Evidence for a New Sub-class of Methionine Sulfoxide Reductases B with an Alternative Thioredoxin Recognition Signature*

Received for publication, July 6, 2004, and in revised form, July 21, 2004
Published, JBC Papers in Press, July 26, 2004, DOI 10.1074/jbc.M407464200

Fabrice Neiers, Alexandre Kriznik, Sandrine Boschi-Muller, and Guy Branlant†
From the UMR CNRS-UHP 7567, Maturation des ARN et Enzymologie Moléculaire, Faculté des Sciences, Bld. des Aiguillettes, BP 239, 54506 Vandoeuvre-lès-Nancy, France

Methionine sulfoxide reductases catalyze the reduction of protein-bound methionine sulfoxide back to methionine via a thioredoxin-recycling process. Two classes of methionine sulfoxide reductases, called MsrA and MsrB, exist that display opposite stereoselectivities toward the sulfoxide function. Although they are structurally unrelated, they share a similar chemical mechanism that includes three steps with 1) formation of a sulfenic acid intermediate with a concomitant release of 1 mol of methionine per mole of enzyme; 2) formation of an intradisulfide Msr bond; and 3) reduction of the oxidized Msr by thioredoxin. In the MsrBs that have been biochemically, enzymatically, and structurally characterized so far, the cysteine involved in the regeneration of the catalytic Cys-117 is Cys-63. Cys-117 is located on a β strand, whereas the recycling Cys-63 is on a loop near Cys-117. The distance between the two cysteines is compatible with formation of the Cys-117/Cys-63 intradisulfide bond. Analyses of MsrB sequences show that at least 37% of the MsrBs do not possess the recycling Cys-63. In the present study, it is shown that Cys-31 in the Xanthomonas campestris MsrB, which is located on another loop, can efficiently substitute for Cys-63. Such a result implies flexibility of the MsrB structures, at least of the loops on which Cys-31 or Cys-63 are located. The fact that about 25% of the putative MsrBs have no recycling cysteine supports other recycling processes in which thioredoxin is not operative.

—

Post-translation oxidation of methionine into methionine sulfoxide (MetSO)² in proteins is known to provoke loss of protein function and, in particular, to be involved in the aging process (for a review see Ref. 1). There exist methionine sulfoxide reductases (Msr) that catalyze reduction of free and peptide-bound methionine sulfoxide back to methionine and therefore restore the function of the modified MetSO proteins (2–6). Two structurally unrelated classes of monomeric Msrs have been described so far (7–10). MrsAs reduce the S-epimer, whereas MsrBs reduce the R-epimer at the sulfur atom of MetSO (11–14). Both classes display a similar new catalytic mechanism with at least three steps (13, 15, 16): 1) a reductase process that includes formation of a sulfenic acid intermediate and a concomitant release of 1 mol of methionine per mole of enzyme, the rate of which is not rate-limiting; 2) a second step that leads to formation of an intradisulfide intermediate with a rate that is at least as fast as that of the sulfenic acid intermediate formation; and 3) a third step that consists of reducing the oxidized Msr under a disulfide state by thioredoxin (Trx) (Scheme 1). This step was shown to be overall rate-limiting for both classes of Msr (16, 17).

Inspection of the alignment of the sequences of MsrBs deduced from the DNA sequences show that, as expected, the catalytic Cys-117 on which the sulfenic acid intermediate is formed, is invariant, including SelR MsrB in which Cys-117 is a Sec residue. In contrast, the recycling Cys-63 from Neisseria meningitidis which was shown to be involved in the regeneration of the Cys-117 through formation of an intradisulfide bond followed by reduction by Trx, is present in only 63% of the MsrB sequences (see Fig. 1). The absence of the recycling Cys-63 in the remaining MsrBs suggests an alternative mechanism of regeneration of the reductase activity for these MsrBs, in vivo. In the crystal structure of the MsrBs from Neisseria gonorrhoeae (9) and N. meningitidis,² Cys-117 is situated on a β strand, whereas Cys-63 is located within a loop. The distance between the sulfur atoms of both cysteines is compatible with the catalytic mechanism and in particular with formation of the Cys-117/Cys-63 intradisulfide bond. Two hypotheses can be postulated to explain the activity of MsrBs devoid of the recycling Cys-63. In the first one, the sulfenic acid on Cys-117 is reduced by either Trx or another reductant. An alternative is the participation of another cysteine that would play a role similar to that of Cys-63. In this case, this implies a conformational flexibility of the MsrB structures and a positioning of the recycling cysteine compatible not only with an efficient formation of an intradisulfide bond with Cys-117 but also with an efficient reduction of the disulfide bond by Trx.

