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Methionine sulfoxide reductase: Chemistry, substrate binding, recycling process and oxidase activity

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ABSTRACT

Three classes of methionine sulfoxide reductases are known: MsrA and MsrB which are implicated stereo-selectively in the repair of protein oxidized on their methionine residues; and fRMsr, discovered more recently, which binds and reduces selectively free L-Met-*R*-O. It is now well established that the chemical mechanism of the reductase step passes through formation of a sulfenic acid intermediate. The oxidized catalytic cysteine can then be recycled by either Trx when a recycling cysteine is operative or a reductant like glutathione in the absence of recycling cysteine which is the case for 30% of the MsrBs. Recently, it was shown that a subclass of MsrAs with two recycling cysteines displays an oxidase activity. This reverse activity needs the accumulation of the sulfenic acid intermediate. The present review focuses on recent insights into the catalytic mechanism of action of the Msrs based on kinetic studies, theoretical chemistry investigations and new structural data. Major attention is placed on how the sulfenic acid intermediate can be formed and the oxidized catalytic cysteine returns back to its reduced form.

1. Introduction

Three classes of methionine sulfoxide reductases (Msr) have been described to date. The MsrA and MsrB reduce more efficiently a Met-O included in a protein with an *S* and *R* configuration at the sulfoxide function, respectively; while the fRMsr reduces selectively a free Met-O with an *R* configuration [1–3]. Catalysis passes through the sulfenic acid chemistry *via* formation of a sulfenic acid intermediate on a catalytic Cys (or a selenenic acid when the catalytic Cys is replaced by a Sec residue) [4,5]. Kinetic studies showed that the rate of formation of the sulfenic acid is high while the recycling process which reduces back the oxidized catalytic Cys is overall rate-limiting, at least for MsrAs and MsrBs [6,7]. The three-dimensional structures (3-D) reveal distinct unrelated folds [8–11]. In particular, the fRMsrs, which are only found in eubacteria and unicellular eukaryotes, exhibit a dimeric GAF-type fold. Based on the 3-D structures determined under different states including Msrs in complex with their substrate, hypotheses were postulated for the role of amino-acids that could be involved in catalysis and substrate specificity. These roles were evaluated in MsrA and MsrB by site-directed mutagenesis, using appropriate kinetic tools including stopped-flow and quenched-flow techniques and the fluorescence property of a tryptophan located in the active site as a probe [12–14]. More recently, theoretical chemistry investigations were carried out on MsrA and MsrB to define in particular the route that leads to formation of the sulfenic acid intermediate [15–17]. Lastly, a reverse activity called oxidase activity was recently described for a subclass of MsrA with two recycling Cys [18].

The present review discusses recent results obtained by our group and others, and focus in particular on how (1) the reduction of Met-O occurs in Msrs and leads to formation of a sulfenic acid intermediate, (2) the substrate binds, and the consequences which result from its binding, (3) the oxidized catalytic Cys returns back to its reduced form; and (4) the oxidase activity of MsrA can be operative *in vivo*.

2. Chemistry of the reduction of Met-O by Msrs

In a previous review, the chemical mechanism of the sulfoxide reduction by thiols was extensively discussed [19]. In short, it was pointed out that an S–O bond is a highly polarized single bond, the strength of which depending mainly on its electrostatic environment with at least a partially positive and negative charge on the S and O atoms, respectively. The chemistry was shown to proceed through nucleophilic attack of the catalytic Cys on the sulfur of the sulfoxide with concomitant protonation of the oxygen of the sulfoxide to give a sulfurane intermediate whose formation is rate-limiting in the reduction process [20]. A similar chemistry occurs in

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Msrs. Analysis of the 3-D structures of the Msrs in complex with their substrate, shows that the oxygen of the sulfoxide is strongly stabilized by a hydrophilic subsite composed of a network of hydrogen bonding interactions including Glu94, Tyr82 and Tyr134 in MsrA (numbering based on the *Escherichia coli* MsrA sequence), His103 and a water molecule which itself interacts with His100, Asn119 and Thr26 in MsrB (numbering based on the *E. coli* MsrB sequence); and Asp143 and Wat99 which is itself hydrogen-bonded to the main chain NH of Ser122 in fRMsr (numbering based on the *E. coli* fRMsr sequence) (Fig. 1) [21–23]. A scenario of formation of the sulfurane for all the Msrs can be proposed in which the hydrogen of the catalytic Cys protonates the oxygen of the sulfurane *via* a hydrogen transfer through an acid/base catalyst i.e. Glu94 in MsrA, His103 in MsrB and Asp143 in fRMsr, respectively. Based on QM/MM models and energy calculations, it was shown that formation of the sulfurane is rate-limiting in formation of the sulfenic acid intermediate in MsrA and MsrB similarly to that shown by chemistry. The consequence of the protonation of the sulfurane is to lengthen the S–O bond while the S–S bond is shortened [15–17]. In terms of evolution, it is interesting to note that the amino-acids which play the role of acid/base catalyst in the transfer of the proton from the catalytic Cys to the oxygen of the sulfurane, are different depending on the class of Msrs.

The sulfurane has a trigonal bipyramidal geometry, with the sulfur and the OH group in apical position. Such a geometry was shown to be incompatible with formation of a sulfenic acid intermediate *via* an intramolecular 1,2 transfer of the OH group to Cys51. In fact, theoretical chemistry investigation done on chemical model [24] and more recently on MsrA [15,17] and MsrB [16] showed that the OH group of the sulfurane has to be protonated to evolve into a sulfonium cation and a water molecule (Fig. 2). Such a scenario implies that the OH group of the sulfurane is not deprotonated and thus has a pK_{app} of at least 9, and the participation of an acid catalyst to favor the release of the OH group as a water molecule in order to break the S–OH bond. In the case of MsrA, one of the two Tyr i.e. either Tyr82 or Tyr134 which interacts

