Functional analysis of free methionine-\(R\)-sulfoxide reductase from \textit{Saccharomyces cerevisiae}

Dung Tien Le*,1, Byung Cheon Lee*,1, Stefano M. Marino*, Yan Zhang*, Dmitri E. Fomenko*, Alaattin Kaya*, Elise Hacioglu#, Geun-Hee Kwak†, Ahmet Koç§, Hwa-Young Kim||, and Vadim N. Gladyshev*§

* Department of Biochemistry and Redox Biology Center, University of Nebraska-Lincoln, Lincoln, NE 68588, USA; # Izmir Institute of Technology, Department of Molecular Biology and Genetics, 35430, Urla, Izmir, Turkey; || Department of Biochemistry and Molecular Biology, Yeungnam University College of Medicine, Daegu 705-717, Republic of Korea

1 - equal contribution

§Corresponding author. Tel. (402) 472-4948. E-mail: vgladyshev1@unl.edu

Methionine sulfoxide reductases (Msrs) are oxidoreductases that catalyze thiol-dependent reduction of oxidized methionines. MsrA and MsrB are the best known Msrs that repair methionine-\(S\)-sulfoxide (Met-S-SO) and methionine-\(R\)-sulfoxide (Met-R-SO) residues in proteins, respectively. In addition, an \textit{Escherichia coli} enzyme specific for free Met-R-SO, designated fRMsr, was recently discovered. In this work, we carried out comparative genomic and experimental analyses to examine occurrence, evolution and function of fRMsr. This protein is present in single copies and two mutually exclusive subtypes in about half of prokaryotes and unicellular eukaryotes, but is missing in higher plants and animals. A \textit{Saccharomyces cerevisiae} fRMsr homolog was found to reduce free Met-R-SO, but not free Met-S-SO or dabsyl-Met-R-SO. fRMsr was responsible for growth of yeast cells on Met-R-SO, and the double fRMsr/MsrA mutant could not grow on a mixture of methionine sulfoxides. However, in the presence of methionine, even the triple fRMsr/MsrA/MsrB mutant could grow on a mixture of methionine sulfoxides.

Among the 20 common amino acids in proteins, methionine (Met) and cysteine (Cys) are the residues most susceptible to oxidation by reactive oxygen species (ROS). Upon oxidation, Met forms a diastereomeric mixture of methionine-\(S\)-sulfoxide (Met-S-SO) and methionine-\(R\)-sulfoxide (Met-R-SO). Met-S-SO and Met-R-SO can be reduced back to Met by Met-S-SO reductase (MsrA) and Met-R-SO reductase (MsrB), respectively [1]. These enzymes have been reported to play important roles in the protection of cells and proteins against oxidative stress [2-8]. Reversible Met oxidation has also been proposed to scavenge ROS thereby protecting cells from oxidative damage [9-11]. Increased expression of MsrA and MsrB can extend the lifespan of yeast cells and fruit flies, whereas deletion of the MsrA gene leads to the reduction in lifespan in mice and yeast [12-14].

Previously, three MsrB isozymes and a single MsrA were found in mammals. MsrB1 (also known as SelR or SelX) is a selenoprotein, which contains selenocysteine (Sec) in the active site and is localized to cytosol and nucleus. MsrB2 and MsrB3 are Cys-containing homologs of MsrB1. MsrB2 resides in mitochondria, whereas human MsrB3 has two alternative splice forms, wherein MsrB3A localizes to the ER and MsrB3B is targeted to mitochondria [15].

The catalytic mechanism of MsrA involves a sulfenic acid intermediate at the catalytic Cys followed by the formation of a disulfide bond between the catalytic and resolving Cys. A third Cys may then form a disulfide with the resolving Cys [16, 17]. The resulting disulfide is reduced by thioredoxin or other oxidoreductases, generating the initial, reduced form of the protein. X-ray
structures of MsrAs from several organisms have been solved [17, 18].

Cys-containing MsrBs (e.g., mammalian MsrB2 and MsrB3) follow the same mechanism, even though the two Msr types have no homology and are characterized by different structural folds [19-21]. Sec-containing mammalian MsrB1 has also been characterized and compared to Cys-containing MsrBs [20]. Interestingly, Cys-containing MsrBs share some active-site features (e.g., conserved residues His77, Val81 and Asn97, numbering based on mouse MsrB1 sequence) which are absent in selenoprotein MsrB1s. When these three residues were introduced into the Sec-containing MsrB1, the enzyme was inactive. However, when the three residues were introduced into the Cys mutant form of MsrB1, the activity was partially recovered [20]. This evidence supports the idea that catalytic Cys and Sec require different active site features.

In addition to MsrA and MsrB functions, previous studies suggested the presence of additional Msr activities in E. coli and yeast cells, which were especially evident in cells deficient in both enzymes [14,21-23]. Recently, Lowther and colleagues discovered a new enzyme, designated fRMsr, which catalyzes the reduction of free Met-R-SO in E. coli [24]. They showed that this activity is associated with a GAF-like-domain-containing protein. Homologs of this enzyme were found in other bacteria as well as in eukaryotes, suggesting that these proteins also could function as fRMrs. However, none of these other proteins have been functionally characterized.

In this work, we cloned a yeast homolog of bacterial fRMsr and functionally characterized it with regard to the in vivo function and catalytic mechanism. In addition, we carried out comparative genomic analyses to examine evolution of this protein family. The data show that fRMsr is the main enzyme responsible for the reduction of free Met-R-SO in both prokaryotes and unicellular eukaryotes.

**Experimental Procedures**

**Materials** - E. coli NovaBlue cells (Novagen) were used for DNA manipulation, and BL21(DE3) cells (Invitrogen) for protein expression. Restriction enzymes were from Fermentas, PCR reagents from Invitrogen, and Talon polyhistidine affinity resin from Clontech.

**Comparative genomic analyses of fRMrs in prokaryotes and eukaryotes** - Sequenced genomes of archaea, bacteria and eukaryotes were retrieved from the NCBI website (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). A total of 540 bacterial, 48 archaeal and 160 eukaryotic organisms were analyzed (as of March 2008). We used E. coli (NP_416346) and S. cerevisiae fRMsr proteins (NP_012854) as our seed sequences to search for homologs in other organisms. TBLASTN [25] was used with default parameters. Orthologous proteins were defined as bidirectional best hits [26].

**Multiple sequence alignment and phylogenetic analysis** - A phylogenetic tree based on concatenation of 31 orthologs occurring in 191 species [27] was used to analyze the distribution of organisms that do or do not contain fRMrs. Multiple sequence alignments were performed using CLUSTALW [28] with default parameters and ambiguous alignments in highly variable regions excluded. Phylogenetic trees were reconstructed with PHYLIP programs [29]. Neighbor-joining (NJ) trees were obtained with NEIGHBOR and the most parsimonious trees were determined with PROTPARS. The robustness of these trees was evaluated by maximum likelihood (ML) analysis with PHYML [30] and Bayesian estimation of phylogeny with MrBayes [31].

**Computational analysis of the active site of yeast fRMsr** – In silico analysis of the active site was carried out using the structure of oxidized yeast fRMsr with PDB code 1F5M. The structure was adjusted with the program VegaZZ 2.2.0 (http://www.vegazz.net/) to reduce the disulfide bond and protonate residues, and minimized (60 steps of 0.05 Å, steepest descent global minimization) with UCSF Chimera (http://www.cgl.ucsf.edu/chimera/). The active site was analyzed with Q-site finder (http://bmbpcu36.leeds.ac.uk/qsitefinder/), which assesses residue accessibility to a small molecular probe and then ranks up to ten potential active sites [32]. The top-ranked predicted active sites...
were considered as the most probable sites of catalysis. The theoretical titration data for each putative catalytic Cys were obtained using the H++ server (http://biophysics.cs.vt.edu/H++/index.php, [35]), which calculates theoretical pKa values and estimates variation of the charge state on the titrable atom of each titrable residue at different pH values. These values were then superimposed with the calculated curves for a typical HH behavior at the corresponding theoretical pKa. The assumptions were that deviating titrable residues more frequently occur in enzyme active sites [33,34,36] and that the theoretical titration curves may indicate the tendency of the residue to be catalytic. Finally, visual inspections of the titration curves were carried out for curves with the largest deviation from the standard HH behavior as candidate residues of the active site [33, 36].

