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Inhibition of methionine sulfoxide reduction by dimethyl sulfoxide

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Dimethyl sulfoxide (DMSO) is widely used in chemistry and biology as a solvent and as a cryoprotectant. It is also used as a pharmaceutical agent for the treatment of interstitial cystitis and rheumatoid arthritis. Previous reports described DMSO as being reduced by methionine-*S*-sulfoxide reductase (MsrA). However, little is known about the DMSO reduction capability of methionine-*R*-sulfoxide reductase (MsrB) or its effect on the catalysis of methionine sulfoxide reduction. We show that mammalian MsrB2 and MsrB3 were unable to reduce DMSO. This compound inhibited MsrB2 activity but did not inhibit MsrB3 activity. We further determined that DMSO functions as an inhibitor of MsrA and MsrB2 in the reduction of methionine sulfoxides via different inhibition mechanisms. DMSO competitively inhibited MsrA activity but acted as a non-competitive inhibitor of MsrB2 activity. Our study also demonstrated that DMSO inhibits *in vivo* methionine sulfoxide reduction in yeast and mammalian cells. [BMB reports 2009; 42(9): 580-585]

INTRODUCTION

Methionine sulfoxide (Met-O) reductases (Msrs) are important enzymes that repair oxidatively damaged proteins and function as antioxidants to protect cells from oxidative stress (1-3). These enzymes have been implicated in a variety of physiological and pathological processes, including aging and neurological degenerative diseases (4-8). The two major Msr families, MsrA and MsrB, differ in sequence and structure. MsrA stereospecifically catalyzes the reduction of the *S*-epimer of Met-O (Met-*S*-O) to Met, whereas MsrB catalyzes the reduction of the *R*-form (Met-*R*-O). MsrA enzymes in yeast and mammals have significant reduction ability for both free and protein-bound Met-*S*-O (9-11). However, MsrB enzymes in mammals reduce protein-bound Met-*R*-O primarily because they have lower activities toward free Met-*R*-O (11). In addition to these two Msr types, Lin et al. recently discovered and characterized a new type of Msr enzyme from *Escherichia coli*, designated fRMsr (12). This enzyme catalyzes the reduction of free Met-*R*-O, but it has no ability to reduce protein-bound Met-*R*-O or free Met-*S*-O. fRMsr is found in many unicellular organisms, including *Saccharomyces cerevisiae*, but it is absent in all multicellular organisms (11-13).

Mammals have a single MsrA gene and three MsrB genes in their genomes (14, 15). MsrB1 is a selenoprotein that contains selenocysteine in the catalytic site and MsrB2 and MsrB3 are cysteine-containing homologs in which cysteine replaces selenocysteine. The three MsrB proteins are localized within different cellular compartments: MsrB1 is found in the cytosol and nucleus, MsrB2 is found in the mitochondria, and MsrB3 is found in the endoplasmic reticulum and mitochondria (14).

Dimethyl sulfoxide (DMSO) is a widely used solvent for water-insoluble compounds and is used as a cryoprotectant in biological systems. It is also used as a therapeutic agent for the treatment of interstitial cystitis, amyloidosis, and rheumatologic disorders (16). In mammalian cells, DMSO has been reported to affect a variety of cellular processes, including the cell cycle, differentiation, apoptosis, inflammation, and lipid metabolism (16). In some bacteria, DMSO is used as a terminal electron acceptor under anaerobic conditions. DMSO reductase, which contains a molybdopterin cofactor, catalyzes the reduction of DMSO to dimethyl sulfide, and is well characterized at the biochemical and molecular levels (17, 18). However, no DMSO reductase is found in yeast, animals, or plants. Previous reports indicated that DMSO can be reduced by the bovine and yeast MsrA enzymes (9, 10), but little is known about the DMSO reduction capability of MsrB or its effect on the catalysis of Met-O reduction by Msrs.

A common sulfenic acid chemistry is utilized in both MsrA and MsrB catalytic mechanisms (19, 20). Following the attack of the sulfoxide moiety of Met-O, a catalytic Cys is oxidized to a sulfenic acid intermediate, which is accompanied by release of the product, Met. Another Cys (called resolving Cys) then interacts with the sulfenic acid intermediate to form an intramolecular disulfide bond. Finally, an *in vivo* reductant thioredoxin reduces this disulfide to make the enzymes active. Dithiothreitol (DTT) can be used as an *in vitro* reducing agent.