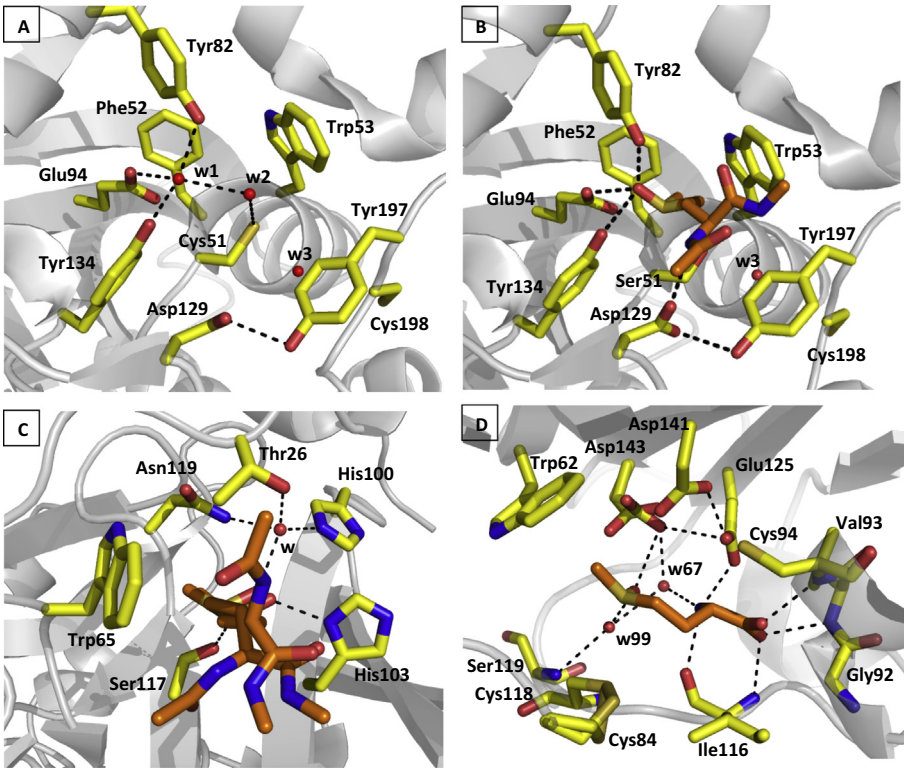

Fig. 1. The active sites of *N. meningitidis* (A) free wild-type MsrA, (B) C51S MsrA in complex with Ac-L-Met-*S*-ONHMe, (C) C117S–C63S MsrB in complex with Ac-L-Met-*R*-ONHMe; and (D) fRMsr in complex with L-Met-*R*-O. The residues are shown in stick representation with gray carbon, water molecules as red balls and the substrate molecules in stick representation with yellow carbon. Hydrogen bonds are shown as black dashed lines. The figures were generated using the PyMol program (Delano Scientific LLC) (A) several water molecules are present in the active site of the MsrA which stabilize it in a conformation similar to that of the active site in complex with the Ac-L-Met-*S*-ONHMe substrate as shown in Fig. 1B. The Wat1 interacts with the side chains of Tyr82 (2.7 Å), Glu94 (2.6 Å) and Tyr134 (2.7 Å). The Wat2 is near Wat1 (3.0 Å) while Wat3 is located in an opposite direction at the entrance of the active site near His199 (see Fig. 3B). The Tyr197 and Asp129 residues form the entrance of the active site and interact *via* a hydrogen bond (2.8 Å) between the OH group of Tyr197 and $O^{\varepsilon 1}$ oxygen of the carboxylate of Asp129. The Phe52 and Trp53 residues form a hydrophobic pocket on one side of the cavity, whereas the side chains of Tyr82, Glu94 and Tyr134 forms a hydrophilic subsite at the opposite side. The distance between Cys51 and Cys198 is 8 Å [21]. (B) The oxygen of the sulfoxide function of Ac-L-Met-*S*-ONHMe occupies the position of Wat1 with the same hydrogen-bonded interactions as Wat1 does. The ε terminal methyl group of Ac-L-Met-*S*-ONHMe points towards the hydrophobic pocket formed by Trp53 and Phe52, and the sulfur atom of the substrate is at 3.3 Å from the O^{γ} of Ser51. The side chains of Tyr197 and Asp129 residues remain in hydrogen-bonded interactions. The carboxyl of Asp129 also interacts with the NH group of the substrate (3.2 Å).The Wat2 is replaced by the γ methylene group of Ac-L-Met-*S*-ONHMe while Wat3 remains at the same position as observed in the reduced structure [21]. (C) The conformation of the active site of the *N. meningitidis* C117S–C63S MsrB in complex with Ac-L-Met-*S*-ONHMe is similar to that of the *N. gonorrhoeae* MsrB in complex with a cacodylate molecule [12]. The oxygen of the sulfoxide function interacts with the N^{δ} of His103 (2.5 Å) and a water molecule (2.6 Å) which is hydrogen-bonded to the N^{ε} of His100 (2.8 Å), the amide group of Asn119 (3.1 Å) and the hydroxyl group of Thr26 (2.6 Å). The sulfur atom of the substrate is at 3.3 Å from the O^{γ} of Ser117. The distance between the N^{δ} of His103 and the O^{γ} of Ser117 is 5.6 Å. The Trp65, *via* its indole group, stabilizes the aliphatic groups of the Ac-L-Met-*S*-ONHMe side chain, in particular the ε methyl group and the γ methylene group. (D) The L-Met-*R*-O is tightly bound within the active site of the fRMsr. The oxygen of the sulfoxide function is hydrogen-bonded to Wat99 (2.7 Å) which is itself hydrogen-bounded to the main chain NH of Ser122 (3.0 Å) and to the $O^{\delta 1}$ of Asp143 (2.9 Å) in its minor conformation (30%) which is likely the conformation catalytically efficient. In the major conformation observed (70%), the $O^{\delta 2}$ of Asp143 interacts with $O^{\delta 2}$ of Asp141 (2.8 Å) while the $O^{\delta 1}$ of Asp141 strongly interacts with the $O^{\varepsilon 2}$ of Glu125 (2.5 Å). The ε methyl group of the substrate is stabilized by the indole moiety of Trp62 whereas the γ and β methylene groups are stabilized by van der Waals interactions with Ile87, Tyr66 and Ile116. An oxyanion binding site composed of the NH of Val93, Cys94 and Ile116 stabilizes the carboxylate of the substrate. Indeed, the O1 is located 2.8 Å from the NH of Cys94, whereas the O2 atom points towards the NH of Val93 and Ile116 (2.8 Å and 2.9 Å, respectively). The NH_3^+ function is in hydrogen-bonded interactions with the $O^{\varepsilon 1}$ (2.9 Å) and the $O^{\varepsilon 2}$ (3 Å) atoms of Glu125.