**Computational docking of Met-R-SO into fRMsr active site and molecular dynamics** - The structure of yeast fRMsr (1F5M) was prepared for docking using VegaZZ 2.2.0, ArgusLab 4.0 (http://www.arguslab.com/) and the AutoDock (http://autodock.scripps.edu/) suite of programs. VegaZZ was also employed to prepare the Met-R-SO substrate (downloaded from the ZINC database). The SP4 force field and the AMMP-mom method were employed to assign charges to Met-R-SO and to minimize the structure. The substrate was then exported to ArgusLab. The yeast fRMsr structure was virtually reduced with VegaZZ building tools. The structure for the reduced protein was treated with Tinker (http://dasher.wustl.edu/tinker/) for extensive minimization dynamics. Using the AMBER99 force field and gradient convergence per atom criterion of 0.01 kcal/mol/Å, the reduced structure employed in the docking calculation was obtained. Molecular dynamics experiments were done with Tinker, using the dynamics option. We conducted first non boundary dynamics starting from the reduced, but not minimized, structure. We sampled time steps in the range 0.01-1.0 fs and overall dynamics time up to 200 ps, and performed calculations at 300 K and 1 atmosphere. After evaluation of the time range (0-1 ps), in which the clear movement of the active site Cys was detectable, we carried out final calculations with the following parameters: time step = 0.1 fs, number of steps = 1,000,000 and the dumps time = 0.05 ps. Analysis of trajectories was done with Tinker, VegaZZ; the root mean square fluctuation (standard definition) of carbon alpha was calculated starting from the structures of our ensemble with in-house programs. For docking, the substrate was analyzed in the intercalation site using a docking box of 20x20x20 Å centered at the approximate center of mass of the two candidate catalytic Cys residues (Cys101 and Cys125). We employed the GAdock docking engine, a genetic algorithm search technique implemented in ArgusLab. To set up the docking parameters, calculations were made with the following values: population size 250, max generations 100,000, mutation rate 0.02, grid resolution 0.1 Å, flexible ligand mode (other parameters were kept with default values). Docking calculations with AutoDock 4 were run in Linux environment making use of the ADT graphical user interface (http://mgltools.scripps.edu/). We imported the same Met-R-SO and yeast fRMsr structures employed for ArgusLab dockings. While with ArgusLab, the protein structure was forced to be rigid during the docking, with AutoDock it was possible to define a subset of flexible amino acids which were free to move during the docking. We defined as flexible the following residues: Tyr70, Leu80, Val89, Ala90, Cys91, Ile94, Gly99, Val100, Cys101, Gly102, Thr103, Ala104, Ala105, Ile123, Ala124, Cys125, and Asp126. These residues can be considered as an extended reaction center around the three Cys (91, 101, and 125). Again, the docking box 20x20x20 Å was centered around the approximate center of mass of the two candidate catalytic Cys residues (Cys101 and Cys125). We employed the genetic algorithm search with the following parameters: population size 250, max generations 50,000, mutation rate 0.02, maximum number of energy evaluation 10000000, grid resolution 0.2 Å, flexible ligand mode. Images were prepared with UCSF Chimera, VegaZZ, and PyMol (http://pymol.sourceforge.net/) packages.

**Preparation and verification of Msr mutant strains** - The yeast strains used in this study were derivatives of wild type BY4741 cells. Mutants missing two or three Msr genes were generated by a one-step gene disruption method using markers described in Table S1 [40]. The HIS3 marker was
amplified from p423 vector with the following primers:
5’-CGGTTTCTTCATGTTTATATGATGACGATTTTCCCAAATGTAAGCAAAACAGGCGACTGCCAGGTATCGTTGAACACG-3’, and 5’-TGGCGGGATTTTCAATGAAATATTGGACAAAGAGACTACTAGTGGAATATTGGTTATGTCGTATGCTGCAGCTTTAAATAATCCG-3’. PCR products were purified from agarose gel and used for yeast transformation. Mutant clones were selected in minimal histidine-free medium. Deletions of each ORF were confirmed by PCR. Yeast strains used in this study are listed in Table S1.

**Cloning, expression and purification of yeast fRMsr -** The gene coding for fRMsr was amplified from yeast genomic DNA with the following primers (restriction enzyme sites *Nde*I and *Not*I are in bold and underlined): fRMrs-F 5’-AAA**CATATG**ATGGGCTCATCAACCGGGTTTC-3’, and fRMrs-R 5’-**AGCGGCCGC**GACACATGATTTATTAATTTAGCAAG-3’. The PCR product was digested with *Nde*I and *Not*I and cloned into pET21b vector (Novagen) and then transformed into *E. coli* NovaBlue cells. The constructs were verified by restriction enzyme digestion and direct sequencing. The plasmid harboring the correct insert was transformed into *E. coli* BL21(DE3) cells for protein expression. These cells were grown in LB medium containing 100 µg/ml ampicillin until OD600 reached 0.8, and IPTG was added to a final concentration of 0.25 mM. Induction of protein synthesis proceeded at 30 °C for 4 h, and cells were then harvested by centrifugation at 4,000 rpm. Cell pellets were washed with PBS and recentrifuged. Harvested cells were stored at -70 °C until use.

To purify recombinant protein, cell pellets were mixed with resuspension buffer (50 mM Tris-HCl, pH 7.5, 15 mM imidazole, 300 mM NaCl), and PMSF was added to a final concentration of 0.5 mM. After sonication, the supernatant was collected by centrifugation at 8,000 rpm for 30 min and loaded on a cobalt Talon resin (Clontech, CA, USA) pre-equilibrated with resuspension buffer. Following washing with the same buffer, the protein was eluted with elution buffer (50 mM Tris-HCl, pH 7.5, 300 mM imidazole, 300 mM NaCl). The eluted fractions were analyzed by SDS-PAGE, and the fractions containing fRMsr were pooled and dialyzed overnight against PBS in a dialysis cassette (Pierce).

**Site-directed mutagenesis of fRMsr -** Substitutions of Cys with Ser at positions 91, 101 and 125 were carried out using QuickChange protocol (Stratagene) with the following primers: C91Sf: 5’-GGAAAGGTGCTAGCCAATGATTC-3’; C91Sr: 5’-GAATCATTTGAGCGACCTTTCC-3’; C101Sf: 5’-GGTGAACTGCCAGGTAAAAC-3’; and C101Sr: 5’-GCTGCAGTTCCGCTAACACCTTTACC-3’; C125Sf: 5’-GTCAATTGCGAGCGGTAAAC-3’; and C125Sr: 5’-GTTTCACCGTCGCAATATGAC-3’. After PCR reactions, wild-type template was digested with *Dpn*I, column-purified, and the PCR products were transformed into *E. coli* NovaBlue cells. Mutations were confirmed by DNA sequencing. Plasmids carrying correct mutations were transformed into *E. coli* BL21(DE3) and protein expression was carried out as described above.

**Determination of fRMsr activity -** The reaction mixture (20 µl in PBS pH 7.4) included 50 mM dithiothreitol (DTT), 1 mM substrate (Met-R-SO4 or Met-S-SO4), and the reaction was initiated by the addition of purified enzyme. The reaction proceeded at 37 °C for 30 min and then was stopped by adding 2 µl of TCA. After further incubation at 4 °C for 10 min and centrifugation at 13,000 rpm for 15 min, the supernatant (5 µl) was mixed with the OPA derivatization reagent to a 100 µl final volume as described [8]. Following a 2 min reaction at room temperature, 50 µl of the mixture were injected onto a Zorbax Eclipse XDB-C8 column (4.6×150 mm) initially equilibrated at room temperature with 20 mM sodium acetate, pH 5.8 (solvent A) and methanol (solvent B) at a ratio of 88:12 (v/v). Met was separated at room temperature at a flow rate of 1.5 ml/min using a linear gradient of 12-40% methanol from 0 to 5 min, 40-47% methanol from 5 to 40 min, and 47-100% methanol from 40 to 45 min. Detection was by fluorescence of Met derivatives using a Waters...
scanning fluorescence detector with excitation at 330 nm and emission at 445 nm.

The reduction of free Met-R-SO was also determined by a coupled assay. Briefly, a 100 µl reaction mixture contained 5 µg of purified fRMsr, 5 µg E. coli thioredoxin (Trx), 0.5 µl E. coli thioredoxin reductase (Sigma), 0.4 mM NADPH and free Met-R-SO. The reaction was allowed to take place at 37 °C for 15 min, 200 µl of PBS was added to the reaction mixture, and NADPH oxidation was analyzed immediately at OD340. The activity was calculated using NADPH extinction coefficient (6220 M⁻¹ cm⁻¹).

