Structural and Kinetic Analysis of Free Methionine-R-sulfoxide Reductase from Staphylococcus aureus: Conformational Changes during Catalysis and Implications for the Catalytic and Inhibitory Mechanisms

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Free methionine-R-sulfoxide reductase (fRMsr) reduces free methionine-R-sulfoxide back to methionine, but its catalytic mechanism is poorly understood. Here, we have determined the crystal structures of the reduced, substrate-bound, and oxidized forms of fRMsr from Staphylococcus aureus. Our structural and biochemical analyses suggest the catalytic mechanism of fRMsr in which Cys-102 functions as the catalytic residue and Cys-68 as the resolving Cys that forms a disulfide bond with Cys-102. Cys-78, previously thought to be a catalytic Cys, is a non-essential residue for catalytic function. Additionally, our structures provide insights into the enzyme-substrate interaction and the role of active site residues in substrate binding. Structural comparison reveals that conformational changes occur in the active site during catalysis, particularly in the loop of residues 97-106 containing the catalytic Cys-102. We have also crystallized a complex between fRMsr and isopropanol, which acts as a competitive inhibitor for the enzyme. This isopropanol-bound structure helps us understand the inhibitory mechanism of fRMsr. Our structural and enzymatic analyses suggest that a branched methyl group in alcohol seems important for competitive inhibition of the fRMsr due to its ability to bind to the active site.

Reactive oxygen species can damage cellular components, including lipids, nucleic acids, and proteins. Damage to proteins by reactive oxygen species is likely due to oxidation of side chains of amino acid residues (1). The sulfur-containing amino acids, methionine and cysteine, are the most sensitive to oxidation. Oxidation of methionine generates a diastereomeric mixture of methionine-S-sulfoxide (Met-S-SO) and methionine-R-sulfoxide (Met-R-SO) (2). Methionine oxidation is associated with a variety of physiological and pathological processes such as cellular signaling, aging, and neurodegenerative diseases (3,4). For example, methionine oxidation activates CaMKII (calcium/calmodulin-dependent protein kinase II) in the absence of calcium (5), regulates the lifespan of yeast, fruitfly, and nematode (6-8) and may advance progression of Alzheimer’s and Parkinson’s diseases (9-12).

However, this oxidation can be reversed by the methionine sulfoxide reductases (Msrs). Two distinct families of Msrs have evolved for the stereospecific reduction of methionine sulfoxides in proteins (13,14). MsrA catalyzes the reduction of Met-S-SO, whereas MsrB reduces Met-R-SO. Most organisms from bacteria to humans possess a methionine sulfoxide reduction system that confers upon them the ability to repair oxidative damage and consequently impacts their longevity in oxidative environments (2,4). In addition, Msrs are involved in the virulence mechanism of some...
bacterial pathogens including *Mycoplasma genitalium* and *Neisseria gonorrhoeae* (15-17). Recently, an enzyme specific for the reduction of free Met-R-SO has been identified from *Escherichia coli* and named fRMsr (18). This protein is found in unicellular organisms including *Saccharomyces cerevisiae*, but absent in multicellular organisms (19). Interestingly, fRMsr contains a GAF-domain, which is ubiquitous motif present in cyclic GMP phosphodiesterases (20). Two variants of fRMsr proteins were detected with different conserved Cys residues (19): type I fRMsrs contain three conserved Cys residues while type II fRMsrs have two.

The structures and catalytic mechanisms of MsrA and MsrB are well characterized (21-24). Although MsrA and MsrB are completely different in sequence and structure, they share a common catalytic mechanism involving formation of a sulfenic acid intermediate on the catalytic Cys, followed by regeneration of the oxidized catalytic Cys. Briefly, a catalytic Cys attacks the sulfur of methionine sulfoxide and forms a sulfenic acid intermediate, with concomitant release of the product, methionine. The catalytic Cys sulfenic acid then forms an intramolecular disulfide bond by interacting with a resolving Cys. The disulfide bond is reduced by reductants and consequently the enzyme becomes active again. Thioredoxin (Trx) is generally considered the *in vivo* reductant while dithiothreitol (DTT) can be used *in vitro*. In contrast, the catalytic mechanism of fRMsr is poorly understood, although previous studies suggested that its catalytic mechanism is similar to those of MsrA and MsrB, involving the common sulfenic acid chemistry.

It is found that *Staphylococcus aureus*, a leading cause of hospital- and community-acquired infections, contains a type I fRMsr, three MsrAs, and an MsrB (19,25). *S. aureus* fRMsr contains three conserved Cys residues (Cys-68, Cys-78 and Cys-102). Two crystal structures of fRMsrs from *E. coli* and *S. cerevisiae* are available (PDB codes 1VHM (18,26) and 1F5M (27), respectively). Both structures contain a disulfide bond between Cys-68 and Cys-102 (numbering is based on the *S. aureus* fRMsr in the active sites, suggesting that fRMsrs use Cys residues for catalysis. The active site is enclosed in a small cavity (18,19,26,27). This enclosed cavity supports the apparent substrate specificity for free Met-R-SO but not for protein-based forms. Previous studies suggested that Cys-78 functions as a catalytic residue, Cys-102 as a primary resolving Cys, and Cys-68 as a secondarily resolving residue (18,19). However, the roles of these three Cys are unclear in the catalysis of fRMsr. Thus, the catalytic mechanism of this enzyme has to be yet elucidated.

