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Analyses of methionine sulfoxide reductase activities towards free and peptidyl methionine sulfoxides

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ABSTRACT

There have been insufficient kinetic data that enable a direct comparison between free and peptide methionine sulfoxide reductase activities of either MsrB or MsrA. In this study, we determined the kinetic parameters of mammalian and yeast MsrBs and MsrAs for the reduction of both free methionine sulfoxide (Met-O) and peptidyl Met-O under the same assay conditions. Catalytic efficiency of mammalian and yeast MsrBs towards free Met-O was >2000-fold lower than that of yeast fRMsr, which is specific for free Met-*R*-O. The ratio of free to peptide Msr activity in MsrBs was 1:20–40. In contrast, mammalian and yeast MsrAs reduced free Met-O much more efficiently than MsrBs. Their k_{cat} values were 40–500-fold greater than those of the corresponding MsrBs. The ratio of free to peptide Msr activity was 1:0.8 in yeast MsrA, indicating that this enzyme can reduce free Met-O as efficiently as peptidyl Met-O. In addition, we analyzed the *in vivo* free Msr activities of MsrBs and MsrAs in yeast cells using a growth complementation assay. Mammalian and yeast MsrBs, as well as the corresponding MsrAs, had apparent *in vivo* free Msr activities. The *in vivo* free Msr activities of MsrBs and MsrAs agreed with their *in vitro* activities.

Introduction

Methionine sulfoxide reductases (Msrs) constitute an important defense system to protect cells against oxidative stress [1,2]. They catalyze the reduction of free and protein-based methionine sulfoxide (Met-O) to methionine. Two distinct Msr families have evolved for the reduction of oxidized methionine with a substrate stereospecificity [3,4]. MsrA is specific for the reduction of the *S*-form of Met-O (Met-*S*-O), whereas MsrB only reduces the *R*-epimer of Met-O (Met-*R*-O). In addition, a new type of Msr, designated fRMsr, was recently discovered [5]. This enzyme catalyzes the reduction of only free form of Met-*R*-O. It cannot reduce protein-based Met-*R*-O residues. MsrA and MsrB are present in all three kingdoms of life from bacteria to humans. However, the occurrence of fRMsr is limited to unicellular organisms [6]. Multicellular organisms including mammals do not contain this enzyme.

Catalytic mechanisms of Msr families are similar, and employ a sulfenic acid chemistry [7]. A catalytic Cys residue attacks the sulfoxide moiety of Met-O, and then is oxidized to the Cys sulfenic acid with concomitant release of the Met product. Another Cys (resolving Cys) interacts with the sulfenic acid of catalytic Cys to form a disulfide bond. The disulfide bond is finally reduced by a reductant and subsequently the Msr enzymes are regenerated for the next cycle of reaction. In the case of Msrs lacking resolving Cys, the sulfenic acid intermediate can be directly reduced by the reductants [8]. Thioredoxin (Trx) is generally thought to be an *in vivo* reductant, while dithiothreitol (DTT) can be used for an *in vitro* reductant.

Although MsrA is active on peptidyl Met-*S*-O, it also has a significant reduction activity towards free Met-*S*-O [9]. In addition to free Met-*S*-O, MsrA can reduce other methyl sulfoxide compounds including dimethyl sulfoxide and sulindac [9–11]. In contrast to MsrA, MsrB appears to act mainly on peptidyl Met-*R*-O with very weak activity towards free Met-*R*-O. MsrB from *Escherichia coli* is much less efficient (1000-fold lower) at reducing free Met-O than *E. coli* MsrA [4]. In addition, MsrB has been reported to be unable to reduce methyl sulfoxide compounds that are reducible by MsrA [11,12]. However, the kinetic data able to directly compare free Msr activity to peptide Msr activity of either MsrA or MsrB remain largely unknown.

One MsrA and three MsrBs (referred to MsrB1, MsrB2, and MsrB3) exist in mammalian cells [13]. Among the three MsrBs, MsrB1 is a selenoenzyme, in which selenocysteine replaces Cys in the active site, while MsrB2 and MsrB3 are Cys-containing homologs. In humans, there are two MsrB3 forms, MsrB3A and MsrB3B, generated by alternative splicing of the first exon [13]. We previously verified that all the three mammalian MsrBs can reduce free Met-*R*-O when assayed with a qualitative method

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[13]. However, a study that employed growth complementation experiments showed that human SK-Hep1 cells are unable to reduce free Met-*R*-O, whereas they are capable of reducing free Met-*S*-O [14]. Thus, it was of interest to quantitatively determine the activities of MsrB and MsrA towards free Met-O and to compare those activities each other.

In this work, we determined the kinetic parameters of mammalian MsrA and MsrBs for the reduction of both free and peptidyl Met-O. In parallel, we also analyzed the kinetic properties of yeast MsrA and MsrB. We used yeast fRMsr to compare the free Msr activities of MsrAs and MsrBs. In addition, we analyzed and compared the *in vivo* free Msr activities of MsrAs and MsrBs using a growth complementation assay in yeast cells.

Materials and methods

Preparation of MsrA, MsrB and fRMsr enzymes

Mouse MsrA, mouse MsrB2, and human MsrB3A(32–191) lacking N-terminal signal peptide were prepared as described previously [13,15]. *Saccharomyces cerevisiae* fRMsr and MsrA were also prepared as described previously [12,16]. *S. cerevisiae* MsrB was PCR-cloned into NdeI/XhoI sites of pET28a vector. The resulting construct was named pET28-yMsrB and coded for the full-length of yeast MsrB with an N-terminal His-tag derived from the vector. The yeast MsrB was overexpressed in *E. coli* BL21(DE3) and purified using a Talon-metal affinity resin (Clontech). All purified Msr proteins were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) to check the purity.