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In this study, we demonstrated that MsrB2 and MsrB3 were unable to reduce DMSO. However, MsrB2 activity was inhibited by DMSO, whereas MsrB3 activity was not. We determined that DMSO functions as an inhibitor of MsrA and MsrB2 in Met-O reduction with different inhibition modes. Furthermore, our study clearly shows that DMSO can inhibit the *in vivo* Met-O reduction.

RESULTS

DMSO inhibits Met-O reduction activities of MsrA and MsrB2

First, we tested if DMSO is reduced by mammalian MsrBs. The reduction of DMSO was determined by an assay analyzing NADPH oxidation at A_{340} in the reaction mixture. MsrA, previously known to have the ability to reduce DMSO, was used for comparison and analysis. As shown in Fig. 1, DMSO was not reduced by either MsrB2 or MsrB3.

Next, we tested the possibility that DMSO could inhibit Met- O reduction because of its role as a substrate of the MsrA enzymes. We performed Met-O reduction assays with multiple DMSO concentrations in the presence of DABSyl-Met-O. The Met-O reduction activity of MsrA decreased as the concentration of DMSO increased (Fig. 2A). At 1% DMSO, the relative activity was 7%. Interestingly, DMSO inhibited the Met-O reduction activity of MsrB2 in a concentration-dependent manner. At 1% DMSO, the relative activity was 60%. However, DMSO did not inhibit the reduction of Met-O by MsrB3 at the concentrations tested.

We then examined the inhibition mechanism of DMSO on the reduction of Met-O by MsrA and MsrB2 by analysis of kinetic parameters. DMSO inhibition of Met-O reduction for both MsrA and MsrB2 was reversible. As shown in Table 1, the apparent K_m values of MsrA increased as the DMSO concentrations increased, but there was little effect on V_{max} values. This indicated that DMSO functions as a competitive inhibitor of MsrA in the reduction of Met-*S*-O. However, the inhibition mechanism for MsrB2 was different. The apparent K_m values for MsrB2 did not change relative to inhibitor concentration, whereas the apparent V_{max} values decreased with increasing DMSO concentrations. These results indicate that DMSO is a non-competitive inhibitor of MsrB2 activity.

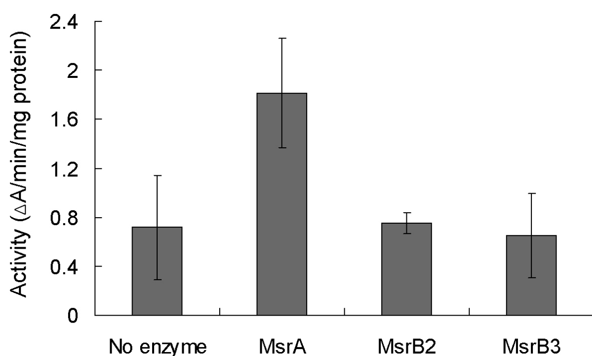

Fig. 1. Mammalian MsrB enzymes are unable to reduce DMSO. To assay the reduction of DMSO, NADPH oxidation coupled with the thioredoxin reduction system was monitored as a decrease in A_{340} at room temperature in the presence of 0.1% DMSO. The rate of decrease was defined as reduction activity.

Yeast MsrA activity is also competitively inhibited by DMSO

We further determined that yeast MsrA activity was inhibited by DMSO (Fig. 2B). DMSO inhibited yeast MsrA activity to a