In the present study, we show that the Xanthomonas campestris MsrB, which does not possess Cys-63, exhibits an efficient Trx-recycling process. Cys-31, which is situated in a loop different from that on which is located Cys-63, is shown to play a role in the Trx-recycling process similar to that of Cys-63 of the N. meningitidis enzyme. Conversely, the C63S MsrB from N. meningitidis, in which a cysteine is introduced at position 31, is shown to be almost as active in the Trx-recycling process as the wild type. These results and those obtained with

² B. Kauffmann, unpublished results.
C31S/T63C MsrB from X. campestris are discussed on the basis of the crystal structure of the N. gonorrhoeae MsrB.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructions, Site-directed Mutagenesis, Production, and Purification of X. campestris and N. meningitidis Wild Type and Mutants—**Plasmid pSKMsrBEx was obtained by cloning the msrB open reading frame synthesized by PCR (GC-rich system, Roche Applied Science; sequences of oligonucleotides not shown) using X. campestris ATCC 13951 genomic DNA, kindly provided by Dr. S. Leduc and Dr. N. D. Lindley, into the plasmid pDB125SNN between the NdeI and SacI sites (13). The other recombinant pSKMsrs were plasmids already described elsewhere (12). Mutated nucleotides were performed using the QuikChange site-directed mutagenesis kit (Stratagene). The E. coli N. D. Lindley, into the plasmid pDB125KSNN between the NdeI and SacI sites (13). The other recombinant pSKMrsB plasmids were already described elsewhere (12). Mutated nucleotides were performed using the QuikChange site-directed mutagenesis kit (Stratagene). The sequence of the corresponding mscrB gene was then cloned into expression vectors. The three-step catalytic mechanism as described by Olry et al. (21) was confirmed by mass spectrometry analyses.

**Preparation of X. campestris and N. meningitidis Wild Type and Mutants—**C117S MsrB did not display any detectable activity. For MsrBs, excepted N. meningitidis S31C/T63C MsrB, the protocol of purification was carried out as previously described for PILB-MsrB (13). In the case of the N. meningitidis S31C/T63C MsrB, the enzyme was found after sonication and centrifugation in the pellet. The pellet was washed with buffer A, followed by a linear gradient of KCl (0.4 M) using a fast protein liquid chromatography system (Amersham Biosciences). The KCl was eluted at 100 mM KCl. Partially purified fractions were pooled and applied onto a phenyl-Sepharose column (Amersham Biosciences) equilibrated with buffer A. At this stage, wild-type and mutant MsrBs were pure, as checked by electrophoresis on 12.5% SDS-PAGE gel followed by Coomassie Brilliant Blue R-250 staining. The predicted mass was confirmed by mass spectrometry analyses.

**Determination of Metal Content—**The zinc and iron contents of wild-type, C63S, C31S/C63S MsrB from X. campestris and Trx—The fluorescence characteristics of 1) the wild-type MsrB in its reduced form and Cys-31,Cys-117 disulfide state, 2) the X. campestris and Trx with saturating concentration of Ac-t-Met-R,S-SO-NHMe and by varying the concentrations of Trxred or with saturating concentration of Trxred and by varying the concentrations of Ac-t-Met-R,S-SO-NHMe. Initial rate data were fitted to the Michaelis-Menten relationship using least squares analysis to determine kcat, value and Km for Trxred and Ac-t-Met-R,S-SO-NHMe. It should be mentioned that Kcat for Ac-t-Met-R,S-SO-NHMe has to be divided by 2, because the S isomer is not a substrate.

**Fluorescence Properties of Wild-type and C31S MsrBs from X. campestris and Trx—**The fluorescence characteristics of 1) the wild-type MsrB in its reduced form and Cys-117/Cys-31 disulfide state, 2) the C31S MsrB in its reduced form and Cys-117 sulfenic acid state, and 3) Trx in its reduced and disulfide state were recorded on a spectrofluorometer (FS SAFAS) thermostatted at 25 °C (see legends of Figs. 2 and 3 for more details).

