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Mechanism of 1-Cys type methionine sulfoxide reductase A regeneration by glutaredoxin

Moon-Jung Kim [a], Jaeho Jeong [b], Jihye Jeong [b], Kwang Yeon Hwang [c], Kong-Joo Lee [b, **], Hwa-Young Kim [a, *]

[a] Department of Biochemistry and Molecular Biology, Yeungnam University College of Medicine, Daegu 705-717, Republic of Korea
[b] Graduate School of Pharmaceutical Sciences, College of Pharmacy, Ewha Womans University, Seoul 120-750, Republic of Korea
[c] Division of Biotechnology, College of Life Sciences & Biotechnology, Korea University, Seoul 136-701, Republic of Korea

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ABSTRACT

Glutaredoxin (Grx), a major redox regulator, can act as a reductant of methionine sulfoxide reductase A (MsrA). However, the biochemical mechanisms involved in MsrA activity regeneration by Grx remain largely unknown. In this study, we investigated the regeneration mechanism of 1-Cys type *Clostridium oremlandii* MsrA (cMsrA) lacking a resolving Cys residue in a Grx-dependent assay. Kinetic analysis showed that cMsrA could be reduced by both monothiol and dithiol Grxs as efficiently as by *in vitro* reductant dithiothreitol. Our data revealed that the catalytic Cys sulfenic acid intermediate is not glutathionylated in the presence of the substrate, and that Grx instead directly formed a complex with cMsrA. Mass spectrometry analysis identified a disulfide bond between the N-terminal catalytic Cys of the active site of Grx and the catalytic Cys of cMsrA. This mixed disulfide bond could be resolved by glutathione. Based on these findings, we propose a model for regeneration of 1-Cys type cMsrA by Grx that involves no glutathionylation on the catalytic Cys of cMsrA. This mechanism contrasts with that of the previously known 1-Cys type MsrB.

1. Introduction

Methionine sulfoxide reductases (Msrs) are important protein repair enzymes that catalyze the reduction of methionine sulfoxide (Met-O) to methionine [1]. These enzymes play a pivotal defensive role against oxidative stress from bacteria to humans [2–5]. Two thiol-dependent enzymes, MsrA and MsrB, are required for complete reduction of the diastereomers of Met-O. MsrA specifically reduces the *S*-form of Met-O, whereas the *R*-form is only reduced by MsrB. Although MsrA and MsrB enzymes are completely different from each other in sequence and structure, they share a similar catalytic mechanism characterized by a common sulfenic acid chemistry [6,7]. The general catalytic mechanism of MsrA and MsrB consists of three steps. (i) A catalytic Cys attacks the sulfur of Met-O and forms a sulfenic acid intermediate, with concomitant release of the product, methionine. (ii) The catalytic Cys sulfenic acid then interacts with a resolving Cys, yielding an intramolecular disulfide bond. (iii) The disulfide bond is reduced by a reductant, resulting in regeneration of the enzyme. Notably, the regeneration of Msr activities is a pivotal process for its enzymatic functions.

MsrAs and MsrBs can be grouped according to the number of Cys residues involved in catalysis [1,7]. 3-Cys MsrAs have three Cys residues, one of which functions as a catalytic residue and the other two as resolving residues [8,9]. 2-Cys MsrAs include a single resolving Cys [10], while 1-Cys MsrAs lack any resolving Cys [11]. Only 2-Cys and 1-Cys type enzymes are found in the case of MsrBs [1]. Thioredoxin (Trx) has been considered the biological reductant for Msrs; however, a growing body of evidence suggests the involvement of glutaredoxin (Grx) in the regeneration of MsrA and MsrB activities [12–15]. We previously found that Grx reduces 1-Cys and 3-Cys MsrAs, while 1-Cys MsrA is more efficiently reduced by Grx than 3-Cys MsrA [15]. In addition, Grx can serve as a reductant for both 1-Cys and 2-Cys MsrBs [15].