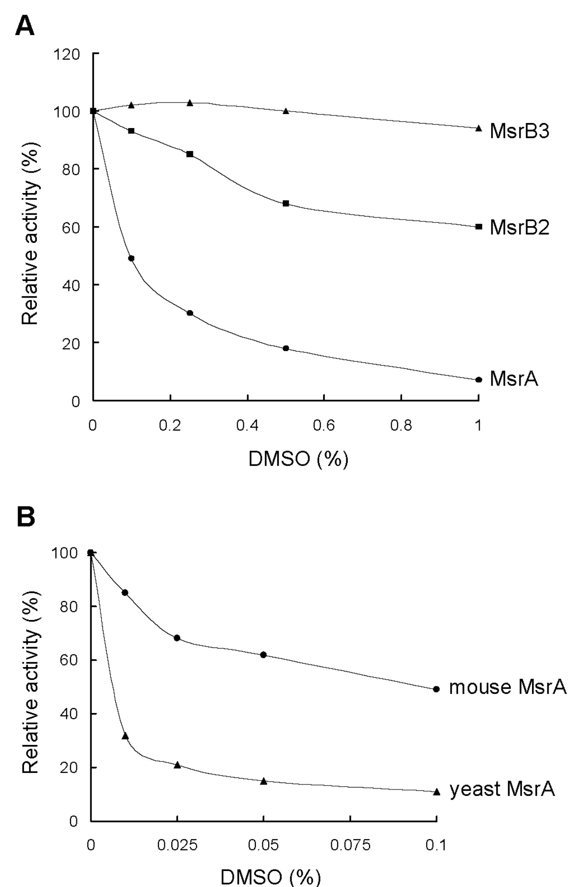

Fig. 2. Effect of DMSO on Met-O reduction by the Msr enzymes. Met-O reduction activities were assayed in a DTT-dependent reaction mixture containing 200 μM DABSyl-Met-*S*-O (for MsrAs), 50 μM DABSyl-Met-*R*-O (for MsrB2), or 200 μM DABSyl-Met-*R*-O (for MsrB3) with multiple concentrations of DMSO. (A) DMSO inhibition of mammalian Msr enzymes. The concentrations of DMSO in the reaction mixture were 0, 0.1, 0.25, 0.5, and 1%. The 100% relative activities of MsrA, MsrB2, and MsrB3 correspond to 206, 196, and 179 nmol min^{-1} (mg protein)$^{-1}$, respectively. (B) DMSO inhibition of yeast MsrA activity. The DTT-dependent reaction mixture contained multiple concentrations of DMSO (0, 0.01, 0.025, 0.05, or 0.1%) and 1 μg yeast MsrA (0.44 μM) or mouse MsrA (0.37 μM). The 100% relative activities of yeast and mouse MsrAs correspond to 136 and 206 nmol min^{-1} (mg protein)$^{-1}$, respectively.

Table 1. Kinetic parameters for inhibition of MsrA and MsrB2 by DMSO

DMSO (%)	MsrA		MsrB2	
	K_m (mM)	V_{max} (mM/min/mg protein)	K_m (mM)	V_{max} (mM/min/mg protein)
0	1.1 ± 0.1	11.6 ± 0.3	0.035 ± 0.005	3.04 ± 0.32
0.1	2.0 ± 0.1	11.4 ± 0.3	0.038 ± 0.004	2.71 ± 0.22
0.5	4.8 ± 0.3	10.7 ± 0.4	0.038 ± 0.005	2.13 ± 0.24

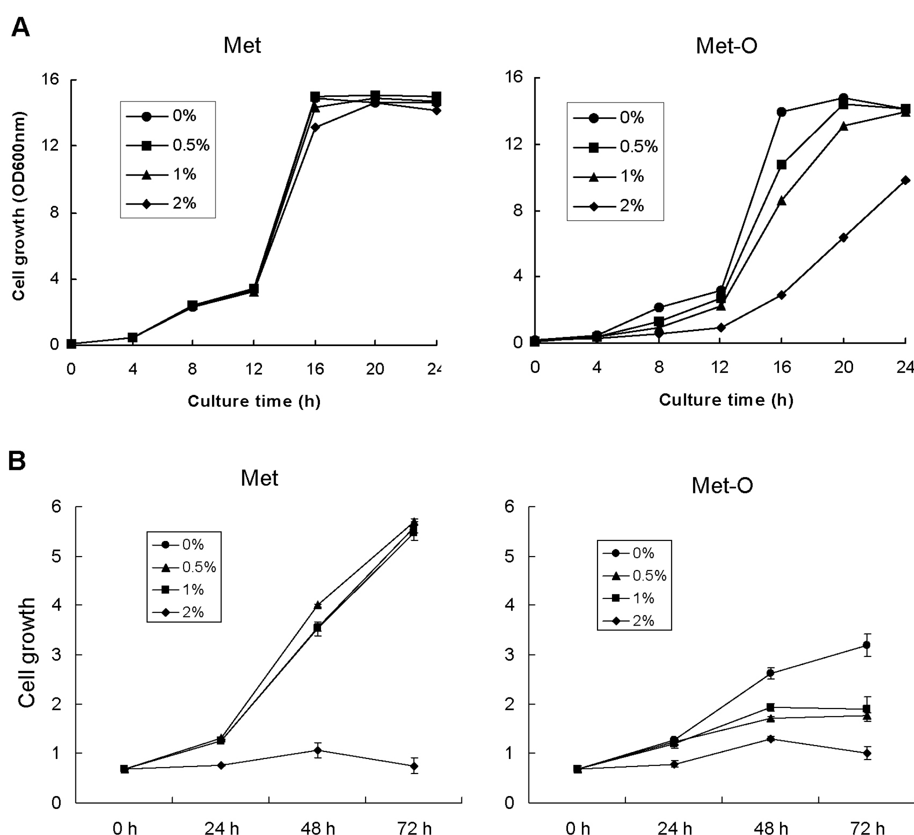

Fig. 3. DMSO inhibition on free Met-O reduction in yeast (A) and mammalian (B) cells. (A) *S. cerevisiae* cells deficient in fRMsr were cultured in YNB minimal media with 0.14 mM Met (left) or 0.28 mM Met-(*R,S*)- O (right) containing the indicated concentrations of DMSO. Cell growth was measured by optical density at 600 nm. Representative data from two independent experiments are shown. (B) SK-Hep1 cells were cultured in Met (left) or Met-(*R,S*)-O (right) medium containing the indicated concentrations of DMSO. Cell growth was analyzed at 0, 24, 48, and 72 h by MTT assay.

