gated. For that, the kinetic parameters and the pH dependence of the rate constant of the reductase step of mutated MsrAs at positions 82, 94, and 134 were determined and compared with those of the wild type. The pK\textsubscript{a} of Cys-51 in the free enzyme was also determined. The results show that Cys-51 is activated upon substrate binding to the active site with a shift of its pK\textsubscript{a} from 9.5 to 5.7. Substitutions at positions 82, 94, and 134 do not modify the apparent affinity for the substrate in the reductase step. In contrast, drastic decrease of the reductase rate is observed for the E94A and Y82F/Y134F MsrAs, whereas E94Q MsrA displays only a small decrease. Moreover, each mutated MsrA is characterized by a shift of the pK\textsubscript{a} of its Cys-51 to higher values compared with wild type. Taking into account all the results, a scenario for the catalysis of the sulfoxide reductase step is proposed in which Glu-94, Tyr-82, and Tyr-134 stabilize the sulfurane transition state formed. In this scenario, the substrate binds to the active site with its sulfoxide function largely polarized via interactions with the side chains of Glu-94, Tyr-82, and Tyr-134 and plays a major role in stabilizing Cys-51 via the positive, or partially positive, charge borne by the sulfur of the sulfoxide function.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis, Production, and Purification of Wild-type and Mutated N. meningitidis MsrAs—**The E. coli strain used for all N. meningitidis MsrA productions was BE002 (MG1655 msrA::spec\textOmega, msrB::α3kana), transformed with the plasmidic construction pSKPILBMsrA containing only the coding sequence of msrA from pILB, under the lac promoter (7). 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 previously described (1). Wild-type and mutated 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 previously described. The molecular concentration was determined spectrophotometrically, using extinction coefficient at 280 nm of 26,200 M\textsuperscript{-1}cm\textsuperscript{-1} for wild-type and mutated MsrAs. In this report, N. meningitidis MsrA amino acid numbering is based on E. coli MsrA sequence.

**Quantification of the Free Cysteine Content with 5,5′-Dithiobis(2-nitro)benzoate—**Cysteine content of MsrA was routinely determined using 5,5′-dithiobis(2-nitro)benzoate under non-denaturing conditions in buffer A (50 mM Tris-HCl, 2 mM EDTA, pH 8) as previously described (8).

**pH Dependence of MsrA Thiol Reaction Rates with 2,2′-Dipyridyl Disulfide (2PDS)—**Because of the high reactivity of Cys-51 and Cys-198 in MsrA, fast kinetic measurements were carried out on an Applied PhotoPhysics SX18MV-R stopped-flow apparatus. MsrA reactions with 2PDS were performed at 25 °C under pseudo-first-order conditions in 30 mM acetic acid, 30 mM imidazole, 120 mM Tris/HCl buffer at constant ionic strength of 0.15 M over a pH range of 6 to 10 (polybuffer B). MsrA and 2PDS concentrations after mixing were 6.2 and 310 μM, respectively. The pseudo-first-order rate constant k\textsubscript{obs} was determined at each pH by fitting the absorbance (A) at 343 nm versus time (t) to a mono-exponential Equation 1, where a is the burst amplitude and c is the end point. The second-order rate constants k\textsubscript{2} were calculated by dividing k\textsubscript{obs} by 2PDS concentration and then fitted to Equation 2, in which k\textsubscript{max} represents the second rate constant for the thiolate form.

\[
A = a(1 - e^{-k_{10}t}) + c \quad \text{(Eq. 1)}
\]

\[
k_2 = \frac{k_{2\text{max}}}{1 + 10^{(pK_a - pH)}} \quad \text{(Eq. 2)}
\]

**Measurement of the Thiol Ionization by Ultraviolet Absorbance—**Absorbance spectra were measured for all enzymes in 1.0-cm path length quartz cuvettes in a SAFAS UV-visible absorbance spectrophotometer. The protein samples were diluted to 23 μM in polybuffer B. Spectra were recorded at 25 °C in 0.5-nm steps from 300 to 200 nm over a pH range of 7 to 10. The buffer solution was scanned relative to air, followed by a protein solution in the same cuvette versus air. The two spectra were then subtracted and the difference converted to molar absorption coefficients at 240 nm (ε\textsubscript{240 nm}). Data were fitted to a model derived from the Henderson-Hasselbach equation as shown in Equation 3 for one apparent pK\textsubscript{a}.

