Methionine sulfoxide reductases (Msrs) are ubiquitous enzymes that catalyze the thioredoxin-dependent reduction of methionine sulfoxide (MetSO) back to methionine. In vivo, Msrs are essential in protecting cells against oxidative damages on proteins and in the virulence of some bacteria. There exists two structurally unrelated classes of Msrs. MsrAs are stereo-specific toward the S epimer on the sulfur of the sulfoxide, whereas MsrBs are specific toward the R isomer. Both classes of Msrs display a similar catalytic mechanism of sulfoxide reduction by thiol via the sulfenic acid chemistry and a better affinity for protein-bound MetSO than for free MetSO. Recently, the role of the amino acids implicated in the catalysis of the reductase step of Neisseria meningitidis MsrA was determined. In the present study, the invariant amino acids potentially involved in substrate binding, i.e. Phe-52, Trp-53, Asp-129, His-186, Tyr-189, and Tyr-197, were substituted. The catalytic parameters under steady-state conditions and of the reductase step of the mutated MsrAs were determined and compared with those of the wild type. Altogether, the results support the presence of at least two binding subsites. The first one, whose contribution is major in the efficiency of the reductase step and in which the L-methyl group of MetSO binds, is the hydrophobic pocket formed by Phe-52 and Trp-53, the position of the indole ring being stabilized by interactions with His-186 and Tyr-189. The second subsite composed of Asp-129 and Tyr-197 contributes to the binding of the main chain of the substrate but to a lesser extent.

Methionine sulfoxide reductases (Msrs) are ubiquitous enzymes that catalyze the thioredoxin-dependent reduction of methionine sulfoxide (MetSO) back to methionine. In vivo, Msrs are essential in protecting cells against oxidative damages on proteins and in the virulence of some bacteria. There exists two structurally unrelated classes of methionine sulfoxide reductases (Msrs) in most organisms, called MsrA and MsrB, which selectively reduce free or protein-bound Met-SO and Met-R-SO, respectively. Msrs are described to exert various biological functions in vivo (3–5). They can (i) repair oxidized proteins, and thus, may regulate their function, (ii) play an antioxidant role as oxidation of surface methionine residues is considered as a mechanism that scavenges reactive oxygen species without modification of the properties of proteins, and (iii) play a role in the virulence of some bacteria.

The catalytic mechanism of both classes of Msrs characterized to date (6–9) is composed of three steps including: 1) a reductase step consisting of a nucleophilic attack of the catalytic Cys on the sulfur atom of the sulfoxide substrate that leads to formation of a sulfenic acid intermediate and release of 1 mol of Met/mol of enzyme, 2) formation of an intradisulfide bond between the catalytic Cys and a recycling Cys with a concomitant release of 1 mol of water, and 3) reduction of the Msr disulfide bond by thioredoxin (Trx) that leads to regeneration of the reduced form of Msr and to formation of oxidized Trx. The catalytic mechanism is in agreement with the kinetic mechanism, which was shown to be of ping-pong type for both classes of Msrs (9, 10). Moreover, for both classes of Msrs, the overall rate-limiting step is associated with the Trx-recycling process, whereas the rate of formation of the intradisulfide bond is governed by that of the reductase step, the rate of which is fast (11, 12).

A theoretical study of the reduction mechanism of sulfoxides by thiol has been recently investigated by quantum chemistry calculations, which supports formation of a sulfurane intermediate (13). The amino acids involved in the catalysis of the reductase step of Neisseria meningitidis MsrA have also recently been characterized by molecular enzymology approaches (14). The invariant Glu-94, and to a lesser extent Tyr-82 and Tyr-134, were shown to play a major role in the stabilization of the transition state of sulfurane type and indirectly in the decrease of the $K_{\text{app}}$ of the catalytic Cys-51. A scenario was 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 to the stabilization of the catalytic Cys.