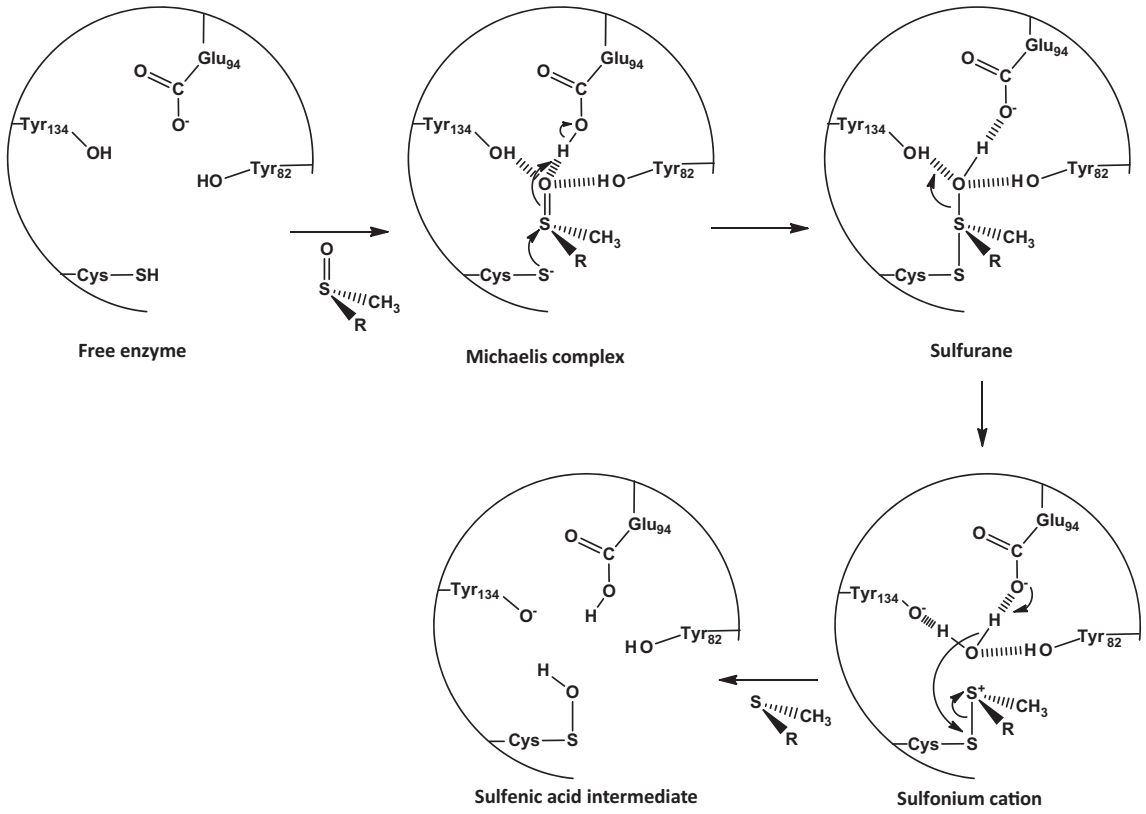

Fig. 2. Proposed scenario of formation of the sulfenic acid intermediate in MsrA. Formation of the Michaelis complex leads to transfer a proton from the catalytic Cys51 to the carboxylate of Glu94. The oxygen of the sulfoxide function of the substrate is stabilized by hydrogen-bonded interactions with Tyr82, Glu94 and Tyr134. The sulfurane is formed *via* a transfer of a proton from the carboxyl form of Glu94 followed by that of a sulfonium cation and a water molecule *via* either Tyr82 or Tyr134 as proton donor. Based on preliminary experiments carried out by our group, the oxygen of the sulfenic acid intermediate is postulated to come from the substrate. This implies that the Cys51 sulfur of the sulfonium cation is then attacked by the sequestrated water molecule coming from the sulfurane. This water molecule can be activated by the carboxylate of Glu94.

via hydrogen bonds to the oxygen of the sulfoxide function in the binary complex MsrA–substrate (Fig. 1B), was proposed to give a proton to the OH group of the sulfurane [15]. This is in accord with the kinetic data obtained with the Tyr mutants which showed that the rate of the reductase step is significantly decreased only when both Tyr are mutated [12]. This was recently confirmed by comparing the relative energy profiles determined from QM/MM models of the wild type and the single mutants which are similar, and the double mutant for which the relative energy is largely higher [17]. For MsrB, it is probably the water molecule in hydrogen-bonded interaction with the imidazole of His100 which plays the role of acid catalyst (Fig. 1C). A similar scenario can be proposed for fRMsr with Wat99 likely involved as an acid catalyst (Fig. 1D). At this stage of the chemical process, it was proposed, based again on comparison of the relative energy profiles, that the Cys117 sulfur of the sulfonium cation in MsrB is directly attacked by the recycling Cys [16]. However, the fact that the sulfenic acid intermediate in MsrB is formed at the same rate i.e. $\sim$85 s^{-1} in the presence or absence of the recycling Cys [4] suggests that the scheme which leads to formation of a sulfenic acid intermediate is operative even in the presence of the recycling Cys. This is reinforced by the fact that MsrBs do not always possess a recycling Cys but use another recycling process in which the sulfenic acid intermediate accumulates (see Section 4.2). In this context, it is reasonable to extend the chemistry of formation of a sulfenic acid intermediate to all the classes of Msrs. The next question which now arises concerns the origin of the water molecule that attacks the sulfur of the sulfonium cation. Two hypotheses can be advanced: the water molecule coming from the sulfurane, either is not involved and thus the oxygen of the sulfenic acid derives from the solvent, or attacks the sulfur of the sulfonium cation and thus is sequestrated into the active site and cannot exchange with the solvent. In this case, the oxygen of the sulfenic acid intermediate would come from the substrate. Anyway, whatever its origin, the water molecule has to be activated by a base catalyst. Recently, mouse MsrA in which the two recycling Cys were mutated, was incubated with Met-O in the presence of $H_2{}^{16}O$ or $H_2{}^{18}O$. Mass spectrometry (MS) analyses for peptide mapping and sequencing, showed that the mass of the 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole derivative was 2 Da heavier when $H_2{}^{18}O$ was used as solvent instead of $H_2{}^{16}O$. Based on these data, it was concluded that the oxygen of the sulfenic acid comes from the solvent [18]. Thus, this result suggested that after formation of the sulfonium cation, the water molecule which attacks the Cys51 sulfur of the sulfonium cation derives from the solvent. The theoretical chemistry investigations recently done on MsrA are rather in accord with this interpretation [17]. Indeed, the attacking water molecule was positioned at the entry of the active site near the recycling Cys198, thus in an opposite side of the active site in which the catalytic residues Glu94, Tyr82 and Tyr134 are located. The attacking water molecule was proposed to be base-activated by the recycling Cys198[1] *via* the invariant Asp129, forming a

[1] As indicated in the Section 4.1.1 relative to the recycling process, a subclass of MsrA with two recycling Cys exists. In this subclass, two mol of Met was shown to be formed in the absence of Trx [4]. Recently, the rate of formation of the second sulfenic acid intermediate was determined and found to be reduced by a factor of only 15-fold compared to that of the first sulfenic acid intermediate [41]. However, when formation of the second sulfenic acid intermediate occurs, Cys198 is not free and is already engaged in a disulfide bond with Cys206. Thus, these data do not support a catalytic role of Cys198 in formation of the sulfenic acid intermediate and rather suggest a minor role of Cys198, if any, in the activation of the water molecule that attacks the Cys51 sulfur of the sulfonium cation.

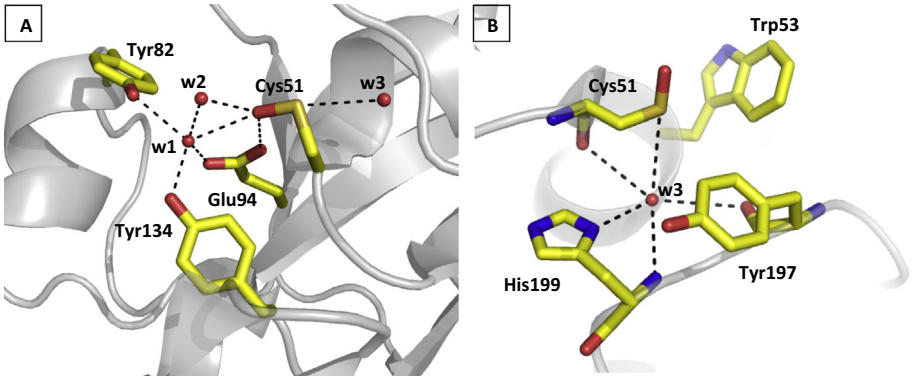

Fig. 3. The active site of the C198S MsrACys51SOH from *N. meningitidis*. (A) The environment of the OH group of the sulfenic acid. No noticeable movement of the side chains of the active-site residues is observed to accommodate the OH group of the sulfenic acid function. The O^{δ} of the Cys51 sulfenic acid is distant by 3.1 Å from Wat1 which is itself hydrogen-bonded to the $O^{\varepsilon 2}$ oxygen of Glu94 (2.6 Å), the hydroxyl of Tyr82 (2.6 Å) and of Tyr134 (2.9 Å). The Wat2 is again present at a distance of 3.1 Å from Wat1, 5.6 Å from the $O^{\varepsilon 1}$ oxygen of Glu94 and 2.7 Å from the O^{γ} oxygen of the sulfenic acid. The Wat3 remains also present at the same position as in the 3-D structures of the reduced form and the C51S MsrA in complex with the substrate (see Fig. 3B) [21]. (B) The protein environment of Wat3. The Wat3, which is located near the oxygen of the main chain of Tyr197 (3.7 Å) and of the main chain of Cys51SOH (3.6 Å) is stabilized by hydrogen-bonded interaction with the imidazole of His199 (2.8 Å) and, is well positioned, at a distance of 3.6 Å, to attack the sulfur of the Cys51SOH. The Wat3 shares the same position as the Cys198 in the Cys51–Cys198 disulfide 3-D structure (see Fig. 4). The side chain of Ser198 has the same orientation of Cys198 in the reduced and the substrate-bound MsrAs with a distance of 7.5 Å between the S^{γ} atom of Cys51 and the O^{γ} atom of Ser198 instead of 8 Å between the S^{γ} atom of Cys51 and the S^{γ} atom of Cys198 as observed in the reduced form [21].