In this study, we resolved four structural forms of the *S. aureus* fRMsr by X-ray crystallography: reduced form (fRMsr_red), complexed form with the substrate (fRMsr_sub), oxidized form (fRMsr_ox), and another complexed form with isopropanol (fRMsr_isopro). The first three structures represent different catalytic states of fRMsr. The last structure, fRMsr_isopro, helps us understand the inhibitory mechanism of fRMsr. We also performed biochemical analyses using the wild-type *S. aureus* fRMsr and single and double mutants, in which the three conserved Cys are replaced with Ser. We studied the inhibitory effect of various alcohols on fRMsr. Our structural and enzymatic studies provide insights into the catalytic mechanism of fRMsr with conformational changes that occur during catalysis and into the inhibitory mechanism involving a branched methyl group of alcohols.

**EXPERIMENTAL PROCEDURES**

**Purification, crystallization and X-ray analysis.** Gene cloning, protein expression, purification, and crystallization of *S. aureus* fRMsr have been described elsewhere for the oxidized and isopropanol-complexed forms of *S. aureus* fRMsr (fRMsr_ox and fRMsr_isopro) (29). The crystal complexed with isopropanol was obtained from a crystallization solution consisting of 2 M ammonium sulfate and 10% (v/v) 2-propanol. For the reduced form of fRMsr (fRMsr_red), cell pellets were resuspended in ice-cold lysis buffer (20 mM Tris–HCl, pH 7.9, 500 mM NaCl, 5 mM imidazole, 10 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride). The purification procedures were similar to those of oxidized form, but 10 mM DTT was used in the final purification procedure of gel filtration. The crystallization condition comprised of 24% PEG 3350 and 0.35 M potassium fluoride. The substrate complex form
of fRMsr (fRMsr sub) was obtained by soaking 9 mM free Met-R-SO in native crystals of mutant C68S fRMsr in which the crystallization condition comprised of 26% PEG 400 and 0.1 M 2-[N-morpholino]-ethanesulfonic acid (MES, pH 6.4).

The crystals were soaked in a solution containing 25% (v/v) ethylene glycol used as cryoprotectant and frozen in liquid nitrogen. X-ray diffraction data were collected with an ADSC Quantum CCD 210 detector at the beamline 6C and 4A at Pohang Light Source (Pohang, South Korea). A total range of 360° was covered with 1.0° oscillation and 30 s exposure per frame. The crystal-to-detector distance was set to 150 mm. The data sets were processed and scaled using HKL 2000 (30). The fRMsr red, fRMsr sub, fRMsr ox and fRMsr isopro crystals diffracted to 1.9, 2.3, 1.5 and 1.7 Å, respectively. The detailed statistics are summarized in Table 1.

Model building and structure refinement. The crystal structures of fRMsr were solved by molecular replacement methods using CNS (28) and Molrep (31) programs. The coordinates of E. coli fRMsr (PDB code 1VHM) (18, 26) were used as the search model. Refinements were performed with several cycles of torsion-angle-simulated annealing, energy minimization, individual B-factor refinement, and manual model rebuilding. The models were completed by iterative cycles of model building with Coot (32) and subsequently by refinement with CNS (28). The final models for fRMsr red, fRMsr sub, fRMsr ox and fRMsr isopro yielded R factor and R free values of 21.6 and 25.6% for fRMsr red, 22.2 and 25.2% for fRMsr sub, 22.1 and 23.4% for fRMsr ox, and 22.0 and 24.2% for fRMsr isopro, respectively. Refinement data were validated by the PROCHECK program (33) and are provided in Table 1. All figures were created using the CCP4mg (34) and the coordinates have been deposited in the Protein Data Bank (PDB codes 3KSF, fRMsr red, 3KSG, fRMsr sub, 3KSH, fRMsr ox, 3KSI, fRMsr isopro).

Measurements of Msr activities. For free Msr activity, NADPH oxidation was monitored as a decrease of A 340 at room temperature for 10 min in the reaction mixture. The reaction mixture (200 µl) contained 50 mM sodium phosphate (pH 7.5), 50 mM NaCl, 0.2 mM NADPH, 10 µg E. coli Trx (Sigma), 14 µg human Trx reductase 1, 0.1 mM EDTA, 1 mM free Met-R-SO or free Met-S-SO, and 2 or 10 µg fRMsr enzyme. Enzyme activity was defined as nmole of oxidized NADPH per min using a molar extinction coefficient of 6220 M -1 cm -1. K m and V max values were determined by non-linear regression using GraphPad Prism 5 software.

