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Structural and biochemical analysis of a type II free methionine-*R*-sulfoxide reductase from *Thermoplasma acidophilum*

Hyun Sook Kim [a,1], Geun-Hee Kwak [b,1], Kitaik Lee [a], Chang-Hwa Jo [a], Kwang Yeon Hwang [a,*], Hwa-Young Kim [b,*]

[a] Department of Biosystems and Biotechnology, College of Life Sciences and Biotechnology, Korea University, Seoul 136-701, Republic of Korea
[b] Department of Biochemistry and Molecular Biology, Yeungnam University College of Medicine, Daegu 705-717, Republic of Korea

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ABSTRACT

Free methionine-*R*-sulfoxide reductase (fRMsr) enzymes only reduce the free form of methionine-*R*-sulfoxide and can be grouped into two types with respect to the number of conserved Cys residues in the active sites. In this work, the crystal structures of type II fRMsr from *Thermoplasma acidophilum* (*Taf*RMsr), which contains two conserved Cys, have been determined in native form and in a complex with the substrate. The overall structure of *Taf*RMsr consists of a central β-sheet encompassed by a two-α-helix bundle flanking on one side and one small α-helix on the other side. Based on biochemical and growth complementation assays, Cys^{84} is demonstrated to be the catalytic Cys. The data also show that *Taf*RMsr functions as an antioxidant protein. Structural analyses reveal insights into substrate recognition and orientation, conformational changes in the active site during substrate binding, and the role of active site residues in substrate binding. A model for the catalytic mechanism of type II *Taf*RMsr is suggested, in which intramolecular disulfide bond formation is not involved. In addition, the biochemical, enzymatic, and structural properties of type II *Taf*RMsr are compared with those of type I enzymes.

Introduction

Reactive oxygen species (ROS),[2] generated as by-products of cellular metabolism in aerobic organisms, oxidize various cellular components. The ROS-mediated oxidation of macromolecules (such as DNA, proteins, and lipids) may lead to the development of diseases and accelerate the aging process. The sulfur atom of methionine is susceptible to oxidation by ROS. Methionine oxidation produces two diastereomers, methionine-*S*-sulfoxide (Met-*S*-O) and methionine-*R*-sulfoxide (Met-*R*-O) [1], reversible through a reductase system.

Methionine sulfoxide reductases (Msrs) are the enzymes responsible for the reduction of Met-O and they play a pivotal defensive role against oxidative stress [2–5]. There are three Msr families, MsrA, MsrB, and fRMsr, with distinct substrate specificity [6–8]. MsrA is specific for Met-*S*-O [9] and has significant reduction activity toward free Met-*S*-O as well as peptidyl Met-*S*-O [10]. MsrB catalyzes the stereospecific reduction of Met-*R*-O [11] but is mainly active on peptidyl Met-*R*-O, with a very low activity toward free Met-*R*-O [10]. The fRMsr enzymes, the most recently identified Msr enzymes, only reduce the free form of Met-*R*-O [12] and cannot act on peptidyl Met-*R*-O. Interestingly, the fRMsr enzymes contain a GAF domain, a ubiquitous motif in cyclic GMP phosphodiesterases [12].

The fRMsr enzymes only exist in unicellular organisms, including *Escherichia coli*, and not multicellular organisms [13]. There are two variants of fRMsr proteins, classified by the number of conserved Cys residues [13]. Type I fRMsrs, such as the enzymes from *E. coli* and *Staphylococcus aureus*, possess three conserved Cys residues (Cys^{50}, Cys^{60}, and Cys^{84}; numbering based on *Thermoplasma acidophilum* fRMsr), whereas type II fRMsrs contain two conserved Cys residues (Cys^{60} and Cys^{84}).

The structures and catalytic mechanism of fRMsrs have only been characterized in type I enzymes [14,15]. The catalytic

* Corresponding authors. Address: Department of Biosystems and Biotechnology, College of Life Sciences and Biotechnology, Korea University, 145 Anam-ro, Seongbuk, Seoul 136-701, Republic of Korea. Fax: +82 2 923 3229 (K.Y. Hwang). Address: Department of Biochemistry and Molecular Biology, Yeungnam University College of Medicine, 170 Hyeonchungno, Namgu, Daegu 705-717, Republic of Korea. Fax: +82 53 620 4341 (H.-Y. Kim).
E-mail addresses: chahong@korea.ac.kr (K.Y. Hwang), hykim@ynu.ac.kr (H.-Y. Kim).