sulfenic acid with its OH group oriented towards the solvent and not inside the active site towards Glu94, Tyr82 and Tyr134. The fact that the recycling Cys198 is located at 15 Å of the Asp129 in a very mobile loop in the binary complex MsrA–substrate however argues against the implication of the Asp129 in the positioning of the Cys198 and thus of the involvement of Cys198 as a base catalyst in the activation of the water molecule that attacks the Cys51 sulfur of the sulfonium cation. Anyway, if the mechanism proposed in [17] is right, it subsequently necessitates a rotation around the CH_2–S or the S–OH bond to position the OH group inside the active site to form the disulfide bond with the recycling Cys. Indeed, four *Neisseria meningitidis* X-ray structures were determined, and in particular the 3-D structures of the sulfenic acid intermediate (Fig. 3) and of the disulfide form (Fig. 4) [21]. Inspection of the 3-D structure of the sulfenic acid intermediate shows that the OH group is oriented inside and not at the entrance of the active site and is stabilized by a water molecule noted Wat2 *via* a network of hydrogen-bonded interactions including Wat1, Glu94, Tyr82 and Tyr134 residues which are the same amino-acids involved in the stabilization of the oxygen of the sulfoxide. Moreover, a water molecule, Wat3, which is always present in all the X-ray structures of the *N. meningitidis* MsrA, except in the disulfide form, is located at the opposite side of the active site towards the solvent and stabilized by hydrogen-bonded interaction with the imidazole of His199, at a position occupied by the sulfur of the recycling Cys in the X-ray structure of the disulfide oxidized form (Figs. 3B and 4). Therefore, Wat3 is in a good position to attack the sulfenic acid with release of the OH group of the sulfenic acid under a water molecule. Such a reaction has to be facilitated by a proton donor catalyst located within the active site. The Glu94 is likely involved indirectly *via* Wat1 (2.6 Å) which is ideally positioned at 3.1 Å from the OH group of the sulfenic acid (Fig. 3A). Thus, an exchange of the OH of the sulfenic acid intermediate with the solvent, when Glu94 is present, might occur before the MS measurements and thus renders the interpretations of the isotopic experiments recently reported questionable [18]. Indeed, experiments done by our group, several years ago with the *E. coli* C86S–C198S–C206S MsrA showed by MS that when the catalytic Cys is oxidized under ^{16}O sulfenic acid state, no significant ^{18}O isotopic exchange occurs at pH 8 with the solvent $H_2{}^{18}O$ when Glu94 is mutated into Ala whereas an exchange occurs when Glu is present (unpublished data). To give a clear-cut answer, experiments using labeled Met-^{18}O as substrate are underway with $H_2{}^{16}O$ as solvent to confirm the role of Glu94 in the ^{18}O isotopic exchange. If its role is confirmed, this means that the oxygen of the sulfenic acid derives from that of Met-O and as a consequence the water molecule which derives from the sulfurane would be caged within the active site and would attack the sulfonium cation as proposed in Fig. 2. Thus, Glu94 would play not only a major role as an acid/base catalyst in formation of the sulfurane and activation of the water that attacks the sulfonium cation, but also could favor the exchange of the OH group of the sulfenic acid with the solvent when the recycling Cys is mutated. In this context, the origin of the oxygen in the sulfenic acid intermediate remains to be determined for MsrB and fRMsr. Moreover, in the absence of X-ray structure of the sulfenic acid intermediate, no structural information is available to date for MsrB and fRMsr on how the water molecule which attacks the catalytic Cys sulfur of the sulfonium cation, would be activated. The fact that the rate of the reductase step of MsrB is similar in the presence or absence of recycling Cys as mentioned above, does not support a catalytic role of the recycling Cys in the activation of the water molecule. For MsrB, a possibility is the implication of the catalytic His103 similarly to the catalytic Glu94 in MsrA.

The MsrBs in which a selenocysteine (Sec) replaces the catalytic Cys is a particular case. For its Cys counterpart, the catalytic active form in the presence of the substrate is the $Cys117^{-}/His103^{+}$ species with pK_{apps} of 6.6 and 8.3, respectively as well illustrated by the bell-shaped curve representing the variation of the rate of the reductase step in function of pH [14]. We have however no clear indication on the way by which the hydrogen of the catalytic Cys117 is transferred to the imidazole of His117. Indeed, the distance between the sulfur of the Cys117 and the N^{δ} of the imidazole of His103 is 6.5 Å in the presence of the substrate in *N. meningitidis* MsrB. Formation of the Michaelis complex leads however to a significant change of the pK_{app} values of Cys117 by −2.7 units and of His103 by +2.6 units from their values in solution. Such pK_{app} modifications favor the attack of the thiolate of the Cys117 on the sulfur of the sulfoxide function, which is highly polarized, with a proton transfer from the imidazolium ring of His103 to the oxygen of the sulfoxide to form the sulfurane. The Sec MsrB should behave likewise, except that in resting Sec MsrB, the Sec residue would be already deprotonated because the intrinsic pK_{app} of a selenol is 5.2. The catalytic active $Sec117^{-}/His103^{+}$ species is likely also formed only when the substrate binds. More difficult is to predict how the selenosulfurane evolves into a selenosulfonium cation and

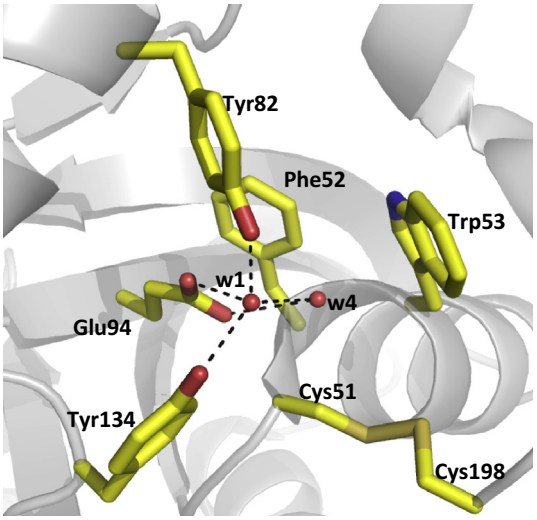

Fig. 4. Schematic view of the X-ray structure of the Cys51–Cys198 disulfide oxidized form of MsrA from *N. meningitidis*. Formation of the Cys51–Cys198 disulfide bond implies a reorientation of the Cys51 side chain with a translation of the Cys51 main chain by 0.6 Å. The consequence is the displacement of the Cys51 S^{γ} by 2.6 Å towards the entry of the active site. A large movement of the Gln122–Thr132 part which covers the active site before formation of the disulfide bond occurs, the consequence of which is to break the hydrogen-bonded interaction between Asp129 and Tyr197 and to expose a hydrophobic surface. The Wat1 in interactions with Ty82, Tyr134 and Glu94 shifts towards the entry of the active site by 1.1 Å. The S^{γ} atom of Cys198 is located at the position occupied by Wat3 in the X-ray structures of the reduced, substrate-bound and sulfenic acid MsrAs. A water molecule, noted Wat4, which is absent in the other X-ray structures of the *N. meningitidis* MsrA, is at 3.0 Å of the $O^{\varepsilon 2}$ atom of Glu94 and at 3.1 Å of Wat1. The amino-acids adjacent to Cys198 are very mobile which likely explains the lack of electron density for residues 199–201 [21].