Viability assays and oxidative stress resistance - Yeast strains were grown in liquid media overnight and then seeded into fresh liquid medium at the OD600 of 0.1. The cultures were allowed to reach OD600 of 0.4-0.5, diluted to OD600 of 0.1-0.2 and used for viability assays. Briefly, 50 µl yeast culture was inoculated into 1 ml of liquid medium, and of that, 50 µl was diluted into 450 µl of medium and used to count colonies at time zero. H2O2 was then added, at indicated concentrations, to 1 ml cultures, and the cells were further incubated at 30 °C. At 30, 60, and 120 min after treatment, 50 µl of each culture was diluted into 450 µl of liquid medium. To count viable colonies, the diluted cultures were mixed with 3 ml of liquid medium supplemented with 0.4% agarose held in a water bath at 42 °C, and the resulting mixtures were poured onto agar plates containing the same medium components as the liquid medium. Viability of wild-type and deletion mutant strains was assayed in YPD or YNB media, and those of wild-type strains overexpressing vector or yeast fRMsr were assayed in YNB medium without histidine.

For plate assays, overnight cultures of wild-type and deletion mutant strains were adjusted to an OD600 of 2.5, 0.25, 0.025, and 0.0025 via serial dilution. Each diluted sample (5 µl) was spotted onto YNB agar medium in the presence or absence of indicated concentrations of H2O2 and cell growth was quantified after incubation for 3 days at 30°C.

Analysis of microarray data – Data on expression levels of fRMsr (GDS777/10627_at/YKL069W), MsrA (GDS777/5697_at/MXR1), and MsrB (GDS777/6899_at/YCL033C) at different nutrient-limiting conditions (i.e., carbon, nitrogen, phosphorus, and sulfur) and at different oxygen availability (i.e., aerobic and anaerobic) were collected from GEO profiles [42]. Data from three single channels were averaged, and standard deviations were calculated.

Preparation of human thioredoxin 1 (hTrx1) Cys35Ser mutant-immobilized resins and Cys targeting – Cys35Ser hTrx1-immobilized resins were prepared to trap resolving cysteines of fRMsr. Briefly, mutant Cys35Ser hTrx1 in 0.1 M sodium carbonate buffer (pH 8.5) containing 0.5 M NaCl was incubated with cyanogen bromide-activated Sepharose 4B (Sigma) that had been swelled according to the manufacturer's instructions. The coupling reaction was stopped by centrifugation, and unreacted side chains on the resin were blocked with 0.2 M glycine (pH 8.0) for 2 h at room temperature. After extensive washing with the basic coupling buffer described above and acetate buffer (0.1 M, pH 4) several times, immobilized Cys35Ser hTrx1 was quantified based on the difference between the amounts of protein initially used and remaining in solution after the coupling reaction. To search for residues targeted by Cys35Ser hTrx1, various fRMsr forms (wild-type, Cys91Ser, Cys101Ser, Cys125Ser, or Cys101/125Ser; 50 µg of each) were incubated with 0.5 ml of Cys35Ser hTrx1-immobilized resin at room temperature for 2 h under gentle stirring. Immobilized Cys35Ser hTrx1 was initially reduced with 1 mM DTT for 30 min and washed with 50 mM sodium phosphate buffer (pH 7.5, 50 mM NaCl). After incubating the Cys35Ser hTrx1-immobilized resin with each fRMsr form at room temperature for 2 h, the resins were suspended in 50 mM sodium phosphate buffer (pH 7.5, 50 mM NaCl, 10 mM DTT) and incubated at room temperature for 30 min. During the whole process, initial samples prior to incubation with the Cys35Ser hTrx1-immobilized resin (I), flow through samples after incubation with the resin (F), and elution samples after 10 mM DTT treatment (E) were collected and 10 µl of each fraction were loaded on SDS-polyacrylamide gels and visualized by Western blot with anti-fRMsr antibodies.
Results

Comparative genomics of fRMsr - Analysis of completely sequenced genomes revealed a restricted (compared to MsrA and MsrB) use of fRMsr (a list of analyzed organisms is shown in Table S2). 41.5% of all examined bacteria, 6% archaea and 44.4% eukaryotes were found to possess this protein, and each of these organisms contained a single copy of fRMsr. The distribution of fRMsr was further analyzed in detail in bacteria (Fig. S1), archaea (Fig. S2), and eukaryotes (Fig. S3) based on a highly resolved tree of life [27] (see supporting information). Interestingly, the occurrence of this protein was limited to unicellular organisms, whereas higher plants and animals lacked this protein.

Two variants (or subtypes) of fRMsr proteins were detected. Both contained two fully conserved Cys residues (i.e., Cys101 and Cys125 in S. cerevisiae fRMsr) which were absent in other GAF-domain-containing proteins. Thus, these two residues represent a signature for the identification of fRMsr sequences. Type I fRMsr possessed an additional conserved Cys (e.g., Cys91 in S. cerevisiae fRMsr) which was invariantly absent in type II fRMsr. Occurrence of the two fRMsr types is shown in Fig. S1-S3, and multiple alignment of fRMsr sequences in Fig. 1. No organism possessing both fRMsr types was detected. Type II fRMsr had lower occurrence and were found in 26 bacteria, 3 archaea and 2 eukaryotes characterized by complete genome sequences. Phylogenetic analysis was used to further examine the evolutionary relationships of the two types of fRMsr in different organisms (Fig. S4).

We also examined domain fusions involving fRMsr in eukaryotes and prokaryotes (Fig. 2). One example involved type I fRMsr that was fused with a homolog of TIP41 (pfam04176, TIP41-like family) [24] in Kinetoplastida (including Leishmania and Trypanosoma, Fig. 2A). TIP41 was reported to be involved in the regulation of yeast type 2A phosphatases [43]. However, the functional relationship between TIP41 and fRMsr is not clear. Similarly, in bacteria, all type II fRMsr in sequenced Thermotogae contained an unknown N-terminal domain (designated Unknown_1, Fig. 2B). In Fervidobacterium nodosum, which belongs to Thermotogae, an additional GGDEF domain (pfam00990, GGDEF domain) was found to be fused at the C-terminus of fRMsr (Fig. 2C).

Overall, functional genomics analysis of fRMsr homologs revealed the presence of these proteins in all three domains of life. fRMsr showed lower occurrence than MsrA or MsrB and also was present in single copies in organisms containing this protein. Our data suggested that all fRMsr sequences may have a similar function: by analogy to the E. coli fRMsr, which naturally reduces free Met-R-SO, other organisms also could use fRMsr for this function.

fRMsr is important for Met-R-SO consumption and growth of yeast cells on Met-R-SO – For functional characterization, we chose a S. cerevisiae fRMsr homolog (YKL069W) which is annotated as a putative protein of unknown function. We prepared mutant cells deficient in fRMsr and its double mutants with MsrA or MsrB. We first examined the growth of these and wild-type cells on YNB agar media in which Met was substituted with Met-R-SO or Met-S-SO. Fig. 3A shows that the deficiency in fRMsr, MsrA or MsrB only slightly decreased the growth of yeast cells on Met-R-SO. The growth was reduced more significantly, when both MsrA and MsrB genes were deleted [44]. Interestingly, deletion of fRMsr and MsrA completely blocked the growth of yeast cells, suggesting that these two proteins were responsible for the reduction of Met sulfoxides in S. cerevisiae. In this regard, the growth of the fRMsr mutant strain on Met-R-SO may be explained by the presence of small amounts of Met-S-SO in the Met-R-SO preparation or by the lower requirement for Met in this assay.

In the media where Met was replaced with Met-S-SO (Fig. 3B), deletion of fRMsr or MsrB had no effect on the growth of yeast cells; however, deletion of MsrA reduced the growth significantly. The combined MsrA and fRMsr deficiency further reduced the growth of yeast cells. Again, a small contribution of fRMsr to the growth of MsrA-deficient cells on Met-S-SO may be explained by trace amounts of Met-R-SO present in the Met-S-
SO preparation. None of the mutant strains showed significant growth defects in Met media (Fig. 3C). The results of the assay in liquid medium containing Met-R-SO instead of Met are shown in Fig. 3D. The complete growth inhibition of the fRMsr mutant in Met-R-SO medium was observed in this liquid culture assay. To further examine Met-SO reduction in *S. cerevisiae*, we prepared and characterized a mutant strain lacking all three enzymes (i.e., MsrA, MsrB and fRMsr). The triple mutant overexpressing fRMsr was shown to restore the growth. These data clearly demonstrate the role of fRMsr in the reduction of free Met-R-SO. Overall, the data show that fRMsr is the main enzyme responsible for Met-R-SO reduction in yeast and likely other organisms, and MsrA is the main enzyme reducing free Met-S-SO.