For peptide Msr activity, dabsylated methionine sulfoxide was used as the substrate in a DTT-dependent reaction. The reaction mixture (100 µl) containing 50 mM sodium phosphate (pH 7.5), 50 mM NaCl, 0.2 mM NADPH, 10 µg E. coli Trx, 14 µg human Trx reductase 1, 0.1 mM EDTA, 1 mM free Met-R-SO, 2 or 10 µg fRMsr enzyme, was incubated at 37°C for 30 min. The reaction product, dabsyl-Met, was analyzed by HPLC.

For inhibition assays of various alcohols on fRMsr activity, the reaction mixture (200 µl) contained 50 mM sodium phosphate (pH 7.5), 50 mM NaCl, 0.2 mM NADPH, 10 µg E. coli Trx, 14 µg human Trx reductase 1, 0.1 mM EDTA, 1 mM free Met-R-SO, 1% various alcohols, and 2 µg fRMsr enzyme. The reaction mixture was incubated at room temperature for 10 min and the decrease of A 340 was monitored.

Preparation of single or double mutant forms of S. aureus fRMsr. C68S, C78S, C102S, C68S/C78S, and C68S/C102S mutants were generated by site-directed mutagenesis using a pET28a-based wild-type construct (29). All constructs were verified by DNA sequencing.

RESULTS AND DISCUSSION

Catalytic activities of wild-type and mutant forms of S. aureus fRMsr. First, we tested substrate specificity of S. aureus fRMsr towards free Met-R-SO, free Met-S-SO, dabsyl-Met-R-SO (mimic to peptide Met-R-SO), dabsyl-Met-S-SO, and dimethyl sulfoxide. The enzyme assay was performed by analyzing NADPH oxidation in the reaction mixture. As expected, S. aureus fRMsr reduced free Met-R-SO, but could not reduce free Met-S-SO, dabsyl-Met-R-SO (mimic to peptide Met-R-SO), dabsyl-Met-S-SO, and dimethyl sulfoxide. The enzyme assay was performed by analyzing NADPH oxidation in the reaction mixture. As expected, S. aureus fRMsr reduced free Met-R-SO, but could not reduce free Met-S-SO, dabsyl-Met-R-SO or dabsyl-Met-S-SO, or dimethyl sulfoxide, showing the same substrate specificity as E. coli and S. cerevisae fRMss characterized previously (18, 19).

To determine the roles of the three conserved Cys residues (Cys-68, Cys-78, and Cys-102; supplementary Fig. S1) in catalysis, we mutated these residues to Ser making single or double mutants (C68S, C78S, C102S, free Met-R-SO or free Met-S-SO, and 2 or 10 µg fRMsr enzyme. Enzyme activity was defined as nmole of oxidized NADPH per min using a molar extinction coefficient of 6220 M -1 cm -1. K m and V max values were determined by non-linear regression using GraphPad Prism 5 software.

For peptide Msr activity, dabsylated methionine sulfoxide was used as the substrate in a DTT-dependent reaction. The reaction mixture (100 µl) containing 50 mM sodium phosphate (pH 7.5), 50 mM NaCl, 0.2 mM NADPH, 10 µg E. coli Trx, 14 µg human Trx reductase 1, 0.1 mM EDTA, 1 mM free Met-R-SO, 1% various alcohols, and 2 µg fRMsr enzyme. The reaction mixture was incubated at room temperature for 10 min and the decrease of A 340 was monitored.
C68S/C78S, and C68S/C102S). We assayed the Trx-dependent activities of these mutant fRMsr and compared them with the wild-type. As shown in Table 2, the activity of C68S was 32% of wild-type. Unexpectedly, the activity of C78S was 75% of wild-type. This Cys residue was previously suggested to be the catalytic residue in E. coli and S. cerevisiae fRMsr (18,19). Interestingly, C102S had no catalytic activity. Consistent with this result, C68S/C102S had no catalytic activity either, while C68S/C78S retained 22% of enzyme activity.

We then analyzed kinetic parameters of C78S, C68S, and C68S/C78S as well as wild-type (Table 2). The $V_{\text{max}}$ value of C78S was slightly higher than that of wild-type; the $K_m$ value was 2-fold higher than that of wild-type. These data indicate that Cys-78 is non-essential for catalysis by fRMsr. The $V_{\text{max}}$ value was significantly reduced in C68S mutant, whereas the $K_m$ value was 4-fold higher, compared to those of wild-type. The double C68S/C78S mutant exhibited more decreased $V_{\text{max}}$ (3-fold lower than wild-type) and more increased $K_m$ (16-fold higher than wild-type).

Thus, in contrast to the previously suggested model, Cys-102 is proposed to be the catalytic Cys, Cys-68 may serve as the resolving Cys that forms a disulfide bond with Cys-102, and Cys-78 is a non-essential residue for catalytic function.