The three-dimensional structures of the MsrAs from Escherichia coli, Bos taurus, Mycobacterium tuberculosis, and poplar...
have been recently obtained by x-ray crystallography (15–18). The MsrA models describe a single-domain protein composed of a central core around which the long N- and C-terminal coils seem to wind. The overall fold is of the mixed α/β type, with the core containing a two-layer sandwich, α-β plaits motif. The active site can be represented as an opened basin easily accessible to the MetSO substrate in which the catalytic Cys-51 is situated at the entrance of the first α-helix of the central core. In all the structures, the active site is occupied by a molecule that is covalently or noncovalently bound to the Cys-51. In particular, in the E. coli MsrA, a dimethyl arsenate is covalently bound, whereas in the M. tuberculosis MsrA, a methionine residue (Met-1) from a neighboring monomer occupies the active site. In both cases, a water molecule is present, the position of which can mimic the oxygen atom of the sulfoxide function of the substrate. One of the methyl groups of the dimethyl arsenate and the e-methyl group of Met-1 are bound to a hydrophobic pocket formed by invariant Phe-52 and Trp-53 residues. The Trp-53 indole ring is in interactions with invariant His-186 and Tyr-189 residues, whereas invariant Asp-129 and Tyr-197 residues are in a position to interact with the NH main chain of the Met-1 that occupies the active site of the M. tuberculosis MsrA (see Fig. 1 and see “Results and Discussion” under the paragraph “Rationale for the Substitutions”).

In view of this information, it was reasonable to postulate that Phe-52, Trp-53, His-186, Tyr-189, directly or indirectly, and Asp-129 and Tyr-197 residues are involved in the binding of the MetSO substrate via the e-methyl group and the main chain, respectively. In the present study, all these residues of MsrA from N. meningitidis were substituted to validate their role. The catalytic parameters under steady-state conditions and of the reductase step of the mutated MsrAs were determined and compared with those of the wild type. Altogether, the results support the presence of at least two subsites involved in substrate binding. The first one, whose contribution is major, is the hydrophobic pocket formed by the side chains of invariant Phe-52 and Trp-53, the position of the latter being stabilized by interactions with His-186 and Tyr-189. The second subsite is composed of the side chains of invariant residues Asp-129 and Tyr-197.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis, Production, and Purification of Mutated N. meningitidis MsrAs—**The E. coli strain used for all N. meningitidis MsrA productions was BE002 (MG1655 msrA::specW, msrB::a3kana), transformed with plasmid pSKPILBMsrA containing only the coding sequence of MsrA from *pilB*, under the *lac* promoter (7). The BE002 strain was kindly provided by Dr F. Barra. 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 (11). Mutated MsrAs were pure, as verified by electrophoresis on a 12.5% SDS-PAGE gel followed by Coomassie Brilliant Blue R-250 staining and by electrospray mass spectrometry analyses.

Enzymes were stored as described previously. The molecular concentration was determined spectrophotometrically, using extinction coefficient at 280 nm of 26,200 M⁻¹·cm⁻¹ for all mutated MsrAs except W53A and W53F MsrAs, which corresponded to an extinction coefficient of 20,480 M⁻¹·cm⁻¹.

**Steady-state MsrA Kinetics in the Presence of the Trx-reductase System—**Steady-state kinetic parameters were determined with the Trx reductase recycling system (*E. coli* Trx reductase (1.2 μM), NADPH (0.3 mM)) with saturating concentration of *E. coli* Trx (100 μM) and by varying the concentrations of AcMetSONHMe. Initial rate measurements were carried out at 25 °C in buffer A (50 mM Tris-HCl, 2 mM EDTA, pH 8) 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 fit to the Michaelis-Menten relationship using least squares analysis to determine *kcat* and *km* for AcMetSONHMe. *E. coli* Trx1 and Trx reductase were prepared following experimental procedures already published (19, 20). Ac-L-Met-R,S-ISO-NHMe was prepared and purified as described previously (12).

**Fluorescence Properties of Mutated MsrAs—**The fluorescence excitation and emission spectra of mutated MsrAs, in their reduced and Cys-51/Cys-198 disulfide states, were recorded on an flx spectrofluorometer (SAFAS) thermostated at 25 °C in buffer A with 10 μM of each protein, as described previously (11). For the determination of λmax, the emission spectrum and of the fluorescence intensity at λmax, the excitation wavelength was 295 nm, and the emission was measured from 300 to 450 nm using a 10-nm band-pass for excitation and emission.

