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Purification and Characterization of Methionine Sulfoxide Reductases from Mouse and *Staphylococcus aureus* and Their Substrate Stereospecificity

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Many organisms have been shown to possess a methionine sulfoxide reductase (MsrA), exhibiting high specificity for reduction the S form of free and protein-bound methionine sulfoxide to methionine. Recently, a different form of the reductase (referred to as MsrB) has been detected in several organisms. We show here that MsrB is a selenoprotein that exhibits high specificity for reduction of the *R* forms of free and protein-bound methionine sulfoxide. The enzyme was partially purified from mouse liver and a derivative of the mouse *MsrB* gene, in which the codon specifying selenocystein incorporation was replaced by the cystein codon, was prepared, cloned, and overexpressed in *Escherichia coli.* The properties of the modified MsrB protein were compared directly with those of MsrA. Also, we have shown that in *Staphylococcus aureus* there are two MsrA and one nonselenoprotein MsrB, which demonstrates the same substrate stereospecificity as the mouse MsrB.

Key Words: **methionine sulfoxide; oxidative stress; methionine sulfoxide reductase; free radicals; methionine oxidation; selenoprotein; stereospecificity.**

Methionine residues of proteins are readily oxidized to methionine sulfoxide (MetO) by most reactive oxygen species However, in contrast to most other oxidative posttranslational modifications, the oxidation of methionine residues is repaired by the action of methionine sulfoxide reductase (MsrA) which catalyzes reduction of MetO to methionine, both *in vitro* and *in vivo* (1, 2). In addition to limiting the steady-state level of oxidized methionine, the cyclic oxidation/reduction of protein methionine residues constitutes a mechanism for the scavenging of ROS, and thereby provides increased resistance to oxidative cellular damage and to enhanced survival under conditions of oxidative stress (3–5). It was shown previously that a mouse strain lacking MsrA is more prone to oxidative stress damage, has shorter life span and exhibits a typical "tip toe" walking behavior starting at six months of age (5). Other studies have shown that the level of MsrA declines with age (6), and that over expression of the enzyme in human T cells increases their survival rate under conditions of oxidative stress. Furthermore, in addition to its repair and antioxidant functions, MsrA may play a regulatory role in regulation of various biological functions (7, 8).

In view of the fact that the ROS-mediated oxidation of protein-bound methionine residues leads to a racemic mixture of the *R* and *S* forms of MetO, it was disturbing that MsrA exhibits high specificity toward the *S* form only (9, 10). Moreover, an enzyme (Fmsr) that catalyzes specifically the reduction of free MetO, is also specific for the *S* isomer (9). Nevertheless, results of other studies indicated that cells have the ability to convert the *R* form of MetO to methionine, by an unknown mechanism (9). In an effort to identify the enzyme(s) responsible for the conversion of *R*-MetO to methionine, we examined the substrate stereospecificity of three different Msrs in *Staphylococcus aureus* (11), and also of the MsrB form of enzyme found in extracts of $MsrA^{-/-}$ mouse (5).

MATERIALS AND METHODS

Methionine sulfoxide reductase activity. Enzymatic activity of methionine sulfoxide reductase was measured using either dabsyl-methionine sulfoxide or free methionine sulfoxide (*R* or *S* form) as substrate, as previously described (9). Briefly, the reaction mixture contained 1 mM substrate, 20 mM DTT, and 25 mM Tris–HCl, pH 7.4, or PBS. Following incubation for 30 min at 37°C, the reaction mixture was injected into a C18 column (Apex, Jones Chromatogra-

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phy, Denver, CO) and the methionine or dabsyl methionine peak was monitored.

