REGENERATION MECHANISMS OF ARABIDOPSIS THALIANA METHIONINE SULFOXIDE REDUCTASES B BY GLUTAREDOXINS AND THIOREDOXINS

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Running title: Regeneration of MSRB activity by Grxs and Trxs
Methionine oxidation leads to the formation of S- and R-diastereomers of methionine sulfoxide (MetSO), which are reduced back to methionine by methionine sulfoxide reductases (MSRs) A and B, respectively. MSRs are classified in two groups depending on the conservation of one or two redox-active Cys: 2-Cys MSRBs possess a catalytic Cys reducing MetSO and a resolving Cys allowing regeneration by thioredoxins (Trxs). The second type, 1-Cys MSRBs, possess only the catalytic Cys. The biochemical mechanisms involved in activity regeneration of 1-Cys MSRBs remain largely elusive. In the present work, we used recombinant plastidial Arabidopsis thaliana MSRB1 and MSRB2, as models for 1-Cys and 2-Cys MSRBs, respectively, to delineate the Trx- and glutaredoxin-dependent reduction mechanisms. Activity assays carried out using a series of cysteinic mutants, combined to measurement of free thiols under distinct oxidation states and to mass spectrometry experiments, show that the 2-Cys MSRB2 is reduced by Trx through a dithiol-disulfide exchange involving both redox-active Cys of the two partners. Regarding 1-Cys MSRB1, oxidation of the enzyme after substrate reduction leads to the formation of a stable sulfenic acid on the catalytic Cys, which is subsequently glutathionylated. The deglutathionylation of MSRB1 is achieved by both mono- and dithiol glutaredoxins (Grxs) and involves only their N-terminal conserved catalytic Cys. This study proposes a detailed mechanism of the regeneration of 1-Cys MSR activity by Grxs, which likely constitute physiological reductants for this type of MSR.

INTRODUCTION

Proteins are prone to oxidative modifications due to the action of reactive oxygen species (ROS). Methionine (Met), one of the most susceptible amino acids to oxidation (1), is converted into methionine sulfoxide (MetSO) resulting in altered conformation and activity for many proteins (1). Methionine sulfoxide reductases (MSRs), that catalyze the reduction of MetSO back to Met, are present in most living organisms. MSRA, the first MSR isolated (2), is specific of the MetSO S-diastereomer and participates in protection against oxidative stress (3). A second MSR type, MSRB, which catalytically reduces the MetSO R-diastereomer, was identified later (4). MSRA and MSRB are monomeric enzymes, that display no sequence or structural homologies, but share a similar three-step catalytic mechanism: i) reduction of MetSO by MSR and formation of a sulfenic acid intermediate on the “catalytic” cysteine (Cys), ii) formation of a disulfide bond between “catalytic” and “resolving” Cys and release of H2O, iii) reduction of the disulfide bond by a reductant (5,6). Thioredoxins (Trxs) have been proposed to be the biological reductant for MSRs (2,7). Trxs are small and ubiquitous disulfide reductases with a Trp-(Gly/Pro)-Pro-Cys active site. They function as electron donors and play essential roles in many processes, through control of protein conformation and activity, by supplying the reducing power needed to reduce disulfide bonds in target proteins.

Most MSRBs, named 2-Cys MSRBs, possess two conserved Cys and are actually reduced by Trxs (7,8). However, in a second class of MSRBs, termed 1-Cys MSRBs and representing ca. 40% of known MSRBs, the “resolving” Cys residue corresponding to Cys-63 in E. coli is replaced by Thr or Ser (8,9). Although some of these MSRBs possess another potential resolving Cys (9), most 1-Cys MSRBs do not have any additional Cys indicating that an alternative mechanism involving no intramolecular disulfide reduction is needed for their regeneration (7). Contrasting data concerning the role of Trxs in providing electrons to these MSRBs have been reported. Several studies showed that cytosolic Trx is not an efficient reductant for human 1-Cys MSRBs (10-12) while mitochondrial Trxs were recently reported to efficiently regenerate mitochondrial 1-Cys MSRBs (13). It has been proposed that regeneration of mammalian and plant 1-Cys MSRBs could involve direct
reduction of the cysteine sulfenic acid form generated during catalysis (10,13-15).

Arabidopsis thaliana possesses two plastidial MSRBs referred as MSRB1 and MSRB2 and related to 1-Cys and 2-Cys MSRB types, respectively. MSRB2 possesses two Cys-Xxx-Xxx-Cys motifs potentially implicated in the coordination of a Zn atom, a Cys in position 187 corresponding to the catalytic Cys-117 of E. coli MSRB, a potential resolving Cys in position 134 and an additional Cys in position 115. MSRB1 contains also the four Cys potentially coordinating Zn, the potential catalytic Cys-186 and a Cys in position 116 while the potential resolving Cys is replaced by a Thr in position 132. Previously, we showed that various types of canonical Trxs are efficient electron suppliers to MSRB2, whereas MSRB1 can only be reduced by the peculiar Trx CDSP32 (Chloroplastic Drought-induced Stress Protein of 32 kDa) and by Grxs (15-17). Grxs are oxidoreductases of the Trx superfamily possessing either a monothiol Cys-Xxx-Xxx-Ser or a dithiol Cys-Xxx-Xxx-Cys active site, and are generally reduced by glutathione (18). Grxs are able to reduce protein disulfides, but also glutathione-mixed disulfides, a reaction termed de-glutathionylation for which Trxs are not efficient catalysts (19,20). Classical dithiol Grxs can reduce disulfide bonds using both Cys of active site, as shown for E. coli ribonucleotide reductase, but can also reduce glutathione-mixed disulfides through a monothiol mechanism which requires only the N-terminal active site Cys (21). Cys-Xxx-Xxx-Ser-type Grxs catalyze de-glutathionylation either through a monothiol mechanism, as recently shown for chloroplastic GrxS12 (Cys-Ser-Tyr-Ser active site) (22), or through a dithiol mechanism as suggested for Cys-Gly-Phe-Ser GrxS (20,23).

