

Cloning and expression of a mammalian gene involved in the reduction of methionine sulfoxide residues in proteins

JACKOB MOSKOVITZ, HERBERT WEISSBACH, AND NATHAN BROTH

Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110-1199

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ABSTRACT An enzyme that reduces methionine sulfoxide [Met(O)] residues in proteins [peptide Met(O) reductase (MsrA), EC 1.8.4.6; originally identified in *Escherichia coli*] was purified from bovine liver, and the cDNA encoding this enzyme was cloned and sequenced. The mammalian homologue of *E. coli* *msrA* (also called *pmsR*) cDNA encodes a protein of 255 amino acids with a calculated molecular mass of 25,846 Da. This protein has 61% identity with the *E. coli* MsrA throughout a region encompassing a 199-amino acid overlap. The protein has been overexpressed in *E. coli* and purified to homogeneity. The mammalian recombinant MsrA can use as substrate, proteins containing Met(O) as well as other organic compounds that contain an alkyl sulfoxide group such as *N*-acetylMet(O), Met(O), and dimethyl sulfoxide. Northern analysis of rat tissue extracts showed that rat *msrA* mRNA is present in a variety of organs with the highest level found in kidney. This is consistent with the observation that kidney extracts also contained the highest level of enzyme activity.

One of the known posttranslational modification of proteins is the oxidation of methionine residues to methionine sulfoxide [Met(O)] (1). This oxidation can readily occur since a variety of reactive molecules that are by-products of aerobic metabolism such as the superoxide anion, hydrogen peroxide, hydroxyl radical, and hypochlorite ion are capable of causing this oxidation (1). It is of interest that this oxidation is reversible. An enzyme, peptide-Met(O) reductase (MsrA, EC 1.8.4.6), which has been detected in virtually all organisms examined, catalyzes the reduction of Met(O) residues in proteins to methionine (2–4).

The gene (*msrA*; also called *pmsR*) for the *Escherichia coli* enzyme has been cloned and sequenced (5) and a *msrA* mutant has recently been shown to be more sensitive to oxidative damage than the parent strain (6). This latter finding is the first *in vivo* demonstration of a possible role of the MsrA protein in protecting cells from oxidative damage.

The presence of this enzyme in a wide variety of organisms and animal tissues suggests that it could have an important function in providing cells with a defense against oxidative stress. In many cases, the oxidation of a specific methionine residue in a protein leads to the loss of biological activity (7), which can be restored by incubation of the oxidized protein with MsrA (2–4). In some cases there is a known pathophysiology. For example, oxidized α -1-proteinase inhibitor [α -1-PI containing Met(O)] is thought to be involved in the etiology of smokers' emphysema (8) and may also play a role in adult respiratory distress syndrome (9) and rheumatoid arthritis (10). *In vitro* MsrA can reduce the Met(O) residues in this protein and restore biological activity (2).

As noted above, enzymatic activity towards Met(O) residues in peptides and proteins has been detected in many eukaryotic tissues (3, 4, 11–13). However, very little is known about the

eukaryotic enzyme and whether it differs from other enzymatic systems that are known to reduce sulfoxide residues in compounds containing a methylsulfoxide moiety including Met(O) (14). Xenobiotics containing sulfoxide residues, such as the antiinflammatory compound sulindac (15), the anthelmintic bithionol sulfoxide (16), and carbophenothion sulfoxide, a metabolite of an organophosphorus insecticide, are also known to be reduced by enzymatic systems in animal tissues (17). Some of the characteristics of these systems, where studied, include high activity in kidney and liver as well as the ability of reduced thioredoxin to supply the reducing potential (14).

In the present study we describe the purification of the mammalian homologue of the *E. coli* MsrA enzyme and the cloning, sequencing, and expression of the mammalian *msrA* gene; for simplicity, we use the *E. coli* symbols throughout. Evidence is presented that the *msrA* gene product has a broad substrate specificity and other characteristics that suggest it is similar to previously reported proteins that reduce methyl sulfoxide compounds (14).

MATERIALS AND METHODS

Substrates. Tetramethylene sulfoxide, diphenyl sulfoxide, and *S*-(-)-methyl *p*-tolyl sulfoxide, were obtained from Aldrich. Sulindac *z*-{*cis*-5-fluoro-2-methyl-1-[(*p*-methylsulfinyl)-benzylidene]indene-3-acetic acid}, L-methionine, D-methionine, L-ethionine, and *N*-acetylmethionine (*N*-AcMet) were obtained from Sigma. To oxidize D- or L-methionine, L-ethionine, and *N*-AcMet, these compounds (30 mM) were treated with 0.3% H₂O₂ for 16 hr at room temperature. After lyophilization the dried material was dissolved in water. Dimethyl sulfoxide was obtained from Fisher Scientific.