To date, the only regeneration mechanism of Msrs by Grx has been suggested in 1-Cys MsrB from *Arabidopsis thaliana* [13]. The model for the regeneration of *Arabidopsis* 1-Cys MsrB involves

* Corresponding author. Department of Biochemistry and Molecular Biology, Yeungnam University College of Medicine, 170 Hyeonchungno, Namgu, Daegu 705-717, Republic of Korea. Fax: +82 53 640 6936.
** Corresponding author. Fax: +82 2 3277 3760.
E-mail addresses: kjl@ewha.ac.kr (K.-J. Lee), hykim@ynu.ac.kr (H.-Y. Kim).

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glutathionylation/deglutathionylation on the catalytic Cys sulfenic acid. The Cys sulfenic acid forms after substrate reduction, and is subsequently attacked by glutathione (GSH), leading to glutathionylation. The glutathionylated catalytic Cys is solved by Grx through a deglutathionylation reaction.

Clostridium oremlandii MsrA (cMsrA) is a selenoprotein containing a selenocysteine (Sec) residue within its active site [16]. cMsrA consists of 209 amino acids with no Cys residues and can thus be classified as 1-Cys type MsrA. Selenoprotein cMsrA shows a higher activity than its Sec-to-Cys version, but the Sec-to-Cys form also has an activity comparable to other Cys-containing MsrAs [16,17]. Trx does not serve as a reductant for cMsrA, whereas Grx can reduce this enzyme [14–16]. Herein, we report a regeneration mechanism of 1-Cys cMsrA by Grx in which no glutathionylation on the catalytic Cys is involved. This mechanism contrasts with that of *Arabidopsis* 1-Cys MsrB.

2. Materials and methods

2.1. Protein preparation

cMsrA (Sec-to-Cys form), *C. oremlandii* Grx2 (cGrx2) and its monothiol mutant (cGrx2/C15S), and *Escherichia coli* Grx1 (eGrx1) proteins were purified from *E. coli* as previously described [14,15].

2.2. MsrA assay and kinetic analysis

The reaction mixture (100 μl) contained 50 mM sodium phosphate (pH 7.5), 50 mM NaCl, 0.05–1.6 mM dabsylated Met-*S*-O, 0.4 μM cMsrA, and 20 mM dithiothreitol (DTT) or a Grx reduction system containing 0.2 mM NADPH, 10 mM GSH, 0.5 unit yeast GSH reductase (Sigma–Aldrich), and 10 μM Grx. The reaction was carried out at 37 °C for 30 min, after which the product, dabsyl-Met, was analyzed by HPLC as previously described [15]. K_m and k_{cat} values were determined by non-linear regression using the Prism 5 software (GraphPad).

2.3. Glutathionylation analysis of cMsrA

Five μM cMsrA in phosphate-buffered saline was incubated with 200 μM dabsylated Met-*S*-O for 20 min at 37 °C, after which the substrate-treated reaction mixture was incubated with 5 mM GSH (or 2.5 mM GSSG, oxidized GSH) for 20 min at 37 °C and then for another 20 min with 40 μM eGrx1. The cMsrA proteins were subjected to non-reducing SDS–PAGE and visualized by Coomassie staining. The protein bands were separated from the gel and subjected to mass spectrometry analysis as described below.

2.4. Determination of MsrA–Grx complex

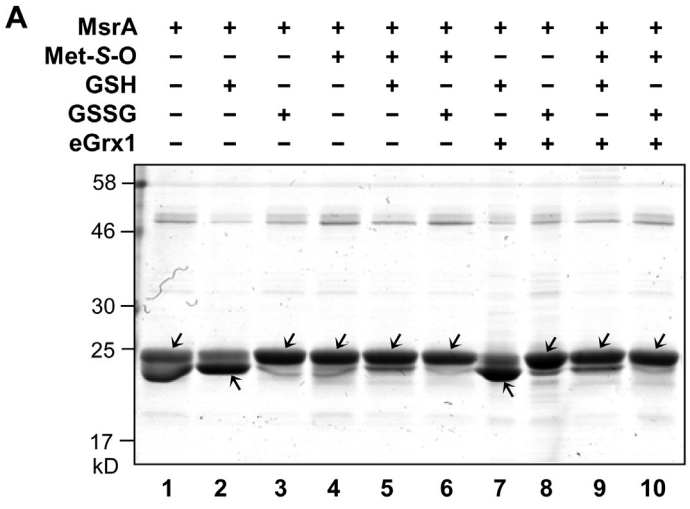
The reaction mixture (100 μl) contained 50 mM sodium phosphate (pH 7.5), 50 mM NaCl, 200 μM dabsyl-Met-*S*-O, 4 μM cMsrA, and 10 μM cGrx2/C15S. After incubation for 1 h at 37 °C, proteins were separated by non-reducing SDS–PAGE and visualized by Coomassie staining. To determine if there was a mixed disulfide bond between MsrA and Grx, a 34 kD protein band corresponding to the size of the cMsrA–cGrx2/C15S complex was subjected to mass spectrometry analysis as described below. In addition, to test whether GSH could resolve the MsrA–Grx complex, the resulting reaction mixture was incubated with 10 mM GSH (or GSSG) for 30 min at 37 °C.