We reported recently the involvement of Grxs in regeneration of MSRB activity (15). Nevertheless, the precise biochemical mechanism underlying regeneration by Grxs remains unknown. In this study, we performed a detailed analysis of the roles of redox-active Cys in reductants (Trxs and Grxs) and in acceptors (plastidial Arabidopsis MSRBs). We provide evidence that reduction of MSRB2 by Trxs is achieved through a classical dithiol-disulfide exchange. The data on MSRB1 reveal that 1-Cys MSRBs are regenerated by Grxs through a glutathionylation step of the catalytic Cys.

EXPERIMENTAL PROCEDURES

Site-directed mutagenesis

Nucleotide substitutions at specific positions in MSRB1 and MSRB2 were performed using the QuickChange mutagenesis method (Stratagene, La Jolla, CA). The pQE-30 expression vector (Qiagen, Valencia, CA) carrying the coding sequence of MSRB2 (17) was used as a template for mutagenic PCR. The MSRB1 coding sequence was cloned into the BamHI and PstI restriction sites of the pQE-30 expression vector and used to generate mutated forms. Primers for site-directed mutagenesis contained a modified restriction enzyme site allowing screening (Supplemental Table 1).

Expression and purification of recombinant proteins

Arabidopsis NADPH thioredoxin reductase B, wild-type (WT) and mutated forms of poplar Trx h1, GrxC4 and GrxS12 were purified as described (22,24-27). Recombinant WT and mutant forms of MSRB1 and MSRB2 proteins fused to an N-terminal 6xHis-tag were produced in M15rep4 E. coli strains and purified as previously described (15). After elution, proteins were desalted in 30 mM Tris-HCl, pH 8.0 using HiTrap™ Desalting Column (GE Healthcare). Proteins were reduced using 10 mM DTT for 30 min at room temperature and excess DTT was removed by desalting using Illustra™ NAP-5 Sephadex G-25 column (GE Healthcare) in the same buffer. Protein concentrations were determined using the bicinchoninic acid assay (BC Assay Reagent, Interchim, Montluçon, France) or spectrophotometrically using molar extinction coefficients at 280 nm of 23,960 M⁻¹.cm⁻¹ for WT MSRB1, C116S MSRB1, T132C MSRB1, C189S MSRB1 and 16,960 M⁻¹.cm⁻¹ for WT...
MSR activity assay

The activity of recombinant MSR proteins was determined by monitoring reduction of the synthetic substrate, dabsyl-MetSO, in the presence or absence of DTE, using a method modified from (15). Dabsyl-Met and dabsyl-MetSO were separated by high performance liquid chromatography (HPLC) using a C18 reverse phase column, SunFire™ 3.5 μm, 3.0 x 50 mm (Waters, Milford, MA) and 29 mM acetate buffer, pH 4.16 and acetonitrile as solvents. Apparent affinity for substrate (K_Dabsyl'MetSO) was determined using substrate concentrations ranging from 7.8 μM to 1 mM and non-linear regression (SigmaPlot 10.0, Systat Software, San Jose, CA, USA). Alternatively, the MSR activity was measured following NADPH oxidation at 340 nm in the presence of a Trx reducing system (either 200 or 400 μM NADPH, 2 μM Arabidopsis NADPH Trx reductase B, and a saturating concentration of poplar Trx h1 or C42S Trx h1 (40-100 μM)) or of a Grx reducing system (400 μM NADPH, 0.5 unit yeast glutathione reductase (Sigma), 10 mM GSH and 0.13 to 50 μM Grx) using a saturating concentration of N-acetyl-MetSO (2 mM) and 1 to 6 μM MSR. The reaction was carried out at 25°C in a 500 μL reaction volume. MSR activity was calculated from the slope due to NADPH consumption, after subtracting background activity in the absence of enzyme and considering that one mole of oxidized NADPH corresponds to one mole of Met formed.

Thiol titration

Free thiols were determined using the 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) procedure (28). Pre-reduced proteins (20-50 μM) were incubated in 30 mM Tris-HCl, pH 8.0, in the presence or not of 0.5 mM N-acetyl-MetSO. After 15 min incubation at room temperature, 100 μM DTNB was added and the absorbance at 412 nm was measured after 30 min incubation in the dark. The free thiol content was estimated using a molar extinction coefficient of 14,150 M⁻¹.cm⁻¹ for TNB.

Mass spectrometry analyses

Recombinant Arabidopsis MSR1 and MSR2 were reduced with 10 mM DTT for 1 h at room temperature and subsequently subjected to desalting on NAP-5 columns equilibrated with 50 mM HEPES-NaOH, pH 7.2. Pre-reduced proteins were treated for 15 min at room temperature with 2 mM N-acetyl-MetSO or for 30 min with 2 mM N-acetyl-MetSO and 2 mM GSH and subjected to desalting as described above. MALDI-TOF mass spectrometry analyses were performed before and after treatment in the presence of 10 mM DTT for 30 min at room temperature. Mass determination of MSR1 and MSR2 proteins was carried out on whole proteins or after tryptic digestion as described in (29).