Purification and Partial Sequence Determination. MsrA activity was routinely assayed as described by Broth *et al.* (18) with *N*-Ac[³H]Met(O) as substrate. About 1 kg of bovine liver was homogenized in 1 liter of buffer containing 25 mM Tris-HCl (pH 7.4) and 150 mM NaCl. Following disruption in a Polytron homogenizer, the suspension was centrifuged at 100,000 × *g*. The supernatant fraction (18 g of protein) was brought to 25% (NH₄)₂SO₄ saturation, and the precipitate was removed by centrifugation and discarded. The supernatant was then brought to 80% (NH₄)₂SO₄ saturation, and after centrifugation the precipitate was dissolved in a buffer containing 25 mM Tris-HCl (pH 7.4) and 1 mM dithiothreitol (buffer A) and was dialyzed against this buffer. This material (15.8 g of protein) was applied to a DE-52 column (5 × 51 cm; Whatman) equilibrated with buffer A. The column was eluted with buffer A, the flow-through was brought to 80% (NH₄)₂SO₄ saturation, and the resultant precipitate (8.3 g of protein) was dissolved in buffer A (90 ml) and applied to an Ultrogel AcA-54 column (6 × 77 cm; IBF Biotechnics Columbia, MD)

Abbreviations: Met(O), methionine sulfoxide; MsrA, peptide-Met(O) reductase (also called *pmsR*); *N*-AcMet(O), *N*-acetyl-Met(O); *N*-AcMet, *N*-acetylmethionine; IPTG, isopropyl β -D-thiogalactoside; α -1-PI, α -1-proteinase inhibitor.

*The bovine sequence reported in this paper has been deposited in the GenBank data base (accession no. U37150).

equilibrated with buffer A. Fractions containing the MsrA activity, eluted from the column at a volume that was consistent with a molecular mass of 20–30 kDa, were pooled (1.1 g of protein). The pooled fractions were then applied to a CM-52 column (4 × 12 cm; Whatman) equilibrated with 25 mM Tris-HCl (pH 6.0) and were eluted with the same buffer. The unbound protein (360 mg of protein) was subjected to chromatofocusing on a Polybuffer exchanger 94 column (1 × 42 cm; Pharmacia) equilibrated with buffer A. The buffer used for elution was Polybuffer 74-HCl (Pharmacia). Fractions containing the MsrA activity were pooled (15 mg of protein) and then applied to an FPLC (fast protein liquid chromatography) Mono-Q high-resolution (HR) column (Pharmacia) and eluted with a 0–300 mM NaCl gradient. About 100 µg of protein from the peak of enzyme activity (1.1 mg of total protein) was run on a preparative SDS/12.5% PAGE column and electroblotted onto an Immobilon-P polyvinylidene difluoride membrane (Millipore). The protein band was cut out, and amino acid sequences were obtained (Harvard Microchemistry Facility, Cambridge, MA). Briefly, after digestion with trypsin, the mixture was fractionated by microbore HPLC. The resultant peptides were detected by differential UV absorbance and matrix-assisted laser desorption mass spectrometry (Lasermat; Finnigan-MAT, San Jose, CA). Selected peptides were then subjected to automated Edman microsequencing (19). The amino-terminal sequences of the four MsrA tryptic peptides were obtained, and in single-letter amino acid code are as follows: (i) VVFQPEHISFEELK, (ii) VLSEHGFLITDIR, (iii) SAIYPTSAEHVGAALK, and (iv) GVVSTQVGFAGGYTPNPTYK.