**Kinetics of the Formation of the Cys-117—S—S—Cys-31 Disulfide Bonds of Wild-type and C31S/T63C MsrBs from X. campestris, Respectively, in the Absence of Trx by Single Turnover Stopped-flow Experiment—**Kinetics of the emission fluorescence intensity increase associated with the formation of the Cys-117/Cys-31 and Cys-117/Cys-63 disulfide bonds of the MsrBs from X. campestris were measured at 25 °C on an SX18MV-R stopped-flow apparatus (Applied PhotoPhysics) fitted for fluorescence measurements. The excitation wavelength was set at 293 nm, and the emitted light was collected above 320 nm, using a cutoff filter. One syringe contained MsrB in buffer A (10 mM final concentration after mixing), and the other one contained Ac-t-Met-R,S-SO-NHMe (100 mM final concentration). An average of six runs was recorded. Rate constants, kcat, were obtained by fitting fluorescence traces with the monoexponential Equation 1 in which c represents the end point, a, the amplitude of the fluorescence increase (<c>), and k<sub>obs</sub> the rate constant.

\[ y = a e^{-k_{ob}t} + c \]

(1) Determination of the Rate of Ac-t-Met-NHMe Formation 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 react for 40 s. Enzymes) were mixed in the aging loop. The mixtures were then allowed to quench for 30 min at room temperature, four shots were done, and the four subsequent quenched samples were collected in a 200-l loop of the quenched samples to precipitate the protein. Samples were centrifuged at 12,000 g for 30 min at room temperature. Ac-t-Met-NHMe quantification in the resulting supernatant was carried out by reverse phase chromatography as described previously (17). In parallel, the quenched samples, which were not treated with 100% trifluoroacetic acid, were used to determine the protein concentration from the absorbance at 280 nm.

For Ac-t-Met-NHMe titration, data were plotted as moles of Ac-t-Met-NHMe added per mole of MsrB as a function of time. The rate of Ac-t-Met-NHMe formation was determined by fitting the curve to the monoeponential Equation 2 in which k represents the maximum fraction of Ac-t-Met-NHMe formed per mole of MsrB and k\textsubscript{obs} the rate constant.

y = a(1 - e\textsuperscript{-kt})  \hspace{1cm} (Eq. 2)

Kinetics of Formation of Oxidized Trx (Trx\textsubscript{ox}) upon the Reduction of the X. campestris MsrB Cys-31/45/97/117 Disulfide Bonds and of the N. meningitidis MsrB Cys-117/94/117 Disulfide Bond under Single Turnover Conditions—Two approaches were used depending on the rate of the kinetics. When the rate was rapid as observed with the X. campestris wild-type and N. meningitidis MsrB, the fluorescence quenching associated with formation of the x-ray structure, a putative Zn\textsuperscript{2+}–binding site is indicated with black stars.

RESULTS

Biochemical Properties of Wild-type, C31S/T63C, and C31ST63M S. typhimurium MsrB from X. campestris and of S31C/G35S MsrB from N. meningitidis—DTNB thiol titrations on the X. campestris wild-type MsrB revealed six cysteines under denaturing conditions. These results are in agreement with the X. campestris ATCC 13951 DNA sequence that indicates six cysteines at positions 31, 45, 48, 94, 97, and 117 (Fig. 1). Under native conditions, only two cysteines were reactive (Table I). Metal analyses of the purified enzyme by atomic emission spectrometry showed the presence of iron at a stoichiometry of 0.9 mol per mole of enzyme. The presence of iron, less than 0.05 mol per mole of enzyme was also detected. As shown from the inspection of the x-ray structure, a putative Zn\textsuperscript{2+}–binding site is
present in the *N. gonorrhoeae* MsrB (9). Assuming a fold similar to that of the *N. gonorrhoeae* MsrB, the metal binding site of the *X. campestris* MsrB is composed of the two CXXC motifs with Cys-45, Cys-48, Cys-94, and Cys-97 and is situated in an opposite direction from the active site. Therefore, the accessible cysteines in the *X. campestris* MsrB are likely Cys-31 and Cys-117, the latter being the catalytic cysteine on which the sulfinic acid is formed. Also two cysteines were reactive in the C31S/T63C MsrB indicating that the Cys-63 is accessible to DTNB. In the case of the C31S MsrB wild type only one cysteine, Cys-117, is shown to be accessible (Table I). Finally, two cysteines are also reactive in the S31C/C63S MsrB wild type from *N. meningitidis*. This showed that the introduced Cys-31 is also accessible, as is the case for Cys-63 in the wild type (13).