RESULTS

Reduction of MSR2 by Trx

To determine the regeneration mechanism of MSR2 by Trxs, we first monitored the activity of MSR2 and C134T MSR2 in the presence of a Trx reducing system, composed of NADPH, NADPH Trx Reductase (NTR) and poplar Trx h1 (Fig. 1). The replacement of the potential resolving Cys, Cys-134, by Thr allows to mimic MSR1 sequence. In this assay, WT MSR2 exhibited a specific activity of 4758 ± 94 nmol Met.min⁻¹.mg protein⁻¹ while no significant consumption of NADPH was observed for C134T MSR2, indicating that the mutation compromises MSR recycling by Trx. Similar results were obtained with a mutated MSR2, in which Cys-134 was replaced by Ser (data not shown). These results are consistent with the absence of recycling by Trx previously reported for MSR1, which naturally possess a Thr at the position corresponding to the resolving Cys-134 (15). To gain further insight into the reduction mechanism, we assayed the capacity of C42S Trx h1, a mutant form lacking the second active site Cys, to supply reducing power to MSR2 (Fig. 1B). No activity was detected using this mutant.
indicating that the resolving Cys-42 of Trx h1 is required for MSRB2 reduction. The free thiol content of pre-reduced WT and C134T MSRB2 was measured in the presence or absence of N-acetyl-MetSO under non denaturing conditions to avoid titration of the four Zn-coordinating Cys (Table 1). In the case of reduced MSRB2, 3.2 thiol groups were titrated, likely corresponding to the two redox-active Cys (Cys-134 and Cys-187) and the additional free Cys (Cys-115). On the other hand, only 1.3 thiols were titrated after incubation with substrate (Table 1). The calculated decrease of 1.9 units of free thiols is consistent with the formation of an intramolecular disulfide bridge. Reduced C134T MSRB2 was found to contain ca. 2 thiols/mol. A decrease of 0.7 thiol was observed after incubation with N-acetyl-MetSO, likely due to oxidation of one unique Cys, Cys-187, to a sulfenic acid intermediate after reaction with substrate, the Cys-115 being still free to react with DTNB. Thiol titration carried out using C134S MSRB2 gave similar values (data not shown). Altogether, these results indicate that reduction of MetSO by MSRB2 involves the formation of a disulfide bond between Cys-134 and Cys-187, which is then reduced by Trxs through a dithiol-disulfide exchange requiring both catalytic and resolving Cys of the two partners.

Catalytic parameters of MetSO reduction by WT and mutated MSRBs

We have further investigated the role of the main residues differing between the two MSRBs, i.e. Thr-132 of MSRB1 and Cys-134 of MSRB2. Using DTE as a reductant, MSRB1 specific activity was 3-fold higher than that of MSRB2 (Table 2), in agreement with previous results (15). The replacement of Thr-132 by a Cys in MSRB1 led to a 14-fold decrease in the specific activity compared to that of the WT form. Interestingly, the specific activity of mutated MSRB2, in which Cys-134 was replaced by a Thr, was higher than that of WT MSRB2 and in the same range of that recorded for MSRB1.

To determine whether the different levels of activity observed for these proteins were due to alterations in substrate reduction or in regeneration of catalytic Cys by reductants, we carried out assays in the absence of DTE to calculate the stoichiometry of the reactions and the apparent affinities ($K_{Dabsyl-MetSO}$) for dabsyl-MetSO (Table 2). For all WT and mutated MSRBs, an expected stoichiometry of one mole of Met formed per mole of enzyme, or slightly higher in the case of C134T MSRB2, was measured. MSRB1 and MSRB2 possess comparable apparent affinities for the substrate, in the range of 2 μM. Using mutated C186S MSRB1, no activity was detected (data not shown), confirming that Cys-186 is the catalytic residue absolutely required for MetSO reduction. In MSRB1, Cys-116 appeared dispensable for MetSO reduction as the C116S mutant form was still able to reduce substrate with a specific activity representing two thirds of that of the WT form. The T132C MSRB1 mutation resulted in a 2-fold increase in the $K_{Dabsyl-MetSO}$ value compared to that of WT MSRB1. This indicates that the mutation does not significantly affect the capacity of MetSO reduction and suggests that the very low activity observed in the presence of DTE likely originates from a modification affecting the reduction of the catalytic Cys. With regard to C134T MSRB2, the $K_{Dabsyl-MetSO}$ value is 9-fold higher than that of WT MSRB2. Despite this lower affinity, C134T MSRB2 exhibits a higher activity than the WT enzyme in the presence of DTE, suggesting that the mutation favors the regeneration of the catalytic Cys.

We used the DNTB procedure to compare the number of free thiols in pre-reduced MSRB1 before or after addition of the substrate, N-acetyl-MetSO. Reduced MSRB1 was found to contain 2.4 thiols, consistent with the expected 2 free thiols, Cys-186 and Cys-116. After addition of N-acetyl-MetSO, 1.6 thiols were measured, corresponding to a decrease of 0.8 free thiols (Table 1). These results are consistent with the oxidation of one single Cys, very likely Cys-186, to a sulfenic acid form. Note that the formation of an intermolecular disulfide can be excluded, as no MSRB1 dimer was observed after oxidation of WT or mutated forms, even in the presence of a large amount of diamide (data not shown). Thiol-titration experiments were also achieved
using T132C MSRB1. The reduced form of T132C MSRB1 contains about 3 thiols, likely corresponding to Cys-116, Cys-186 and the Cys replacing Thr in position 132 (Table 1). The decrease of ca. 1 thiol after oxidation by substrate is consistent with the oxidation of only the catalytic Cys-186, Cys-116 and Cys-132 being still free to react with DTNB.