Isolation and Sequence of the *msrA* cDNA Clone. mRNA was isolated from 50 mg of bovine adrenal medulla using a micro RNA isolation kit (Stratagene). First- and second-strand cDNA synthesis was performed with the cDNA synthesis kit from Amersham (Arlington Heights, Illinois). To construct a cDNA library, cDNAs were subcloned by blunt-end ligation to the *EcoRV* site of the pBluescript II SK(+) vector and transformed into supercompetent XL2-Blue MRF' cells (Stratagene). A partial cDNA (228 bp) was isolated by PCR by using as primers the sense and antisense oligonucleotides 5'-GTGGTGTTCAGCCCGAGCACAT-3' and 5'-CCGG-ATGTCGGTGGTGATCAGGCCGAA-3', which encode residues 1–8 and 7–15 of tryptic peptides I and II, respectively (see above). PCR was performed for 30 cycles of 30 sec at 94°C, 60 sec at 50°C, and 90 sec at 72°C, with the first-strand cDNA as template. The gel-purified PCR product was labeled by using a random primer labeling kit (Boehringer Mannheim) and [α -³²P]dCTP. XL2-Blue MRF' cells harboring a recombinant plasmid (pJM200) carrying the complete *msrA* gene were identified by colony hybridization (20) using the ³²P-labeled PCR product. A plasmid preparation was obtained by using the Wizard Minipreps DNA purification system (Promega), and the *msrA* DNA was sequenced by using the *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems).

Overexpression and Purification of the MsrA Protein. The *msrA* open reading frame was amplified by PCR using pJM200 as template, a 5' sense primer containing a *Bam*HI site (5'-CGCGGATCCATGCTCTCGATCACCAGG), and a 3' reverse complement primer with an *Eco*RI site (5'-GGGGAGAATTC-ACITTTTAATACCCAGGGAC). Reaction conditions were as described above. Both the amplified product and pGEX-2T were digested with *Bam*HI and *Eco*RI, and the PCR fragment encompassing the complete bovine *msrA* coding region was ligated into the restricted pGEX-2T by using phage T4 DNA ligase (Boehringer Mannheim). *E. coli* mutant cells lacking the *msrA* gene (SK8779, *msrA1::kan*) (6) were transformed with an aliquot of the ligation mixture and grown in LB medium containing ampicillin (50 µg/ml). When cells reached an A_{600} of 0.4, isopropyl β -D-thiogalactoside (IPTG) was added to a final concentration of 1 mM, and the growth continued for an additional

4 hr. The cells were collected by centrifugation, suspended in buffer containing 50 mM Tris-HCl (pH 7.4), and 150 mM NaCl, and sonicated. The lysate was centrifuged, the supernatant was applied to a glutathione-agarose column (Sigma), and the MsrA protein was purified as described (21). Minor contaminating proteins were removed from the preparation by chromatography on a DE-52 column, which was eluted with 100 mM NaCl in buffer A. The purity of the protein was analyzed by SDS/PAGE.

RNA Blot Analysis. Rat and human multiple tissue RNA blots were purchased from Clontech and were probed with the ³²P-labeled PCR product and used to detect the expression of the *msr* gene in these tissues. Hybridization conditions were according to the Clontech protocol for DNA probes.

Determination of MsrA Activity in Rat Tissues. Rat heart, brain, spleen, lungs, liver, and kidneys were each disrupted by using a Dounce homogenizer in 3 ml of buffer A. The homogenates were centrifuged (30,000 × g), and the MsrA activity of each supernatant was assayed by using *N*-Ac[³H]Met(O) as substrate, as described elsewhere (18). Specific activity is defined as pmol of *N*-AcMet formed per µg of protein per hr.

MsrA Activity with Other Substrates. The activity of MsrA with Met(O), *N*-AcMet(O), or other sulfoxide-containing compounds was measured by incubating the enzyme (2.4 µg) in 50 mM Tris-HCl (pH 7.4) containing 50 nmol of NADPH, 1.5 µg of thioredoxin reductase, 2.5 nmol of thioredoxin, and 3 µM of the various substrates in a final volume of 0.5 ml. A unit of activity is defined as nmol of NADPH oxidized per min, as determined by the change in absorbance at 340 nm. The activity using oxidized α -1-PI as substrate was determined as described by Abrams *et al.* (2). Sulindac reduction could not be assayed spectrophotometrically because of its high absorbance at 340 nm. Therefore, it was tested for its ability to act as a competitor in the standard assay with *N*-Ac[³H]Met(O) as substrate.