\[
\epsilon_{240\,\text{nm}} = \epsilon_{SH} + \frac{\epsilon_S}{1 + 10^{(pK_a - pH)}} \quad \text{(Eq. 3)}
\]

**Steady-state MsrA Kinetics in the Presence of the Trx Recycling System—**Steady-state kinetic parameters were determined with the Trx reductase recycling system (E. coli Trx (100 μM), E. coli Trx reductase (4.8 μM), NADPH (1.2 mM)) and by varying the concentrations of AcMetSONHMe. AcMetSONHMe was prepared and purified as previously described (2). Initial rate measurements were carried out at 25 °C in buffer A or polybuffer B 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\textsubscript{cat} and K\textsubscript{m} for AcMetSONHMe. E. coli Trx1 and 

---

**SCHEME 1. Schematic representation of the catalytic mechanism of MsrA and MsrB from N. meningitidis.** The mechanism consists of three steps. In step 1, called the reductase step, a sulfenic acid intermediate is formed on the catalytic Cys-X with a concomitant release of one mol of Met/mol of enzyme. In step II, a disulfide bond is formed between the Cys-X and the recycling Cys-Y with a release of a water molecule. In step III, return of the active site to a fully reduced state proceeds via reduction of the Mr sulfide bond by reduced Trx. RS\textsubscript{SH} and RSH\textsubscript{r} represent MetSO and Met, respectively. For N. meningitidis MsrA, Cys-X = Cys-51 and Cys-Y = Cys-198.
Catalytic Mechanism of MsrA Reductase Step

Trx reductase were prepared following experimental procedures already published (7).

Preparation of MsrA under Oxidized Disulfide State—MsrA oxidation was achieved by mixing 100 μM 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. 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 MsrA in the disulfide state was checked by titration with 5,5′-dithiobis(2-nitro)benzoate.

Fluorescence Properties of Wild-type and Mutated MsrAs—The fluorescence excitation and emission spectra of wild-type and mutated MsrAs in their reduced and Cys-51/Cys-198 disulfide state were recorded on a flx spectrofluorometer (SAFAS) thermostated at 25 °C in buffer A with 10 μM of each protein as previously described (1).

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 double mixing and adapted to recover the quenched samples as previously described (1). The apparatus worked in a pulsed mode. Under the conditions used, a minimum aging time of ~25–40 ms was determined. Equal volumes (57.5 μl) of a solution containing 550 μM Glu-94-mutated MsrA in buffer A and a solution containing AcMetSONHMe in buffer A were mixed in the aging loop. The mixture was then allowed to react for the desired time before being mixed with 115 μl of a quenched aqueous solution containing 2% of trifluoroacetic acid. Quenched samples were then collected in a 200-μl loop. For each aging time, four shots were done and the four corresponding quenched samples were pooled in a volume of 700 μl and then analyzed.

After protein precipitation and centrifugation, Ac-L-MetNHMe (AcMetNHMe) quantification in the resulting supernatant was carried out by reverse phase chromatography as previously described (2): 100 μl were injected onto a 4.6 × 250-mm Atlantis dC18 reverse phase column (Waters) on an AKTA explorer system (Amersham Biosciences) equilibrated with H2O/0.1% trifluoroacetic acid. AcMetNHMe was eluted after AcMetSONHMe with a linear gradient of acetonitrile.

The other part of the quenched samples that was 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 2PDS 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 μM, and 2PDS concentration was 665 μM. The amount of pyridine-2-thione formed was calculated using an extinction coefficient at 343 nm of 8,080 M−1 cm−1.

Data were plotted as mole of AcMetNHMe formed/mole of MsrA and as free remaining thiols/mole of MsrA, both as a function of time. The rate of Met formation was determined by fitting the curve to the monoexponential Equation 4 in which a represents the fraction of Met formed/mole of MsrA and kMet represents the rate constant.

\[ y = a(1 - e^{-k_{\text{Met}}t}) \]  

(Eq. 4)

The rate of loss in free thiols was determined by fitting the curve to the monoexponential Equation 5 in which y0 represents the number of free remaining thiols, a the number of oxidized thiols, and \( k_{\text{SS}} \) the rate constant.

\[ y = y_0 + ae^{-k_{\text{SS}}t} \]  

(Eq. 5)