Reduction of mutated MSRBs by Grxs

We investigated the capacity of Trxs and Grxs to reduce mutated forms of MSRBs. Despite the presence of Cys instead of Thr in position 132 corresponding to the potential resolving Cys of MSRB2, we observed that T132C MSRB1 does not use canonical Trxs as electron donors (data not shown). This is in agreement with the absence of disulfide bond suggested by thiol titrations. The capacity of GrxC4 (Cys-Pro-Tyr-Cys active site) and GrxS12 (Cys-Ser-Tyr-Ser active site) to provide electrons to mutated MSRB1s was determined by measuring specific activities following NADPH consumption in the presence of GSH and GR as described in experimental procedures. No significant activity could be detected when using T132C MSRB1 (data not shown). This result is consistent with DTE regeneration assays and affinity measurements, which indicated that the T132C mutation likely affects the regeneration of MSRB1 activity. Interestingly, in the case of C116S MSRB1, the specific activity values measured when using GrxC4 and GrxS12 as electron donors were 1193 ± 406 and 1756 ± 61 nmol Met.min\(^{-1}\).mg protein\(^{-1}\), respectively. These values relatively comparable to those measured for the WT form (Fig. 2) show that Cys-116 is not involved in the regeneration of MSRB1 activity by Grxs. Thus, since no other Cys can act as a resolving residue, these data indicate that regeneration of MSRB1 activity involves only the catalytic Cys-186.

Regarding MSRB2, no activity was recorded using Grxs as electron donors (Fig. 2; (15)). But very interestingly, in the case of C134T MSRB2, substantial specific activities (1,113 ± 52 and 1,333 ± 212 nmol Met.min\(^{-1}\).mg protein\(^{-1}\)) were recorded using GrxC4 and GrxS12, respectively, and were in the same range as those obtained for MSRB1 (1,731 ± 67 and 2,077 ± 38 nmol Met.min\(^{-1}\).mg protein\(^{-1}\), respectively) (Fig. 2, Table 3). These results demonstrate that the replacement of the resolving Cys by a Thr in 2-Cys MSRB2 is sufficient to allow regeneration by Grxs.

Reduction of MSRB1 by mutated Grxs

To delineate the mechanism used by Grxs for regenerating MSRB1 activity, we compared the ability of WT and mutated forms of GrxC4, possessing a classical dithiol active site Cys-Pro-Tyr-Cys but no extra-Cys, and of GrxS12, possessing an atypical monothiol active site Cys-Ser-Tyr-Ser and a Cys in the C-terminal part (Cys-87), to supply electrons to MSRB1. First, we determined MSR activity using HPLC quantification of dabsyl-MetSO reduction. In the presence of NADPH and GR only, the specific activity of MSRB1 was close to 7 nmol Met.min\(^{-1}\).mg protein\(^{-1}\). Addition of either Grx or GSH alone did not significantly increase dabsyl-Met production (data not shown). Addition of the whole system, containing both Grx and GSH, resulted in an MSRB1 specific activity around 140 nmol Met.min\(^{-1}\).mg protein\(^{-1}\), using either GrxC4 or GrxS12 as reductants (data not shown). This result indicates that both GSH and Grxs are required for MSRB1 regeneration. Then, specific activities were determined using the NADPH coupled system (Table 3). In the presence of 50 µM GrxC4 or GrxS12, MSRB1 was efficiently reduced since it exhibited a specific activity nearly 10-fold higher than the basal activity measured when adding all components, except Grx. On one hand, C27S GrxC4 and C29S GrxS12, in which the first redox active Cys was changed to Ser, lost their ability to supply electrons to MSRB1. On the other hand, mutations to Ser of the other Cys, Cys-30 in GrxC4 or Cys-87 in GrxS12, did not prevent the capacity of Grxs to reduce MSRB1 (Table 3). Regarding kinetic parameters, the saturation curves obtained when varying Grx concentrations were found to follow Michaelis-Menten kinetics (data not shown) in agreement with previous data (15). The apparent affinity constants between WT MSRB1 and Grxs (\(K_{Grx}\)), measured in steady-
state conditions, were found to be in the low µM range, with a value higher for GrxC4 than for GrxS12, while comparable turnover numbers (kcat), about 0.5 s⁻¹, were calculated for both Grxs. These differences result in a 6-fold higher catalytic efficiency (kcat/Km) for GrxS12. The C30S mutation in GrxC4 leads to a 7-fold increase of Km, and a 2-fold increase of kcat, resulting in a 4-fold decrease in catalytic efficiency consistent with previous observations (26). For GrxS12, the C87S mutation did not significantly alter its catalytic parameters. This is consistent with the recent biochemical characterization of GrxS12 revealing that Cys-87 is dispensable for activity and does not form a disulfide bridge with the catalytic Cys-29 (22). Altogether, these results demonstrate that both GrxC4 and GrxS12 use only the N-terminal active site Cys to regenerate MSRB1 activity, and that GSH is absolutely required for this process.

Mass spectrometry analyses

We used MALDI-TOF mass spectrometry to determine the masses of MSRBs in the reduced form or after oxidation by MetSO. The mass measured for reduced MSRB2 was very close to the expected one (-1.1 Da), while the experimental mass of reduced MSRB1 was slightly inferior (-9.0 Da) to that theoretically deduced from the sequence, but inconsistent with an amino-acid cleavage (Table 4, Fig. 3A). After oxidation in the presence of 2 mM N-acetyl-MetSO, the masses of MSRB1 and MSRB2 were found to increase by 15.3 Da and 4.3 Da, respectively (Table 4, Fig. 3B). While the increase is not significant in the case of MSRB2, the increase observed for MSRB1 is significantly higher than the experimental error (±0.05%, ca ±8 Da) and affects most of the protein pool. The mass increase is in the range of 16 Da and corresponds to the addition of an oxygen atom in the protein (Fig. 3). These results argue for the formation of a stable sulfenic acid on Cys-186 of MSRB1 after oxidation by the substrate.