Purification of the MsrB (SelR) protein. Fifteen livers from *MsrA*$^{-/-}$ mice (1 g of protein), were homogenized in 50 mM Tris–HCl, pH 7.4, buffer and following ammonium sulfate precipitation (30–60%), the sample was dialyzed versus the same buffer. The soluble material was subjected to various columns and fractions, containing the reducing activity using dabsyl-methionine sulfoxide as substrate, were pooled at each step. The following columns and methods were used sequentially: DEAE-5PW Column (TosoHaas, Montgomeryville, PA) using 0–500 mM NaCl gradient in 50 mM Tris–HCl, pH 8.0; sizing column Ultrogel AcA-54 (Amersham–Pharmacia Biotech) using 50 mM Tris–HCl, pH 7.4 (buffer A), as the running solvent; MonoP column using poly buffer exchanger 74 (Amersham–Pharmacia Biotech); MonoP column using poly buffer exchanger 96 (Amersham–Pharmacia Biotech); sizing column G3000PW (TosoHaas, Montgomeryville, PA) using buffer A as running solvent. To identify which band of the five major proteins corresponds to the Msr activity, an SDS/PAGE analysis without adding SDS to the sample was performed on the peak fraction of activity monitored in the last step of purification. Only one band of protein showed activity and its estimated molecular weight from the gel was between 12–13 kDa. The band was further digested with LysC and following mass spectrometry analysis reviled identity to SelR protein from mouse.

Overexpression and purification of the recombinant MsrB (SelR) protein in which the selenocystein residue was changed to Cys. To express the protein to its full length, the selenoCys (SeCys) coded at the first stop codon appearing in the protein (TGA at position 95 of the protein) was changed to Cys to allow translation of the protein till its second and final stop codon (21 amino acids down stream of the TGA). To achieve that goal two 5′ and 3′ primers were designed as follows: The 5′ forward primer was designed to fit the 6His reading frame in the pQE30 vector (Qiagen) using an *Sph*I site (underlined) in front of the first methionine: 5′-CCCGTCGCATGC-ATGTCGTTCTGCAGCTTCTTCGGAG. The 3′ prime reverse complement primer was designed to change the TGA (CESIC) to TGC (Cys) (bolded) using *Sal*I site (underlined) at the 3′-untranslated region 5′-GCAGTCGACGTCCTAGTGCCCCTGGGAGGCAGCAG-CTTCTTTGCCTTTAGGGACGAACTTCAGTGAGCTGCTAAATA-**TGC**AG. PCR was performed using these two primers and liver mouse cDNA as template.

Following digestion with the restriction enzymes *Sph*I and *Sal*I the PCR product was cloned into pQE30 at its compatible restriction sites to create expression construct. *Escherichia coli* cells (XL1blue) were transformed with the construct and the protein was expressed and purified following induction with isopropyl-β-thiogalactopyranoside (IPTG) and affinity purification on a nickel column, according to the procedure described by Clontech. The His-tagged protein was then dialyzed against 50 mM Tris–HCl, pH 7.4, and kept at −70°C.

Overexpression and purification of recombinant His-tagged Msrs from S. ureus. Three Msrs have been recently identified in *S. aureus* (11). One is a homologue of the *E. coli msrA* gene and is the first gene in a polycistronic message. Downstream of it, there is a homologue to PilB gene that also has been shown to posses an Msr activity. Both enzymes were expressed as His-tagged proteins according to the description in our recent publication (11) and in this study they are being referred to as MsrA1 and PilB proteins, respectively. The third enzyme has 50% homology to MsrA1 (GI 13701158) and it is being referred to as MsrA2 protein. The latter one was overexpressed and purified as a His-tagged protein as follows: DNA fragment (1341 bp) was PCR amplified using *S. aureus* RN450 genomic DNA as the template, a 5′ sense primer containing a *Bam*HI site (underlined) (5′-GGATCCATGTCAAAAATGAATATTAATAC-AGC-3′) and a 3′ reverse complement primer containing a *Hin*dIII site (underlined) (5′-AAGCTTAACGCCGTGGATTTAGAG-3′). The PCR product was then cloned into pCR2.1 vector (Invitrogen, Carlsbad, CA) and subsequently subcloned in frame at the *Bam*HI and *Hin*dIII sites of the overexpression pRSETa vector (Invitrogen) resulting into the construct pRSETa-*msrA2*. *E. coli* cells [BLR(DE3)pLysS] (Novagen, Madison, WI) were transformed with the construct and the protein was expressed and purified following induction with IPTG and affinity purification on a nickel column, according to the procedure described by Clontech. The His-tagged protein was then dialyzed against 50 mM Tris–HCl, pH 7.4, and kept at −70°C.