**Determination of the Rate of AcMetNHMe Formation by Single Turnover Quenched-flow Experiments for W53F MsrA—**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 described previously (11). The apparatus operated in a pulsed mode. Under the conditions used, a minimum aging time of about 25–40 ms was determined. Equal volumes (57.5 μl) of a solution containing 550 μM of W53F 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 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 centrifugation, AcMetNHMe quantification in the resulting supernatant was carried out by reverse phase chromatography; 100 μl were injected onto a 4.6 × 250-mm Atlantis dC18 reverse phase column (Waters) in an AKTA explorer system (Amersham Biosciences), equilibrated with H2O/0.1% trifluoroacetic acid. AcMetNHMe was eluted after AcMetSONHMe, with a linear gradient of acetonitrile.

Data were plotted as mol of AcMetNHMe formed per mol of MsrA as a function of time. The rate of AcMetNHMe formation was determined by fitting the curve to the monoexponen-
Substrate Specificity of MsrA

RESULTS AND DISCUSSION

Rationale for the Substitutions

As indicated in the Introduction, invariant Phe-52 and Trp-53 form a hydrophobic pocket in which one of the methyl groups of dimethyl arsenate in E. coli MsrA and the ϵ-methyl group of Met-1 in M. tuberculosis MsrA is bound (Fig. 1). Therefore, this strongly supported binding of the ϵ-methyl group of MetSO to this pocket. Inspection of the MsrA active sites also shows the existence of (i) a hydrogen bond between the NH group of the Trp-53 indole ring and the N of His-186 (distance of 2.9 Å) and (ii) a stacking of the Trp-53 indole ring with the phenyl ring of Tyr-189, the two aromatic rings being parallel at 3.4 Å from each other. These interactions are likely essential to stabilize the positioning of the indole ring adequate for efficient binding of the MetSO substrate via its ϵ-methyl group. Thus, two types of substitutions were performed: 1) those that are expected to significantly perturb the binding of the ϵ-methyl of the substrate, either directly (F52L, W53F, and W53F) or indirectly (H186A and Y189A MsrAs), and thus, can lead to a KS and a kobs max effects; and 2) those that conserve the ability to position efficiently the indole ring of Trp-53 (H186N by hydrogen bond via the amide function and Y189F by stacking via the phenyl ring) and therefore are expected to have little effect on the kinetic parameters of the reductase step.

As mentioned in the Introduction, Asp-129 and Tyr-197 were postulated to stabilize the main chain of the substrate. Indeed, the inspection of the M. tuberculosis MsrA active site, which is occupied by the Met-1 residue, shows that the carboxylate group of Asp-129 makes two hydrogen bonds: 1) one with...
the NH\textsubscript{2} of Met-1 engaged in a peptide bond with His-0 and 2) the other with the hydroxyl group of Tyr-197. Moreover, the Tyr-197 phenyl ring also forms a stacking interaction with the peptide bond between His-0 and Met-1. Thus, to determine their role, Asp-129 and Tyr-197 residues were both substituted: 1) by Ala to disrupt all the stabilizing interactions that can exist and 2) by Asn and Phe, respectively, to conserve one putative stabilizing interaction.