**Enzymatic Properties of the Wild-type and C31S MsrBs from *X. campestris***—The activity of the wild type was tested with Ac-1-Met-R-S-SO-NHMe as a substrate to evaluate the kinetics of the reductase step at saturating concentrations of the substrate. Surprisingly, a turnover activity was observed in the presence of Trxred and Trx reductase. This raised the question of the MR activity of the wild type. For example, it is possible that the sulfenic acid intermediate, which can be attainable through the C31S MsrB, decreased and was moreover insignificant compared with the increase observed upon the disulfide formation (Fig. 2). At pH 8.0 a *k*<sub>obs</sub> value of 11 s<sup>-1</sup> was found that is in the range of the *k*<sub>obs</sub> value of 8 s<sup>-1</sup> determined for Ac-1-Met-NHMe formation (at a concentration of 100 mM Ac-1-Met-R-S-SO-NHMe, which is saturating; data not shown) (Table III). Therefore, similar to that found for MsrA and MsrB from *N. meningitidis*, the sulfinic acid intermediate does not accumulate. As soon as it is formed on Cys-117, it is attacked by Cys-31. Together, the data obtained for the wild-type MsrB from *X. campestris* supported: 1) a rate-determining step associated with formation of the sulfinic acid intermediate within the two-step process leading to formation of the Cys-117/Cys-31 disulfide bond and 2) an overall rate-limiting step associated with the Trx-recycling process. To better characterize the rate-limiting step, the rate of formation of Trxox upon reduction of MsrB<sub>ox</sub> was evaluated by following selectively the quenching of the fluorescence emission intensity of Trx upon going from the reduced to the oxidized disulfide form using the same approach as that described for MsrA from *N. meningitidis* (17).

**Table I**

| MsrB                | No. of Cys | Without Ac-1-Met-R-S-SO | With Ac-1-Met-R-S-SO | Decrease in free thiol<sup>a</sup> Calculated | Theoretical |
|---------------------|------------|-------------------------|---------------------|---------------------------------------------|-------------|
| *N. meningitidis* wild type<sup>b</sup> | 2          | 2.0                     | 0.1                 | 1.9                                         | 2           |
| *N. meningitidis* S31C/C63S | 2          | 2.0                     | 0.1                 | 1.9                                         | 2           |
| *X. campestris* wild-type | 2          | 2.1                     | 0.1                 | 2.0                                         | 2           |
| *X. campestris* C31S | 1          | 0.9                     | 0.0                 | 0.9                                         | 1           |
| *X. campestris* C31S/T63C | 2          | 2.1                     | 0.0                 | 2.1                                         | 2           |

<sup>a</sup> The difference in the number of free cysteine thiols upon treatment with Ac-1-Met-R-S-SO<sup>b</sup> versus no treatment.

<sup>b</sup> From Olry, et al. (13).
**Table II**

Kinetic parameters of wild-type and mutant MsrB from *N. meningitidis and X. campestris*

Reactions under steady-state conditions were carried out in buffer A at pH 8.0, at 25 °C as described under “Experimental Procedures.” Enzyme concentrations were 1 μM for S31C/C63S *N. meningitidis* MsrB, 0.5 μM for wild-type *X. campestris* MsrB, and 50 μM for C31S/T63C *X. campestris* MsrB. The substrate used was Ac-L-Met-R,S,S- SO-NHMe. Data presented were obtained by fitting the experimental data to the Michaelis-Menten equation. The kinetic parameters of Trxox formation under single turnover conditions were determined using the change of the Trx emission fluorescence intensity. The final concentrations of the S31C/C63S MsrBox from *N. meningitidis*, the wild-type, and the C31S/T63C MsrBos from *X. campestris* varied from 10 to 25 μM, 5 to 20 μM, and 5 to 40 μM, respectively. The corresponding concentrations of the Trxox were varied from 50 to 800 μM, 20 to 500 μM, and 20 to 800 μM, respectively (for more details, see “Experimental Procedures”). Data were then fit to Equation 3 by least-squares regression, which gave $k_c$, $k_f$, and $K_m$ values.

| MsrB                     | Steady-state kinetic parameters | Trxox formation upon MsrBox reduction |
|--------------------------|---------------------------------|--------------------------------------|
|                           | $K_m$, μM | $k_{cat}$ | $K_s$, μM | $k_f$ |  |
| *N. meningitidis* wild type | 1.2 ± 0.4 | 34 ± 6 | 0.46 ± 0.04 | 440 ± 75 | 5.2 ± 0.4 |
| *N. meningitidis* S31C/C63S | 0.5 ± 0.2 | 13 ± 3 | 0.12 ± 0.02 | 390 ± 44 | 40 ± 3  |
| *X. campestris* wild type | 6 ± 1     | 5.7 ± 0.5 | 2.5 ± 0.4 | 30 ± 6 | 13.8 ± 0.6 |
| *X. campestris* C31S/T63C | 3 ± 2     | ND $^a$ | (1.8 ± 0.2) × 10$^{-3}$ | 40 ± 15 | (1.9 ± 0.2) × 10$^{-3}$ |

$^a$ From Olyry, et al. (13, 17).