Kinetics of the Formation of the Cys-51/Cys-198 MsrA Disulfide Bond in the Absence of Reductant by Single Turnover Stopped-flow Experiment at pH 8—Kinetics of the Trp-53 fluorescence variation associated with the formation of the Cys-51/Cys-198 disulfide bond were measured for E94Q, Y82F, and Y134F MsrAs at 25 °C on a SX18MV-R stopped-flow apparatus (Applied PhotoPhysics) fitted for fluorescence measurements as described previously (1). 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 μM final concentration after mixing), and the other one contained AcMetSONHMe at various concentrations in buffer A. An average of at least six runs was recorded for each AcMetSONHMe concentration. Rate constants, \( k_{\text{obs}} \), were obtained by fitting fluorescence traces with the monoexponential Equation 6 in which c represents the end point, a the amplitude of the fluorescence increase (<0), and \( k_{\text{obs}} \) the rate constant.

\[ y = ae^{-k_{\text{obs}}t} + c \]  

(Eq. 6)

Data were fitted to Equation 7 using least square analysis to determine \( k_{\text{max}} \) and \( K_s \) for AcMetSONHMe. S represents the AcMetSONHMe concentration and \( K_s \) the apparent affinity constant.

\[ k_{\text{obs}} = \frac{k_{\text{max}}S}{K_s + S} \]  

(Eq. 7)

Kinetics of the Trp-53 fluorescence variation associated with the formation of the Cys-51/Cys-198 disulfide bond were measured for Y82F/Y134F and Y82F/Y134F/E94Q MsrAs at 25 °C on a flx spectrofluorometer (SAFAS). The excitation wavelength was set at 284 nm, and the fluorescence emission at 340 nm was recorded versus time after enzyme addition. Data were then treated as described above to obtain \( k_{\text{obs}} \), \( k_{\text{max}} \), and \( K_s \) values.

pH Dependence of the Reductase Step Rate Constant—Determination of \( k_{\text{max}} \) and \( K_s \) as a function of pH was carried out for wild-type MsrA by single turnover pre-steady-state fluorescence stopped-flow spectroscopy, using the same procedure as described in the previous section but replacing buffer A with polybuffer B. \( k_{\text{obs}} \) values for E94Q, Y82F, Y134F, Y82F/Y134F, and Y82F/Y134F/E94Q MsrAs were determined at saturating concentration of AcMetSONHMe as a function of pH. Kinetics of Trp-53 fluorescence variation were recorded either with the stopped-flow apparatus or the spectrofluorometer depending on the mutated MsrA, as described in the previous section. The pH dependence of the reductase step rate constant for E94A and E94D MsrAs was determined under steady-state conditions using the Trx recycling system. \( k_{\text{max}} \) (or \( k_{\text{obs}} \)) values were plotted against pH and fitted to Equation 8, deriving from a
RESULTS

Determination of $pK_{\text{app}}$ of the Cys Residues

The $pK_{\text{app}}$ of both Cys-51 and Cys-198 were determined in the reduced free enzyme by two methods. The first one involved determining the second-order rate constant of the reaction with the Cys-specific reactivity probe 2-PDS as a function of pH. The second one took advantage of the variation of the thiolate UV absorbance as a function of pH.

Kinetics of Reaction of Reduced Wild-type, C51S, and C198S MsrAs with 2PDS—Reaction of 2PDS with wild-type MsrA obeyed pseudo-first-order kinetics, with formation of 2 mol of pyridine-2-thione/mol of MsrA as determined from the absorbance change at 343 nm. This result was expected as two Cys are present in *N. meningitidis* MsrA at positions 51 and 198. For all pH used, stopped-flow traces fitted to monoexponential Equation 1, with amplitude corresponding to the release of 2 mol of pyridine-2-thione. pH-$k_2$ profile fitted to monosigmoidal Equation 2 with a $pK_{\text{app}}$ value of 9.7 and $k_{2\text{max}}$ value of $(2.4 \pm 0.3) \times 10^5 \text{ M}^{-1} \text{s}^{-1}$ (Fig. 1A). The product of 2PDS reaction with wild-type MsrA is the disulfide-oxidized enzyme and not the thiopyridine adducts. Indeed, no release of pyridine-2-thione was observed when 10 mM dithiothreitol was added to the purified protein (data not shown).