[1] These authors contributed equally to this work.

[2] *Abbreviations used:* ROS, reactive oxygen species; Met-*S*-O, methionine-*S*-sulfoxide; Met-*R*-O, methionine-*R*-sulfoxide; Msrs, methionine sulfoxide reductases; DTNB, 5,5′-dithiobis(2-nitro)benzoate; SDS, sodium dodecyl sulfate.

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mechanism of type I fRMsr involves a sulfenic acid chemistry [14,15], similar to the mechanisms of MsrA and MsrB, in which Cys^{84} functions as a catalytic residue (Scheme 1). After its attack on the sulfoxide moiety of the substrate, the catalytic Cys^{84} is converted into a sulfenic acid intermediate with the concomitant release of the product Met. Cys^{50} acts as a resolving residue and interacts with the Cys^{84} sulfenic acid, forming an intramolecular disulfide bond. The disulfide Cys^{84}–Cys^{50} bond is then reduced by reductants. Thioredoxin (Trx) is considered the *in vivo* reductant, while dithiothreitol (DTT) is used as the *in vitro* reductant. However, the structure and catalytic mechanism of type II fRMsr enzymes are unknown.

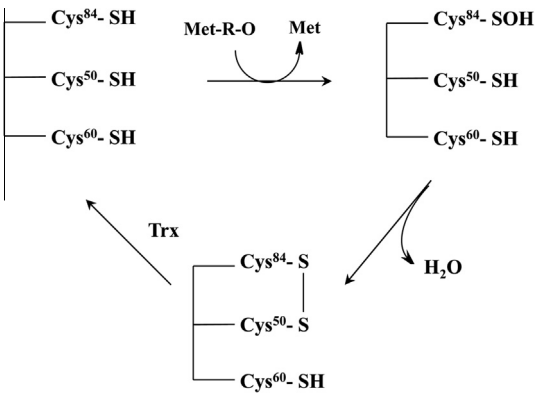
In this study, we determined the crystal structures of a type II fRMsr from *T. acidophilum* (*Taf*RMsr), including the native and substrate-bound forms. Biochemical and kinetic analyses of wild type *Taf*RMsr and mutants, in which Cys residues were replaced with Ser, were performed. A catalytic mechanism of *Taf*RMsr is proposed, in which no intramolecular disulfide bond formation is involved. In addition, differences in the structures and catalytic mechanisms between type I and type II fRMsr enzymes are discussed.

Materials and methods

Cloning and purification of TafRMsr

The coding region of the *Taf*RMsr was PCR-amplified using its genomic DNA and cloned into NdeI/XhoI sites of pET21b (Novagen). The resulting construct, named pET-TAfRMsr, encoded the full-length *Taf*RMsr with a C-terminal His-tag (LEHHHHHH). C15S, C60S, and C84S mutants, in which Cys is replaced with Ser, were generated by site-directed mutagenesis using the construct pET-TAfRMsr. All constructs were verified by DNA sequencing.

The expression plasmid was introduced into *E. coli* BL21(DE3) cells. After growing the cells in LB medium containing 100 μg/ml ampicillin at 37 °C to an OD_{600} of 0.8, protein overexpression was induced overnight by adding 0.5 mM IPTG at 18 °C. The cells were then pelleted, resuspended in an extraction buffer (30 mM Tris–HCl, pH 8.0, 10% glycerol, and 40 mM imidazole), and sonicated for disruption. After centrifugation at 17,000×*g* for 20 min, the supernatant of the lysate was loaded onto a HiTrapTM column (GE Healthcare) and washed with the extraction buffer. The protein was eluted by a linear gradient of imidazole (40–500 mM). The *Taf*RMsr protein was further purified by size-exclusion chromatography on a HiLoad 26/60 SuperdexTM 200 (Amersham Biosciences).