Then, we performed mass analyses to determine whether the catalytic Cys-186 of MSRB1 could undergo glutathionylation after MetSO reduction. After incubation in the presence of N-acetyl-MetSO and GSH, a shift in molecular mass was observed for MSRB1, but not for MSRB2 (Fig. 3C, Table 4). This shift of 304.8 Da is perfectly consistent with the presence of one glutathione adduct per molecule of MSRB1 (theoretical additional mass: 305 Da) and affects most of the protein pool. Moreover, after DTT treatment of MetSO/GSH-treated MSRB1, the molecular mass of the protein shifted back to the mass of the reduced form, showing that glutathionylation of the protein is fully reversible. In contrast, no mass increase was observed after treatment with GSH alone. These results suggest that, following the formation of the sulfenic acid upon MetSO reduction, Cys-186 is the residue undergoing glutathionylation. This hypothesis was validated by peptide mass fingerprinting after tryptic digestion of glutathionylated MSRB1 (Fig. 4). The peptide profile of glutathionylated MSRB1 reveals an additional peak compared to the profile of the reduced protein. This peak corresponds to the peptide [R184-K193], containing the catalytic Cys-186, shifted by 305 Da. These data unambiguously reveal that the mass increase is due to the formation of a mixed disulfide between GSH and the thiol of Cys-186. The modification appeared to be reversible since when adding a disulfide reductant, DTT, the additional peak was no longer detected.

DISCUSSION

The goal of the present study was to delineate the mechanisms used by Grxs for the reduction of the Arabidopsis plastidial 1-Cys MSRB1, and to compare it to the Trx-dependent reduction of the 2-Cys MSRB2. Despite, the distinct number of redox-active Cys, MSRB1 and MSRB2 display very similar biochemical capacities and an expected stoichiometry of one mole of Met formed per mole of enzyme. Although the Kdabyl-MetSO values, measured with reduced enzymes, appear somewhat lower than those calculated in the presence of DTE, the data reported here are consistent with previous results (15,17), and with the catalytic parameters of other
mammalian and prokaryote MSRBs determined in similar conditions (4,5,10,13,30). In the presence of DTE, MSRB1 was found to be 3-fold more efficient than MSRB2 (Table 2). The substitution of Thr-132 by Cys in MSRB1 resulted in a substantial loss of activity, whereas in the case of MSRB2, the mutation of Cys-134 to Thr led to a substantial increase (2.3-fold) in specific activity up to the value range recorded for MSRB1 (Table 2). Interestingly, most MSRB proteins from bacteria and animal cells belonging to the 1-Cys type display a Thr or a Ser in place of the resolving Cys (7,10). In agreement with our data on T132C MSRB1 activity, Kim and Gladyshev (10) reported that replacement of Ser by Cys resulted in a strong decrease in the MetSO reductase activity of mammalian MSRB3 in the presence of DTT or of E. coli Trx as electron donors. These data provide evidence that the presence of Thr/Ser in the position corresponding to the resolving Cys in 1-Cys MSRBs could be essential for the catalytic mechanism and help maintaining a high capacity of MetSO reduction. From an evolutionary point of view, the presence of a single MSRB gene in most prokaryotes suggests that a prototypic enzyme exists and that various types of enzymes evolved from this ancestor. Regardless which enzyme was the ancestor, the 1-Cys or the 2-Cys MSRB, it is worth mentioning that the single change from Cys to Thr or from Thr to Cys, in addition to changing the kinetic parameters of the enzymes, also drastically modifies the regeneration system used (Trx or Grx).

Using site-directed mutagenesis, we investigated the role of Cys-134 of MSRB2 in the Trx-mediated recycling process. Activity measurements, combined to the titration of free thiol groups in various oxidation states (Table 1) and mass spectrometry analyses (Table 4) lead us to propose that the sulfenic acid formed on Cys-187 is reduced by the nucleophilic attack of the sulfur atom of Cys-134, resulting in the formation of an intramolecular disulfide bridge. This bond is reduced subsequently by Trx through a classical dithiol-disulfide exchange involving both catalytic and resolving Cys of the two partners. The proposed mechanism of MSRB2 reduction by canonical Trxs is in agreement with those described for other 2-Cys MSRBs (5,7) that possess two redox-active Cys or a catalytic selenocysteine and a resolving Cys (10). Our data indicate that this mechanism is likely conserved in 2-Cys MSRBs of higher plants and green algae, which are very similar to Arabidopsis MSRB2 (8).