C51S and C198S MsrAs behaved similarly to wild-type MsrA, except that only 1 mol of pyridine-2-thione/mol of MsrA was formed. $pK_{\text{app}}$ value of 9.3 $\pm$ 0.1 and a $k_{2\text{max}}$ value of $(3.1 \pm 0.7) \times 10^4 \text{ M}^{-1} \text{s}^{-1}$ for Cys-51 and a $pK_{\text{app}}$ of 9.8 $\pm$ 0.1 and a $k_{2\text{max}}$ value of $(2.6 \pm 0.6) \times 10^4 \text{ M}^{-1} \text{s}^{-1}$ for Cys-198 were determined (Fig. 1B). Altogether, the data support a $pK_{\text{app}}$ value of both Cys-51 and Cys-198 in the reduced free wild-type enzyme close to 9.5.

Direct Thiolate UV Absorbance of Reduced Wild-type, C51S, and C198S MsrAs—The thiolate absorbance of wild-type, C51S, and C198S MsrAs was monitored between pH 6 and 10. Analysis of the spectra and of the $o$-240 nm as a function of pH yielded monosigmoidal plots for all three MsrAs. Data fitted to $pK$ values of 9.7, 9.8, and 9.7, associated with $\Delta o$-240 nm of $3.1 \times 10^4$, $2.3 \times 10^4$, and $2.6 \times 10^4 \text{ M}^{-1} \text{cm}^{-1}$ for wild-type, C51S, and C198S MsrAs, respectively (Fig. 2). These $pK_{\text{app}}$ values of Cys-51 and Cys-198 in the reduced free enzyme are in good agreement with those obtained with 2PDS.

Kinetic Characterization with Identification of the Rate-limiting Step of the Mutated MsrAs at pH 8

Steady-state catalytic constants of mutated MsrAs at positions 82, 94, and/or 134 were determined at pH 8, which is the optimum pH for the wild type (1). AcMetSONHMe was used instead of MetSO because MsrA displays a better affinity for AcMetSONHMe (9). As shown in Table 1, Y82F, Y134F, and E94Q MsrAs exhibited slight modifications of $k_{\text{cat}}$ compared with wild-type MsrA, with $k_{\text{cat}}$ values from 0.9 to 2.2 s$^{-1}$ and a $K_m$ increase from 0.8 to 25 mM. In contrast, E94A, E94D, Y82F/
In the wild type, the rate of the reductase step is largely higher than the \( k_{\text{cat}} \) value (1). Therefore, to interpret the eventual kinetic consequences of the substitutions at positions 82, 94, and 134 at the level of the reductase step, it was first necessary to attain this rate. This was determined for E94Q, Y82F, Y134F, and Y82F/Y134F MsrAs by following the variation of the Trp-53 fluorescence intensity under single turnover conditions, i.e. in the absence of Trx. This was done at a saturating concentration of 300 mM AcMetSONHMe. E94D analysis required the use of a rapid mixing apparatus, whereas E94A study was possible by manual mixing. Formation of 0.9 mol of AcMetNHMe/mol of enzyme was observed for both E94D and E94A MsrAs. Rate constant for AcMetNHMe formation \((k_{\text{cat}})\) was 0.27 s\(^{-1}\) and 2.9 \times 10^{-2} s\(^{-1}\) for E94D and E94A MsrAs, respectively. The free thiol content profile fitted to a monoeponential model, with concomitant loss of Cys-51 and Cys-198 thiols, with rate constant \((k_{\text{cat}})\) of 0.31 s\(^{-1}\) and 4.10 \times 10^{-2} s\(^{-1}\) for E94D and E94A MsrAs, respectively. The fact that \( k_{\text{cat}} \) values determined under steady-state conditions represent the \( K_s \) value of the reductase step. As observed for the other mutated MsrAs, the \( K_s \) values are similar to that of the wild type (see Table 1).

### pH Dependence of the Kinetic Parameters of the MetSO Reductase Step

The kinetic parameters \( k_{\text{max}} \) and \( K_s \) of the reductase step for the wild type were determined at different pH values by fluorescence stopped-flow spectroscopy under single turnover conditions, i.e. in the absence of Trx. The pH-\( k_{\text{max}} \) plot, presented in Fig. 3, exhibits a monosigmoidal profile governed by the contribution of an ionizable group of \( pK_{\text{app}} \) value of 5.7 ± 0.1 that must be deprotonated for efficient MetSO reduction. This ionized species is characterized by a \( k_{\text{max opt}} \) value of 730 s\(^{-1}\).