greater degree than the mouse MsrA activity. Kinetic analysis also revealed that DMSO competitively inhibited yeast MsrA activity (data not shown).

DMSO can inhibit the Met-O reduction *in vivo*

Because DMSO inhibits *in vitro* activities of mouse and yeast MsrAs and MsrB2, it is of interest whether this compound can inhibit the *in vivo* Met-O reduction. Recently, we found that MsrA is responsible for the reduction of free Met-*S*-O in *S. cerevisiae*, while fRMsr is the enzyme responsible for the reduction of free Met-*R*-O (13). Based on these findings, we used an *S. cerevisiae* strain deficient in the fRMsr gene to investigate the *in vivo* effect of DMSO on the Met-*S*-O reduction. This strain can grow in a Met-*S*-O medium but cannot grow in a Met-*R*-O medium. We cultured the fRMsr mutant cells in medium with Met or Met-(*R,S*)-O containing 0, 0.5, 1, or 2% DMSO. Notably, the MsrA activity able to reduce free Met-*S*-O to Met contributes solely to cell growth of this mutant strain in the Met-(*R,S*)-O medium. As shown in Fig. 3A, when the yeast cells were grown in the Met media, there was no inhibition effect of DMSO on the cell growth in all concentrations tested. In contrast, in the Met-(*R,S*)-O media, cell growth was clearly inhibited by DMSO in a concentration-dependent manner. A previous study illustrated that transcriptional expression of the MsrA gene of *S. cerevisiae* was slightly elevated in the presence of DMSO (21). Thus, the data suggest that DMSO sup-

pressed the *in vivo* Met-*S*-O reduction in *S. cerevisiae* by inhibiting the MsrA activity.

We also confirmed that DMSO inhibits *in vivo* Met-O reduction in mammalian cells. SK-Hep1 cells were recently discovered to reduce free Met-*S*-O with MsrA, but are unable to reduce free Met-*R*-O (11). Thus, these cells can grow in a Met-(*R,S*)-O medium owing solely to the MsrA activity. SK-Hep1 cells were cultured in a Met- or Met-(*R,S*)-O medium containing 0, 0.5, 1, or 2% DMSO. As shown in Fig. 3B, when the SK-Hep1 cells were grown in the Met medium, there was no inhibition effect of DMSO on the cell growth at 0.5 and 1%. However, at 2% DMSO cell growth was severely inhibited, indicating cytotoxicity of DMSO at this concentration. In the Met-(*R,S*)-O medium without DMSO, cells grew ∼40% less than in the corresponding Met medium. This cell growth was consistent with previous results (11). However, cell growth was significantly inhibited at 0.5 and 1% DMSO. There was no significant change in MsrA expression level at these DMSO concentrations (data not shown). Thus, these results clearly indicate that DMSO inhibited the *in vivo* Met-*S*-O reduction by MsrA.

DISCUSSION

Met-O reduction is an essential pathway that protects cells against oxidative stress and regulates lifespan. This pathway is also involved in the progression of neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases. Deletion of the MsrA gene in mice results in the shortening of lifespan by 40% (6). Overexpression of bovine MsrA in *Drosophila* leads to a 70% increase in lifespan (7). A recent study reported that the overexpression of MsrA had a profound effect on preventing the development of Parkinson's-like symptoms in a *Drosophila* model (22). Therefore, the Met-O reduction catalyzed by Msrs is an attractive target for therapeutic approaches against aging, neurodegenerative diseases, and other diseases caused by oxidative stress.

Bovine and yeast MsrAs can catalyze the reduction of DMSO to dimethyl sulfide (9, 10). Here, we show that mouse MsrB2 and human MsrB3 cannot reduce DMSO. It is not surprising that DMSO competitively inhibited the Met-O reduction ability of MsrA as it serves as a substrate for this enzyme. However, it is interesting that DMSO could non-competitively inhibit MsrB2 enzyme, although with lower inhibition effects compared to MsrA. These results suggest that DMSO binds to a site distinct from the substrate (Met-*R*-O) binding site in MsrB2, whereas it competes with Met-*S*-SO for the active sites of MsrAs.