**Yeast fRMsr serves as an antioxidant protein –** Wild-type and fRMsr mutant strains, as well as cells transformed with control vector or the vector expressing fRMsr were tested for sensitivity to oxidative stress. Strains deleted for MsrB gene or overexpressing this protein were included for comparison. As shown in Fig. 4A, in the presence of 1 mM H$_2$O$_2$, deletion of the fRMsr gene resulted in reduced viability at 30 min. Fig. 4B shows that, in the presence of 1 mM H$_2$O$_2$, the yeast cells overexpressing fRMsr showed an increased resistance to peroxide treatment (compared to control cells). The antioxidant role of fRMsr was further confirmed in plate assays (Fig. 4C). The fRMsr mutant was more sensitive to H$_2$O$_2$-mediated cell death compared to wild-type cells. However, the deletion of fRMsr less affected sensitivity to oxidative stress than that of MsrA or MsrB. Thus, fRMsr as well as MsrA and MsrB serve as antioxidants in yeast cells.

A recent study examined global gene expression in yeast cells under the limitation of nutrients, such as carbon, nitrogen, phosphorus and sulfur, as well as examined the effect of oxygen availability [42]. Expression data for fRMsr, MsrA, MsrB were retrieved from GEO Profiles and are shown in Fig. S5. Under aerobic conditions, fRMsr gene expression was elevated when sulfur was limiting in the growth medium. MsrA was also elevated, but MsrB was not. However, under anaerobic conditions, neither fRMsr nor MsrA gene expression was affected when sulfur was limiting in the medium. These data suggest that, in the presence of oxygen (when ROS levels are higher), MsrA and fRMsr (but not MsrB) likely contribute to sulfur acquisition by reducing Met-S-SO and Met-R-SO, respectively.

**Roles of yeast fRMsr in aging –** Wild-type and mutant strains deficient in individual Msrs and their combinations were analyzed for lifespan in a replicative assay of yeast aging. Deletion of MsrB did not influence lifespan, whereas fRMsr and MsrA mutant strains showed a reduced lifespan (18% and 30%, respectively) (Fig. 5A). To further elucidate the role of fRMsr in aging, the lifespans of ∆fRMsr∆MsrA, ∆fRMsr∆MsrB and ∆fRMsr∆MsrA∆MsrB cells were determined. These strains showed 20% reduction in lifespan compared to wild-type cells, which was not significantly different from the lifespan of cells lacking only fRMsr. fRMsr overexpression did not extend yeast lifespan in cells grown in minimal YNB media (Fig. 5B).

**Characterization of catalytic properties of fRMsr -** To explore enzymatic properties of yeast fRMsr, we expressed this protein in *E. coli* as a His-tagged protein and examined its catalytic properties. The recombinant protein had robust Met-R-SO reductase activity, but was inactive with Met-S-SO as well as dabsylated Met-R-SO. Kinetic parameters derived by fitting the experimental data onto Michaelis-Menten equation are shown in Fig. 6. The enzyme had a $V_{\text{max}}$ value of 443 nmol/min/mg protein and its $K_m$ value for free Met-R-SO was 230 µM.

**Identification of catalytic Cys in fRMsr –** We searched for catalytic Cys in *S. cerevisiae* fRMsr by analyzing properties of the active site in this protein. The Q-site finder prediction placed three Cys residues (Cys91, Cys101 and Cys125) in the most probable enzymatic cleft for fRMsr. The same three Cys residues were also identified in *E. coli* fRMsr [24]. However, while Cys101 and Cys125 provided their sulfur atoms for possible substrate binding, only the backbone atoms of Cys91 were involved. The best-scoring predicted active site was composed of Trp66, Tyr70, Ala90, Ile94, Glu99, Val100, Cys101, Val116, His112, Ile122, Ala124, Cys125, Asp126, Thr129, Glu132, Asp149, and Asp151 (Fig. S6).
We further analyzed the theoretical titration behavior for Cys91, Cys101, and Cys125 using the H++ server to fit into the standard HH curve (Fig. S7). The deviating fitting was more pronounced for Cys125 and Cys101, but Cys91 showed a non-deviating curve similar to most residues in proteins [35] including Cys177 in fRMsr. On the assumption that highly deviating titrable residues are often located in enzyme active sites [33,34,36], these results suggested Cys125 or Cys101, but not Cys91, as the residues directly involved in reducing free Met-R-SO. Cys125 was in a close proximity to two other titrable residues showing HH deviating behavior, Tyr70 and Asp151, while Cys101 clustered with highly deviating Glu132 and Asp149. Altogether, computational analyses suggested that Cys125 and Cys101 were candidate catalytic residues, while Cys 91 was not.

To further analyze Cys residues participating in catalysis and to characterize the reaction mechanism of fRMsr, we carried out computational docking of Met-R-SO into the active site of the enzyme as described in Experimental Procedures. Employing different methodologies, we consistently observed the same Met-R-SO-binding site in yeast fRMsr (shown as cluster of scoring models in Fig. S6). Among these models, the best scoring candidate (interaction energy of -8.1 kcal/mol) is shown in Fig. 7. This binding structure was detected by both AutoDock and ArguLab, with the substrate close to both Cys101 and Cys125 and pointing the sulfoxide moiety toward the sulfur atom of Cys101 (at a distance of 2.4 Å). Cys125 appeared to orient the substrate to react with Cys101. Given that this model was reproduced with different docking methods, and received good scores, our data suggested that Cys101 is the catalytic residue, and Cys125 the initial resolving Cys. In addition, we observed that Cys125 was remarkably more mobile than either Cys91 or Cys101 (the latter is least mobile of the three) (Fig. 8). Following reduction of the disulfide bond, Cys125 moves away from Cys91 (from 2.1 Å to 6.9 Å), coming closer to Cys101 and Asp151 (from 7.1 Å to 4.6 Å).

Our reduced structure model (i.e., the most stable reduced form of the protein) presents a Cys125-Asp151 distance of 3.6 Å, taken as the shortest atomic distance between the two residues (Fig. 8), which is very similar to the previously observed distance (3.5 Å) of the reduced form of yeast fRMsr [45]. In this work, it was found that Cys125 occurs in two different conformations when in oxidized and reduced forms, the latter positioned near Asp151 and away from Cys91. Thus, our data are consistent with the previous structural analysis [45] and further suggested that Cys125 is the most mobile Cys in the active site.

We carried out molecular dynamics calculations to further address Cys125 mobility in a short time scale (0 to 200 ps). The movement of Cys125 was very quick (within 1 ps, Fig. S8). At the same time, other Cys were stationary preserving their initial positions. Following the initial movement, Cys125 mobility was not significantly different compared to the other active site residues (i.e., its fluctuations were comparable to those of Cys91 and Cys101). Thus, Cys125 is initially the most mobile active site Cys, in agreement with the expected properties of the resolving Cys.

Catalytic activity of fRMsr mutants and Cys targeting by hTrx1 - To directly determine the roles of Cys residues in fRMsr catalysis, we separately mutated the three conserved Cys in the yeast enzyme to Ser. Following affinity isolation, wild-type and mutant fRMsr proteins were pure as assessed by SDS-PAGE. Mutation of each of Cys dramatically affected enzyme activity (the remaining activity was within background). Thus, all three Cys were found to be critical for the catalytic activity of fRMsr.

Wild-type fRMsr (WT) and Cys mutants (Cys91Ser, Cys101Ser, Cys125Ser, Cys101/125Ser) were further subjected to Cys targeting by hTrx1 (Cys35Ser mutant)-immobilized resin (Fig. 9A). Since hTrx1 is an efficient reductant of fRMsr in in vitro assays, we separated and mutant fRMsr proteins were pure as assessed by SDS-PAGE. Mutation of each of Cys dramatically affected enzyme activity (the remaining activity was within background). Thus, all three Cys were found to be critical for the catalytic activity of fRMsr.
hTrx1. Cys101Ser mutant, but not other mutants, could still form a disulfide between Cys91 and Cys125, reducible by hTrx1. Our model of the fRMsr reaction mechanism is shown in Fig. 9B.

