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An anaerobic bacterial MsrB model reveals catalytic mechanisms, advantages, and disadvantages provided by selenocysteine and cysteine in reduction of methionine-*R*-sulfoxide

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ABSTRACT

We verified and generalized the catalytic features that selenocysteine (Sec) and cysteine (Cys) contribute to the reduction of methionine-*R*-sulfoxide using an anaerobic bacterial MsrB from *Clostridium* sp. OhILA as a model protein. The Sec-containing *Clostridium* MsrB form exhibited 100-fold higher activity than its Cys-containing form, revealing that Sec provided the catalytic advantage of higher activity. However, a resolving Cys was required for the thioredoxin (Trx)-dependent recycling process of the Sec-containing form. Thus, Trx could reduce the selenenylsulfide bond, but its Trx-dependent recycling process was much less efficient compared to that for the disulfide bond in the Cys-containing form, demonstrating an obvious catalytic disadvantage. These data agreed well with our previous data on mammalian MsrBs, and therefore suggested that the catalytic mechanisms, as well as the catalytic advantages and disadvantages provided by the Sec and Cys residues, are most likely conserved from anaerobic bacteria to mammals. Taken together, we propose that the use of Sec in MsrB may depend on a balance between the catalytic advantage of higher activity and the disadvantage of a less efficient regeneration process provided by this residue.

Free and protein-bound methionine residues can be readily oxidized to methionine sulfoxides by various reactive oxygen species. This methionine oxidation in proteins may inflict structural and functional damage unless it is reversed by repair enzymes, the methionine sulfoxide reductases (Msrs)[1] [1]. Msrs are subcategorized into two distinct families, MsrA and MsrB. MsrAs specifically reduce the *S*-epimer of methionine sulfoxide, whereas MsrBs only reduce the *R*-form. These proteins protect cells from oxidative stress, and thus they are viewed as antioxidant and protein repair enzymes [2–4].

Both MsrA and MsrB employ sulfenic acid chemistry in their catalytic mechanisms [5–8]. Following the attack of the sulfoxide moiety within the substrate, a catalytic Cys residue within the enzyme forms a sulfenic acid intermediate, accompanied by a release of the reduced product, methionine. The sulfenic acid of the catalytic Cys then interacts with another Cys (a so-called resolving Cys) to form an intramolecular disulfide bond. A reductant subsequently regenerates the active form of the enzyme by reducing this disulfide bond. Thioredoxin (Trx) is generally accepted as the principal *in vivo* reducing agent, but dithiothreitol (DTT) is commonly used as an *in vitro* reductant. When enzymes lack a resolving Cys, this reductant can also directly reduce the sulfenic acid intermediate.

Selenocysteine (Sec) is translationally inserted into proteins in response to a UGA codon, which normally serves as a stop codon [9]. Sec is frequently found within the catalytic sites of oxidoreductase selenoproteins, in which this residue is essential for catalytic activity [2,10–12]. Selenoprotein MsrBs are found in invertebrates and vertebrates, but not in bacteria or plants [13,14]. In contrast, selenoprotein forms of MsrA are also found in bacteria and lower eukaryotes, including a unicellular green alga [14–16]. Mammals have one selenoprotein MsrB (MsrB1) and two Cys-containing homologs (MsrB2 and MsrB3) [17]. We previously demonstrated that the Sec residue is essential for MsrB1 catalytic activity, and we suggested that higher catalytic activity is per se the advantage conveyed by this Sec residue [17,18]. MsrAs also exhibit this Sec-mediated catalytic advantage [16].

Msr genes exist in most organisms, including bacteria, archaea, and eukaryotes [13,19]. These genes are even found in anaerobic organisms, but to date, little is known about Msrs from anaerobes. *Clostridium* sp. (also known as *Alkaliphilus oremlandii*) OhILA is an anaerobic bacterium whose genome has recently been sequenced (GenBank accession no. CP000853). The *Clostridium* MsrB contains only two Cys residues, each fulfilling respective catalytic and resolving functions. We propose that this structural simplicity

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[1] *Abbreviations used:* Sec, selenocysteine; Cys, cysteine; Trx, thioredoxin; Msrs, methionine sulfoxide reductases; DTT, dithiothreitol.

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makes the *Clostridium* MsrB enzyme a reasonable model to study the catalytic mechanisms for Cys- and Sec-containing MsrBs.

In this study, we characterized the catalytic features of Cys- and Sec-containing forms of *Clostridium* MsrB. The data herein agreed well with our previous work on mammalian MsrBs. These results therefore confirmed that the catalytic mechanisms, as well as the catalytic advantages and disadvantages provided by the Sec and Cys residues, are most likely conserved from anaerobic bacteria to mammals, and that they are probably characteristics of methionine-*R*-sulfoxide reduction catalysis in general, rather than specific properties of mammalian MsrB enzymes.

Materials and methods

Cloning, expression, and purification of the wild-type and mutant forms of Clostridium MsrB in Escherichia coli

A coding region of the MsrB gene from *Clostridium* sp. OhILAs was PCR-amplified using genomic DNA (kindly provided by Dr. John Stolz at Duquesne University; Pittsburgh, PA, USA) and cloned into the *Nde*I/*Xho*I sites of pET21b (Novagen; Madison, WI, USA). To amplify the coding region, forward 5′-GCGCCATATGAGCGAAAAAT ATTTAAAGC-3′ and reverse 5′-CGCGCTCGAGTTCAAAAATTTTAAG ATATTC-3′ primers were used. The resulting plasmid, designated pET-CLOS-MsrB, encoded the full-length MsrB with a C-terminal His-tag (LEHHHHHH). We also generated a C67S mutant form, in which resolving Cys67 was replaced with Ser by site-directed mutagenesis. The resulting construct was named pET-CLOS-MsrB/C67S.