DMSO has a protective effect on arsenic-induced cytotoxicity by reducing intracellular reactive oxygen species production in human-hamster hybrid cell lines (23) and mouse embryonic mesenchymal cells (24). However, DMSO was also reported to have toxic effects on human lens epithelial cells, effectively decreasing cell viability and increasing cellular apoptosis at certain concentrations (25). Cell viability of SK-Hep1 was also markedly decreased when treated with 2% DMSO. Thus, it is important to further investigate and evaluate the toxic effects of DMSO on cell viability.

In this work, DMSO was found to inhibit the *in vivo* reduction of free Met-O in yeast and mammalian cells. Thus, it is likely that in these cells DMSO directly affects the physiological functions of Msr enzymes that repair oxidatively damaged proteins and protect cells from oxidative stress. Further studies will be needed to verify these possible roles of DMSO in the Msr system.

MATERIALS AND METHODS

Preparation of purified Msr enzymes

Mouse MsrA, mouse MsrB2, and human MsrB3 lacking an N-terminal signal peptide were prepared as described previously (14, 26). For *S. cerevisiae* MsrA, a coding region of the MsrA gene was amplified by PCR using a p423GPD-based construct (27) and cloned into *Nhe*I/*Xho*I sites of pET21b. The resulting plasmid, designated pET21b-yMsrA, encoded the full-length yeast MsrA with a C-terminal His-tag (LEHHHHHH). The yeast MsrA protein was purified from *E. coli* BL21(DE3) cells transformed with the pET21b-yMsrA construct using a Talon-metal affinity resin (Clontech), as described previously (14).

Assay for DMSO reduction

The reaction mixture (200 μl) contained 50 mM sodium phosphate (pH 7.5), 50 mM NaCl, 0.1% (v/v) DMSO, 0.2 mM NADPH, 10 μg rat mitochondrial thioredoxin, 5.8 μg human cytosolic thioredoxin reductase, and 2 μg Msr enzyme. The reaction was carried out at 25°C for 20 min and NADPH oxidation was analyzed at A_{340}.

Inhibition assay of DMSO on Met-O reduction activity

Met-O reduction activities were assayed in a DTT-dependent reaction mixture in the presence of DMSO in multiple concentrations. The reaction mixture (100 μl) contained 50 mM sodium phosphate (pH 7.5), 50 mM NaCl, 20 mM DTT, substrate (200 μM DABSyl-Met-*S*-O for MsrAs, 50 μM DABSyl-Met-*R*-O for MsrB2, or 200 μM DABSyl-Met-*R*-O for MsrB3), 0-1% (v/v) DMSO, and 1 μg Msr enzyme. The reaction was carried out at 37°C for 30 min. The reaction product, DABSyl-Met, was analyzed by HPLC, as described previously (28).

Determination of inhibition mechanisms of DMSO on Met-O reduction

K_m and V_{max} values for Met-O reduction were determined from Lineweaver-Burk plots in the DTT-dependent reaction with increasing DMSO concentrations.

Assay for DMSO inhibition on Met-O reduction *in vivo*

The cell growth assay was performed to examine the *in vivo* DMSO inhibition effect on the Met-O reduction in yeast and

mammalian cells.

For the yeast cell growth assay, *S. cerevisiae* cells deficient in fRMsr gene (*MATa his3 leu2 met15 ura3 ⊿fRMsr::KAN*) were grown aerobically at 30°C in yeast nitrogen base (YNB) minimal medium in the presence of 0.14 mM Met or 0.28 mM Met-(*R,S*)-O. The media contained multiple concentrations of DMSO (0-2%). Cell growth was monitored by optical density at 600 nm.

For the mammalian cell growth assay, human SK-Hep1 cells were cultured in a medium containing Met or Met-O prepared as described (11) at 37°C in a 5% CO_2 incubator. The medium contained Met-free DMEM (Invitrogen) including 0.2 mM L-cystine, 4 mM L-glutamine, and 1 mM sodium pyruvate, 10% dialyzed fetal bovine serum, antibiotics, 0.1 mM Met or 0.2 mM Met-(*R,S*)-O, and multiple concentrations of DMSO (0-2%). The cells in regular DMEM were plated in 12-well plates at a density of 3×10^4 cells/well. After 20 h plating, the regular DMEM media were replaced with the Met- or Met-O-containing media with DMSO. Cell growth was analyzed at 0, 24, 48, and 72 h using colorimetric MTT assay.

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