a water molecule in the absence of knowledge of a Sec MsrB 3-D structure, in particular of the selenenic acid intermediate structure. Indeed, the amino-acid sequence of the Sec MsrB shows the absence of His100 which is a good candidate *via* a water molecule to favor the release of the water molecule from the sulfurane as postulated for its Cys counterpart. We can only hypothesize that His103 is likely the base which activates the water molecule that attacks the selenium of the selenosulfonium cation. From a chemical point of view, it can be predicted that the rate of reduction of Met-O is not too different when comparing a Sec MsrB to its Cys counterpart. Indeed, in both cases, the Sec and the Cys residues are under anionic form in the Michaelis complex and it is described that the difference in nucleophilicities between Se^- and S^- is relatively modest in the range of six to ten-fold [25–28]. If we postulate that formation of the selenosulfurane remains rate-limiting as shown for its Cys counterpart with protonation of the oxygen of the selenosulfurane by His103, an increase of the limiting rate by a factor of maximum 10-fold is expected for formation of the selenenic acid intermediate. This is in apparent contradiction with the kinetic data published for Sec MsrBs, the activity of which is found to be 100-fold higher than its Cys-containing form [29,30]. In fact, the kinetics which were done with DTT correspond to the rate of the recycling process that is overall-rate limiting. To be conclusive, the kinetic data of the reductase step of a Sec MsrB has to be determined by rapid kinetic approaches in the absence of reductant.

Some organisms express a MsrA with a catalytic Sec residue like in *Clostridium* sp. [31] or in *Clamydomonas reinhardtii* [32]. Recently, the X-ray structure of the Cys mutant of the *Clostridium oremlandi* MsrA was described [33]. These MsrAs share the equivalent catalytic Glu94, and Tyr82 and Tyr134 but have no recycling Cys. It is described that in the presence of DTT used as reductant, the rate is at least 20-fold higher than that of its Cys mutant. Based on the kinetics, it was concluded that the presence of the Sec residue provides a substantial catalytic advantage. In fact, it is also possible that the active site is not adapted to efficiently activate a Cys residue, the consequence of which would be to decrease the rate of the reductase step of the Cys mutant. Moreover, as pointed out above, the rate measured with DTT does not correspond to that of the reductase step.

3. The substrate binding

Previous studies showed that MsrA, MsrB and fRMsr were Met-O stereospecific. The MsrA reduces the *S* epimer of the sulfoxide function while MsrB and fRMsr reduces the *R* epimer as indicated in the Introduction. Moreover, MsrA and MsrB have a preference for Met-O included in a polypeptide chain as deduced from the catalytic efficiency of the reductase step which is about 10-fold higher compared to that determined with free Met-O. In contrast, the fRMsr only recognizes free Met-O. These differences in substrate binding selectivity are likely related to the *in vivo* role of Msrs. The physiological role of MsrA and MsrB is to repair oxidized Met included in a protein while that of fRMsr is not clearly defined to date. Inspection of the 3-D structures of MsrA and MsrB shows that their active sites are located at the surface of each protein. This can explain why the neighboring amino-acids that surround the Met-O have no influence on the catalytic efficiency of the reductase step. Moreover, the Met-O residue, in particular the CH_2–CH_2–$SOCH_3$ side chain, has to be accessible in the protein target [34,35]. In MsrA and MsrB, the binding site of the $-SOCH_3$ function is composed of a hydrophilic subsite which stabilizes the oxygen of the sulfoxide function as already described and a hydrophobic subsite in which the ε methyl group binds i.e. Trp53 and Phe52 in MsrA, and Trp65 in MsrB. In the absence or presence of substrate, residues Asp129 and Tyr197 in MsrA which are located at the entrance of the active site, are in hydrogen-bonded interactions (Fig. 1A and B). Inspection of the X-ray structure of *Mycobacterium tuberculosis* MsrA in complex with protein-bound Met shows that Asp129 can be hydrogen-bonded to the backbone amide of Met [36]. This is confirmed by the X-ray structure of the C51S MsrA-AcMet-*S*-ONHMe complex in which Asp129 is hydrogen-bonded to the NH group of the substrate (Fig. 1B). This interaction can therefore be a factor that discriminates in favor of the binding of a peptidic Met-O versus a free Met-O. However, mutation of the Asp129 of the *N. meningitidis* MsrA has a relative modest effect on the affinity not only for a substrate which mimics a peptidic substrate but also for a free Met-O i.e. a K_S maximum effect of 10 [13]. Thus, the relative selectivity of MsrA for protein-bound Met-O likely arises from other structural factors. In MsrBs, we have no structural information for the implication of selective hydrogen-bonded interactions which could favor the binding of a peptidic substrate versus a free Met-O although kinetic data also indicate a better catalytic efficiency for peptidic substrate [7]. The situation is totally different for dimeric fRMsr. Indeed, only the free L-Met-*R*-O isomer is accepted efficiently as a substrate [3,23]. The absence of binding and of activity of a Met-O included in a polypeptide is due to the architecture of the active site. The substrate is strongly bound in a small cavity, capped by a flap which is very flexible and contains the catalytic Cys118. The sulfoxide function is also stabilized by a hydrophilic subsite like in MsrA and MsrB while the ε methyl group is stabilized by an interaction with in particular Trp62 (Fig. 1D). What is different is the binding of the free carboxyl function and the amino group. In particular, it is the carboxylate which is bound with its charge totally delocalized and buried at the bottom of the active site. Its binding is strongly favored by the presence of an oxyanion binding site composed of the backbone amide of three residues i.e. Val93, Cys94 and Ile116 which excludes the binding of a peptidic Met-O (Fig. 1D). The NH^{3+} group is stabilized by a hydrogen-bonding network including

the side chain of the invariant Glu125 and the main chain oxygen of Ile [23] (Fig. 1D). The situation is different in the X-ray structure of the *Staphylococcus aureus* fRMsr in complex with Met-O [37]. In this case, the sulfoxide function occupies the position of the carboxylate in the *N. meningitidis* fRMsr structure and vice versa. This position excludes a competent catalytic binding of the substrate [38]. Indeed, the position of the catalytic Cys118 is not appropriate to attack the sulfur of the sulfoxide function. Moreover, the presence of the oxyanion hole prevents protonation of the oxygen of the sulfurane and thus formation of the sulfonium cation and of the water molecule which is a prerequisite to form a sulfenic acid intermediate. Formation of a non-productive binary complex is likely the consequence of the architecture of the active site and of its high conformational flexibility. These differences in substrate binding could be indicative that the fRMsr–substrate binary complexes formed in solution would be not all catalytically competent.