$^b$ ND, not determined.

**Table III**

Rates of Ac-L-Met-NHMe formation measured by quenched-flow experiment and of disulfide bond formation by fluorescence stopped-flow experiment under single turnover kinetics

The quenched-flow experiments were carried out as described under “Experimental Procedures” (see for more details). For the S31C/C63S *N. meningitidis* MsrB, the experiment was done in 50 mM MES, pH 5.5. The final concentration was 350 mM, and that of Ac-L-Met-R,S,S-SO-NHMe was 300 μM. For the *X. campestris* MsrBs, the experiments were carried out in buffer A at pH 8.0. The final concentrations were 300 μM, and that of Ac-L-Met-R,S,S-SO-NHMe was 100 mM. Data of Ac-L-Met-NHMe quantification were fit to Equation 2 and gave rate constants and $K_{obs}$ values. The fluorescence stopped-flow experiments were done on the *X. campestris* MsrBs in buffer A at pH 8.0 as described under “Experimental Procedures.” Final concentrations of MsrBs and Ac-L-Met-R,S,S-SO-NHMe were 10 μM and 100 μM, respectively. Excitation wavelength was set at 293 nm, and emitted light was collected above 320 nm via a cutoff filter. Rate constants and $K_{obs}$ were obtained by fitting fluorescence traces with Equation 1.

| MsrB                     | Ac-L-Met-NHMe formation | Disulfide bond formation, $k_{obs}$ |
|--------------------------|-------------------------|-----------------------------------|
|                           | $k_{obs}$, s$^{-1}$ | mol Ac-L-Met-NHMe/mol of enzyme |  |
| *N. meningitidis* wild type | 18 ± 1   | 0.9 ± 0.1 | 13 ± 1  |
| *N. meningitidis* S31C/C63S | 12 ± 1   | 0.9 ± 0.1 | ND $^b$ |
| *X. campestris* wild type | 8 ± 1     | 0.8 ± 0.1 | 11 ± 1  |
| *X. campestris* C31S | 11 ± 2     | 1.0 ± 0.1 | No disulfide bond |
| *X. campestris* C31S/T63C | 1.1 ± 0.2 | 1.0 ± 0.1 | 1.9 ± 0.2 |

$^a$ From Olyry, et al., in 50 mM MES, pH 5.5, at a 350 μM final concentration of Ac-L-Met-R,S,S-SO-NHMe (17).

$^b$ ND, not determined.

disulfide exchange can be considered as irreversible, and therefore, the $k_{obs}$ value presumably measures the rate of the two-electron chemical exchange. The values of $k_f$ and $K_m$ are 5.5-fold and 5.5-fold higher than the $k_{cat}$ and $K_m$ values determined under steady-state conditions, respectively. Therefore, as suggested for the *N. meningitidis* MsrB (17), the rate-limiting step is likely associated with release of Trxox. However, compared with the *N. meningitidis* enzyme, the $K_s$ value for Trxox is 15-fold decreased. This suggests better productive interactions between Trxox and *X. campestris* MsrBox.
Enzymatic Properties of the C31S/T63C MsrB from *X. campestris*—In the presence of Trxred, a recycling activity was observed but with a 1000-fold decrease in the *k*<sub>cat</sub> compared with the wild type (Table I). This value is, however, significant because no turnover activity was observed in the absence of Trx and the activity is proportional to enzyme concentrations. Due to the high concentration of MsrB needed to measure activity, the *K*<sub>m</sub> value for Trxred was not attainable. In the absence of Trx, under single turnover conditions, the rate of Ac-L-Met-NHMe formation was studied using the same approach as already described for MsrA and MsrB from *N. meningitidis* (16, 17). The study was done at 100 mM Ac-L-Met-R,S,SO-NHMe in buffer A at pH 8.0. A *k*<sub>obs</sub> value of 1.1 s<sup>-1</sup> and a stoichiometry of 1.0 mol of Ac-L-Met-NHMe per mole of enzyme were determined (Table III). The interpretation of the fluorescence message was not ambiguous because the fluorescence message due to formation of the sulfenic acid intermediate, which can be attainable through the C31S MsrB, decreased and was moreover insignificant compared with the increase observed upon Cys-117/Cys-63 disulfide bond formation (spectra not shown). A *k*<sub>obs</sub> value of 1.9 s<sup>-1</sup> was found that is in the range of the *k*<sub>obs</sub> value of 1.1 s<sup>-1</sup> determined for Ac-L-Met-NHMe formation (at a concentration of 100 mM Ac-L-Met-R,S,SO-NHMe concentration for the two experiments) (Table III). Therefore, the data demonstrated that the rate-limiting step in the C31S/T63C MsrB is again associated with the Trx-recycling process as in the wild type. The fact that the rate of formation of Ac-L-Met-NHMe is 7-fold decreased compared with the wild type remains to be explained.