The constructs, pET-CLOS-MsrB (wild-type) and pET-CLOS-MsrB/C67S (C67S mutant), were separately transformed into *E. coli* BL21(DE3) cells. The transformed cells were grown in LB media containing 100 μg/ml ampicillin at 37 °C with shaking until their optical density at 600 nm reached 0.6–0.8; at that point, 0.1 mM IPTG was added to induce protein expression. The cells were cultured for another 4–6 h at 30 °C, harvested, and stored at −20 °C until use.

The cell pellets were resuspended in an extraction buffer, consisting of 50 mM sodium phosphate (pH 7.0), 300 mM NaCl, 10 mM imidazole, and 1 mM phenylmethylsulfonyl fluoride, and lysed by sonication. The supernatant protein samples were separated by centrifugation, loaded on a Talon metal-affinity column (Clontech; San Jose, CA, USA), washed with the extraction buffer, and eluted with a buffer containing 50 mM sodium phosphate (pH 7.0), 50 mM NaCl, and 150 mM imidazole. The eluted proteins were dialyzed against 50 mM sodium phosphate (pH 7.5) and 50 mM NaCl. The purity of the eluate was analyzed by SDS–PAGE and consisted almost exclusively of the ectopic protein.

Construction, expression, and purification of the wild-type and selenoprotein forms of Clostridium MsrB in mammalian cells

Full-length MsrB with a C-terminal His-tag was PCR-amplified using pET-CLOS-MsrB as a template and cloned into the *Sal*I/*Xba*I sites of pSelExpress1, a vector designed to overexpress selenoproteins in mammalian cells [20]. The resulting plasmid, pSel-CLOS-MsrB, encoded a wild-type Cys-containing MsrB with a C-terminal His-tag (LEHHHHHH). Through site-directed mutagenesis, we generated a selenoprotein form (C122U) in which the catalytic Cys122 was replaced with Sec. We also prepared a C122U/C67S form, in which the resolving Cys in the selenoprotein form was mutated to Ser.

To express the wild-type and selenoprotein forms of *Clostridium* MsrB in the human embryonic kidney-derived AD-293 cells (Invitrogen; Carlsbad, CA, USA), the constructs were separately introduced into the cells in large-scale transfections (5–8 plates, 100-mm diameter each) using calcium phosphate. The transfected cells were cultured in Dulbecco's modified Eagle medium containing 10% fetal bovine serum and antibiotics for 2–3 days at 37 °C under 5% CO_2. For cells transfected with selenoprotein constructs, 0.2 μM sodium selenite was added to the media to enhance expression of the selenoprotein form of MsrB. The C-terminally His-tagged proteins were purified using a Talon metal-affinity resin, as previously described [18], concentrated using Centricon YM-10 (Amersham Biosciences; Buckinghamshire, UK), and dialyzed against 50 mM sodium phosphate (pH 7.5) and 50 mM NaCl. Because of small amounts of the affinity-purified wild-type and selenoprotein forms, the purity of the concentrated samples was analyzed by Western blots using anti-His antibodies (Rockland; Gilbertsville, PA, USA). The samples largely contained endogenous contaminant proteins which correspond to non-specific bands on the Western blots (>40% in the wild-type and >80% in the selenoprotein form samples).

Determination of protein concentration

Concentrations of the purified proteins expressed in *E. coli* were determined by the Bradford method using a Bio-Rad protein assay reagent (Bio-Rad; Hercules, CA, USA) and bovine serum albumin as a standard. Concentrations of proteins expressed in AD-293 cells were determined by western blotting using anti-His antibodies, followed by quantifying the western blot signals with a densitometric analysis (ImageJ, NIH, USA). The bacterially-expressed His-tagged wild-type protein was used as an internal standard.

MsrB enzyme assay and analysis of kinetics

MsrB activity was measured in the presence of DTT or Trx. In the DTT-dependent reaction, the reaction mixture (100 μl) contained 50 mM sodium phosphate (pH 7.5), 50 mM NaCl, 20 mM DTT, 200 μM DABSyl-methionine-*R*-sulfoxide, and the purified proteins. In the Trx-dependent reaction, the reaction mixture (100 μl) contained 50 mM sodium phosphate (pH 7.5), 50 mM NaCl, 0.2 mM NADPH, 6 μg *E. coli* Trx (Sigma; St. Louis, MO, USA), 2.9 or 5.8 μg human Trx reductase 1 [21], 200 μM DABSyl-methionine-*R*-sulfoxide, and the purified proteins. The reactions were performed at 37 °C for 30 min, and the product (DABSyl-Met) was analyzed by HPLC using a C_{18} column (Agilent Eclipse XDB-C18, 4.6 × 150 mm, 5 μm; Agilent; Santa Clara, CA, USA) as described previously [5].

K_m and k_{cat} values were determined by fitting the experimental data to the Michaelis–Menten equation in the DTT-dependent reaction.

Analysis of oxidized proteins formed with intermolecular disulfide bonds

Reduced and non-reduced samples of the purified wild-type and C67S mutant proteins, respectively, were prepared for SDS-PAGE analysis. After adding NuPAGE LDS sample buffer (Invitrogen), the samples were supplemented with 2-mercaptoethanol or without the reducing agent, heated at 70 °C for 10 min, and subjected to SDS–PAGE using a NuPAGE 4–12% Bis–Tris gel. The protein bands were stained with Coomassie reagent, and the corresponding bands to dimer and multimers formed with intermolecular disulfide bonds were analyzed.

Results

In our previous study of the mammalian MsrBs [18], we suggested that the Sec-containing MsrB1 and the Cys-containing MsrB2 and MsrB3 possessed different catalytic mechanisms for