2.5. Mass spectrometry analyses

To identify glutathionylation of cMsrA and the presence of a mixed disulfide bond between cMsrA and cGrx2/C15S, proteins separated by non-reducing SDS–PAGE were in gel digested and the resulting peptides were extracted as previously described [18]. Briefly, each gel band was excised with a scalpel, destained with 25 mM NH_4HCO_3/50% acetonitrile, and washed to remove destaining reagent. The pH was then adjusted to 8.0 with 50 mM NH_4HCO_3 to facilitate trypsin digestion. The gels were subsequently incubated at 37 °C for 16 h, after which the peptides were extracted and evaporated to dryness in a SpeedVac for MS analysis. Concentrated peptides were analyzed by sequencing with nanoUPLC-ESI-q-TOF tandem MS (SYNAPT™ HDMS™, Waters). Peptides were separated using a C18 reversed-phase 75 μm i.d. × 250 mm analytical column (1.7 mm particle size, BEH130 C18, Waters) with an integrated electrospray ionization PicoTip™ (±10 μm, New Objective). The mass spectrometer was programmed to record scan cycles composed of one MS scan followed by MS/MS scans of the 3~4 most abundant ions in each MS scan. The MS/MS spectra were processed using the Micromass ProteinLynx Global Server (PLGSTM) 2.3 data processing software and output was collected as a single peak list (.pkl) file. The peak list files were applied to query the SwissProt database using Mascot (global search engine) and DBond algorithm for disulfide bond assignment (Korea, http://prix.hanyang.ac.kr/) [19], with the following parameters: peptide mass tolerance, 0.2 Da; MS/MS ion mass tolerance, 0.2 Da; up to 1 missed trypsin cleavage sites allowed. Redundant peptides were excluded in the next run analysis using SEMSA to increase sequence coverage of each protein [20]. Reported assignments were verified by manual interpretation of spectra from Mascot and DBond.

3. Results and discussion

To investigate the regeneration mechanism of cMsrA by Grx, we first analyzed whether glutathionylation on the catalytic Cys16 of cMsrA was involved. The cMsrA protein was sequentially incubated with the substrate, GSH (or GSSG), and eGrx1, which acts as a good reductant of cMsrA enzyme [15]. Peptide sequencing with tandem mass spectrometry was then used to identify and quantify modified species of Cys16 of the tryptic peptide (^{7}LSIAVFALGCFWGPDAQFGSIK^{28}) (Fig. 1). Since mass spectrometry analysis was conducted on a signal intensity-dependent mode, the number of peptides counted can represent the amounts of modified peptides [21]. Free sulfhydryl and trioxidation (sulfonic acid) forms of Cys16 were detected from the MsrA only sample (lane 1). These two species were also detected in the reaction sample with the substrate (lane 4). No sulfenic acid form was detected due to its reactive and unstable properties during preparation of the samples. In the reaction sample that contained substrate followed by GSH, no glutathionylated form was identified (lane 5), even though free and sulfonic acid species were detected. These data suggest no involvement of glutathionylation on the catalytic Cys16 sulfenic acid led by the attack of GSH during catalysis. We also confirmed that no glutathionylated form was present in the protein sample incubated simultaneously with the substrate and GSH. Interestingly, the glutathionylated form was primarily found in the protein sample incubated with GSSG (lane 3). This form was also identified in the reaction sample with the substrate followed by GSSG (lane 6), although the number detected was reduced by up to 80%. Reactions of these samples with eGrx1 completely depleted the glutathionylated forms (lanes 8 and 10), indicating the occurrence of deglutathionylation reaction by Grx. Dehydroalanine form of Cys16 was detected in the protein sample incubated with the substrate