### Justification of the Methods Used to Characterize the Properties of the Mutated MsrAs

As indicated in the Introduction, the rate of the reductase step in MsrA is fast and largely higher than the \( k_{\text{cat}} \) value. Therefore, to interpret the consequences of a substitution on the kinetic parameters of the reductase step, it was necessary to attain the rate of the reductase step for each mutated MsrA. For that, we took advantage of the Trp-53 fluorescence emission signal intensity of all the mutated MsrAs, except for W53F MsrA, which varied from the reduced to the oxidized disulfide form. In fact, formation of the disulfide bond led to an increase in the Trp-53 fluorescence emission signal intensity of all the mutated MsrAs, except for W53F MsrA, which is due to an increase of the fluorescence intensity of the reduced form of these mutated MsrAs (see below, paragraph “Fluorescence Properties of F52L, H186A, and W53F MsrAs”). For all mutated MsrAs, the rate of the reductase step was determined under single turnover conditions, i.e. in the absence of reductant as already described for the wild type (11). In that context, we have assumed that the reductase step was still rate-determining in the process, leading to formation of the Msr disulfide bond, as demonstrated previously for the wild type (11). For all mutated MsrAs, \( k_{\text{cat}} \) values that are representative of the \( k_{\text{obs,max}}/K_{S} \) values were determined under subsaturating concentrations of AcMetSONHMe. When saturating concentration of AcMetSONHMe was observed, \( K_{S} \) and \( k_{\text{obs,max}} \) values were also determined.

In the absence of the fluorescent probe Trp-53, the kinetics of the reductase step for the W53F MsrA was attained by following the rate of formation of AcMetNHzMe under single turnover conditions, i.e. in the absence of reductant (11). This was done only at two concentrations of AcMetSONHMe (300 and 600 mM) with the use of a rapid mixing apparatus. Formation of ~0.9 mol of AcMetNHzMe/mol of W53F MsrA was observed for both substrate concentrations.

The kinetic experiments were performed with AcMetSONHMe as a substrate for all mutated MsrAs. The rationale is that the wild-type MsrA was previously shown to exhibit a better affinity for AcMetSONHMe (\( K_{S} \) value of 55 mM) when compared with MetSO, i.e. by a factor of at least 20, in the reductase step (9, 11). Thus, AcMetSONHMe can be considered as a better mimic of MetSO included in proteins when compared with free MetSO. For mutated MsrAs at positions 129 and 197, \( k_{S} \) values were determined not only with AcMetSONHMe but also with MetSO and Me\textsubscript{2}SO. Indeed, comparison of the \( k_{S} \) values can provide information on the structural factors that are responsible for the enhanced affinity of MsrA for AcMetSONHMe.

### Characterization of the amino acids involved in the recognition of the e-methyl group of Ac-L-MetSONHMe

**Kinetic Properties of F52L and W53F/W53A MsrAs—**As shown in Table 1, the substitution of Phe-52 by Leu and Trp-53 by Phe or Ala resulted in a significant increase of the \( K_{S} \) value for AcMetSONHMe from 60- to 1300-fold, whereas the \( k_{\text{cat}} \) value decreased in the range of only 4-11-fold. Under single turnover conditions, no saturating kinetic effects with respect to AcMetSONHMe were observed for F52L MsrA up to 800 mM substrate (Fig. 2A). The \( k_{\text{obs}} \) value determined at 800 mM substrate was 16-fold higher than the \( k_{\text{cat}} \) value for F52L MsrA, indicating a rate-limiting step still associated with the Trx-recycling step. The \( k_{S} \) value of 0.02 mM\textsuperscript{−1}s\textsuperscript{−1} decreased 745-fold when compared with that of the wild type. For W53F MsrA, \( k_{\text{obs}} \) values of 5.2 and 8.5 s\textsuperscript{−1} for AcMetNHMe formation were determined at 300 and 600 mM AcMetSONHMe, respectively (Fig. 3). These data indicated: 1) a rate-limiting step still associated with the Trx-recycling step and 2) a substrate saturating effect not yet attained at 600 mM AcMetSONHMe. A \( k_{S} \) value of ~0.015 mm\textsuperscript{−1}s\textsuperscript{−1} can be estimated that is ~1000-fold smaller than the \( k_{S} \) value of the wild type (Table 1). For both W53F and F52L MsrAs, the \( k_{S} \) value at a substrate concentration of 600 and 800 mM, respectively, is at least 80-fold lower than the \( k_{\text{obs,max}} \) of the wild type. Therefore, the 750–1000-fold decrease in \( k_{S} \) reflects both a \( k_{\text{obs,max}} \) effect and a \( K_{S} \) effect. Therefore, it is probable that substituting Phe for Trp-53 and Leu for Phe-52 disrupts, in part, the hydrophobic pocket, and as a consequence, not only decreases the affinity of the substrate by a factor that can be estimated to be at least 20 but also perturbs the positioning of the sulfoxide function relative to that of the catalytic amino acids involved in the sulfoxide reduction. The situation is probably the same and even more pronounced