The titration of free thiol groups in oxidized MSRB1 and C134T MSRB2 showed that only one Cys is oxidized by the substrate (Table 1). Furthermore, the mutated MSRB1, in which the Cys-116 was replaced by a Ser, was found to be almost as active as WT MSRB1 and to retain its capacity to be reduced by Grxs. This demonstrates that Cys-116 cannot act as a resolving Cys and that MSRB1 uses only Cys-186 for the catalytic activity and the Grx-dependent regeneration mechanism. Moreover, the fact that the replacement of resolving Cys-134 of MSRB2 by a Thr allows to generate an “MSRB1-like” enzyme regenerated by Grxs leads us to propose that the distinct regeneration mechanisms for MSRB1 and MSRB2 originate from the presence or not of a stable sulfenic acid intermediate, which is linked to the absence or presence of a resolving cysteine. Hence, in MSRB2, the resolving Cys-134 very likely rapidly attacks the sulfenic acid formed after MetSO reduction, whereas in MSRB1 and in C134T MSRB2, the sulfenic acid form of catalytic Cys is much more stable due to the absence of a resolving Cys. The mass spectrometry results validate this hypothesis. Indeed, after incubation with an excess of N-acetyl-MetSO, an increase of nearly 16 Da was observed for most of the MSRB1 protein pool assayed. In comparison, no significant mass difference was recorded for MSRB2. The formation of a transient sulfenic acid was shown in Drosophila 2-Cys MSRB after MetSO reduction: mass spectrometry of dimedone-treated WT 2-Cys MSRB, after MetSO reduction, revealed a small peak with a +138 Da mass shift, suggesting the presence of a dimedone-reacted sulfenic acid, but the major fraction of the enzyme was converted by MetSO to an oxidized form not able to bind dimedone, suggesting a very fast reduction of the sulfenic acid.
acid intermediate by the resolving Cys (7). A similar result was observed for E. coli MSRA (31). In contrast, our data reveal the presence of a stable sulfenic acid intermediate in a 1-Cys MSRB after MetSO reduction.

The mass spectrometry results acquired on MSRB1 incubated with N-acetyl-MetSO and GSH showed that the sulfenic acid formed on Cys-186 is attacked by glutathione. The fact that GSH is absolutely required for the Grx-dependent MSRB1 regeneration, as shown by activity assays, is also consistent with the observation that Grxs alone are unable to reduce the sulfenic acid on MSRB1. The use of monothiol and dithiol Grxs and of Cys to Ser mutated forms showed that only the catalytic Cys is required to provide MSRB1 with electrons (Table 3). These results indicate that reduction of MSRB1 by Grxs is very likely performed through a monothiol mechanism involving only the N-terminal active site Cys. Based on our data, we propose a model (Fig. 5), in which the catalytic mechanism for 1-Cys MSRB1 involves: i) formation of a sulfenic acid on Cys-186 due to MetSO reduction, ii) glutathionylation of Cys-186 by reaction of the sulfenic acid with GSH and iii) regeneration of reduced MSRB1 by deglutathionylation mediated by Grxs. Then, the thiol group of an external reduced glutathione reduces glutathionylated Grx, since it is well established that Grxs can catalyze protein de glutathionylation using only the N-terminal active site Cys (21,22,32).

Our results show for the first time a glutathionylation step in the regeneration of the activity of MSRB enzymes. Interestingly, these results are related to those reported on plant type II peroxiredoxins (Prxs). These are thiol-dependent peroxidases, which also use the sulfenic acid chemistry and the GSH/Grx system for their regeneration. Nevertheless, a major difference is that these type II Prxs also accept the Trx system as an alternative reducing system (33). The study of the Grx-mediated Prx regeneration mechanism also showed that only the N-terminal catalytic Cys of Grxs is required. However, the order in which GSH and Grx are involved in this process is still unclear, because covalent heterodimers can be formed between Prx and Grx in the absence of GSH (33). Interestingly, the regeneration mechanism of human 1-Cys Prx by glutathione-S-transferase has been shown to implicate glutathionylation of the oxidized catalytic Cys (34,35). The data acquired in the present work extend the participation of glutathionylation in activity regeneration of another type of enzyme involved in protection against oxidative modification, i.e. 1-Cys methionine sulfoxide reductases B. In other respects, several studies proposed that reversible glutathionylation of Cys could be a protective mechanism during oxidative stress. For instance, Zaffagnini et al. (36) reported that plant chloroplastic glyceraldehyde-3-phosphate dehydrogenase is transiently inactivated in vitro by glutathionylation of the catalytic Cys following an oxidative treatment. Similar results were recently reported for the 20S proteasome of yeast (37). In conclusion, glutathionylation appears to fulfill at least two distinct roles during oxidative stress: i) regeneration of the activity of stress-specific enzymes such as MSRs and Prxs, ii) transient inactivation and protection of metabolic enzymes. In addition, as glutathionylation has also been shown to alter, either positively or negatively, the activity of many signaling proteins, including several members of the NF-kappaB pathway and protein tyrosine phosphatases, to cite only few examples, it is tempting to speculate that glutathionylation also participates to the signaling pathways in response to oxidative stress (38-40).

The GSH/Grx system is not the only possible reducing system for 1-Cys MSRB. Mammalian Trxs have been shown to reduce 1-Cys MSRB2 and MSRB3 (13), and we previously reported that the peculiar plant Trx CDSP32, which participates in the tolerance to oxidative stress and interacts with MSRB1 in plant extracts (41,42), regenerates the activity of this 1-Cys MSRB (14,15) without addition of GSH or of another thiol compound (data not shown). This process might involve direct reduction of the sulfenic acid and the formation of a heterodimeric disulfide complex. Several other compounds have been proposed as potential reducing agents for 1-Cys MSRBs. Indeed, Sagher and co-workers
reported that selenocystamine, selenocysteine (11) and thionein, the reduced apoprotein of Zn-metallothionein (12), are able to reduce human 1-Cys MSRB2 and MSRB3. However, the in vivo significance of these data remains largely unclear. Our data promote the involvement of GSH, the major low molecular weight thiol in cell, and Grxs as a physiological direct reducing system for 1-Cys MSRBs. This is in agreement with the roles of GSH in protection against oxidative stress and redox homeostasis that are clearly established in plants (reviewed in (18)) as well as in other organisms (reviewed in (43)). Preliminary results acquired with Arabidopsis plants lacking both MSRB1 and MSRB2 genes and identification of their potential targets reveal that the two plastidial MSRBs could have redundant functions in protecting plants against oxidative damage (data not shown). In contrast, the distinct specificity observed for their reductants reveals a difference in electron supply (NADPH for the GSH/Grx system, ferredoxin for the Trx system), which could be associated with a preservation of the plastidial MSRB capacity under various environmental conditions.