B

Species of Cys16	Lane No. (1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
Free sulfhydryl	2	0	1	1	1	1	0	0	1	1
Glutathionylated	0	0	5	0	0	1	0	0	0	0
Trioxidation	2	0	1	1	3	1	1	1	1	1
Dehydroalanine	0	0	0	0	0	1	0	0	0	0

Fig. 1. Identification of modified species of catalytic Cys16 by mass spectrometry analysis. Substrate-treated or -untreated cMsrA proteins were incubated sequentially with GSH (or GSSG) and eGrx1 for 20 min each at 37 °C. The samples were then subjected to non-reducing SDS-PAGE, after which the protein bands were visualized by Coomassie staining (A). cMsrA proteins appeared as doublet bands, and arrowed cMsrA bands from each lane were subjected to mass spectrometry analysis to identify species of catalytic Cys16. The identified species of Cys16 on the tryptic peptide ^{7}LSIAVFALGCFWGPDAQFGSIK^{28} and the number of identified peptides are shown (B).

followed by GSSG (lane 6). The dehydroalanine form could be generated by cleavage of thiosulfonate [18].

We determined the kinetic parameters of cMsrA using cGrx2, which is a dithiol Grx, and then compared them with those of the DTT-dependent assay (Table 1). Notably, a saturated concentration (10 μM) of cGrx2 for the assay was used as previously reported [15]. The k_{cat} value of the cGrx2-dependent assay was 2-fold lower than that of the DTT-dependent assay. However, the catalytic efficiency of the Grx-dependent assay was 1.5-fold higher than that of the DTT-dependent assay, indicating that cGrx2 can serve as an efficient reductant for cMsrA enzyme. We also analyzed the reduction ability of the monothiol mutant form of cGrx2. The K_m and k_{cat} values of the cGrx2/C15S-dependent assay were similar to those of the cGrx2-dependent assay. Collectively, the data suggest that both the dithiol and monothiol forms of cGrx2 can efficiently reduce cMsrA.

To investigate whether Grx can directly attack the catalytic Cys16 sulfenic acid of cMsrA, we attempted to identify the cMsrA–Grx reaction intermediate complex and disulfide bond formation. To accomplish this, we used monothiol cGrx2/C15S in which the C-terminal resolving Cys15 of the active site was replaced with Ser. As described in the materials and methods, the reaction mixtures contained no GSH. We assumed that during the reaction with the substrate, Cys12 of cGrx2/C15S would first directly form a disulfide bond with the Cys16 sulfenic acid intermediate of cMsrA. However, this mixed disulfide bond could not be resolved due to the absence of resolving Cys of the active site in cGrx2/C15S protein. Therefore, protein bands of the cMsrA–cGrx2 would be detected on non-reducing SDS-PAGE gels.

The molecular masses of recombinant cMsrA and cGrx2/C15S proteins were calculated to be 23 and 11 kDa, respectively, on the non-reducing gel. As shown in Fig. 2A, the reaction mixture with cGrx2/C15S and the substrate contained an apparent protein band of 34 kDa corresponding to the size of the cMsrA–cGrx2 complex (lane 3). In addition, at least two more protein complex bands above 50 kDa also appeared when compared with the sample in the absence of cGrx2/C15S (lane 1). In the reaction mixture without the substrate, these protein complex bands were hardly detectable (lane 4). The sample shown in lane 3 was treated with a reducing agent and subjected to SDS-PAGE gels. All protein bands between 30 and 60 kDa disappeared (Fig. 2B), indicating that these protein complexes are likely formed by disulfide bonds. Mass spectrometry analysis was conducted to further identify the 34 kDa protein band. cMsrA and cGrx2/C15S proteins were well identified and as shown in Fig. 2C, disulfide bond formation between Cys12 of cGrx2/C15S and Cys16 of cMsrA was clearly identified. Taken together, these data suggest that cGrx2 can directly attack the Cys16 sulfenic acid of cMsrA in the presence of the substrate Met-*S*-O.