The above enzymatic data are consistent with our recent findings from S. cerevisiae fRMsr that Cys-125 (corresponding to Cys-102 in S. aureus fRMsr) functions as the catalytic residue, as determined by enzyme and in vivo growth complementation assays (35).

Crystal structure of the reduced form of fRMsr. Previously known structures from both E. coli and S. cerevisiae fRMsr are oxidized forms with a disulfide bond between Cys-68 and Cys-102 (26,27). In addition, the E. coli fRMsr structure contains a complex with MES in the active site. Here, we have resolved the structure of a reduced form of S. aureus fRMsr (fRMsr$_{\text{red}}$) (Fig. S2A). The crystal of fRMsr$_{\text{red}}$ comprises of four dimers in the asymmetric unit. There are several hydrogen bond interactions in the interface region of the dimer structure (Fig. 1A). To assess on quantitative grounds the possibility that these hydrogen bond interactions may stabilize an fRMsr$_{\text{red}}$ dimer, the dimer interface was evaluated by using the program PISA (36). This widely used program estimates a dimeric state for fRMsr$_{\text{red}}$ (complexation significance score = 1). In particular, this analysis shows that the buried area upon formation of the dimeric assembly is 932.7 Å$^2$, which accounts for 11.8% of the total surface area for each molecule. It should be noted that S. cerevisiae fRMsr is also a dimer in solution (27).

The carboxamide groups of Asn-32 and Gln-63 from one subunit form hydrogen bonds with the backbones of Ala-67 and Phe-62 from the other subunit, respectively. In addition, the side chain of Gln-63 of one subunit interacts with the backbone of Gly-64 of the other subunit. The overall one subunit structure of fRMsr$_{\text{red}}$ is composed of six antiparallel $\beta$-strands ($\beta_1$-$\beta_6$) and four $\alpha$-helices ($\alpha_1$-$\alpha_4$) (Fig. S2A). The active site contained Trp-46, Tyr-50, Leu-59, Cys-68, Cys-78, Cys-102, Asp-103, Ala-104, Ser-106, Glu-109, Asp-125, and Asp-127 in five antiparallel $\beta$-strands, two loops, and one $\alpha$-helix, where Cys-78 is located. (Figs. 2C and S2A). The side chains of Cys-102 and Cys-68 are located into the active site. The distances between the sulfur atoms of Cys-68 and Cys-102, Cys-68 and Cys-78, and Cys-102 and Cys-102 are 5.7, 10.1, and 10.2 Å, respectively. On one side of the cavity of the active site, Trp-46 and Ala-104 form a hydrophobic region, while the opposite side displays a hydrophilic region consisting of Tyr-50, Glu-109, Asp-125 and Asp-127. The structure of fRMsr$_{\text{red}}$ contains several water molecules (Wat) in the active site. Particularly, Wat-4 interacts with the side chains of Tyr-50 (3.4 Å) and Asp-125 (2.9 Å). Also, Wat-104 interacts with the side chains of Glu-109, Asp-125, and Asp-127; Wat-491 interacts with the side chain of Cys-78, Glu-109 and Asp-125, respectively (Fig. 2C). These interactions involving water molecules in the active site may stabilize the conformation of reduced fRMsr.

Structure of fRMsr in complex with the substrate. Here, we have resolved the first structure of S. aureus fRMsr complexed with the substrate free Met-R-SO$_2$ fRMsr$_{\text{sub}}$ using C68S fRMsr, which shows a Michaelis-like complex (Fig. S2B). The sulfoxide moiety of the substrate was clearly shown in the omit electron density map of the active site (Fig. 1B). This structure could lead us to understand the catalytic mechanism of fRMsr, the mode of binding to the substrate, and the roles of the active site residues during catalysis. The structure of fRMsr$_{\text{sub}}$
comprises of a dimer with the substrate in each subunit of the asymmetric unit. The overall conformation of fRMsr_{sub}, in which Ser replaces Cys-68 in the loop of the active site, is conserved with the reduced form of wild-type fRMsr (Fig. S2). However, there are significant conformational changes around the active site as discussed in below sections.

The substrate Met-R-SO is positioned by several hydrogen bonds and stacking interactions. The acidic side chains of Glu-109, Asp-125 and Asp-127 in the hydrophilic region form hydrogen bonds with nitrogen of the substrate (Fig. 1B). In addition, the residue Tyr-50 forms a hydrogen bond with the carboxylate group of Met-R-SO. The sulfoxide of the substrate is located close to Cys-102, pointing toward the sulfur atom of Cys-102 (6.7 Å). The thiol of Cys-78 points toward the carboxylate of the substrate (4.3 Å). Our structural analysis along with the above enzymatic data suggests that Cys-102 is the catalytic residue of fRMsr. The hydrophobic region involving Ala-101 and Asp-103 move away from the corresponding residues of E. coli fRMsr in a distance with 4.2 and 6.1 Å, respectively. Thus, the structural comparison revealed that the catalytic Cys-containing loop region is quite flexible in fRMsr proteins.