4. The recycling process

4.1. A recycling Cys is implicated

4.1.1. The activation of the recycling Cys

When a recycling Cys is present, a disulfide bond is formed by reaction between the catalytic Cys oxidized under sulfenic acid and the recycling Cys which is then reduced by Trx. The question which arises is how the disulfide bond is efficiently formed. Kinetic measurements carried out on MsrA and MsrB from *N. meningitidis* showed that the rate of formation of the disulfide bond is high at pH 8 and limited by that of formation of the sulfenic acid intermediate i.e. ~800 and 85 s^{-1}, respectively. This indicates that the "intrinsic" rate of formation of the intradisulfide bond is very high. A prerequisite to efficiently form a disulfide bond from the sulfenic acid intermediate and a recycling Cys is to favor the release of the OH group from the sulfenic acid under a water molecule. First, this implies that the sulfenic acid is not deprotonated within the active site and thus has a pK_{app} of at least 7 as determined from chemistry [39]. Second, an assisted acid catalysis should occur to favor the release of the water molecule. The Glu94 and His103 residues could be involved *via* water molecules which form a network of hydrogen-bonded interactions in the active site of MsrA and MsrB, respectively. The recycling Cys reacts under its thiolate form. So the next question which arises is whether – or not – the recycling Cys has to be activated by its protein environment. An attractive hypothesis is that the recycling Cys can give its proton to the OH group of the sulfenic acid *via* a concerted mechanism. However, inspection of the 3-D structure of the sulfenic acid intermediate in MsrA shows that the positioning of the recycling Cys198 and of the OH group of the sulfenic acid is not favorable for a direct proton transfer [21]. In the case of the C198A MsrA variant, the water molecule, Wat3, is ideally placed to be activated in particular by the imidazole of His199 and thus to attack efficiently the sulfenic acid intermediate when the recycling Cys198 is absent. Note, as already mentioned, that the recycling Cys198 has the position of the Wat3 molecule in the structure of the disulfide form. Thus, it is tempting to postulate a similar activation of the Cys198, even though NMR and X-ray structures have shown a great mobility of Cys198. However, His199 is not invariant, and moreover other subclasses of MsrA exist for which the location of the recycling Cys is different i.e. positions 54 and 206 (see Section 4.1.3). Recently, a computational model was proposed for formation of the Cys51–Cys198 disulfide bond [17]. In this model, Asp87 plays a major role in the activation of the Cys198 *via* a water molecule and in the release of the OH group of the sulfenic acid intermediate. Moreover, the rate-limiting step appears to occur after formation of the sulfenic acid. This is in apparent contradiction with our kinetic data which show no accumulation of the sulfenic acid intermediate and a small kinetic effect when Asp129 is mutated into Ala. In this context, it is important to note that the rate of the reaction between a sulfenic acid and a thiolate is very high i.e. 10^5–10^8 - M^{-1} s^{-1} [40]. Considering a pK_{app} of ~9 for Cys198 in the sulfenic acid form, similar to that determined in the free MsrA, and the fact that (1) the reaction is intramolecular and thus should afford a significant enhancement of the rate of formation of the intradisulfide bond, and (2) the recycling Cys198 is located on a very mobile loop, the concentration of the thiolate form of the recycling Cys198 at pH 8 within the active site could be sufficient to reach a rate of 800 s^{-1}. In the case of MsrBs, whatever the subclasses considered i.e. with a recycling Cys either at position 63 or 31, we have no kinetic and structural information to date to determine whether the recycling Cys is activated – or not – by its protein environment. In this context, nothing is also known on how the recycling Cys84 is activated – or not – in fRMsr upon formation of the Cys118–Cys84 disulfide which is the bond reduced by Trx.

A particular subclass of MsrAs exists which possesses two recycling Cys at position 198 and 206. Two successive reductase steps can occur with formation of two mol of Met. The Cys51–Cys198 disulfide species is first formed but does not accumulate and evolves into the Cys198–Cys206 disulfide species. It is the second disulfide bond which is reduced by Trx [41]. The rate of formation of the Cys198–Cys206 is thus governed by that of formation of the sulfenic acid on Cys51. So, in this case, the Cys206 which is located on the same flexible loop as Cys198 reacts at a high rate i.e. ~800 s^{-1}, on the Cys198 engaged in a disulfide bond with Cys51. As already discussed for Cys198, the question which arises is how the Cys206 is activated – or not. The situation is however different from the subclass of MsrA with only one recycling Cys. Indeed, the second recycling Cys attacks a Cys involved in a disulfide bond instead of a sulfenic acid. If no activation occurs, this means that the kinetic advantage brought by the intramolecular process which liberates Cys51 from the Cys51–Cys198 disulfide bond combined with the high flexibility of the loop on which Cys206 is located would be sufficient to reach a rate of ~800 s^{-1}.

4.1.2. The kinetics of the Trx-recycling process

In the case of MsrA from *N. meningitidis* with a recycling Cys at position 198, and MsrB from *N. meningitidis* with a recycling Cys at position 63, the overall rate-limiting step was studied and determined to be associated with the Trx-recycling process. More precisely, using Trx as a fluorescent probe, we showed that the rate-limiting step is associated with the release of oxidized Trx (Trx_{ox}) from the Msr_{red}/Trx_{ox} complex rather that the chemical process involving the transfer of two electrons and two protons by a kinetic factor of 7- and 5-fold for MsrA and MsrB, respectively [7,19]. Such kinetic study was not carried out with Sec MsrB to date. What is described is that the introduction of a catalytic Sec in a bacterial MsrB which possesses a catalytic and a recycling Cys gives a substantial kinetic advantage using DTT as the reductant but any kinetic advantage with Trx [30]. Such results were expected. In particular, the rate-limiting step with Trx as the reductant likely remains the same i.e. associated with the release of the Sec Msr_{red} from the Sec Msr_{red}/Trx_{ox} complex. What should be significantly higher is the rate of the chemical process of transfer of the two electrons and two protons from the selenenylsulfide to the Trx_{red} as described for selenoglutaredoxin (Grx) [42]. When larger quantity of Sec MsrB will be available, such a study could be undertaken with Trx as a fluorescent probe. For fRMsrs, the kinetics of the recycling process by Trx have not been yet determined.

4.1.3. The interactions of Msr with Trx

Only one X-ray structure of a covalent interdisulfide complex between a MsrA from Yeast and Trx2 was determined to date [43]. The interface between both partners buries a small area of

1000 Å^2 from solvent which is typically a sized interface for redox partners having short-lived interactions. Although the overall 3-D structures of Trx2 and of MsrA are similar in the complex to those of Trx2 and MsrA alone, a substantial structural disorder of the segment from amino-acids 122–132, which covers the MsrA active site that includes Asp129 postulated to be involved in the substrate binding, occurs. The consequence is to expose not only a hydrophobic area as suggested from NMR studies [44] but also the C-terminal loop of the MsrA including the recycling Cys198, which can now directly interact with Trx2 and permits the reduction of the Cys51–Cys198 disulfide bond. As already mentioned, comparison of the amino-acid sequences and of the 3-D structures, when available, shows that the recycling Cys can be located in different protein environments. In *Bacillus subtilis* MsrA, the recycling Cys is at position 54 in an α-helix which also contains the catalytic Cys51 at the N-terminus; and it was shown that the catalytic efficiency of the recycling process which includes reduction of the Cys51–Cys54 disulfide bond is similar to that of a Cys51–Cys198 disulfide bond [5]. In the case of the *E. coli* and bovine MsrAs, a second recycling Cys is present at position 206 located in the same loop as for Cys198. In this case, it is the Cys198–Cys206 disulfide bond which is efficiently reduced by Trx [41]. Thus, due to the different signatures surrounding the recycling Cys54, Cys198 and Cys206, the stabilizing interactions between the different subclasses of MsrAs and Trx will be not the same.