We next examined whether GSH could resolve the disulfide bond of cMsrA–cGrx2. The sample shown in lane 3 of Fig. 2A was treated with GSH or GSSG and then subjected to non-reducing SDS-PAGE. As shown in Fig. 3, the cMsrA–cGrx2 complex band completely disappeared in the presence of GSH, whereas it remained in the presence of GSSG. In addition, the protein complexes above 50 kDa disappeared in response to treatment with GSH. Our data suggest that GSH acts as a resolving agent to reduce the disulfide bond between Cys16 of cMsrA and Cys12 of cGrx2/C15S.

Table 1
Kinetic parameters of cMsrA.

Reductant	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} s^{-1})
DTT	10.0 ± 0.8	0.84 ± 0.12	12
cGrx2	4.5 ± 0.3	0.25 ± 0.05	18
cGrx2/C15S	4.6 ± 0.2	0.27 ± 0.04	17

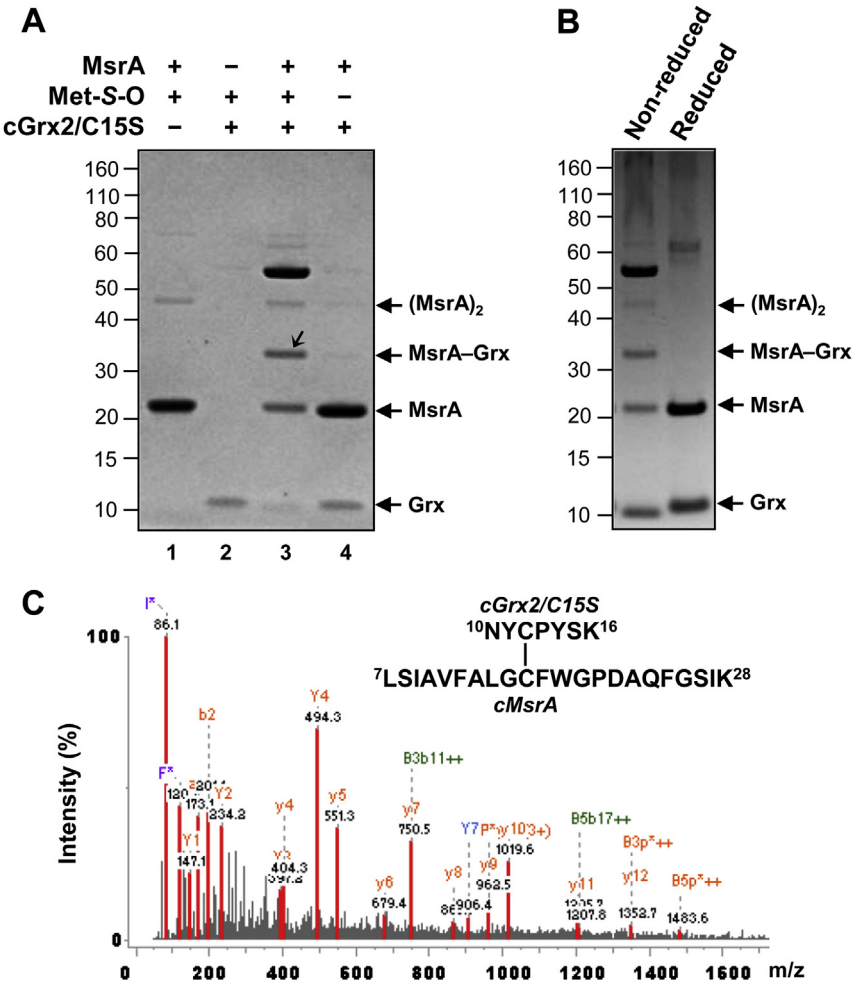


Fig. 2. Identification of cMsrA–cGrx2 complex and its disulfide bond. C15S mutant cGrx2 was incubated with cMsrA as described in the materials and methods. The samples were subjected to non-reducing SDS-PAGE and the protein bands were analyzed by Coomassie staining (A). The sample from lane 3 was treated with the reducing agent β-mercaptoethanol, which resulted in disappearance of the protein complexes (B). The cMsrA–cGrx2 complex band indicated by an arrow in A was subjected to mass spectrometry analysis. A disulfide bond between Cys12 of cGrx2 and Cys16 of cMsrA was identified (C).