Scheme 1. Catalytic mechanism of type I fRMsr. The catalytic Cys^{84} attacks the sulfoxide of Met-*R*-O to form a sulfenic acid intermediate, with concomitant release of Met. The resolving Cys^{50} then reacts with the Cys^{84} sulfenic acid to form a disulfide bond, which is reduced by the reductant Trx.

Yeast constructs and growth complementation assay

Wild type and mutants of *Taf*RMsr with a C-terminal His tag were PCR-amplified using the pET-based constructs and cloned into *Bam*HI/*Sal*I sites of p425 GPD yeast vector. All constructs were verified by DNA sequencing. The yeast expression constructs were introduced into *Saccharomyces cerevisiae* cells deficient in triple *msrA/msrB/fRmsr* genes (*MATa his3 leu2 met15 ura3 ΔmsrA::URA3 ΔmsrB::KAN ΔfRmsr::HIS3*) [13] using the lithium acetate method. Transformants were selected for leucine prototrophy. Growth complementation assays for the cells containing *Taf*RMsr constructs were performed on a yeast nitrogen base minimal medium supplemented with 2% glucose (YNBD) agar medium in the presence of 0.14 mM Met or 0.28 mM Met-(*R,S*)-O, as previously described [16]. At least two biological replicates were done.

Antioxidant assay

The triple *msrA/msrB/fRmsr*-deleted *S. cerevisiae* cells containing the p425 vector alone or p425-based *Taf*RMsr constructs were grown aerobically at 30 °C in YNBD media. The overnight cultures were each adjusted to an OD_{600} of 2.5, 0.25, and 0.025 via serial dilution. Each diluted sample (5 μl) was spotted onto YNBD agar medium in the presence of 0.3 mM H_2O_2. The spotted cells were incubated for 2 days at 30 °C and cell growth was monitored.

Activity and kinetic analysis of TafRMsr

The reaction mixture (200 μl) contained 50 mM sodium phosphate (pH 7.5), 50 mM NaCl, 0.2 mM NADPH, 0.1 mM EDTA, 10 μg *E. coli* Trx, 14 μg human Trx reductase 1, 0.05–1.6 mM free Met-*R*-O, and 3 μg *Taf*RMsr. The reactions were carried out at 25 °C for 5 min, and a decrease in the absorbance of NADPH was monitored at 340 nm using a spectrophotometer UV-160A (Shimadzu). A control for normalization purposes was the reaction mixture without fRMsr enzyme. Enzyme activity was calculated using the molar extinction coefficient of NADPH (6220 M^{-1} cm^{-1}) and expressed as nmole of oxidized NADPH per min. K_m and k_{cat} values were determined by non-linear regression using Prism 5 software (GraphPad).

Measurements of the free thiol content of TafRMsr

The free Cys content of wild type *Taf*RMsr and mutants were determined using 5,5′-dithiobis(2-nitro)benzoate (DTNB) under denaturing conditions after treatment or no treatment of Met-O, as described elsewhere [17]. Briefly, 10 μM *Taf*RMsr enzyme in a buffer (50 mM sodium phosphate, pH 7.5, and 50 mM NaCl) was treated with or without 20 mM Met-(*R,S*)-O for 30 min at 37 °C. Sodium dodecyl sulfate (SDS) was added to a final concentration of 10 mM and the enzyme solution was heated for 10 min for 70 °C. DTNB was treated with a final concentration of 300 μM and progress curves of thionitrobenzoate (TNB^-) production were monitored at 412 nm at 25 °C. The amount of TNB^- formed was calculated using the extinction coefficient of 13,600 M^{-1} cm^{-1} [17].

Crystallization, data collection, and structure determination

The *Taf*RMsr used for crystallization was concentrated to 10 mg/ml in a buffer (30 mM Tris–HCl, pH 8.0, and 5 mM DTT). The crystals of the native *Taf*RMsr were obtained by the hanging-drop vapor diffusion method by mixing 1 μl protein with 1 μl well solution containing 0.17 M ammonium acetate, 0.085 M sodium