The kinetics of the Trx-recycling process was then studied under single turnover conditions. Again, the rate was determined by following the rate associated with a change of the Trx fluorescence intensity upon going from the reduced to the oxidized forms during the reduction of the Cys-117/Cys-63 MsrB disulfide bond as already described for MsrA and MsrB from *N. meningitidis* (16, 17). When the excitation was done at 310 nm, the contribution of the MsrB to the quenching of the fluorescence is indeed small compared with that of Trx (spectra not shown). From the curve of *k*<sub>obs</sub> versus Trxred concentration, assuming binding of Trxred to MsrBox is in rapid equilibrium and using Equation 3, a *K*<sub>s</sub> value of 40 μM for Trxred and *k*<sub>1</sub> and *k*<sub>2</sub> values of 1.9 × 10<sup>-3</sup> s<sup>-1</sup> and −0.8 s<sup>-1</sup> can be determined, respectively (Table II). The value of *k*<sub>1</sub> is in the same range as the *k*<sub>cat</sub> value. Therefore, these results strongly support a rate-limiting step in C31S/T63C MsrB that either precedes or is concomitant to formation of Trxox.

Properties of S31C/C63S MsrB from *N. meningitidis*—Catalytic constants of S31C/C63S *N. meningitidis* MsrB determined under steady-state conditions were obtained with Ac-L-Met-R,S,SO-NHMe as a substrate. For Ac-L-Met-R,S,SO-NHMe *K*<sub>m</sub>, Trxred, and *k*<sub>cat</sub> Values were 0.5 mM, 13 μM, and 0.12 s<sup>-1</sup>, respectively, at pH 8.0 (Table II).

Kinetic characterizations of the rate of formation of Ac-L-Met-NHMe and of the reduction of the Cys-117/Cys-31 disulfide bond by Trxred were then done by using the same methods as those described for the wild type. For the rate of Ac-L-Met-NHMe formation at pH 8.0, a burst of Ac-L-Met-NHMe formation was observed with a stoichiometry of 0.9 mol of Ac-L-Met-NHMe per mole of MsrB<sub>red</sub>. However, the rate was too fast to be determined with the apparatus adapted for quenched-flow experiments. In contrast, at pH 5.5, a *k*<sub>obs</sub> value of 12 s<sup>-1</sup> was attainable with a stoichiometry of 0.9 mol of Ac-L-Met-NHMe per mole of enzyme (Table III). Therefore, the data demonstrated that the rate-limiting step in the S31C/C63S MsrB takes place after formation of the sulfenic acid intermediate and is probably associated with the Trx-recycling process as in the wild type. The kinetics of this process was then studied under single turnover conditions using the Trxox fluorescence message as already described for the wild type. When the excitation was done at 310 nm, the contribution of MsrB to the quenching of the fluorescence signal was indeed small compared with that of Trx (spectra not shown). Assuming binding of Trxox to MsrB<sub>ox</sub> is in rapid equilibrium and using Equation 3, a *K*<sub>s</sub> value of 390 μM for Trxox and *k*<sub>1</sub> and *k*<sub>2</sub> values of 40 s<sup>-1</sup> and −0.8 s<sup>-1</sup> can be determined from the curve of *k*<sub>obs</sub> versus Trxox concentration, respectively. The fact that the values of *K*<sub>s</sub> and of *K*<sub>cat</sub> for Trxox are 330-fold and 30-fold higher than those determined under steady-state conditions, respectively, strongly suggests that the rate-limiting step is associated with release of Trxox similar to that proposed for the wild-type enzyme (17).

**DISCUSSION**

In a previous study carried out on the *N. meningitidis* MsrB, the recycling reductase process was shown to be Trx-dependent via formation of a disulfide bond between Cys-117 and Cys-63 (13). In the present study, we show that Cys-31 in the *X. campestris* MsrB plays a role similar to that of the Cys-63 in the *N. meningitidis* MsrB. Several data argue in favor of this interpretation. First, Trx-dependent recycling activity was observed for the *X. campestris* MsrB, which does not possess a cysteine at position 63. Second, a loss of two cysteines was observed under the reduction step in the absence of Trx. Third, when Ser was substituted for Cys-31, *X. campestris* C31S MsrB was as efficient in the reductase step as the wild type but displayed no recycling activity with Trxox. Fourth, S31C/C63S MsrB from *N. meningitidis* showed recycling Trx-dependent activity similar to the wild type, in contrast to the C63S MsrB, which did not show any recycling activity with Trxox (13).