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**Table 1**

| Enzyme                  | Steady-state \( k_{\text{cat}} \) (s\textsuperscript{−1}) | Steady-state \( K_{S} \) (mM) | Reductase step \( k_{\text{cat,AcMetSONHMe}} \) (s\textsuperscript{−1}mM\textsuperscript{−1}) |
|-------------------------|----------------------------------------------------------|--------------------------------|------------------------------------------------------------------------------------------------|
| Wild type               | 3.4 ± 0.2                                               | 0.6 ± 0.2                      | 14.9 ± 0.9                                                                                           |
| F52L                    | 0.5 ± 0.1                                               | 0.02 ± 0.01                    | ND                                                                                                 |
| W53A                    | 0.3 ± 0.1                                               | 0.015                          | ND                                                                                                 |
| W53F                    | 0.8 ± 0.1                                               | 0.012 ± 0.01                   | 1.0 ± 0.5                                                                                           |
| H186A                   | 2.6 ± 0.3                                               | 0.041                          | 0.3 ± 0.1                                                                                           |
| H186N                   | 3.1 ± 0.3                                               | 0.001                          | 0.3 ± 0.1                                                                                           |
| Y189A                   | 2.5 ± 0.2                                               | 0.036                          | 0.5 ± 0.1                                                                                           |
| Y189F                   | 1.8 ± 0.1                                               | 0.015                          | 0.3 ± 0.1                                                                                           |
| D129A                   | 1.3 ± 0.1                                               | 0.001                          | 0.3 ± 0.1                                                                                           |
| D129N                   | 1.8 ± 0.1                                               | 0.040                          | 0.040                                                |
| Y197A                   | 3.5 ± 0.3                                               | 0.015                          | 0.3 ± 0.1                                                                                           |
| Y197F                   | 3.5 ± 0.3                                               | 0.015                          | 0.3 ± 0.1                                                                                           |

*The \( k_{S} \) value was an estimation obtained from only two substrate concentrations (300 and 600 mM, see “Results,” paragraph “Kinetic Properties of F52L and W53F/W53A MsrAs”).
when Trp-53 is substituted by Ala, as suggested by the very high $K_m$ value for the substrate (800 mM when compared with 0.6 mM for the wild type).

**Kinetic Properties of H186A/H186N and Y189A/Y189F Msrs**

The kinetic constants determined for the H186A, H186N, F52L, H186A/H186N, Y189A/Y189F, D129A/D129N, and Y197A/Y197F MsrAs measured by fluorescence stopped-flow under single turnover kinetics. The MsrA fluorescence variation was recorded on a stopped-flow apparatus at 25 °C. Final concentration of MsrA was 10 mM. Excitation wavelength was set at 284 nm, and emitted light was collected above 320 nm using a cut-off filter. For each substrate concentration, experimental data were analyzed by nonlinear regression against Equation 2 to obtain $k_{obs}$. In A and B, the substrate range was from 10 to 800 mM. For H186N and Y189F MsrAs, saturating concentrations of AcMetSONHMe were observed, and $k_{obs}$ data were fit to Equation 3, which gave $k_{obs max}$ and $K_s$ values as follows: Y189F MsrA (A, solid line, $k_{obs max} = 800 \pm 30$ s$^{-1}$ and $K_s = 130 \pm 10$ mM), H186N MsrA (A, solid line, $k_{obs max} = 590 \pm 10$ s$^{-1}$ and $K_s = 200 \pm 10$ mM), and Y197A/Y197F MsrAs, no saturating concentrations of AcMetSONHMe were observed, and $k_{obs}$ data were fit to the linear equation as follows: F52L MsrA (A, solid line), H186A, Y189A, D129A/D129N, and Y197A/Y197F MsrAs. No saturating concentrations of AcMetSONHMe were observed, and $k_{obs}$ data were fit to the linear equation as follows: F52L MsrA (A, solid line), H186A, Y189A, D129A/D129N, and Y197A/Y197F MsrAs. No saturating concentrations of AcMetSONHMe were observed, and $k_{obs}$ data were fit to the linear equation as follows: F52L MsrA (A, solid line), H186A, Y189A, D129A/D129N, and Y197A/Y197F MsrAs. No saturating concentrations of AcMetSONHMe were observed, and $k_{obs}$ data were fit to the linear equation as follows: F52L MsrA (A, solid line), H186A, Y189A, D129A/D129N, and Y197A/Y197F MsrAs.
Y189A, and Y189F MrAs under steady-state conditions showed that the $k_{\text{cat}}$ is not modified for all mutated MrAs (Table 1). The $K_m$ values varied slightly for the H186N and Y189F MrAs (4-fold increase and 2-fold decrease, respectively) but were strongly increased for the H186A and Y189A MrAs (165- and 57-fold, respectively) when compared with that of the wild type.