For E94Q, Y82F, Y134F, Y82F/Y134F, and Y82F/Y134F/E94Q MsrAs, the rate of the reductase step was also measured by fluorescence spectroscopy under single turnover conditions, either on a stopped-flow apparatus or a conventional spectrofluorometer, depending on the value of the rate. In the case of E94A and E94D MsrAs, the rate of the reductase step was determined under steady-state conditions. For each substituted MsrA, the observed rate constant \( k_{\text{obs}} \) of the MetSO reductase step was measured, at each pH, with only one concentration of AcMetSONHMe (300 mM). This concentration was shown to be saturating over the pH range investigated except for E94Q MsrA (data not shown). Therefore, \( k_{\text{obs}} \) value can be considered

#### Table 1: Steady-state and reductase step kinetic parameters of wild-type and mutated MsrAs, with associated \( pK_{\text{app}} \)

| MsrA    | \( k_{\text{cat}} \) \( \text{s}^{-1}, \text{pH} 8.0 \) | \( K_s \) \( \text{mM}, \text{pH} 8.0 \) | \( K_{\text{app}} \) \( \text{pH} 8.0 \) | \( k_{\text{max opt}} \) \( \text{pH} 8.0 \) |
|---------|--------------------------------|----------------|----------------|----------------|
| Wild type | 3.7 ± 0.5 | 0.6 ± 0.2 | - | - |
| E94A | (1.5 ± 0.1) \times 10^{-2} | 119 ± 27 | - | - |
| E94D | 0.25 ± 0.3 | 161 ± 42 | - | - |
| E94Q | 0.88 ± 0.07 | 25 ± 6 | - | - |
| Y82F | 2.2 ± 0.1 | 3.7 ± 0.7 | - | - |
| Y134F | 1.5 ± 0.1 | 0.8 ± 0.2 | - | - |
| Y82F/Y134F | (1.5 ± 0.2) \times 10^{-2} | 24 ± 5 | - | - |
| Y82F/Y134F/E94Q | (1.0 ± 0.1) \times 10^{-3} | 62 ± 22 | - | - |
| E94Q | 790 ± 10 | 55 ± 2 | 730 ± 10 | 5.7 ± 0.1 |
| Y82F | 12.2 ± 0.3 | 151 ± 8 | 28 ± 3 | 8.0 ± 0.1 |
| Y134F | 51 ± 1 | 72 ± 4 | 46 ± 1 | 7.6 ± 0.1 |
| Y82F/Y134F | 750 ± 8 | 70 ± 10 | 380 ± 10 | 7.7 ± 0.1 |
| Y82F/Y134F/E94Q | (3.4 ± 0.1) \times 10^{-2} | 26 ± 2 | (7.2 ± 0.7) \times 10^{-2} | 8.0 ± 0.1 |

\( a \) Steady-state parameters were deduced from nonlinear regression of initial rates to the Michaelis-Menten relationship (see “Experimental Procedures”).

\( b \) Kinetic parameters of the reductase step were obtained from nonlinear regression of \( k_{\text{cat}} \) to Equation 7 (see “Experimental Procedures”), except for E94A and E94D MsrAs. For these two latter substituted MsrAs, \( k_{\text{max}} \) and \( K_s \) values correspond to \( k_{\text{cat}} \) and \( K_{\text{app}} \) values determined under steady-state conditions (see “Results”).

\( c \) Kinetic parameters, \( k_{\text{max opt}} \) at optimum pH and \( pK_{\text{app}} \) values, were deduced from nonlinear regression of \( k_{\text{obs}} \) to Equation 8 (see also Fig. 4).
as a \( k_{\text{max}} \) value. In the case of E94Q MsrA, 300 mM AcMetSONHMe was not saturating at pH >8, and consequently \( k_{\text{obs}} \) values were determined only up to pH 8. The \( k_{\text{obs}} \) profile remains monosigmoidal for all substituted MsrAs, with increasing \( k_{\text{obs}} \) value with increasing pH. Data fitted to a single \( pK_a \) model (see Fig. 4). E94D MsrA has a \( pK_a \) of 6.7 with a \( k_{\text{max opt}} \) value of 0.19 s\(^{-1}\). E94A, E94Q, Y82F, Y134F, and Y82F/Y134F MsrAs displayed a more pronounced \( pK_a \) shift, with values ranging from 7.5 to 8.0 and with \( k_{\text{max opt}} \) values of 2\( \times \)10\(^{-2}\), 28, 46, 380, and 7\( \times \)10\(^{-2}\) s\(^{-1}\), respectively (see Fig. 4 and Table 1). It is noteworthy that substitutions of Tyr-82 or Tyr-134 induced a similar \( pK_a \) shift to 7.6 and that double substitution of these two Tyr led to a higher shift to 8.0. The triple substituted MsrA Y82F/Y134F/E94Q displayed the most highly shifted \( pK_a \) with a value of 9.5 and the lowest \( k_{\text{max opt}} \) value of 1.1\( \times \)10\(^{-2}\) s\(^{-1}\) (see Fig. 4 and Table 1).