**Comparison and conformational changes of reduced, substrate-bound, and oxidized forms of fRMsr.**

We compared the reduced (fRMsr_{red}), substrate-bound (fRMsr_{sub}), and oxidized (fRMsr_{ox}) structures of S. aureus fRMsr (Fig. 2), which are representative of the catalytic steps of the fRMsr reaction.

The backbone structure of fRMsr_{sub} could be superimposed on the fRMsr_{red}, with an r.m.s.d of 1.4 Å (Fig. 2A). There were significant conformational changes particularly in the loop consisting of residues 97-106 (Fig. 2B). Cys-102 and Asp-103 of fRMsr_{sub} are the most displaced residues in the loop, shifted by 4.7 and 10.9 Å, respectively. The positions of Ile-100, His-99 and Lys-97 lie at 2.4, 5.1 and 2.2 Å, respectively, from the corresponding residues of fRMsr_{ox}. However, the position of Cys-78 in fRMsr_{sub} and fRMsr_{ox} remains relatively unchanged. Glu-109 residue in fRMsr_{sub} places at a distance of 3.6 Å from Asp-127, while in fRMsr_{ox} it places at a distance of 4.7 Å. Cys-68 and Cys-102 residues in fRMsr_{sub} place from Cys-78 at a distance of 9.7 and 11.7 Å, respectively, while in fRMsr_{red} they place at a distance of 10.1 and 10.2 Å, respectively. Water
molecules in the active site of fRMsr red interact with Glu-109, Asp-125, Asp-127 and Tyr-50 residues that form the hydrogen bonds with nitrogen and carboxylate group of the substrate (Fig. 2C). When compared the structure of fRMsr sub with fRMsr red, the substrate Met-R-SO in the active site replaces the water molecules occupied in the fRMsr red (Fig. 2B-C).

We next compared the structures of fRMsr sub with fRMsr ox. The backbone structure of fRMsr sub could be superimposed on the fRMsr ox, with an r.m.s.d of 1.4 Å (Fig. 2A). Significant conformational changes are observed in the loop of residues 97-106 between these two structures (Fig. 2D). Cys-102 and Asp-103 residues are shifted by 6.7 and 7.8 Å, respectively, between fRMsr sub and fRMsr ox. Specifically, in the substrate complex form the loop moves into the active site compared with the oxidized form, resulting in positioning the thiol group of the catalytic Cys-102 toward the entrance of active site. The positions of Ile-100, His-99 and Gly-98 in fRMsr ox lie at 6.6, 7.7 and 3.4 Å, respectively, from the corresponding residues of fRMsr sub. However, the position of Cys-78 in fRMsr sub and fRMsr red remains relatively unchanged.

We finally compared fRMsr red structure with fRMsr ox structure. The backbone structure of fRMsr red could be superimposed on the fRMsr ox with an r.m.s.d of 1.5 Å (Fig. 2A). Although the overall structures were well superimposed, there were significant differences in the loop of residues 94-106 (Fig. 2E). The loop in fRMsr red moves into the active site, which results in the positioning of the His-99, Cys-102, Asp-103 and Ala-104 residues towards the entrance of active site. Large movements occur in the catalytic residue Cys-102 and its neighboring residues Asp-103 and Ala-104. Cys-102 residue in the oxidized and reduced forms of fRMsr, places at a distance of 4.7 Å from each other. Asp-103 and Ala-104 in the oxidized and reduced forms place at a distance of 8.7 and 6.5 Å, respectively, from each other. The side chains of Glu-109 and Asp-127 in the active site place farther from each other in fRMsr red (4.9 Å) than in fRMsr ox (3.7 Å). Moreover, the distance between Cys-78 and Asp-127 is changed from 5.8 Å in fRMsr red to 8.3 Å in fRMsr ox. In addition, the fRMsr red structure shows movement of the side chain of Cys-68 towards the entrance of the active site. Together, the movements of the active site residues (particularly Cys-102) determine the conformations of fRMsr red and fRMsr ox, leading to an open conformation in fRMsr red and a closed conformation in fRMsr ox (Fig. 2F).

**Catalytic mechanism.** Our crystal structures of fRMsr red, fRMsr sub, and fRMsr ox help in understanding the mode of binding of the substrate Met-R-SO to the active site of fRMsr, the roles of the active site residues, and the conformational changes of fRMsr during catalysis. Significant conformational changes of the active site, particularly in the loop including the catalytic Cys, occur in each catalytic step. The reduced form has an open conformation to allow access to the substrate, the substrate-bound form takes a closed conformation after accommodation of Met-R-SO, and the oxidized form is turned to a more closed conformation after catalysis (Fig. 2F).