Under single turnover conditions, a saturating kinetics effect with respect to AcMetSONHMe concentration was only observed for H186N and Y189F MrAs, whereas no saturating effect was observed for H186A and Y189A MrAs up to 800 mM (Fig. 2, A and B). The $k_{\text{obs}}$ values determined at 800 mM substrate were 11-, 52-, 190-, and 444-fold higher than the $k_{\text{cat}}$ values for H186A, Y189A, H186N, and Y189F MrAs, respectively, indicating a rate-limiting step still associated with the Trx-recycling step. The $K_s$, $k_{\text{obs max}}$, and $k_2$ values determined for H186N and Y189F MrAs were in the range of those determined for the wild type (Fig. 2B and Table 1). This was not the case when His-186 or Tyr-189 was substituted by Ala, as probed by the 124- and 25-fold $k_2$ decrease observed, respectively. The $k_2$ decreases reflect both a $K_s$ effect and a $k_{\text{obs max}}$ effect since the $k_{\text{obs}}$ values determined at 800 mM AcMetSONHMe were decreased by 27- and 6-fold for H186A and Y189A MrAs, respectively, when compared with the $k_{\text{obs max}}$ value of the wild type. When compared with the W53F MrA, the $k_2$ values for H186A and Y189A are decreased less. Altogether, the kinetic data are thus in accord with the postulated role of His-186 and Tyr-189 that favored an efficient positioning of the Trp-53 indole ring via a hydrogen bond and a stacking interaction, respectively. This also agrees with the fact that the conservative substitutions of His-186 and Tyr-189 by Asn and Phe, respectively, have no significant effect on the catalytic efficiency of the reductase step.

**Fluorescence Properties of F52L, H186A/H186N, and Y189A/Y189F MrAs**—Previous work showed that the fluorescence properties of wild-type MsrA, when excitation was performed at 295 nm, were only due to the contribution of Trp-53 and consequently reflect the microenvironment of the Trp-53 indole ring (11). Comparison of the emission spectra of all mutated MrAs with that of the wild-type, of either the reduced or the oxidized disulfide forms, showed no difference except for the reduced form of F52L, H186A, and Y189A MrAs (spectra not shown). For these mutated MrAs, the $\lambda_{\text{max}}$ is shifted from 338 to 346 nm, which reflects a microenvironment of the indole side chains of Trp-53 more polar than in the reduced forms of the wild type and the other mutated MrAs. This is likely due to an increased accessibility of Trp-53 to solvent. Moreover, in contrast to other mutated MrAs, in particular to H186N and Y189F MrAs, F52L, H186A, and Y189A MrAs display a 3.8-, 2.3-, and 2.7-fold increase of the fluorescence intensity at $\lambda_{\text{max}}$, respectively, when compared with that of the wild type (spectra not shown). This reflects a lesser quenching of the Trp-53 fluorescence. As already mentioned, inspection of the x-ray MrA structure shows a $\pi-\pi$ stacking interaction between the phenyl ring of Tyr-189 and the indole ring of Trp-53. Therefore, Tyr-189 is a good candidate as the quencher of the Trp-53 fluorescence in the wild type. This is in accord with the increase of the Trp-53 fluorescence intensity, which is observed in Y189A MrA but not in Y189F MrA that still possesses a phenyl ring. The increase of the fluorescence intensity observed for H186A MrA and F52L MrA suggests a separation taking place between Trp-53 and the phenyl ring of Tyr-189 in both mutated MrAs. In both cases, it is probable that the positioning of the indole ring relative to that of Tyr-189 is perturbed. For H186A, it is likely the consequence of the loss of a hydrogen bond with the indole ring of Trp-53. As a support, H186N MrA, which conserves the capacity of forming a hydrogen bond, displays similar $\lambda_{\text{max}}$ and fluorescence intensity as the wild type. In the case of F52L, it is probable that substituting Leu for Phe-52 disrupts, in part, the hydrophobic pocket, and in return, destabilizes the positioning of Trp-53 relative to Tyr-189. These interpretations are in accord with the $K_s$ effects observed for F52L, H186A, and Y189A MrAs.