TABLE 1

Specific Activity of the Partially Purified MsrB and the Recombinant SelR Cys Mutant and Their Substrate Stereospecificity toward R-MetO and dabsyl-R-MetO

	Specific activity (pmol Met formed/mg protein/min)			
Enzyme	*R*-MetO	*S*-MetO	Dabsyl-*R*-MetO	Dabsyl-*S*-MetO
MsrB[a]	37,925	0	40,808	0
HSelR(Cys)	11,324	0	11,735	0

[a] Partialy purified.

Note. The residual Msr activity from livers of *MsrA*$^{-/-}$ mice (MsrB) was partially purified, as previously described under Materials and Methods, and was denoted as MsrB. HSelR(Cys) represents the recombinant His-tagged SelR in which its SeCys residue was replaced by Cys. The procedures for making the recombinant protein and Msr activity analysis are described under Materials and Methods.

RESULTS AND DISCUSSION

Tissues of a mouse strain lacking MsrA activity were found to contain another Msr activity which we referred to as MsrB. Following SDS/PAGE analysis, an apparently homogeneous protein fraction with a mass of 12–13 kDa possessing MsrB, was isolated as described under Materials and Methods. Based on the mass spectral analysis of Lys C digest, it was found to correspond to a selenoprotein of unknown function (SelR in mouse and SelX in human) (12, 13). This protein contains a single selenoCys (SeCys) residue at position 95, which is encoded by a TGA codon. To facilitate its isolation and overexpression in *E. coli*, a full length His-tagged recombinant protein analog was made by replacing the SeCys codon with the Cys codon, as described under Materials and Methods. As expected, the purified Msr analog exhibited lower specific activity than its selenoprotein counterpart (MsrB), but both forma exhibited absolute *R*-stereospecificity for both free and protein-bound MetO (Table 1). To investigate the possibility that homologues of SelR protein in other organisms posses the same stereospecificity, we examined the Msr activity of the homologous PilB protein from *S. aureus* (11). As shown in Table 2, this enzyme is also stereospecific for *R* isomers of both the free and peptide MetO derivatives. For comparison, the MsrA homologues from *S. aureus* were also examined and were found to be specific for the *S* isomers (Table 2). It is noteworthy, that MsrA2 exhibited ~4-fold higher specific activity than MsrA1. This difference

TABLE 2

Specific Activity of MsrA1, MsrA2, and Pilb Homolog of *S. aureus* toward Free MetO and Their Substrate Stereospecificity

	Specific activity (pmol Met formed/mg protein/min)	
Enzyme	*R*-MetO	*S*-MetO
MsrA1	0	63,544
MsrA2	0	235,396
PilB	1,779,232	0

Note. The three His-tagged Msr enzymes of *S. aureus* were checked for their stereospecificity toward free *S* and *R* MetO, according to the procedures described under Materials and Methods.

could reflect a naturally occurring point mutation in MsrA1 in which the last Gly of the conserved MsrA active site (GCFWG) is changed to Cys (GCFWC). In some strains of bacteria the *msrA* and *msrB* are arranged as separate genes [e.g., *E. coli* and *Streptococcus pneumoniae* (3, 14)]; as one fusion protein (e.g., *Neisseria Gonorrhoeae* (14); or in an operon [e.g., *S. aureus* (11)]. Abolishing the expression of MsrA could therefore lead to increased sensitivity to oxidative stress or adhesion problem of the organism, or both (3, 14–17). Although likely, further studies are needed to determined if organisms lacking both *msrA* and *msrB* genes are far more sensitive to oxidative stress, exhibit shorter life span, and are less infectious in comparison to their parent strains.

To assess the effects of MsrA on MsrB expression in mammals, we checked the ability of tissue extracts from wild-type (WT) and $MsrA^{-/-}$ mice to reduce the *R* or *S* forms of free MetO. As shown in Table 3, the ability of various tissues to reduce *R*-MetO was decreased by the disruption of the *MsrA* gene. The loss of *R*-MetO activity was greatest in the liver (52%) followed by cerebellum (43%), kidney (33%), and brain other than cerebellum (10%). The results suggest that in mice, MsrA may be implicated in the regulation of MsrB expression, and that of all tissues tested the brain (excluding cerebellum) is the most protected (similar results were obtained with *S* and *R* forms of dabsyl-MetO, data not shown). This result is consistent with the demonstration that the brains of $MsrA^{-/-}$ mice were more resistant to oxidative damage under conditions of oxidative stress (5). The fact that the MsrB activity in cerebellum is more severely affected by the loss of MsrA activity suggests that damage to this organ may be implicated in the development of ataxia-like symptoms ("tip-toe" walking) of the $MsrA^{-/-}$ mice (5). The residual MsrA activity observed in tissues of $MsrA^{-/-}$ mice mouse (Table 3) is likely due to the activity of the free Msr (FMsr) which has been shown to be specific for free *S*-MetO (9). Significantly, tissue extracts of the $MsrA^{-/-}$ mice exhibited no ability to