Among the three conserved Cys, Cys-102 was the most mobile, whereas Cys-78, the previously suggested catalytic residue from *E. coli* and *S. cerevisiae* fRMsr (18,19), was the most immobile. Our enzymatic studies concluded that Cys-102 is the catalytic residue. Cys-68 is suggested to be the resolving Cys by structural and kinetic analyses. Cys-78 had no catalytic function, but this residue may play a role in substrate binding, as judged by the kinetic data (i.e. an increase in *K*<sub>m</sub> value in C78S mutant). Here, we propose that the catalytic mechanism of fRMsr consists of three steps: (1) Cys-102 attacks the sulfoxide moiety of Met-R-SO and is then oxidized to Cys sulfenic acid. (2) Cys-68 interacts with the sulfenic acid intermediate to form a disulfide bond. (3) Finally, the Cys-102-Cys-68 disulfide bond is reduced by a reductant (typically by Trx) and the fRMsr enzyme activity gets regenerated (Fig. 3).

It should be noted that in contrast to type I fRMsr s, type II enzymes contain only the conserved Cys-78 and Cys-102. They lack Cys-68. Since our studies revealed no direct function for Cys-78 in the catalysis of type I fRMsr, it is questionable whether this residue plays any role in the catalysis of type II fRMsr. It is possible that this Cys-78 would function as a resolving Cys replacing the role of Cys-68 in type I enzymes. Thus, biochemical and structural studies of type I fRMsr would be interesting.

**Implications of the mechanism of action of fRMsr using a competitive inhibitor.** We
determined another complex structure (fRMsrs_{isoopro}) that contains isopropanol in the active site pocket of fRMsr. This crystal was obtained from a crystallization solution consisting of 2 M ammonium sulfate and 10% (v/v) isopropanol and had one subunit of protein in the asymmetric unit like fRMsrx. The fRMsrs_{isoopro} structure also contains a disulfide bond formed by Cys-68 and Cys-102. Interestingly, the binding pattern of isopropanol is expected to define the location of the substrate binding site (Fig. 4). The hydroxyl group of isopropanol interacts by hydrogen bonding with Glu-109, Asp-125 and Asp-127, respectively. This is similar to the hydrogen bond interactions of these residues with the nitrogen of the substrate in fRMsrs_{sub}. This structural analysis suggests that isopropanol could act as a competitive inhibitor of fRMsr enzyme. To test this hypothesis, we assayed the enzyme activities in the presence of 1% ethanol, n-propanol, or isopropanol (Table 3). Relative activities of fRMsr in the presence of ethanol and n-propanol were 91%. However, in the presence of isopropanol the enzyme activity significantly decreased to 69%. We further determined kinetic parameters in the presence of 1% isopropanol. The $K_m$ value was 100 ± 30 μM, which is 2-fold higher than that in the absence of isopropanol. The $V_{max}$ value was 420 ± 30 nmol•min$^{-1}$•mg protein$^{-1}$, similar to that without isopropanol. These results indicated that isopropanol can competitively inhibit fRMsr activity. Moreover, a branched methyl group in isopropanol may play a role in this inhibitory effect.

We further tested the inhibitory effect with 1% n-butanol and isobutanol (Table 3). In the presence of n-butanol the relative activity was 87%, similar to that with ethanol or n-propanol. However, the enzyme activity was significantly inhibited by 50% in the presence of isobutanol. Together, our results indicate that a branched methyl group in alcohols seems important for competitive inhibition of fRMsr enzyme activity. The branched methyl group of alcohols may be crucial for binding to the active site in order to competitively inhibit the fRMsr activity, suggesting that the methyl group of the substrate may be important for binding affinity to the enzyme shown in the structure of fRMsrs_{sub}.

In summary, we have determined the crystal structures of reduced, substrate-bound, oxidized, and inhibitor-bound fRMsrs at atomic resolution levels. Our structural and biochemical studies suggest the catalytic mechanism of fRMsr, where Cys-102 acts as the catalytic residue and Cys-68 as the resolving Cys. Our structures show the mode of binding of the substrate free Met-R-SO, roles of active site residues in catalysis, and the conformational changes of the active site during catalysis, particularly by the loop containing the catalytic Cys-102. In addition, our studies with a competitive inhibitor, isopropanol, predict the mechanism of action of fRMsr where the methyl group in the substrate or a branched methyl group in alcohols seems important for interaction with the enzyme.

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The abbreviations used are: DTT, dithiothreitol; fRMsr, free methionine-R-sulfoxide reductase; Msr, methionine sulfoxide reductase; Met-S-SO, methionine-S-sulfoxide; Met-R-SO, methionine-R-sulfoxide; Trx, thioredoxin.