In MsrBs, the recycling Cys can be also either located in different positions or absent in ~30% of the MsrBs. When a recycling Cys is operative, a subclass of MsrB including the *Xanthomonas campestris* enzyme was found where the recycling Cys is located at the N-terminal part at position 31 at a distance of at least 13 Å from the catalytic Cys117 instead of 3.2 Å for the recycling Cys63 in *N. meningitidis* MsrB which belongs to the major subclass [45]. As shown by the inspection of the X-ray structure, formation of the Cys117–Cys31 disulfide bond induces a substantial reorganization of the fold which is the consequence of the great flexibility of the MsrB [22] (Fig. 5). In particular, the loop that contains the Trp65 which is essential for the substrate binding, has to be withdrawn from the active site and thus Trp65 no longer constitutes the hydrophobic face of the active site in contrast to the Cys63 subclass. Thus, it is probable that Trx which reduces efficiently both the Cys117–Cys31 and Cys117–Cys63 bond, does not interact with the same hydrophobic interface. The mouse Sec MsrB1 has a recycling Cys located at a position equivalent to that of Cys31 and thus likely belongs to the same subclass of MsrB than *X. campestris* MsrB. Therefore, similarly to the Cys31 subclass, formation of the selenenyldisulfide bond should also withdraw the Trp65 from the active site even though the N-terminal region is shortened [46]. The fact that Trx can reduce efficiently the Cys117–Cys63 and Cys117–Cys31 disulfide bonds located in different protein environments is another example of the role of Trx as a protein "ménage" which has the ability to recognize a large variety of substrates but nevertheless with a structural specificity *à minima* [47]. In this context, the knowledge of the 3-D structure of covalent MsrB–Trx binary complexes from the two subclasses of MsrB could be another illustration of the ability of Trx to efficiently interact with different hydrophobic interfaces. Similarly, the determination of the 3-D structure of a fRMsr–Trx complex would be also informative.

4.2. The recycling process in the absence of recycling Cys

Various reductants were described to regenerate monocysteinic MsrB (1-Cys MsrB) *in vitro* i.e. thionein, seleno compound, glutathione (GSH) and Trx [48–53]. But no evidence has been yet obtained for their *in vivo* implication. Whatever the reductant, except for Trx, no binding site of these reductants exist in MsrB. This means that formation of a MsrB-adduct is a second-order kinetic process that will depend on the effective concentration of the nucleophile. This is an indication that GSH, when it is synthetized in cells, could be the recycling agent because its *in vivo* concentration is higher compared to those of thionein and seleno compounds. Moreover, an MsrB-GS adduct which likely has its GS moiety at the surface of the protein can be easily recognized by a Grx that should facilitate the recycling process. However, although the concentration of GSH is high *in vivo*, e.g. 1–5 mM, the reaction between MsrB and GSH will depend on the *in vivo* conditions. Indeed, the pK_{app} of GSH is 8.9 [54]. So if the *in vivo* pH is between 7.9 and 6.9 as measured in the stroma of plants, the effective concentration of the thiolate form of GSH will be only 5–25 μM at pH 6.9. The experiments done on *Arabidopsis thaliana* gave a k_{cat} of 0.075 s^{-1} at a concentration of 10 mM of GSH, at pH 8 [51]. Assuming a bimolecular rate of the sulfenic acid and GS$^-$ between 10^5 and 10^8 M^{-1} s^{-1} [40], the catalytic process will be thus rate-limited by the Grx-recycling process. In our case, we have studied the reduction of a 1-Cys MsrB from *Mycoplasma pulmonis*, an organism which does not synthetize GSH. In our hands, the GSH/Grx system is capable to reduce the sulfenic acid with a k_{cat} of 0.1 s^{-1} at a concentration of 10 mM of GSH and at pH 8, that is in the range of that published for other 1-Cys MsrBs. Similarly, when the bacterial MsrB from *N. meningitidis* has its recycling Cys mutated, the GSH/Grx system can be also operative (unpublished data). In other words, when a sulfenic acid accumulates and provided that GSH is synthetized, 1-Cys MsrBs can be recycled by the GSH/Grx system. However, the rate of the recycling process is low and at least 10–100-fold lower compared to that with Trx on Msrs with a recycling Cys. Thus, the GSH/Grx system cannot be functional in Msrs in which a disulfide bond is formed and reduced by Trx. This is in accord with the fact that a Met auxotrophic *E. coli* strain is unable to grow in the presence of Met-O when the genes coding for Trx1 and Trx2 are inactivated (55 and refs therein), thus demonstrating that glutathione cannot supply Trx for the regeneration of the Msr activity in *E. coli*, including the MsrA, MsrB and fRMsr activities. When GSH is not synthetized like in *M. pulmonis*, another reductant has to be involved, implying a recycling process to regenerate the reductant which forms a disulfide intermediate with the Msr.

Another possibility is to reduce the sulfenic acid intermediate directly by Trx. Some data were published which favor this possibility [50–52]. In particular, the Trx-like protein CPDSP32 from plastidial *A. thaliana* reduces MsB1 with a k_{cat} of 0.035 s^{-1}. In the same time, it was also described that the GSH/Grx system can be operative [53]. In our hands, Trx1, but not Trx2, from *M. pulmonis*, was shown to reduce the sulfenic acid intermediate with a k_{cat} of only 0.004 s^{-1} and a K_M of 40 μM for Trx, that corresponds to a low catalytic efficiency of 10^2 M^{-1} s^{-1} (unpublished data). Clearly, in all the cases, the rate is low compared to the Trx-recycling process when a recycling Cys is present, by at least a factor of 100. We can note that, in our hands, we never detected a significant recycling activity with Trx for MsrA or MsrB in which the recycling Cys was mutated. This is likely due to the low accessibility of the sulfenic acid within the active site to Trx which prevents formation of a catalytic competent complex between Msr and Trx.

In this context, fRMsr is an interesting example. Indeed, the sulfenic acid intermediate formed in the triple mutant in which only the catalytic Cys is present, is efficiently catalytically reduced i.e. 6×10^4 M^{-1} s^{-1} with a K_M value for Trx of 16 μM and a k_{cat} of 1 s^{-1} [23]. This strongly suggests that Trx is the physiological reductant for the subclass of fRMsrs with no recycling Cys84 which represents less than 6% of the subclasses of fRMsrs [23]. The value of 6×10^4 M^{-1} s^{-1} is in the range of those described for reduction of the disulfide bond by Trx in MsrA and MsrB. This is likely the consequence of the high flexibility of the fRMsr, in particular of the lid which bears the catalytic Cys118, and permits formation of an efficient catalytic binary complex of the fRMsr mutant with Trx.