REFERENCES


**ACKNOWLEDGMENTS**

This work was supported by Agence Nationale de la Recherche, ANR-Génoplante Grant GNP05010G to PR, EL and NR and Grant ANR-JC45751 to MZ and SDL. Financial support to LT from Région Provence-Alpes-Côtes-d’Azur is acknowledged. We are very grateful to Patricia Henri (CEA, IBEB, SBVME, LEMP) for technical assistance, Noelle Becuwe (CEA, IBEB, SBVME, LEMP) for mutagenesis and Stéphan Cuiné (CEA, IBEB, SBVME, LB3M) and Rémy Puppo (CEA, IBEB, SBVME, LEMP) for helpful advices in protein purification and HPLC experiments.

The abbreviations used are: MSR: methionine sulfoxide reductases, Trx: thioredoxin, Grx: glutaredoxin, GSH: reduced glutathione, GR: glutathione reductase, DTT: reduced dithiothreitol, DTE: reduced dithioerythritol, MetSO: methionine sulfoxide, DTNB: 5,5'-dithiobis-2-nitrobenzoic acid.
FIGURE LEGENDS

Fig 1. Reduction of MSRB2 by Trx h1. A. Reduction of WT and C134T MSRB2 by WT Trx h1. For the first two min, the assay mixture contained the Trx reducing system (200 mM NADPH, 2 µM NTR and a saturating concentration of Trx h1 (100 µM)). The arrows indicate addition of 2 mM N-acetyl-MetSO (1), 2 µM C134T MSRB2 (2), C134T MSRB2 a second time to a final concentration of 4 µM (3), and 2 µM MSRB2 (4). B. Reduction of WT MSRB2 by WT and C42S Trx h1. For the first min, the enzymatic mixture contained 200 µM NADPH, 2 µM NTR, 2 mM N-acetyl-MetSO and 40 µM Trx h1 or C42S Trx h1. Addition of MSRB2 at a final concentration of 2 µM is indicated by the arrow.

Fig 2. Activity of MSRBs using GrxC4 and GrxS12 as electron donors. The reaction mixture contained 2 mM N-acetyl-MetSO, 400 µM NADPH, 0.5 GR units, 10 mM GSH and 25 µM Grx in 30 mM Tris-HCl, pH 8.0. The reaction was started by adding MSRB to a final concentration of 5 µM. Measurements were carried out following NADPH oxidation at 340 nm at 25°C in 500 µl reaction medium. Data are represented as means ± SD (n = 3).

Fig 3. MALDI-TOF mass spectrometry of MSRB1 incubated with N-acetyl-MetSO and GSH. Mass spectra of reduced MSRB1 (A), of MSRB1 incubated with either N-acetyl-MetSO (B) or N-acetyl-MetSO and GSH (C). After N-acetyl-MetSO treatment, an increase of 15.3 Da was recorded (B) while an increase of 304.8 Da was observed after treatment with N-acetyl-MetSO in the presence of GSH (C). The shift observed in C is fully reversed by DTT treatment. Accuracy of the measurement is ± 8 Da.

Fig 4. MALDI-TOF mass spectrometry of MSRB1 tryptic peptides incubated with N-acetyl-MetSO and GSH. MALDI-TOF spectra of tryptic peptides before (A) or after (B) N-acetyl-MetSO treatment in the presence of GSH. After the treatment, the molecular mass of the peptide [R₁₈₄YCLNSAALK₁₉₃], containing the catalytic Cys-186, is partially shifted by 305 Da. The shift is fully reversed by DTT treatment.

Fig 5. Model for the regeneration of MSRB1 by Grxs. The first step consists in MetSO reduction with the concomitant release of one mole of Met and the formation of a stable sulfenic acid intermediate on catalytic Cys-186 (1). The oxidized Cys is then attacked by GSH leading to the liberation of one molecule of water and to the formation of a glutathione adduct (2), which is subsequently solved by Grxs through the N-terminal Cys of the active site (3).
# Tables

## Table 1. Free thiol content in WT and mutated MSRB proteins in the absence or presence of $N$-acetyl-MetSO.
The content of free thiols in pre-reduced MSRBs was titrated using a standard DTNB assay in the absence or presence of a saturating concentration of $N$-acetyl-MetSO (0.5 mM). Data are expressed in mol SH.mol enzyme$^{-1}$. Data presented are the means ± SD (n = 3).