FIGURE LEGENDS

Fig. 1. The overall structure of a reduced form of *S. aureus* fRMsr (A) and structure of substrate-bound active site of C68S fRMsr in stereo (B). In A, a dimer is shown by electrostatic surface and ribbon models (C subunit, blue; D subunit, light green). Close-up view represents the dimer interface region. In the interface region Asn-32, Gln-63, Phe-62 and Ala-67 of C subunit interact by hydrogen bonding, respectively, with Ala-67, Gly-64/Phe-62, Gln-63 and Asn-32 of the other subunit. In B, the ligand, free Met-R-SO, is depicted as a light green stick model. The omit electron density of substrate is shown at 1.5 σ. Hydrogen bond interactions between the substrate and the active site residues are indicated by black dotted lines (for details, see the text).

Fig. 2. Structural comparison of reduced, substrate-bound and oxidized *S. aureus* fRMrsrs. (A) Stereoscopic views showing comparison of overall structures of reduced, substrate-bound and oxidized *S. aureus* fMRsrs. The backbone models for reduced (fRMsrred), substrate-bound (fRMsrsub), and oxidized (fRMsrox) forms are shown in green, light blue, and light yellow, respectively. (B-C) Comparison of active sites between fRMsrred (green) and fRMsrsub (light blue). The active site residues of fRMsrred and fRMsrsub are superimposed (B) and those of fRMsrred are independently shown (C). In C, hydrogen bond interactions among water molecules and active site residues are indicated by dotted lines. (D) Comparison of active sites between fRMsrsub (light blue) and fRMsrux (light yellow). The active site residues of fRMsrsub and fRMsrux are superimposed. (E) Comparison of active sites between fRMsrred (green) and fRMsrux (light yellow). The active site residues of fRMsrred and fRMsrux are superimposed. In figures, disulfide bond between Cys-68 and Cys-102 in fRMsrux is represented by a yellow stick and substrate Met-R-SO in fRMsrsub is shown by a light green stick. (F) Conformational changes of fRMsrred, fRMsrsub and fRMsrux. The active site is shown with electrostatic surface models. The surfaces are colored according to the electrostatic potentials from −21 kT/e (red) to +21 kT/e (blue). The electrostatic surface potentials were calculated by using APBS (37).

Fig. 3. Proposed catalytic mechanism of fRMsr. (A) A schematic representation. Catalytic Cys-102 attacks free Met-R-SO and is then oxidized to sulfenic acid. Cys-68 acts as a resolving Cys and thus interacts with the Cys sulfenic acid to form a disulfide bond. The resulting Cys-102-Cys-68 disulfide bond is reduced by a reductant (typically by Trx in vivo or by DTT in vitro), and finally the enzyme becomes active. Cys-78 has no catalytic function. (B) A structural representation. Reduced fRMsr initially displays an open conformation in the active site and after binding of the substrate, the enzyme becomes active. Formation of a disulfide bond between Cys-102 and Cys-68 makes the enzyme more closed.

Fig. 4. Structure of isopropanol-bound *S. aureus* fRMsr and its comparison with substrate-bound form. (A) The active site of fRMsr_isopro. Interactions between isopropanol and active site residues are represented by dotted lines. (B) Comparison of active sites between fRMsr_isopro (light blue) and fRMsrsub (light grey). The active site residues of fRMsr_isopro and fRMsrsub are superimposed. Disulfide bond between Cys-68 and Cys-102 and isopropanol in fRMsr_isopro are shown by yellow and light pink sticks, respectively, and substrate Met-R-SO in fRMsrsub is shown by a light green stick.
Table 1 Data collection statistics and refinement statistics