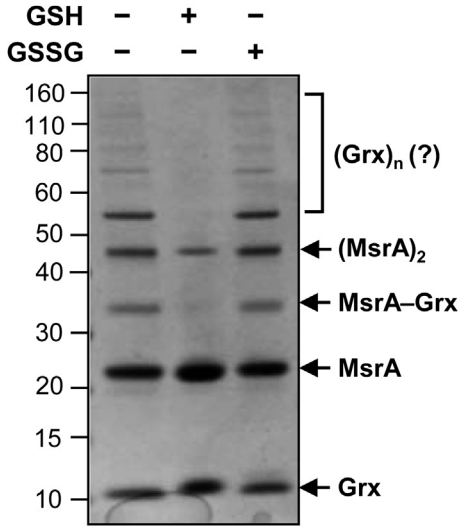


Fig. 3. Resolution of cMsrA–cGrx2 complex by GSH. The reaction mixture containing cMsrA, Met-*S*-O, and cGrx2/C15S was treated with GSH or GSSG and subjected to non-reducing SDS-PAGE. The protein bands were analyzed by Coomassie staining.

Based on these findings, we propose a model for regeneration of 1-Cys cMsrA by monothiol Grx, in which glutathionylation is not involved (Fig. 4). The first step is the formation of a sulfenic acid intermediate on catalytic Cys16 of cMsrA after Met-*S*-O reduction.

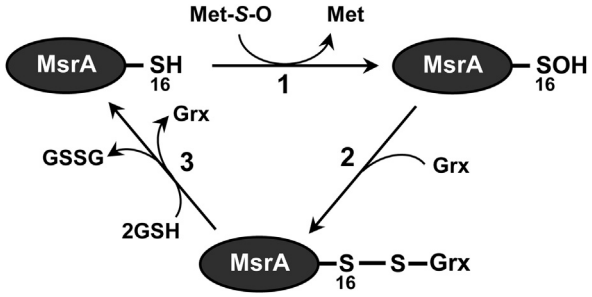


Fig. 4. Proposed regeneration mechanism of cMsrA by Grx. Catalytic Cys16 attacks the sulfoxide moiety of the substrate to form a sulfenic acid intermediate with the concomitant release of the product Met (step 1). The catalytic Cys16 sulfenic acid is then attacked by Grx, leading to the formation of cMsrA–cGrx2 complex through a mixed disulfide bond (step 2). The mixed disulfide bond between monothiol Grx and cMsrA is reduced by GSH, leading to cMsrA regeneration (step 3). In the case of dithiol Grx, the mixed disulfide bond may be resolved by a thiol-disulfide exchange reaction using the C-terminal resolving Cys of the active site of Grx, and the oxidized Grx may subsequently be reduced by GSH.

The sulfenic acid is then attacked by Grx, which leads to the formation of MsrA–Grx complex through a disulfide bond. GSH subsequently resolves the disulfide bond, leading to liberation of reduced MsrA. In the case of regeneration of cMsrA by dithiol Grx, the disulfide bond of the MsrA–Grx complex may first be attacked by the resolving Cys of the active site of Grx, leading to MsrA regeneration, after which the oxidized Grx may be reduced by GSH.

In summary, we report for the first time a regeneration mechanism of 1-Cys type MsrA by Grx. In contrast to the previously reported glutathionylation-mediated regeneration of 1-Cys type *Arabidopsis* MsrB [13], our proposed model suggests no involvement of glutathionylation on the catalytic Cys of 1-Cys cMsrA.

Conflict of interest

The authors have no conflict of interest.

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Transparency document

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