**Discussion**

MsrA and MsrB are the best known Msrs that catalyze the reduction of Met-S-SO and Met-R-SO residues in proteins, respectively. While these enzymes are well characterized structurally and functionally and their critical roles in the repair of oxidized Met residues in proteins have been established, how free Met-SO are reduced by cells is not fully understood. MsrA does show activity towards free Met-S-SO, but the activity of MsrB towards free Met-R-SO is low. Previous studies suggested the occurrence of an additional Msr that catalyzes the reduction of Met-R-SO [14, 22, 23]. Recently, Lowther and co-workers purified and characterized a third Msr catalyzing Met-SO reduction, designated fRMsr [24]. This *E. coli* enzyme specifically reduced free Met-R-SO in in vitro assays and its close homologs occurred in other organisms, including *S. cerevisiae*, suggesting that these homologs may function as fRMsr. The mechanism used by the *E. coli* protein to catalyze the reduction of free Met-R-SO is not known. It is also unclear whether *E. coli* fRMsr homologs indeed possess the same activity and whether fRMsr homologs are responsible for the reduction of bulk of free Met-R-SO in cells. Our work has addressed these important questions.

Comparative genomics analysis of fRMsr homologs in sequenced genomes showed a mosaic distribution of this protein. It formed a clearly defined protein family whose members occurred in single copies and were present in organisms in all three domains of life. Approximately half of the sequenced bacterial genomes contained fRMsr (mostly various proteobacteria and Firmicutes) (Fig. S1). The Firmicutes appear to comprise the earliest branching phylum which may be a Gram-positive ancestor for all bacteria [27]. Therefore, although many phyla lacked this gene, it is possible that fRMsr could have been used in the common ancestor of bacteria. In contrast, it was only detected in three closely related archaeal organisms, suggesting either a recent acquisition of fRMsr (e.g., by horizontal gene transfer from bacteria) in Thermoplasmales or, less likely, a complete loss of it in other archaeal phyla. In eukaryotes, although about half of the sequenced organisms possess fRMsr, it is absent from all multicellular organisms, including higher plants and animals. It appears that either fRMsr is not essential for these organisms or that alternative pathways evolved for Met-R-SO reduction. Besides, two variants of fRMsr were identified on the basis of the presence/absence of a conserved Cys (Cys91 in yeast fRMsr). Both subtypes had two additional conserved Cys residues that were implicated in catalysis [24].

Using *S. cerevisiae* as a model organism, we characterized fRMsr function in vivo and in vitro. The analysis of mutant cells lacking fRMsr or combinations of fRMsr and MsrA and/or MsrB deletions (i.e., double and triple Msr mutants) revealed that fRMsr was responsible for the reduction of free Met-R-SO in yeast cells, whereas MsrA was the main protein that reduced free Met-S-SO in this organism.

Consistent with this function, the fRMsr deletion strain also showed increased sensitivity to oxidative stress as well as a reduced lifespan. In addition, even though overexpression of fRMsr had almost no effect on yeast lifespan, it protected cells from oxidative stress caused by H$_2$O$_2$ treatment. Furthermore, microarray analyses revealed elevated expression of fRMsr and MsrA under the conditions of sulfur limitation in cells grown aerobically, whereas MsrB expression did not change. We also found that expression of yeast fRMsr in mammalian cells provided them with the ability to reduce free Met-R-SO, whereas the initial cells were not able to consume this compound [8]. Altogether, these data established an important role of fRMsr in the reduction of free Met-R-SO, which is a process by which this protein provides Met for cellular metabolism. Taking into account the finding that *E. coli* fRMsr specifically reduced free Met-R-SO [24], we conclude that fRMsr is the main, and may be the only free Met-R-SO reductase and that it functions in supplying cells with Met by salvaging oxidized Met. Perhaps, the loss of fRMsr in plants and animals can be explained by lower levels of free Met sulfoxides in these organisms that evolved...
It can be further inferred that fRMsr, as well as other Msrs, are needed primarily under stress conditions. While the triple fRMsr/MsrA/MsrB mutant cells showed a reduced lifespan and increased sensitivity to H$_2$O$_2$ treatment, in the absence of stress this mutant was viable and grew on the Met-containing media similarly to wild-type cells. These data are also consistent with the previous proposal that Msrs and Met contribute to the cellular antioxidant system by scavenging ROS via reversible Met oxidation [9].

To further examine fRMsr, we characterized the recombinant yeast enzyme. As expected, it showed specificity for the reduction of free Met-R-SO, but was not active with either free Met-S-SO or dabsyl-Met-R-SO residues (the latter mimicking Met-R-SO residues in proteins). No fRMsr mutant proteins have previously been characterized, but a prediction for the catalytic mechanism was made, based on the structural analysis of the *E. coli* fRMsr, that the three Cys residues are involved in catalysis [24]. Indeed, the role of Cys residues in the catalysis by fRMsr is consistent with the fact that both MsrA and MsrB utilize Cys residues for Met-SO reduction. In some MsrA and MsrB forms, these Cys are replaced with Sec.

Through molecular modeling and substrate docking, as well as by employing methodologically diverse tools for active site detection, we located the active site in the area of Cys101 and Cys125, and both of these residues were implicated in catalysis by multiple methods. The specific roles of these residues in catalysis could not be resolved by site-directed mutagenesis as both residues were essential for the catalytic activity of the enzyme. However, we found that Cys101 is the best candidate catalytic Cys through experimental analysis, computational substrate-docking modeling and Cys targeting search. We tracked the best scoring models for the binding of Met-R-SO in the catalytic site of yeast fRMsr (Fig. S6 and Fig. 7). Interestingly, the sulfoxide group of Met-R-SO protrudes toward sulfur of Cys101, probably leading to oxidation of this residue when it attacks the sulfoxide. In contrast, Cys125 may play a supportive role by arranging Met-R-SO to interact with Cys101 in our docking model. Furthermore, protein dynamics suggested that Cys125, in its stable conformation, comes close to Asp151, in agreement with the previously observation on the crystal structure of the reduced active site of yeast fRMsr [45].

Our computational analysis suggested that Cys125 is flexible and moves between two different conformers (Fig. 8), one close to Cys91 (in the oxidized protein) and the other near Asp151 (in the reduced enzyme). These observations clearly support the model (Fig. 9) wherein the flexible Cys125 is the principal resolving Cys. Its two conformations suggest a role in transferring oxidizing equivalents resulting in the Cys91-Cys125 disulfide. Consistent with this model, Cys targeting in fRMsr by mutant Trx demonstrated that Cys125 and Cys91 could interact with this reductant, whereas Cys101 could not. Surprisingly, mutation of either of these residues inactivated the enzyme even in a DTT-dependent reaction, suggesting that the catalytic residue is not accessible for DTT. We propose that the three Cys (Cys91, Cys101 and Cys125) follow classical disulfide exchange reactions, initiated by oxidation of Cys101 [46].

Cys91 is present only in one of two fRMsr forms (Type I), suggesting that it cannot be universally essential for catalysis. However, this residue is essential for the catalysis by yeast fRMsr. Additional fRMsr forms (Type II) will need to be investigated and compared with Type I enzymes to confirm differences in resolving residues.

Finally, it is clear that fRMsr is the main enzyme responsible for the reduction of free Met-R-SO in *S. cerevisiae* and potentially in other organisms from the three domains of life. It is a third member of the group of enzymes that reduce Met sulfoxides. Whereas MsrA is responsible for the reduction of both free and protein-based Met-S-SO, the reduction of Met-R-SO is carried out by two proteins: MsrB that reduces Met-R-SO residues in proteins and fRMsr that accounts for the reduction of free Met-R-SO. All three enzymes are widespread indicating importance of catalytic Met sulfoxide reduction in biology.
References