**DISCUSSION**

The methionine sulfoxide reductase step of the MsrA mechanism was previously shown to be very fast (1) and postulated to imply the formation of a sulfurane transition state of bipyrimal geometry on the basis of the theoretical chemistry study (6). According to these data, the reductase step should imply 1) the formation of an enzyme-substrate complex, 2) the deprotonation of Cys-51, 3) the involvement of an acid catalyst to protonate the sulfoxide substrate and favor the sulfurane-type transition state formation, and 4) the rearrangement of the sulfurane-type transition state to obtain Met and sulfenic acid. The invariant residues Glu-94, Tyr-82, and Tyr-134 are correctly positioned in the three available x-ray MsrA structures to interact via H-bond with a water molecule that is located at the place of the oxygen of the sulfoxide function (Fig. 5). Based on these structural features, a reasonable hypothesis supports Glu-94 as the presumably required acid catalyst. This proposition is reinforced by the observation that no other acidic residue is present in the close proximity of the sulfoxide function. The two phenolic side chains of Tyr-82 and Tyr-134 could be involved in substrate binding (i.e. an affinity contribution) and/or in substrate positioning and transition state stabilization (i.e. a chemical catalysis contribution).

**Cys-51 Activation within the Active Site**—In the free wild-type enzyme, 2-PDS titration and direct thiolate UV absorbance titration revealed a single \( pK_a \) value of 9.5 for both Cys-51 and -198. Moreover, the 2PDS chemical reactivity of MsrA Cys is
the contribution of a single pK of 8 and 9.5, respectively. Such results support the attribution of the pK app of 5.7 to Cys-51 and not to Glu-94. Thus, formation of the MsrA-AcMetSONHMe complex provokes an activation of Cys-51 by decreasing its pK app by 3.8 units.

**Glu-94, Tyr-82, and Tyr-134 Contributions in the Reductase Step**—Substitution of Glu-94 by Ala or Asp drastically decreased the k max opt rate of the reductase step of the MsrA mechanism by factors of 3.6×10^4 and 3.8×10^3, respectively, with no significant K s effect at pH 8 and caused the shift of the rate-limiting step from the Trx-recycling process to the sulfoxide reduction step. These data support the implication of Glu-94 in the catalysis of sulfoxide reduction, but not in substrate binding, and identify its side chain as a critical catalyst. However, the kinetic parameters of the reductase step obtained with E94Q MsrA revealed a rather minor k max opt decrease of only 26-fold compared with that of the wild type. As Glu-94 cannot be a proton donor, it is tempting to conclude that Glu-94 does not directly play a role as a general acid catalyst but likely stabilizes, via H-bonding, the sulfuran transition state leading to sulfenic acid formation (see the last paragraph under “Discussion”). Indeed, substitution of Glu by Gln retains an H-bonding ability. This is reinforced by the fact that no pK app of an acidic catalyst is observed on the pH-k max profile of the wild-type MsrA. In addition to this effect on the rate constant of the sulfoxide reduction, substitution of Glu-94 induced an increase of the pK app governing this step and assigned to Cys-51. Thus, Glu-94 is, directly or not, also involved in the activation of Cys-51 upon substrate binding.

Substitution of Tyr-82 and Tyr-134, or both, by Phe did not affect K s values. Moreover, the k max opt constant of the sulfoxide reduction was slightly decreased by the absence of one of the phenolic hydroxyls, in particular of Tyr-134, but drastically decreased when both were removed. The fact that substitutions of both Tyrs is required to observe a strong decrease of k max opt suggests that in single Tyr-substituted MsrA the remaining Tyr governing this step and assigned to Cys-51. Thus, Glu-94 is, directly or not, also involved in the activation of Cys51 upon substrate binding.