As for the *N. meningitidis* MsrB, the rate-limiting step in the *X. campestris* enzyme was associated with the Trx-recycling process. The fact that Cys-31 can efficiently substitute for Cys-63 in the recycling process raises the question of how the Cys-117/Cys-31 disulfide bond of the *X. campestris* MsrB is efficiently formed and reduced by Trxred. Inspection of the x-ray structures of the *N. meningitidis* and *N. gonorrhoeae* (9) MsrBs shows a core domain composed of two anti-parallel β sheets from strands β1, β2, β9, and β3–β7 decorated by three α-helices. The active site that contains Cys-117 and Cys-63 is on the surface of the protein. Cys-117 is situated on the β8 strand. Its sulfur atom is positioned at least 11.3 Å apart the oxygen atom of Ser-31, whereas it is at 3.2 Å from the sulfur atom of Cys-63 and thus is in a favorable position to form the Cys-117/Cys-63 disulfide bond. The two positions 63 and 31 are situated into two distinct loops. Therefore, two hypotheses could be postulated to explain the formation of the Cys-117/Cys-31 disulfide bond. In the first one, the two Msr belong to two distinct structural sub-classes. In that case, each loop would be located differently in the two MsrBs such that the distance between Cys-117 and the cysteine in-
IZATION OF THE DISULFIDE BOND FORMED, I.E. CYS-117/CYS-63 OR CYS-117/CYS-31 OR BOTH, IN A N. MENINGITIDIS MSR B IN WHICH A CYSTEINE WOULD BE INTRODUCED AT POSITION 31 WILL ALSO BE INFORMATIVE.

THE FACTS THAT THE TRX-RECYCLING PROCESS IS EFFICIENT IN THE N. MENINGITIDIS MSR B AND X. CAMPESTRIS MSR B AS WELL AND IS LIKELY RATE-LIMITED BY TRX ox RELEASE SUPPORT PRODUCTIVE INTERACTIONS BETWEEN MSR B ox AND TRX red, thus permitting efficient two-electron chemical exchange. In the case of the C31S/T63C X. CAMPESTRIS MSR B, THE RATE OF FORMATION OF TRX ox IS 7000-FOLD DECREASED COMPARED WITH THE WILD TYPE AND BECOMES RATE-LIMITING IN THE TRX-RECYCLING PROCESS. ALTHOUGH THE Kcat VALUE FOR TRX red IS SIMILAR TO THAT OF THE WILD TYPE, IT IS POSSIBLE THAT THE DRASTIC DECREASE IN Kcat IS DUE TO NONPRODUCTIVE INTERACTIONS BETWEEN MSR B ox AND TRX red, thus preventing an efficient two-electron chemical exchange. THIS CAN BE DUE TO DIFFERENCES IN THE AMINO ACID ENVIRONMENT NEAR THE INTRODUCED CYS-63 COMPARED WITH THAT NEAR CYS-31. ANOTHER CAUSE COULD HAVE BEEN THE PRESENCE OF THE Zn2+ BINDING SITE IN THE X. CAMPESTRIS MSR B. ABOUT 50% OF THE MSR BS, INCLUDING THE X. CAMPESTRIS, E. coli, AND DROSOPHILA (22) MSR BS, CONTAIN THE TWO CXCX MOTIFS INVOLVED IN THE BINDING OF Zn2+, WHEREAS THE OTHERS SUCH AS THE N. MENINGITIDIS AND N. GONORROhoe MSR BS DO NOT POSSESS CYS-45, CYS-48, CYS-94, AND CYS-97 AND THEREBY HAVE NO Zn2+ ATOM BOUND. THIS IS, HOWEVER, UNLIKELY BECAUSE RECENT STUDIES DONE ON THE E. coli MSR B, WHICH ALSO POSSESSES CYS-63 AND A METAL-BINDING SITE, HAVE SHOWN THAT T31C/C63S E. coli MSR B EXHIBITS AN EFFICIENT TRX-RECYCLING ACTIVITY SIMILAR TO THE WILD TYPE.