1. Weissbach, H., Resnick, L., and Brot, N. (2005) *Biochim. Biophys. Acta* **1703**, 203-212
2. Weissbach, H., Etienne, F., Hoshi, T., Heinemann, S. H., Lowther, W. T., Matthews, B., St John, G., Nathan, C., and Brot, N. (2002) *Arch. Biochem. Biophys.* **397**, 172-178
3. Slyshenkov, V. S., Shevalye, A. A., Liopo, A. V., and Wojtczak, L. (2002) *Acta Biochim. Pol.* **49**, 907-916
4. Kantorow, M., Hawse, J. R., Cowell, T. L., Benhamed, S., Pizarro, G. O., Reddy, V. N., and Hejtmancik, J. F. (2004) *Proc Natl Acad Sci USA* **101**, 9654-9659
5. Picot, C. R., Petropoulos, I., Perichon, M., Moreau, M., Nizard, C., and Figuet, B. (2005) *Free Rad. Biol. Med.* **39**, 1332-1341
6. Boschi-Muller, S., Olry, A., Antoine, M., and Branlant, G. (2005) *Biochim. Biophys. Acta* **1703**, 231-238
7. Cabreiro, F., Picot, C. R., Perichon, M., Mary, J., Friguet, B., and Petropoulos, I. (2007) *Biochimie* **89**, 1388-1395
8. Lee, B. C., Le, D. T., and Gladyshev, V. N. (2008) *J. Biol. Chem.* **283**, 28361-28369
9. Levine, R. L., Berlett, B. S., Moskovitz, J., Mosoni, L., and Stadtman, E. R. (1999) *Mech. Ageing Dev.* **107**, 323-332
10. Stadtman, E. R., Moskovitz, J., Berlett, B. S., and Levine, R. L. (2002) *Mol. Cell. Biochem.* **234/235**, 3-9
11. Ezraty, B., Grimaud, R., Hassouni, M. E., Moinier, D., and Barras, F. (2004) *EMBO J.* **23**, 1868-1877
12. Moskovitz, J., Bar-Noy, S., Williams, W. M., Requena, J., Berlett, B. S., and Stadtman, E. R. (2001) *Proc Natl Acad Sci USA* **98**, 12920-12925
13. Ruan, H., Tang, X. D., Chen, M.-L., Joiner, M.-L. A., Sun, G., Brot, N., Weissbach, H., Heinemann S. H., Iverson, L., Wu, C.-F., and Hoshi, T. (2002) *Proc. Natl. Acad. Sci. USA* **99**, 2748-2753
14. Koc, A., Gasch, A. P., Rutherford, J. C., Kim, H.-Y., and Gladyshev, V. N. (2004) *Proc. Natl. Acad. Sci. USA* **101**, 7999-8004
15. Kim, H.-Y., and Gladyshev, V. N. (2004) *Mol. Biol. Cell* **15**, 1055-1064
16. Boschi-Muller, S., Azza, S., Sanglier-Cianferani, S., Talfournier, F., Dorsselar, A. V., and Branlant, G. (2000) *J. Biol. Chem.* **275**, 35908-35913
17. Lowther, W. T., Brot, N., Weissbach, H., Honok, J. F., and Matthews, B. W. (2000) *Proc. Natl. Acad. Sci. USA* **97**, 6463-6468
18. Taylor, A. B., Benglis, D. M. Jr., Dhandayuthapani, S., and Hart, P. J. (2003) *J. Bacteriol.* **185**, 4119-4126
19. Lowther, W. T., Weissbach, H., Etienne, F., Brot, N., Matthews, B. W. (2002) *Nat. Struct. Biol.* **9**, 348-352
20. Kim, H.-Y., and Gladyshev, V. N. (2005) *PLoS Biol.* **3**, e375
21. Boschi-Muller, S., Gand, A., and Branlant, G. (2008) *Arch. Biochem. Biophys.* **474**, 266-273
22. Etienne, F., Spector, D., Brot, N., and Weissbach, H. (2003) *Biochem. Biophys. Res. Commun.* **300**, 378-382
23. Spector, D., Etienne, F., Brot, N., and Weissbach, H. (2003) *Biochem. Biophys. Res. Commun.* **302**, 284-289
24. Lin, Z., Johnson, L. C., Weissbach, H., Brot, N., Lively, M. O., and Lowther, W. T. (2007) *Proc. Natl. Acad. Sci. USA* **104**, 9597-9602
25. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). *J. Mol. Biol.* **215**, 403–410
26. Tatusov, R. L., Galperin, M. Y., Natale, D. A., and Koonin, E. V. (2000) *Nucleic Acids Res.* **28**, 33-36
27. Ciccarelli, F. D., Doerks, T., von Mering, C., Creevey, C. J., Snel, B., and Bork, P. (2006) *Science* **311**, 1283-1287
28. Higgins, D., Thompson, J., Gibson, T., Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) *Nucleic Acids Res.* **22**, 4673-4680
29. Felsenstein, J. (1989) *Cladistics* **5**, 164-166
30. Guindon, S., and Gascuel, O. (2003) *Syst. Biol.* **52**, 696-704
31. Ronquist, F., and Huelsenbeck, J. P. (2003) *Bioinformatics* **19**, 1572-1574
32. Laurie, A.T., Jackson, R.M. (2005). *Bioinformatics* **21**, 1908-1916
33. Ondrechen, M.J., Clifton, J.G. and Ringe, D. (2001). *Proc. Nat. Acad. Sci. USA* **98**, 12473-12478 (33)
34. Wei, Y., Ko, J., Murga, L.F. and Ondrechen, M.J. (2007). *BMC Bioinformatics* **8**, 119
35. Gordon, J. C., Myers, J. B., Folta, T., Shoji, V., Heath, L. S., and Onufriev, A. (2005) *Nucl. Acids Res.* **33**, W368-W371
36. Ko, J., Murga, L.F., Andre, P., Yang, H., Ondrechen, M.J., Williams, R.J., Agunwamba, A., and Budil, D. E. (2005). *Proteins: Structure Function Bioinformatics* **59**, 183-195
37. Le, D. T., Lee, H.-S., Chung, Y.-J., Yoon, M.-Y., and Choi, J.-D. (2007) *Bull. Korean. Chem. Soc.* **28**, 947-952
38. Richards, F. M. (1977) *Ann. Rev. Biophys. Bioeng.* **6**, 151-176
39. Irwin, J. J., and Shoichet, B. K. (2005) *J. Chem. Inf. Model.* **45**, 177-182
40. Goldstein, A. L., and McCusker, J. H. (1999). *Yeast* **15**, 1541-1553
41. Le, D. T., Liang, X., Fomenko, D. E., Raza, A. S., Chong, C.-K., Carlson, B. A., Hatfield, D. L., and Gladyshev, V. N. (2008) *Biochemistry* **47**, 9985-6694
42. Tai, S. L., Boer, V. M., Daran-Lapujade, P., Walsh, M. C., de Winde, J. H., Daran, J. M., and Pronk, J. T. (2005) *J. Biol. Chem.* **280**, 437-447
43. Smetana, J. H., and Zanchin, N. I. (2007) *FEBS J.* **274**, 5891-5904
44. Kryukov, G. V., Kumar, R. A., Koc, A., Sun, Z., and Gladyshev, V. N. (2002) *Proc. Natl. Acad. Sci. USA* **99**, 4245-4250
45. Ho, Y. S., Burden, L. M., and Hurley, J. H. (2000) *EMBO J.*, **19**, 5288-5299.
46. Boschi-Muller, S., Azza, S., Sanglier-Cianferani, S., Talfournier, F., Van Dorsselear, A., Brantlant, G. (2002) *J. Biol. Chem.*, **17**, 35908-35913

**Footnotes**

This study was supported by NIH grant AG021518 (to VNG) and the KRF (Korea Research Foundation) grant KRF-2007-331-C00198 (to H.-Y.K). Nebraska Redox Biology Center is supported by NIH grant RR017675.

Abbreviations used: Met-R-SO, methionine-R-sulfoxide, Met-S-SO, methionine-S-sulfoxide; fRMsr, free Met-R-SO reductase; Met-SO, methionine sulfoxide; Met, methionine; MsrA, Met-S-SO reductase; MsrB, Met-R-SO reductase; Trx, thioredoxin
Figure Legends

Fig. 1. Multiple sequence alignment of fRMsr proteins. The alignment shows regions containing the three conserved Cys in both types of fRMsr. Conserved residues are highlighted. The predicted functional Cys (C) residues are shown in red. Distant fRMsr homologs, GAF-domain proteins, are shown for comparison.

Fig. 2. Domain fusions involving fRMsr proteins. (A) fRMsr fused with TIP41-like domain (pfam04176) in Kinetoplastida. (B) fRMsr fused with an unknown domain Unknown_1 in Thermotogae; (C) fRMsr fused with both Unknown_1 and GGDEF domain (pfam00990) in Fervidobacterium nodosum.