As stated above, Cys-51 has to be deprotonated to allow its efficient attack on the sulfoxide function. The observed high rate constant of the reductase step at pH 8.0 implies a large, mandatory shift in Cys-51 pK a from near 9.5 to at least somewhere below 7. The pH dependence of k max displays a single pK app of 5.7, the rate of sulfoxide reduction increasing with pH. Moreover, the E94Q and Y82F/Y134F/E94Q MsrAs still show
vides a good model of the substrate-MsrA complex (3). Inspection of the structure shows that no residue bearing a positively charged side chain is present in close proximity to Cys-51 (Fig. 5). Moreover, on one hand the distance between the carboxylate of Glu-94 and the sulfur atom of Cys-51 (5.1 Å) is by far too large to allow a direct interaction between these two functions. On the other hand, the sulfur atom of the Met residue is positioned between one oxygen atom of the carboxylate and the thiol group of Cys-51. Thus, stabilization of the thiolate form of Cys-51 by the protonated form of Glu-94 is rather unlikely. The direct implication of Tyr-82 and Tyr-134 is also unlikely, as the distance between their hydroxyl groups and the thiol of Cys-51 is too large (at least 6.6 Å, Fig. 5). Therefore, the decrease in $pK_{\text{app}}$ of 3.7 units of Cys-51 in the Michaelis complex is likely due to a substrate-assisted mechanism. In the MsrA-substrate complex, the polarization of the sulfur-oxygen bond should be favored by the presence of the side chains of Glu-94, Tyr-82, and Tyr-134. Such a polarization was already described for the sulfur-oxygen bond of the Me$_2$SO by using theoretical chemistry method (11–13) and experimental approaches that gave a dipole moment of 3.96 D (14, 15). The close proximity of a positive, or a partially positive, charge on the sulfur of the sulfoxide function near the thiol group of Cys-51 (3.4 Å between the sulfur of Met and the thiol of Cys-51 in the M. tuberculosis binary complex MsrA-Met, Fig. 5) likely stabilizes the thiolate form of Cys-51 and thus is believed to be the driving force that favors the shift of the Cys-51 $pK_a$ from 9.5 to 5.7 upon substrate binding. In the case of the E94Q MsrA, the same scenario occurs but the polarization of the sulfur-oxygen bond could be lesser developed, leading to a smaller positive partial charge on the sulfur atom and therefore to the shift of the Cys-51 $pK_{\text{app}}$ from 5.7 to 8. It is likely that the proton initially borne by Cys-51 is transferred to the oxygen of the sulfoxide function via a concerted mechanism concomitantly with the attack of the thiolate of Cys-51 on the sulfoxide sulfur atom, leading to the formation of the sulfurane-type transition state. In this context, a proton shift that transfers the proton coming from the thiol of Cys-51 to the oxygen of the sulfoxide must occur. It is seductive to postulate that this proton transfer could be catalyzed via Glu-94. However, as already mentioned, in the structure of M. tuberculosis MsrA, the distance of 5.1 Å between the thiol of Cys-51 and the nearest oxygen of Glu-94 is too far for a direct interaction unless a shortening of this distance occurs or a water molecule is transiently present between the carboxylate and the thiol.

**Proposed Scenario for the Sulfoxide Reductase Step of the MsrA Mechanism**—MetSO likely binds to the active site of MsrA with its sulfoxide largely in its polarized form. The spatial proximity between the sulfur of the sulfoxide function and the thiol of Cys-51 leads to a stabilization of the thiolate form due to the presence of the positive, or partially positive, charge borne by the sulfur. Concomitantly to the substrate binding, the proton of Cys-51 is transferred to the oxygen of the sulfoxide function and the positively, or partially charged, sulfur of MetSO undergoes a nucleophilic attack of the Cys-51 thiolate leading to the formation of a sulfurane transition state of trigonal bipyramidal geometry, as suggested from quantum chemistry calculations (6) (see Scheme 2). How the rearrangement occurs for forming the sulfinic acid intermediate and Met remains to be determined. The mechanism is likely concerted rather than stepwise. In this scenario, Glu-94, Tyr-82, and Tyr-134 have two roles. First, they favor the binding of the polarized form of the sulfoxide function that is the form that is also present in solution. Second, they stabilize the sulfurane transition state. In that context, Glu-94 has the most important contribution and probably intervenes under its carboxylate form, whereas Tyr-82 and Tyr-134 form a hydrogen bond with the two lone pairs of the hydroxyl group.