Preparation of Trxs

S. cerevisiae cytosolic Trx1 (yTrx1), human cytosolic Trx1 (hTrx1), and rat mitochondrial Trx2 (rTrx2) were prepared as described previously [8,16]. *S. cerevisiae* mitochondrial Trx3 (yTrx3; YCR083W) was PCR-cloned into NdeI/XhoI sites of pET21b and the resulting construct was named pET21-yTrx3. The yTrx3 was overexpressed in *E. coli* BL21(DE3) and purified using the Talon-metal affinity resin. The purity of all purified Trxs was analyzed by SDS–PAGE.

Assays for free and peptide Msr activities and kinetic analysis

Free Msr activity was assayed in the Trx-dependent reaction. 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 yTrx1, and 14 μg human Trx reductase 1, 0.1–4 mM free Met-(*R*,*S*)-O (Sigma–Aldrich), and either 30–50 μg MsrBs or 10 μg MsrAs or 1 μg fRMsr. The mixture of (*R*,*S*)-Met-O has also been used for the kinetic studies of MsrA, MsrB, and fRMsr enzymes as described elsewhere [4,17]. The reactions were carried out at 25 °C for 5 min, and a decrease in absorbance of NADPH at 340 nm was monitored using a spectrophotometer UV-160A (Shimadzu). A control for normalization purposes was the reaction mixture without Msr enzyme. Peptide Msr activity was measured by the above assay procedure with the reaction mixture (200 μl) containing 50 mM sodium phosphate (pH 7.5), 50 mM NaCl, 0.2 mM NADPH, 0.1 mM EDTA, 10 μg yTrx1, and 14 μg human Trx reductase 1, 0.025–0.4 mM either dabsyl-Met-*S*-O for MsrA or dabsyl-Met-*R*-O for MsrB, and either 3 μg MsrA or 10 μg MsrB. Enzyme activity was calculated using a 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 GraphPad Prism 5 software.

Measurement of free Msr activity of yeast MsrB with various Trxs

The reaction mixture (200 μl) contained 50 mM sodium phosphate (pH 7.5), 50 mM NaCl, 2 mM free Met-(*R*,*S*)-O, 30 μg MsrB, 0.2 mM NADPH, 0.1 mM EDTA, 14 μg human Trx reductase 1, and 10 μg of various Trxs. The activity was measured following the above assay protocol.

Yeast constructs and transformation

Mouse MsrB2(24–175) lacking N-terminal signal peptide and human MsrB3B were PCR-amplified using p423-based constructs [13] and cloned into SpeI/HindIII sites of p425 GPD yeast vector. The N-terminal signal peptide of MsrB2 was removed to make the protein be expressed in the cytosol of yeast cells. The human MsrB3B is also expressed in the cytosol of yeast cells as previously described [18]. Mouse MsrA and *S. cerevisiae* MsrB in p423-based constructs [18] were cut with BamHI/XhoI and cloned into the same sites of p425 GPD vector. Constructs of p425-based *S. cerevisiae* MsrA and fRMsr have been described elsewhere [16,19]. The p425-based constructs were transformed into *S. cerevisiae* cells deficient in triple *msrA/msrB/fRmsr* genes (*MATa his3 leu2 met15 ura3 ΔmsrA::URA3 ΔmsrB::KAN ΔfRmsr::HIS3*) [6] using the lithium acetate method. Transformants were selected for leucine prototrophy.

Growth complementation assay

The triple *msrA/msrB/fRmsr*-deleted *S. cerevisiae* cells containing p425 vector only or p425-based Msr constructs were grown aerobically at 30 °C in yeast nitrogen base minimal medium supplemented with 2% glucose (YNBD). The overnight cultures were each adjusted to an optical density of 2.5, 0.25, 0.025, and 0.0025 at 600 nm via serial dilution. Each diluted sample (5 μl) was spotted onto YNBD agar medium in the presence of 0.14 mM Met or 0.28 mM Met-(*R*,*S*)-O. The spotted plates were incubated at 30 °C and the cell growth was monitored. At least two biological replicates were done.

Western blot analysis

The triple *msrA/msrB/fRmsr*-deleted *S. cerevisiae* cells containing p425 vector only or p425-based Msr constructs were grown aerobically at 30 °C in YNBD medium containing Met. Whole cell extracts were prepared by alkali treatment and boiling as described elsewhere [18]. Polyclonal antibodies against mouse MsrB2, human MsrB3, mouse MsrA, or yeast fRMsr were used for Western blot analysis.

Measurement of peptide Msr activity from yeast cell extracts

The triple deletion mutant *S. cerevisiae* cells containing p425 vector only or p425-based Msr constructs were grown aerobically for 2 days at 30 °C in YNBD medium containing Met. Yeast cell extracts were prepared as previously described using glass beads [18]. The reaction mixture (100 μl) contained 50 mM sodium phosphate (pH 7.5), 50 mM NaCl, 20 mM DTT, 200 μM dabsyl-Met-*S*-O for MsrA or dabsyl-Met-*R*-O for MsrB, and 300 μg crude protein. The reaction was carried out at 37 °C for 30 min. The product Met was analyzed by high-performance liquid chromatography (HPLC) as described elsewhere [20].

Results and discussion

We first determined the kinetic parameters of the mammalian MsrBs, MsrB2 and MsrB3, using a mixture of free Met-(*R*,*S*)-O for