<table>
<thead>
<tr>
<th>Number of free Cys</th>
<th>Number of free thiol measured</th>
<th>Before $N$-acetyl-MetSO treatment</th>
<th>After $N$-acetyl-MetSO treatment</th>
<th>Decrease in free thiols (Before-After)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSRB1</td>
<td></td>
<td>2.4 ± 0.1</td>
<td>1.6 ± 0.1</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>T132C MSRB1</td>
<td></td>
<td>3.3 ± 0.5</td>
<td>2.4 ± 0.4</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>MSRB2</td>
<td></td>
<td>3.2 ± 0.1</td>
<td>1.3 ± 0.1</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>C134T MSRB2</td>
<td></td>
<td>1.9 ± 0.1</td>
<td>1.2 ± 0.2</td>
<td>0.7 ± 0.1</td>
</tr>
</tbody>
</table>

## Table 2. Catalytic parameters of WT and mutated MSRBs.
The specific activity was determined following the reduction of dabsyl-MetSO to dabsyl-Met using an HPLC-based method. Specific activities were measured using 1 µM MSRB and 0.5 mM dabsyl-MetSO in the presence of 20 mM DTE. Measurements of stoichiometry were carried out using 5 µM MSRB and 0.1 mM dabsyl-MetSO. Considering that only the $R$-diastereomer is reduced by MSRBs, $K_{Dabsyl-MetSO}$ values were divided by two.

<table>
<thead>
<tr>
<th>Specific activity (nmol Met formed.min$^{-1}$.mg MSRB$^{-1}$)</th>
<th>Stoichiometry (mol Met formed.mol MSRB$^{-1}$)</th>
<th>$K_{Dabsyl-MetSO}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSRB1</td>
<td>272 ± 64</td>
<td>2.2 ± 0.6</td>
</tr>
<tr>
<td>C116S MSRB1</td>
<td>165 ± 33</td>
<td>9.2 ± 2.2</td>
</tr>
<tr>
<td>T132C MSRB1</td>
<td>19 ± 1</td>
<td>5.2 ± 1.1</td>
</tr>
<tr>
<td>MSRB2</td>
<td>103 ± 25</td>
<td>2.1 ± 0.6</td>
</tr>
<tr>
<td>C134T MSRB2</td>
<td>237 ± 46</td>
<td>18.1 ± 3.2</td>
</tr>
</tbody>
</table>

## Table 3. Kinetic parameters of MSRB1 using WT and mutated GrxC4 and GrxS12 as electron donors.
Measurements were carried out following NADPH oxidation at 340 nm as described in experimental procedures. Data are represented as means ± SD (n = 3). ND, non determined.

<table>
<thead>
<tr>
<th>Grx</th>
<th>Specific activity (nmol Met.min$^{-1}$.mg MSRB1$^{-1}$)</th>
<th>$K_{cinox}$ (µM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_{cinox}$ (M$^{-1}$.s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Grx</td>
<td>196 ± 35</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GrxC4</td>
<td>1,731 ± 67</td>
<td>6.8 ± 0.8</td>
<td>0.496 ± 0.019</td>
<td>73,373 ± 10,821</td>
</tr>
<tr>
<td>C27S GrxC4</td>
<td>244 ± 10</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>C30S GrxC4</td>
<td>3,001 ± 256</td>
<td>47.1 ± 8.5</td>
<td>0.860 ± 0.073</td>
<td>18,259 ± 4,354</td>
</tr>
<tr>
<td>GrxS12</td>
<td>2,077 ± 38</td>
<td>1.3 ± 0.1</td>
<td>0.595 ± 0.011</td>
<td>444,030 ± 42,613</td>
</tr>
<tr>
<td>C29S GrxS12</td>
<td>165 ± 27</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>C87S GrxS12</td>
<td>1,740 ± 30</td>
<td>1.5 ± 0.1</td>
<td>0.499 ± 0.009</td>
<td>330,601 ± 26,310</td>
</tr>
</tbody>
</table>

## Table 4. Molecular masses of MSRB1 and MSRB2.
Molecular masses were determined using MALDI-TOF mass spectrometry. Masses correspond to major peaks and are in Daltons. The differences were calculated between the measured masses of treated and untreated proteins. Accuracy of the measurement is ± 8 Da.

<table>
<thead>
<tr>
<th>Reduced Theoretical</th>
<th>Measured</th>
<th>+ $N$-acetyl-MetSO Difference</th>
<th>Measured</th>
<th>+ GSH Difference</th>
<th>Measured</th>
<th>+ $N$-acetyl-MetSO + GSH Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSRB1</td>
<td>16,623.6</td>
<td>16,614.6</td>
<td>+ 15.3</td>
<td>16,615.4</td>
<td>+ 0.8</td>
<td>16,919.4</td>
</tr>
<tr>
<td>MSRB2</td>
<td>16,859.8</td>
<td>16,858.9</td>
<td>+ 4.3</td>
<td>16,866.4</td>
<td>+ 7.5</td>
<td>16,859.2</td>
</tr>
</tbody>
</table>

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Figure 1.
Figure 2.

![Graph showing specific activity (nmol NADPH.min⁻¹.mg MSRB⁻¹) for different protein variants.](image-url)
Figure 3.
Figure 4.

A

% Intensity

R184-K193 1138.598
T147-R158 1433.834
R81-R91 1439.772
N68-K79 1453.655

% Intensity

R184-K193 1138.595
T147-R158 1433.829
R184-K193 1443.675
N68-K79 1453.660

Mass (m/z)

+ N-acetyl-MetSO + GSH

+ DTT

+ GSH (305 Da)
Figure 5.

\[
\text{MSRB1}^{\text{SH}}_{186} + \text{MetSO} \xrightleftharpoons{1} \text{MSRB1}^{\text{S\text{-}SOH}}_{186} + \text{GSH}
\]

\[
\text{MSRB1}^{\text{S\text{-}SG}}_{186} \xrightarrow{2} \text{H}_2\text{O}
\]

\[
\text{GS\text{-}S}^{\text{Grx}} \xrightarrow{3} \text{MSRB1}^{\text{S\text{-}SG}}_{186}
\]