However, questions remain to be addressed on the mechanism that allows the formation of a sulfinic acid of tetrahedral geometry from a sulfurane transition state of trigonal bipyramidal geometry. Indeed, recent theoretical study of the reductive mechanism of Me$_2$SO by thiols supports the formation of a sulfurane transition state with the sulfur of the thiol and the OH group in apical position and the two methyl groups and the lone pair in equatorial position (6). Such a geometry is, however, not compatible with a direct shift of the OH group to the sulfur atom of the thiol. A possibility that has also been considered is the formation of a transition state with the sulfur of the thiol into equatorial position and the OH group into apical position. The S=S–O bond angle is near 90°. Such a geometry necessitates higher activation energy to attain the transition state (~20 kcal/mol) that leads to shift of the OH group to the thiol group (6). An alternative that has also been proposed is to form a transition state of epoxide type. In that case, the geometry is more favorable to a shift of the OH group to the thiol group but the penalty in terms of energy of activation is higher (~40 kcal/mol) (6). Another question concerns the way by which the proton of the sulfur of Cys-51 is transferred to the sulfoxide function. An evident candidate is Glu-94. However, as already pointed out, the distance between Cys-51 and Glu-94 is at least 5 Å. In this context, the studies that are underway by theoretical approaches and taking into account the structure of the MsrA active site will be of particular interest.
Catalytic Mechanism of MsrA Reductase Step

Acknowledgments—We thank Prof. W. W. Cleland, Dr. M. F. Ruiz-Lopez, and Dr. G. Monard for helpful discussions. We thank Dr. F. Barras for the kind gift of the BE002 E. coli strain, C. Gauthier and A. Križnik for their very efficient technical help and AcMetSONHMe synthesis, Dr. G. Chevreux and Dr. S. Sanglier-Cianferani for mass spectrometry analysis, and Dr. S. Sonkaria for reviewing the manuscript.

REFERENCES
1. Antoine, M., Boschi-Muller, S., and Branlant, G. (2003) J. Biol. Chem. 278, 45352–45357
2. Olry, A., Boschi-Muller, S., and Branlant, G. (2004) Biochemistry 43, 11616–11622
3. Taylor, A. B., Benglis, D. M., Jr., Dhandayuthapani, S., and Hart, P. J. (2003) J. Bacteriol. 185, 4119–4126
4. Tete-Favier, F., Cobessi, D., Boschi-Muller, S., Azza, S., Branlant, G., and Aubry, A. (2000) Structure Fold Des. 8, 1167–1178
5. Lowther, W. T., Brot, N., Weissbach, H., and Matthews, B. W. (2000) Biochemistry 39, 13307–13312
6. Balta, B., Monard, G., Ruiz-Lopez, M. F., Antoine, M., Gand, A., Boschi-Muller, S., and Branlant, G. (2006) J. Phys. Chem. A 110, 7628–7636
7. Olry, A., Boschi-Muller, S., Marraud, M., Sanglier-Cianferani, S., Van Dorsselaer, A., and Branlant, G. (2002) J. Biol. Chem. 277, 12016–12022
8. Boschi-Muller, S., Azza, S., Sanglier-Cianferani, S., Talfournier, F., Van Dorsselaer, A., and Branlant, G. (2000) J. Biol. Chem. 275, 35908–35913
9. Boschi-Muller, S., Olry, A., Antoine, M., and Branlant, G. (2005) Biochim. Biophys. Acta 1703, 231–238
10. Marchal, S., and Branlant, G. (1999) Biochemistry 38, 12950–12958
11. Dobado, J. A., Martinez-Garcia, H., Molina, J. M., and Sundberg, M. R. (1999) J. Am. Chem. Soc. 121, 3156–3164
12. Stener, M., and Calligaris, M. (2000) J. Mol. Struct. (Theochem.) 497, 91–104
13. Mrazkova, E., and Hobza, P. (2003) J. Phys. Chem. A 107, 1032–1039
14. Cotton, F. A., and Francis, R. (1960) J. Am. Chem. Soc. 82, 2986–2991
15. Dreizler, H., and Dendl, G. (1964) Z. Naturforsch. 19a, 512–514