AS DEDUCED FROM FIG. 1, 32% OF THE MSR B AMINO ACID SEQUENCES NEITHER POSSESS CYS-63 NOR CYS-31. THEREFORE, THIS RAISES THE QUESTION AS TO HOW THESE MSR BS REGENERATE THEIR CYS-117 WHEN IT IS OXIDIZED IN THE SULFenic ACID FORM. SEQUENCE ALIGNMENTS SHOW THAT SOME MSR BS HAVE A CYSTEINE AT POSITION 60 (FIG. 1). THIS POSITION IS SITUATED ON THE SAME LOOP NEAR WHICH CYS-63 IS LOCATED, AND THUS CYS-60 COULD EVENTUALLY PLAY A ROLE SIMILAR TO THAT OF CYS-63. THIS HAS BEEN CONFIRMED BY RECENT STUDIES THAT SHOW THAT INTRODUCING CYS-60 IN THE C63S N. MENINGITIDIS MSR B ALSO GENERATES A MSR B WITH TRX-RECYCLING ACTIVITY, WITH A Kcat VALUE IN THE SAME RANGE AS THAT OF THE Kcat OF THE WILD TYPE. AGAIN, THIS ARGUES FOR A GREATER FLEXIBILITY OF THE MSR B STRUCTURES. ANYWAY, 25% OF THE PUTATIVE MSR BS HAVE NO CYSTEINE THAT CAN SUBSTITUTE FOR CYS-63, CYS-60, OR CYS-31.

References
1. Schoneich, C. (1999) Exp. Gerontol. 34, 19–34
2. Moskovitz, J., Rahman, M. A., Strassman, J., Yancey, S. O., Kushner, S. R., Brot, N., and Weissbach, H. (1995) J. Bacteriol. 177, 502–507
3. Moskovitz, J., Weissbach, H., and Brot, N. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 2095–2099
4. Moskovitz, J., Berlett, B. S., Poston, J. M., and Stadtmann, E. R. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 9585–9589
5. Moskovitz, J., Flescher, E., Berlett, B. S., Azare, J., Poston, J. M., and Stadtmann, E. R. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 14071–14075
6. Sun, H., Gao, J., Ferrington, D. A., Biesiada, H., Williams, T. D., and Squier, T. C. (1999) Biochemistry 38, 105–112
7. Tete-Favier, F., Cobessi, D., Boschi-Muller, S., Azza, S., Branlant, G., and Aubry, A. (2000) Struct. Fold Des. 8, 1167–1178
8. Lowther, W. T., Brot, N., Weissbach, H., and Matthews, B. W. (2000) Biochemistry 39, 13307–13312
9. Lowther, W. T., Weissbach, H., Eitienne, F., Brot, N., and Matthews, B. W. (2002) Nat. Struct. Biol. 9, 348–352
10. Taylor, A. B., Benglis, D. M., Jr., Dhandayuthapani, S., and Hart, P. J. (2003) J. Bacteriol. 185, 4119–4126
11. Grimaud, R., Estrat, B., Mitchell, J. K., Lafitte, D., Briand, C., Derrick, P. J., and Barras, F. (2001) J. Biol. Chem. 276, 48915–48920
12. Sharov, V. S., Ferrington, D. A., Squier, T. C., and Schoneich, C. (1999) FEBS Lett. 455, 247–250
13. Giry, A., Boschi-Muller, S., Marraud, M., Sanglier-Cianferani, S., Van Dorsselear, A., and Branlant, G. (2002) J. Biol. Chem. 277, 12016–12022
14. Moskovitz, J., Poston, J. M., Berlett, B. S., Nosworthy, N. J., Szczepanowski, R., and Stadtmann, E. R. (2000) J. Biol. Chem. 275, 14167–14172
15. Boschi-Muller, S., Azza, S., Sanglier-Cianferani, S., Talfournier, F., Van Dorsselear, A., and Branlant, G. (2000) J. Biol. Chem. 275, 35908–35913
16. Antione, M., Boschi-Muller, S., and Branlant, G. (2003) J. Biol. Chem. 278, 45352–45357
17. Giry, A., Boschi-Muller, S., and Branlant, G. (2004) Biochemistry 43, 11616–11622
18. Scopes, R. R. (1974) Anal. Biochem. 59, 277–282
19. Mulrooney, S. B. (1997) Protein Expr. Purif. 9, 372–378
20. Moshansky, E., Huber-Wunderlich, M., and Glockshuber, R. (1998) Protein Sci. 7, 1233–1244
21. Silver, M. (1979) Methods Enzymol. 62, 135–137
22. Kryukov, G. V., Kumar, R. A., Koc, A., Sun, Z., and Gladyshev, V. N. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 4245–4250