Fig. 3. Roles of Msrs in utilization of Met-SO by S. cerevisiae cells. Yeast strains were analyzed for growth on Met-free YNB medium supplemented with 20 mg/l Met-R-SO (A), Met-S-SO (B) or Met (C). Cells were initially grown on YNB liquid medium until OD$_{600}$ reached 0.6, harvested, washed, diluted to OD$_{600}$ of 0.1 in water and serially spotted on agar YNB plates containing respective Met or Met-SO sources. The plates were incubated at 30 °C and pictures were taken after 48 h. Designation of yeast strains is as follows: ∆A, cells with MsrA gene deleted; ∆B, cells missing the MsrB gene; ∆F, cells with deletion of fRMsr gene. Cells missing multiple Msrs were also analyzed as shown as well as mutant cells containing a vector (+v) or expressing fRMsr (+pF). (D) Growth of wild-type and mutant strains of S. cerevisiae in liquid media (Met-free YNB supplemented with 20 mg/l Met-R-SO). The growth of yeast cells was monitored by measuring OD$_{600}$. Designations of WT and mutant cells as in panels A-C.

Fig. 4. Sensitivity of wild-type and mutant strains to oxidative stress. Indicated strains of S. cerevisiae were grown in liquid media containing 1 mM H$_2$O$_2$ and after the indicated periods of time the viability was assayed as described in Experimental Procedures. (A) Remaining viable fraction of WT and mutant strains in YPD media. (B) Remaining viable fraction of WT cells expressing fRMsr or a vector (control). (C) Plate assay. Wild-type and mutant yeast cells were incubated on YNB agar medium, treated or not with 1.2 mM or 1.5 mM H$_2$O$_2$, and the pictures were taken after 3 days at 30 °C.

Fig. 5. Lifespan analysis of yeast cells deficient in Msrs or overexpressing fRMsr. Replicative assays were used to determine lifespan of indicated mutant and WT yeast cells by counting the number of daughter cells produced by mother cells. Designation of yeast strains is as follows: (A) ∆A, MsrA mutant; ∆B, MsrB mutant; ∆F, fRMsr mutant. Cells lacking fRMsr and MsrA or MsrB, as well as cells with deletion of all three Msrs were analyzed in YPD media. (B) Lifespan of cells overexpressing fRMsr and control cells containing an empty vector was analyzed in minimal medium.

Fig. 6. Substrate saturation curve of fRMsr. $V_{\text{max}}$ and $K_{m}$ values for free Met-R-SO were determined by fitting experimental data, which were obtained by a coupled assay, on the Michaelis-Menten equation.

Fig. 7. Active site of yeast fRMsr. Met-R-SO was docked into the active site as described in Experimental Procedures. This figure was created with UCSF Chimera package. Cys91 and Cys125 are shown in stick representation; Cys101 and Met-R-SO (also highlighted with green contour) are shown in ball and stick representation. The rest of the protein is shown in ribbon. The model shows that that the substrate is oriented by both Cys101 and Cys125; at the same time the reactive sulfoxide group points toward Cys101 sulfur atom (at a distance of 2.4 Å), which is a predicted catalytic Cys.

Figure 8. Mobility of Cys91, Cys125 and Cys101 residues following reduction of the disulfide between Cys125 and Cys91. The initial position (oxidized fRMsr, PDB 1F5M) is shown in grey stick representation, while green sticks show the residues after minimization. An early intermediate structure (after the first 60 steps of minimization) during the MD minimization trajectory is also shown in grey sticks. Cys125 moves much more extensively than the other two Cys (moving away from Cys91 and
closer to Cys101) supporting its role as the resolving Cys. Cys101 is least mobile among the three Cys, supporting its role as the catalytic residue. In our reduced and minimized model, the Cys125-Asp151 distance is 3.6 Å (6.6 Å in the oxidized form).

**Fig. 9.** Cys targeting in fRMsr by mutant Trx and the proposed reaction scheme of yeast fRMsr. (A) Wild-type fRMsr and its four mutants (Cys91Ser, Cys101Ser, Cys125Ser, Cys101/125Ser) were applied onto a resin immobilized with the Cys35Ser form of hTrx1. Equal amounts (10 µl) of initial (I), flow-through (F), and DTT-eluted (E) fractions were analyzed by western blots with anti-yeast fRMsr antibodies. (B) Proposed catalytic mechanism of yeast fRMsr (Type I). Cys101 is proposed to be a catalytic Cys directly oxidized by Met-R-SO. Cys125 serves as the resolving Cys that initially forms a disulfide with Cys101 and then transfers this bond to Cys91 via thiol-disulfide exchange. The resulting Cys91-Cys125 disulfide is then reduced by Trx or DTT.
### Figure 1

**fRMsr (Type I)**

| Organism                  | Conserved Residues |
|---------------------------|--------------------|
| Saccharomyces cerevisiae  | 62                  |
| Aspergillus niger         | 66                  |
| Candida glabrata          | 58                  |
| Fichia stipitis           | 60                  |
| Magnaporthe grisea        | 56                  |
| Leishmania braziliensis   | 65                  |
| Trypanosoma brucei        | 68                  |
| Dictyostelium discoideum  | 50                  |
| Glucosinobacter oxydans   | 45                  |
| Haemophilus influenzae    | 60                  |
| Halobacterium sp.         | 60                  |
| Salmonella typhimurium     | 71                  |
| Xenorhabdus autotrophus   | 43                  |
| Vibrio angustum           | 43                  |
| Shewanella putrefaciens   | 41                  |
| Bacillus anthracis         | 50                  |
| Bacillus coeliclus        | 58                  |
| Oenococcus oeni           | 66                  |
| Escherichia coli          | 68                  |
| Bacillus subtilis          | 50                  |
| Leucobacter mallei        | 51                  |
| Xanthomonas axonopodii     | 52                  |
| Pseudomonas aeruginosa    | 49                  |
| Pseudomonas entomophila   | 49                  |
| Pasteurella multocida     | 39                  |
| Clostridium beijerincki   | 52                  |
| Enterococcus faecium      | 46                  |
| Streptococcus pneumonia   | 45                  |
| Bacteroides thetaiotaomicron | 50          |
| Neisseria gonorrhoeae     | 57                  |
| Lactobacillus fermentum   | 48                  |
| Halobacterium salinarum   | 53                  |
| Salinibacter ruber       | 53                  |
| Stigmatella aurantiaca   | 61                  |

**fRMsr (Type II)**

| Organism                  | Conserved Residues |
|---------------------------|--------------------|
| Crociobacter atlanticus   | 46                  |
| Gramella forestii         | 39                  |
| Psychrobacter arcticus    | 39                  |
| Docioides duodenalis      | 39                  |
| Unidentified subterium    | 39                  |
| Escherichia coli          | 50                  |
| Bacteroides thetaiotaomicron | 50          |
| Entrococcus faecium       | 46                  |
| Streptococcus pneumonia   | 45                  |
| Fisorobacter johnsonii    | 39                  |
| Robococcus bicoforata     | 38                  |
| Alkaliphilus metallidiges | 45                  |
| Thermotoga terrestris     | 78                  |
| Thermoplasma acidophilum  | 25                  |
| Paracoccus tectaelela     | 47                  |

**Other families containing GAF domain**

| Domain                    | Conserved Residues |
|---------------------------|--------------------|
| Diguanylate cyclase       | 37                  |
| Putative GDBP protein     | 55                  |
| Serine phosphatase        | 52                  |
| Signal transduction protein | 49               |
Figure 2

A. fRMsr(I)/TIP41-like fusion

Kinetoplastida: Leishmania, Trypanosoma

B. Unknown_1/fRMsr(II) fusion

Thermotogae

C. Unknown_1/fRMsr(II)/GGDEF domain fusion

Thermotogae: Fervidobacterium nodosum
Figure 3
Figure 4

(A)  
Viability vs. Time (min.):  
- WT  
- ΔMsrB  
- ΔfRMsr

(B)  
Viability vs. Time (min.):  
- WT  
- WT+MsrB  
- WT+ΔMsr

(C)  
WT  
ΔMsrB  
ΔfRMsr  
ΔMsrA  
ΔMsrAΔMsrB  
0 mM H₂O₂  
1.2 mM H₂O₂  
1.5 mM H₂O₂
Figure 6

![Graph showing the relationship between specific activity and free Met-R-SO (µM).](image)

- $R^2 = 0.97919$
- $V_{\text{max}} = 443 \pm 62$
- $K_m = 230 \pm 64$
Figure 7
Figure 9

(A) 

|       | WT | C91 | C101 | C125 | C101/125 |
|-------|----|-----|------|------|----------|
|       | I  | F   | E    | I    | F        |

WB: fRMsr

(B) 

SH | SH | SH | 101 | 125 | 91
SH | SH | SH | 101 | 125 | 91

Met-R-SO | Met
SH | SH | SH | 101 | 125 | 91

Yeast fRMsr (Type I)

S | S | SH | 101 | 125 | 91

H₂O
Supplementary information

The distribution of fRMsr in bacteria, archaea and eukaryotes - In bacteria (Fig. S1), except for phyla containing less than three sequenced genomes (for example, Planctomycetes and Acidobacteria), fRMsr was found to be utilized by all or almost all organisms in certain Firmicutes (Lactobacillales and Bacillales), Betaproteobacteria (Bordetella, Burkholderiaceae and Neisseriaceae), Gammaproteobacteria (Enterobacteriales, Vibrionaceae, Pseudomonadaceae and Xanthomonadaceae) and several other bacterial subdivisions. In contrast, this protein was not detected in many other phyla such as Chlamydiae, Cyanobacteria, Spirochaetes, Thermotogae, Epsilonproteobacteria and certain Alphaproteobacteria (Rickettsiales and Rhizobiaceae). The limited distribution of fRMsr observed in the present study contrasted with the more widespread occurrence of MsrA or MsrB.