**Role of Asp-129 and Tyr-197 in the recognition of the main chain of the substrate**

The kinetic parameters of D129A, D129N, Y197A, and Y197F MrAs determined under steady-state conditions showed that the $k_{\text{cat}}$ value was not modified, whereas the $K_m$ value was increased 16-, 10-, 2.5-, and 5-fold, respectively, when compared with those of the wild type (Table 1). Under single turnover conditions in the absence of reductant, no kinetic saturation effect was observed up to 800 and 200 mM AcMetSONHMe for D129A/D129N and Y197A/Y197F MrAs, respectively (Fig. 2C). The $k_{\text{obs}}$ values determined at substrate concentration of 800 mM for D129A and D129N MrAs and of 200 mM for Y197A and Y197F MrAs were 141-, 133-, 208-, and 110-fold higher than their corresponding $k_{\text{cat}}$ values, indicating a rate-limiting step still associated with the Trx-recycling step for all these mutated MrAs.

The $k_2$ values were 37-, 19-, 8-, and 3-fold decreased for D129A, D129N, Y197A, and Y197F MrAs, respectively, when compared with that of the wild type (Table 1). These decreases in $k_2$ reflect essentially a $K_s$ effect since the $k_{\text{obs}}$ values determined at 800 and 200 mM AcMetSONHMe decreased only 2–3-fold for D129A/D129N and Y197A/Y197F MrAs, respectively, when compared with the $k_{\text{obs max}}$ value of the wild type.

The fact that Asp-129 and Tyr-197 were predicted from the three-dimensional structure of MrA from *M. tuberculosis* complexed with a Met residue to stabilize the main chain of the substrate raised the question whether they can discriminate in favor of the binding of a protein-bound MetSO against the binding of free MetSO. Indeed, MrA displays a better affinity of at least 20-fold for AcMetSONHMe when compared with free MetSO. As shown in Table 2, substituting Asp-129 by Ala or Asn and Tyr-197 by Ala or Phe also affected the $k_2$ values with MetSO as a substrate with the same magnitude as with AcMetSONHMe, in contrast to Me$_2$SO, for which the $k_2$ values are not significantly modified except for D129A MrA. Altogether, the data support: 1) a nondiscriminating role of Asp-129 and Tyr-197 in the recognition of the NH main chain of the MetSO with the amino and carboxyl groups of MetSO either

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5 For Y197A/Y197F MrAs, the variation of the fluorescence signal as a function of time becomes, for an unknown reason, not significant for substrate concentration higher than 200 mM.
Substrate Specificity of MsrA