TABLE 3

Differential Activity toward *S* and *R* Forms of Free MetO in Various Mouse Tissues

	Specific activity (pmol Met formed/mg protein/min)	
Tissues	*R*-MetO	*S*-MetO
WT		
Liver	1924	2316
Kidney	678	1420
Brain–cerebellum	77	236
Cerebellum	160	131
$MsrA^{-/-}$		
Liver	925	136
Kidney	452	117
Brain–cerebellum	70	47
Cerebellum	91	44

Note. Msr stereospecific activity in various tissues from wild-type (WT) and *MsrA* null mutant mice ($MsrA^{-/-}$) was checked according to the procedures described under Materials and Methods. Tissues were taken from five animals and homogenized in PBS, and their supernatants were checked for activity.

reduce the *S* form of dabsyl-S-MetO (data not shown). In earlier studies, we did not detect a dabsyl-MetO reducing activity in *msrA* null mutants of *E. coli* and *S. cerevisiae* (2, 3). In light of the preset results with mice, and the possibility that the levels of cell extracts examined were too small to detect low levels of other Msr activities, we reexamined the Msr activity of these mutants, using larger amounts of cell extracts. Indeed, as shown in Table 4, extracts of these mutants possessed about 25% as much activity as was measured in the WT strains. Moreover, it was confirmed that this activity was specific for the *R* form of dabsyl-MetO (data not shown). In addition, genome search for PilB/SelR homologues in *E. coli* (GI 7429279) and *S. cerevisiae* (ORF YL033C) revealed in each genome a putative homologous protein.

In summary, we have demonstrated that mammals contain two major enzymes that catalyze the reduction

TABLE 4

Specific Activity toward dabsyl-MetO in Extracts from *S. cerevisiae* and *E. coli* and Their *msrA* Null Mutants

Organism extract	Specific activity, dabsyl-MetO (pmol Met formed/mg protein/min)
S. cerevisiae H9 (WT)	83
S. cerevisiae H9Δ*msrA*	20
E. coli MC1061 (WT)	639
E. coli SK8776 (Δ*msrA*)	147

Note. The activity was checked in wild-type (WT) and *msrA* null mutant (Δ*msrA*) strains from each species (2,3) using 1 mg of protein of soluble material, following extraction in Tris–HCl, pH 7.4. Activity was monitored according to the assay described under Materials and Methods.

of both free and protein-bound MetO. One (MsrA) is stereospecific for the *S* isomers and the other (MsrB) is stereospecific for the *R*-isomers. In mammals, the latter is a selenoprotein previously known as SelR or SelX, but some microorganisms contain homologues in which the selenocysteine residue is replaced by cysteine residue. It is noteworthy, that because the oxidation of both free and protein-bound methionine residues by many different forms of ROS leads to racemic mixtures of the *S* and *R* forms of MetO, it is understandable that cells posses enzymes capable of reducing each isomer. Finally, in view of the fact that enzymes which are stereospecific for each isomer are widely distributed in nature, we propose that a change in nomenclature is needed. We suggest: Msr that can reduce free and protein-bound *S*-MetO be denoted as pSMsr; Msr that can reduce free and protein-bound *R*-MetO will be denoted as pRMsr; and enzymes that reduce specifically free *S*- or *R*-MetO, will be denoted as FSMsr or FRMsr, respectively. Furthermore, in cases where more than one isozyme for each species is identified, we propose that they be designated in a capital letter format (A, B, C, etc.) as an extension of the proper name. Thus, the isozyme for pSMsr might be designated as pSMsrA.

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