In archaea, fRMsr was only detected in Euryarchaeota/Thermoplasmatales (Fig. S2). Approximately 94% of sequenced archaeal organisms lacked this protein. Among eukaryotes, 71 (44.4%) organisms, which belonged to Kinetoplastida, Stramenopiles and several major phyla of fungi (such as Pezizomycotina, Saccharomycotina and Basidiomycota), were found to contain fRMsr (Fig. S3, further details are shown in Table S2). On the other hand, algae, parasites, some fungi (e.g., Schizosaccharomycetes) and all multicellular organisms including animals and land plants lacked fRMsr.
Table S1. Yeast strains used in this study

| Strain  | Description | Genotype                          | Source           |
|---------|-------------|-----------------------------------|------------------|
| BY4741  | WT          | MATa his3 leu2 met15 ura3         | SGDP*            |
| GY2     | ΔMsrB       | MATa his3 leu2 met15 ura3, ΔmsrB:KAN | SGDP*           |
| GY4     | ΔMsrA       | MATa his3 leu2 met15 ura3, ΔmsrA:KAN | SGDP*           |
| GY3     | ΔfRMsr      | MATa his3 leu2 met15 ura3, ΔfRMsr:KAN | SGDP*           |
| GY5     | ΔMsrAΔMsrB  | MATa his3 leu2 met15 ura3, ΔmsrB:KAN ΔmsrA:ura3 | [14] in the main text |
| GY200   | ΔMsrA ΔfRMsr| MATa his3 leu2 met15 ura3, ΔmsrA:KAN ΔfRMsr:his3 | This work        |
| GY201   | ΔMsrBΔfRMsr | MATa his3 leu2 met15 ura3, ΔmsrB:KAN ΔfRMsr:his3 | This work        |
| GY202   | ΔMsrAΔMsrBΔfRMsr | MATa his3 leu2 met15 ura3, ΔmsrB:KAN ΔmsrA:ura3 ΔfRMsr:his3 | This work        |

SGDP - *Saccharomyces* Genome Deletion Project (http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html)
Fig. S1. Occurrence of fRMsr proteins in bacteria. The tree is based on the bacterial segment of a highly resolved phylogenetic tree of life. Occurrence of type I (containing 3 conserved Cys residues) and type II (containing 2 conserved Cys residues) fRMsr is shown separately.
Fig. S2. Occurrence of fRMsr proteins in archaea. All three fRMsr-containing organisms possess type II fRMsr.

| Phylum                                      | Organisms | fRMsr (Type II) |
|---------------------------------------------|-----------|-----------------|
| Nanoarchaeota                               | 1         | -               |
| Crenarchaeota/Thermoproteales               | 6         | -               |
| Crenarchaeota/Desulfurococcales             | 4         | -               |
| Crenarchaeota/Sulfolobales                 | 4         | -               |
| Euryarchaeota/Thermoplasmales              | 4         | 3               |
| Euryarchaeota/Archaeoglobales              | 1         | -               |
| Euryarchaeota/Halobacteriales              | 5         | -               |
| Euryarchaeota/Methanosarcinales            | 5         | -               |
| Euryarchaeota/Thermococcales               | 4         | -               |
| Euryarchaeota/Methanococcales              | 6         | -               |
| Euryarchaeota/Methanopyrales               | 1         | -               |
| Euryarchaeota/Methanobacteriales           | 3         | -               |
| Euryarchaeota/Methanomicrobiales           | 4         | -               |

Total 48 3
Fig. S3. Occurrence of fRMsr proteins in eukaryotes. Occurrence of type I and type II fRMsr is shown separately.
Fig. S4. Phylogenetic tree of fRMsr in prokaryotes and eukaryotes. 100 representative sequences were selected from a larger set of homologs. Non-fRMsr GAF-containing protein branches were compressed and are represented by family names. The measurement of distance for the branch lengths (shown by a bar) is indicated.
Fig. S5. Changes in expression levels of three Msr genes under the limitation of different nutrients and oxygen availability. (A) fRMsr, (B) MsrA, (C) MsrB. The data were retrieved from GEO profiles. C, carbon; N, nitrogen; P, phosphorus; S, sulfur.
**Fig. S6. Consensus cluster of Met-R-SO models docked into yeast fRMsr.** The cluster in the Figure refers to 5 models obtained as described in Experimental Procedures. This cluster is includes the most favorable models (i.e., interaction energy value, -8.1 kcal/mol, short S-S distance, 2.4 Å) we found in all our calculations (see Figure 8 in the main text). Cys101 (right part of the figure) and Cys125 (left) are shown in sticks, and the substrate Met-R-SO is represented in balls and sticks, while other nearby residues are shown in wireframe.
Fig. S7. Theoretical titration curves of Cys residues in yeast fRMsr. Theoretical titration curves (shown in red), calculated via the H++ server, were superimposed with the corresponding standard Henderson-Hasselbach (HH) behaving titration curves (shown in blue). The y-axis refers to the calculated charge on the Cys sulfur atom. The deviation from a typical HH behavior is considered to be an important feature of active site residues. Most residues in proteins have HH-like titration curves, like those of Cys91 and Cys177 in the figure. Cys125 is the most perturbed Cys residue in yeast fRMsr. As can be seen, the H++ calculated pKa of both Cys 125 and Cys 101 are high. However, this property is not as relevant as the deviation from the HH behavior in analysis of active sites under the THEMATICS assumptions [1-3].
Fig. S8. Structural analysis of fRMsr. Carbon alpha root mean square fluctuations (rmsf) of different fRMsr structures of the molecular dynamic trajectory in the 1 ps (curve green), 1-10 ps (blue curve) and 10-100 ps (red curve) simulation time scale are shown (A); rmsf (in Å) is plotted with the amino acid sequence of the active site containing the region of interest (residues 80-140). Higher deviation in the short time scale (0.1-1 ps) was observed for Cys125 (indicated by the third arrow, corresponding to position 125 in the X-axis), while the other two active site Cys were much less mobile. After 1 ps, Cys125 almost arrived in its final position (Fig. 8, main text), as can be seen in (B). Here, the molecular structure of the three active site Cys for 5 different dynamics steps in the range 0-1 ps is shown. The most stable position for Cys125 (grey ball and stick representation) was reached quickly, with a considerable displacement of all atoms of the residue. After 100 ps, Cys125 was still found in the 1 ps model illustrated in (B).

Supplementary references
1. Ondrechen, M. J., Clifton, J. G., and Ringe, D. (2001) Proc. Nat. Acad. Sci. USA 98, 12473-12478.
2. Bashford, D., and Karplus, M. (1990) Biochemistry 29, 10219-10225.
3. Bashford, D., and Gerwert, K. (1992) J. Mol. Biol. 224, 473-486.
Functional analysis of free methionine-R-sulfoxide reductase from Saccharomyces cerevisiae

Dung Tien Le, Byung Cheon Lee, Stefano M. Marino, Yan Zhang, Dmitri E. Fomenko, Alaattin Kaya, Elise Hacioglu, Geun-Hee Kwak, Ahmet Koc, Hwa-Young Kim and Vadim N. Gladyshev

J. Biol. Chem. published online December 2, 2008

Access the most updated version of this article at doi: 10.1074/jbc.M805891200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2008/12/02/M805891200.DC1