### Table 2

| Enzyme | $k_{2\text{AcMetSONHMe}}$ | $k_{2\text{MetSO}}$ | $k_{2\text{MeSO}}$ |
|--------|---------------------------|--------------------|------------------|
| Wild type | 14.9 ± 0.9 (100) | 1.40 ± 0.01 (100) | 0.50 ± 0.01 (100) |
| D129A | 0.40 ± 0.01 (27) | 0.03 ± 0.01 (2) | 0.18 ± 0.01 (36) |
| D129N | 0.80 ± 0.01 (54) | 0.12 ± 0.01 (9) | 0.42 ± 0.01 (84) |
| Y197A | 1.90 ± 0.04 (13) | 0.40 ± 0.01 (29) | 0.36 ± 0.01 (72) |
| Y197F | 4.8 ± 0.3 (32) | 0.50 ± 0.01 (36) | 0.30 ± 0.01 (60) |

free, and thus, charged, or engaged in amide bonds, as is the case for a protein-bound MetSO; and 2) a higher contribution of Asp-129 to the substrate binding when compared with Tyr-197. Nevertheless, the fact that a phenyl ring can make interaction with a peptidic bond as well as with a protonated amine (21, 22) prevents discrimination between a direct role of Tyr-197 in substrate binding or an indirect role, via the orientation of the carboxylate group of Asp-129. Anyway, the contribution of Asp-129 and Tyr-197 remains modest when compared with that of Phe-52 and Trp-53 in terms of $K_v$ values.

The selectivity of MsrA for protein-bound MetSO should thus arise from other structural factors (either a stabilizing effect for protein-bound MetSO via interactions with the amide bonds or a destabilizing effect for MetSO due to repulsive interactions with the charges on the amino and/or carboxyl groups) located near the active site of the MsrA. These factors, if existing, remain to be characterized. In that context, determination of the three-dimensional structure of a complex between a MsrA and a protein MetSO substrate could provide additional information to those obtained from the three-dimensional structure of MsrA from *M. tuberculosis* complexed with a Met residue.

**Conclusion**

We have shown that for MsrA: 1) the hydrophobic pocket, formed by the side chains of invariant Phe-52 and Trp-53, is the major factor that contributes to MetSO binding via interactions with the ε-methyl group; 2) invariant His-186 and Tyr-189 are key residues for orienting the Trp-53 indole ring, the positioning of which is essential for an efficient reductase activity to occur; and 3) the side chains of invariant residues Asp-129 and Tyr-197 participate in the substrate binding but are not discriminating in terms of binding between a MetSO either engaged in amide bonds or with amino and carboxyl groups free. The $K_v$ value of MsrA for AcMetNHMe is at least 2 M (data not shown). This shows a low affinity of MsrA for AcMetNHMe, although the ε-methyl group is present. As indicated in the Introduction, Glu-94, Tyr-82, and Tyr-134 are involved in catalysis but not in substrate binding. Moreover, a MetSO substrate such as AcMetSONHMe is bound to the active site with its sulf oxide function largely polarized via interactions with Glu-94, Tyr-82, and Tyr-134. Therefore, the sulfur of the sulf oxide function bears a positive charge, or at least a partial charge, and thus, participates by a substrate-assisted mechanism to interacting and stabilizing the thiolate form of Cys-51 (14). In contrast, the sulfur of AcMetNHMe is rather negatively charged (23, 24). Thus, a repulsive electrostatic interaction is expected to occur between the sulfur of Cys-51 and the sulfur of the product, which can be considered as an anti-determinant factor that disfavors binding of product such as AcMetNHMe. The consequence is either a higher rate of dissociation of the product from the MsrA-product complex when compared with that of a MsrA-MetSO complex and/or a lower rate of association of the product to MsrA when compared with that of MetSO substrate, and thus, would explain the low affinity of MsrA for AcMetNHMe.

Recent structural and dynamical NMR studies of both reduced and oxidized forms of *E. coli* MsrA (25) show the existence of a conformational switch that would be a prerequisite to form the Cys-51/Cys-198 bond. The consequence is a solvent exposition of the hydrophobic surface of the active site of the oxidized MsrA, which could permit an efficient interaction with Trx. The switch only concerns two segments that contain Asp-129 and Tyr-197 residues, respectively. In the oxidized form, the distance between Asp-129 and Tyr-197 is too large for hydrogen bond interaction between both residues in contrast to what is observed in reduced form. It is probable that the release of the product for which MsrA displays low affinity is the driving factor responsible for the conformational switch.

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