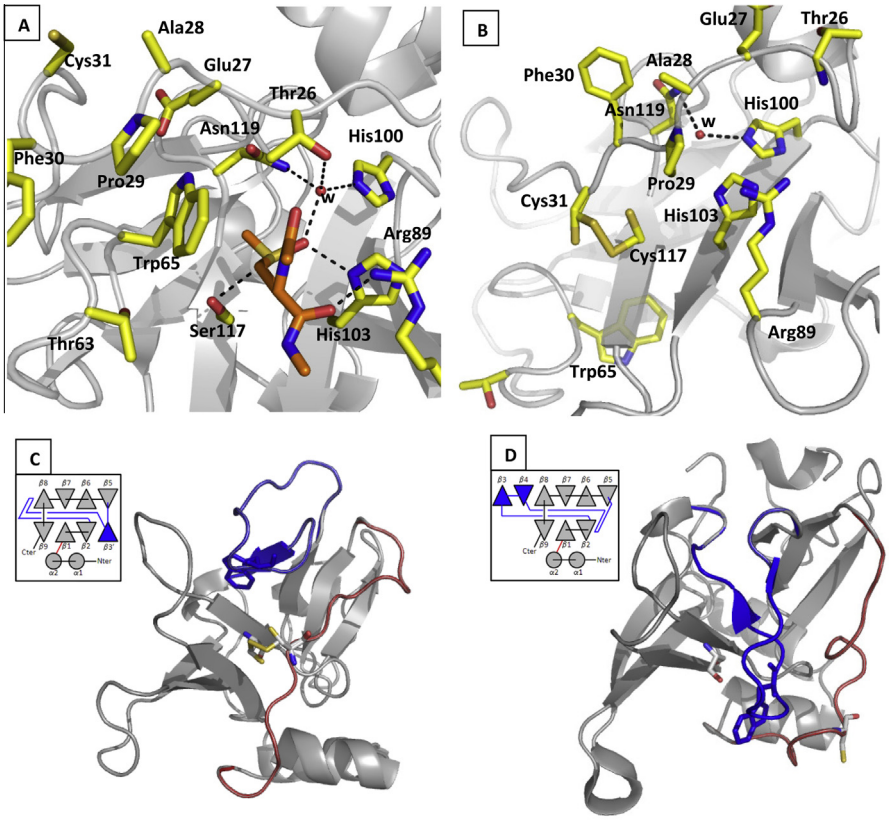

Fig. 5. The active sites of *X. campestris* MsrB. (A) The Cys117S MsrB in complex with Ac-L-Met-*R*-ONHMe. The conformation of the active site is similar to that observed for the *N. meningitidis* MsrB (see Fig. 1C). The Cys31 resides on another loop than that of Cys63 in *N. meningitidis* MsrB. The distance between Ser117 and Cys31 is 13.5 Å. This distance is not compatible with formation of the Cys31–Cys117 disulfide bond. Moreover, Trp65, which is essential for the binding of the ε methyl group of the substrate is inserted between Ser117 and Cys31. (B) The Cys31–Cys117 oxidized form. A drastic conformational change has to take place upon formation of the Cys31–Cys117 disulfide bond. The consequence is that one-third of the residues of the MsrB moves with the withdrawal of Trp65 from the active site concomitantly with a reorganization of the β-sheets of the disulfide oxidized MsrB structure (Fig. 5C) compared to MsrB in complex with the substrate (Fig. 5D). Such significant conformational change implies a high flexibility of the loop on which Cys31 and Trp65 are located.

5. The oxidase activity of MsrA with two recycling Cys

As indicated at the end of the Section 4.1.1 a subclass of MsrAs with two recycling Cys exists like the mouse, *E. coli* or human MsrA. Recently, it was shown that in the absence of Trx, a stereospecific oxidase activity can be operative *in vitro* with the mouse MsrA [18]. Indeed, under Cys51SOH, Cys198–Cys206 disulfide oxidized state, the MsrA oxidizes a Met residue to give a Met-*S*-O. The catalytic efficiency of the oxidase activity compared to that of the reductase activity is very low i.e. 240 fold-lower and is essentially due to a low affinity for Met compared to Met-O. The *in vivo* relevance of the oxidase activity in the presence of Trx remained to be demonstrated. Recently, we have shown by kinetic approaches with the *E. coli* MsrA used as a model of the mouse MsrA that the oxidase activity could be operative in the presence of Trx that is the conditions *in vivo*. Indeed, we demonstrated that (1) the Cys51–Cys198 disulfide intermediate does not accumulate and, (2) the rate of formation of the oxidized MsrA under Cys51SOH and Cys198–Cys206 disulfide state, which corresponds to the rate of formation of the second mol of Met, is largely higher than that of the Trx-recycling process [41]. Moreover, inspection of the X-ray structure of the covalent complex between MsrA from yeast with an equivalent Cys198 and Trx shows that Trx covalently bound to the MsrA *via* Cys198 is positioned outside the active site and thus does not prevent the binding of a molecule of Met [42]. Added to the facts that the cellular concentration of Trx is high e.g. 75 μM for Trx1 in *E. coli* [55] and the apparent affinity constant of MsrA for Trx1 is in the range of 10 μM [5,56], this reinforces the conclusion that the oxidase activity can be operative *in vivo* without the action of a regulatory protein in order to block the binding of Trx. It is also important to note that the oxidase activity needs not only the accumulation of the sulfenic acid intermediate to be revealed but also a high ratio of protein Met versus protein Met-O to counteract the low affinity of Met compared to Met-O. This is likely the case *in vivo*. Anyway, such oxidase activity is restricted to the subclass of MsrA with two recycling Cys which represents less than 10% of the MsrAs. Indeed, in the subclasses of MsrAs with only one recycling Cys, the sulfenic acid intermediate does not accumulate but forms a disulfide bond.

6. Concluding remarks

Since the last decade, significant progresses have been done to decorticate the chemical mechanism of reduction of a Met-O by Msrs. Thanks to the knowledge of the 3-D structures and theoretical chemistry investigations, it is now well established for MsrA and MsrB that formation of the sulfurane is rate-determining and is formed *via* an acid/base catalysis and then evolves into a sulfonium cation. The sulfonium cation is then attacked by an activated water molecule to give a sulfenic acid intermediate. Some debate remains on the origin of the oxygen of the sulfenic acid in MsrA which likely derives from the substrate. In this context, no information on the origin of the oxygen in formation of the sulfenic acid intermediate in MsrB and fRMsr has not been yet obtained. Moreover, for the fRMsr, the amino-acids suggested to be involved in

formation of the sulfenic acid and in the recognition of the substrate remain to be characterized at the kinetic level.

The regeneration of the reductase activity passes through formation of a disulfide bond for the Msrs which possess a recycling Cys. This is the case for most of the MsrAs, 70% of the MsrBs, and most of the fRMsrs. The fact that the rate of formation of the disulfide bond between the recycling and the catalytic Cys is high, raises the question of whether the recycling Cys has to be activated by a protein catalyst. Based on kinetic and structural data and the fact that a thiolate reacts efficiently with a sulfenic acid, we propose that the activation of the recycling Cys by the protein environment in MsrA would be not a necessity, a situation which remains to be evaluated for MsrB and fRMsr.

The overall rate-limiting step in MsrAs and MsrBs is associated with the Trx-recycling process, more particularly to the release of the Trx_{ox} from the binary complex Msr_{red}–Trx_{ox}. In this context, adapted kinetic tools including stopped-flow and quenched-flow approaches have to be used to conclude definitively whether – or not – the presence of a catalytic Sec residue in MsrB affords a kinetic advantage for the Trx-recycling process and for the reductase step, as well. In the case of the fRMsr, the rate-limiting step has to be determined.

Only one 3-D structure of a covalent MsrA–Trx complex was determined to date. The interface between both partners is typical of redox partners having short-lived interactions. Such situation remains to be evaluated for MsrBs and fRMsrs. The fact that the recycling Cys in MsrAs and MsrBs are located at various positions indicate that the hydrophobic surfaces which interact with Trx are different but are however selective.

In the subclass of MsrB which possesses only a catalytic Cys, the recycling process likely uses the GSH/Grx system to reduce the sulfenic acid because the *in vivo* concentration of the thiolate form of the GSH is sufficient even at pH 7. When GSH is not synthetized *in vivo* in bacteria like *M. pulmonis*, another reductant has to be implicated.

In the presence of high concentration of Met, an oxidase activity which is the reverse of the reductase activity, can occur provided that the catalytic Cys accumulates under sulfenic acid state. This is the case for the subclass of MsrA with two recycling Cys. Recent kinetic studies support that the oxidase activity can be operative in the presence of Trx, thus excluding a regulatory process to block the action of Trx *in vivo*.

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