<table>
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<tr>
<th></th>
<th>fRMsr&lt;sub&gt;red&lt;/sub&gt;</th>
<th>fRMsr&lt;sub&gt;sub&lt;/sub&gt;</th>
<th>fRMsr&lt;sub&gt;ox&lt;/sub&gt;</th>
<th>fRMsr&lt;sub&gt;isopro&lt;/sub&gt;</th>
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<td>Wavelength (Å)</td>
<td>1.23986</td>
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<td>1.23986</td>
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<td>Space group</td>
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<td>P2&lt;sub&gt;1&lt;/sub&gt;</td>
<td>P6&lt;sub&gt;1&lt;/sub&gt;22</td>
<td>P6&lt;sub&gt;1&lt;/sub&gt;22</td>
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<td>Unit cell</td>
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<td>a=b=90.0</td>
<td>a=b=89.8</td>
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<tr>
<td></td>
<td>b=119.6</td>
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<td></td>
<td>c=80.3</td>
<td>c=42.9</td>
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<td>Resolution range (Å)</td>
<td>50.0-1.9 (1.93-1.9)</td>
<td>50.0-2.3 (2.34-2.3)</td>
<td>50.0-1.5 (1.55-1.5)</td>
<td>50.0-1.7 (1.76-1.7)</td>
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<td>Observed reflections</td>
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<td>Unique reflections</td>
<td>98,600</td>
<td>13,036</td>
<td>33,994</td>
<td>23,645</td>
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<td>Redundancy</td>
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<td>4.9 (3.2)</td>
<td>28.9 (6.2)</td>
<td>20.3 (7.6)</td>
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<td>Completeness (%)</td>
<td>98.1 (94.5)</td>
<td>97.5 (92.7)</td>
<td>98.6 (87.1)</td>
<td>99.1 (94.8)</td>
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<tr>
<td>R&lt;sub&gt;sym&lt;/sub&gt; (%)</td>
<td>7.5 (33.6)</td>
<td>7.0 (23.6)</td>
<td>5.9 (33.0)</td>
<td>6.6 (28.7)</td>
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<tr>
<td>I/sigma (I)</td>
<td>19.2 (2.7)</td>
<td>13.4 (4.1)</td>
<td>14.5 (3.2)</td>
<td>16.0 (5.5)</td>
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<tr>
<td>Refinement statistics</td>
<td></td>
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<tr>
<td>R&lt;sub&gt;factor&lt;/sub&gt;/R&lt;sub&gt;free&lt;/sub&gt; (%)</td>
<td>21.6/25.6</td>
<td>22.2/25.2</td>
<td>22.1/23.4</td>
<td>22.0/24.2</td>
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<td>r.m.s.d bond (Å)</td>
<td>0.005</td>
<td>0.006</td>
<td>0.004</td>
<td>0.005</td>
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<td>r.m.s.d angles (*)</td>
<td>1.2</td>
<td>1.2</td>
<td>1.1</td>
<td>1.2</td>
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<td>Mean B-factor</td>
<td>26.4</td>
<td>49.7</td>
<td>22.8</td>
<td>23.9</td>
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<td>Ramachandran plot</td>
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<td>Most allowed region (%)</td>
<td>88.9</td>
<td>85.0</td>
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<td>91.2</td>
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<td>Additional allowed region (%)</td>
<td>10.9</td>
<td>12.0</td>
<td>9.6</td>
<td>8.8</td>
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<tr>
<td>Generously allowed region (%)</td>
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<td>0</td>
<td>0</td>
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<td>Disallowed region (%)</td>
<td>0</td>
<td>0</td>
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</table>

Values in parentheses represent the highest resolution shell. 

\( R_{sym} = \frac{\sum_{hkl} I_{hkl} - \langle I_{hkl} \rangle}{\sum_{hkl} I_{hkl}} \), where \( I \) is the observed intensity, \( \langle I \rangle \) is the average intensity, and \( i \) is counts through all symmetry-related reflections. The crystallographic R factor is based on 95% of the data used in refinement and R free is based on 5% of the data withheld for cross-validation test. r.m.s.d., root-mean-square deviation.
Table 2. Specific activities and kinetic parameters of wild-type and mutant forms of *S. aureus* fRMsr

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Specific activity</th>
<th>$K_m$</th>
<th>$V_{\text{max}}$</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>(nmol/min/mg protein)</td>
<td>(μM)</td>
<td>(nmol/min/mg protein)</td>
</tr>
<tr>
<td>Wild-type</td>
<td>85 ± 5 (100)</td>
<td>50 ± 10</td>
<td>360 ± 10</td>
</tr>
<tr>
<td>C68S</td>
<td>27 ± 2 (32)</td>
<td>210 ± 20</td>
<td>280 ± 10</td>
</tr>
<tr>
<td>C78S</td>
<td>64 ± 20 (75)</td>
<td>110 ± 50</td>
<td>440 ± 50</td>
</tr>
<tr>
<td>C102S</td>
<td>0 ± 0.3 (0)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>C68S/C78S</td>
<td>19 ± 3 (22)</td>
<td>830 ± 240</td>
<td>130 ± 20</td>
</tr>
<tr>
<td>C68S/C102S</td>
<td>0 ± 0.3 (0)</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Enzyme assays were performed using 10 μg purified proteins as described in Experimental Procedures. Parenthesis represents the relative activity to wild-type. $K_m$ and $V_{\text{max}}$ values were determined by fitting the data to the Michaelis-Menten equation. NA, not assayed.
Table 3. Relative activity of fRMsr enzyme with various alcohols

<table>
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<tr>
<th>Alcohols</th>
<th>Relative activity (%)</th>
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<tbody>
<tr>
<td>None</td>
<td>100</td>
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<tr>
<td>Ethanol</td>
<td>91</td>
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<tr>
<td>n-Propanol</td>
<td>91</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>69</td>
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<tr>
<td>n-Butanol</td>
<td>87</td>
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<tr>
<td>Isobutanol</td>
<td>52</td>
</tr>
</tbody>
</table>

Enzyme assays were performed in the presence of 1% various alcohols as described in Experimental Procedures.
Figure 3

A.

B. 
Structural and kinetic analysis of free methionine-R-sulfoxide reductase from staphylococcus aureus: conformational changes during catalysis and implications for the catalytic and inhibitory mechanisms
Seoung Min Bong, Geun-Hee Kwak, Jin Ho Moon, Ki Seog Lee, Hong Seok Kim, Hwa-Young Kim and Young Min Chi

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