RESULTS

Nucleotide Sequence of Bovine MsrA-Encoding cDNA and Its Deduced Amino Acid Sequence. Mammalian MsrA was purified from bovine liver (see *Materials and Methods*). Gel electrophoresis of the peak fractions from the FPLC step showed the presence of several major protein bands, one of which migrated with an apparent molecular mass of \approx 26,000 Da (data not shown). This band was shown to possess MsrA enzymatic activity after elution from a preparative gel. The protein in this band was then digested with trypsin, and the amino-terminal sequences of four tryptic peptides were determined by sequential Edman degradation. By using primers selected from regions of low codon degeneracy within peptides I and II (see *Materials and Methods*), a partial *msrA* cDNA was obtained by PCR amplification of bovine adrenal medulla cDNA. The PCR product was sequenced and found to encode amino acid sequences predicted to be inside of peptides I, II, and III (228 bp). This PCR product was then used to screen an adrenal medulla cDNA library. Several cDNA clones were isolated, and an insert of 1370 bp was sequenced. The sequence contains an open reading frame that encodes a protein of 255 amino acids with a calculated molecular mass of 25,846 Da and possesses sequences corresponding to all four tryptic peptides (Fig. 1). The translation termination codon TAA is followed by the conventional polyadenylation signal AATAAA 581 bp downstream. Alignment of the *E. coli* and bovine MsrA amino acid sequences with the TFasta program (22) reveals a 61% identity in a 199-amino acid overlap region (Fig. 2).

Expression, Purification, and Characterization of the Mammalian MsrA Protein. To confirm that the *msrA* cDNA encodes the MsrA protein, the entire open reading frame was subcloned into the pGEX-2T vector to create a fusion protein with glutathione S-transferase (GST). A culture of SK8779 containing the plasmid was induced with IPTG, and an S-30

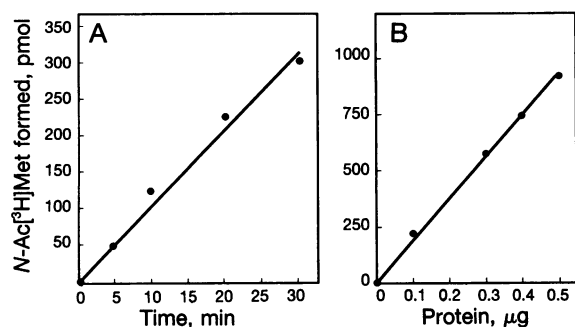


FIG. 4. Effects of time (*Left*) and protein concentration (*Right*) on the formation of *N*-Ac³H]Met. Details of the assay are described elsewhere (18) and in Table 1, except that for one assay 0.2 μg of purified bovine MsrA was added (*A*), and for the other the reaction was carried out for 30 min at 37°C (*B*).

sequences of a 1370-bp cDNA fragment containing the gene has been determined. It contains an open reading frame of 699 bp, starting 64 bp downstream of the cloning site. The *msrA* cDNA contains 601 bp of untranslated region downstream of the termination codon before the start of the deduced poly(A) tail. The deduced amino acid sequence reveals a 61% identity with a 199-amino acid overlap of the *E. coli* gene. Although antibodies raised against either the *E. coli* or bovine MsrA do not cross-react with the other protein (data not shown), in general the characteristics of the two enzymes and the specific activities of the purified enzymes are similar. For example, both enzymes can use either dithiothreitol or reduced thioredoxin as the reductant.

In analyzing the substrate specificity, it was found that eukaryotic MsrA could use as substrate Met(O) in proteins, *N*-AcMet(O), *L*-Met(O), *D*-Met(O), and a variety of sulfoxide-containing compounds including tetramethylene sulfoxide, dimethyl sulfoxide, *S*-(-)-methyl *p*-tolyl sulfoxide, *L*-ethionine sulfoxide, and, from competition results, sulindac. It appears that the enzyme prefers methyl sulfoxide residues, whereas more bulky residues attached to the sulfoxide moiety tend to inhibit the activity. For example, diphenyl sulfoxide was not a substrate for the enzyme, while *L*-ethionine sulfoxide was a substrate but less active relative to other methyl sulfoxide-containing compounds.

The substrate specificity, requirement for thioredoxin and the tissue distribution suggest that this protein is similar to the higher molecular mass (24 kDa) methyl sulfoxide reductase

Table 1. Requirements for the reduction of *N*-acetylMet(O) and of oxidized α-1-PI by bovine MsrA

Substrate	System	Activity, %
<i>N</i> -AcMet(O)	Complete	100
	-Enzyme	0
	-DTT	0
	-DTT/2-mercaptoethanol	0
	-DTT/thioredoxin system	232
Oxidized α-1-PI	Complete	100
	-Enzyme	0
	-DTT	0

The complete system contained 20 nmol of *N*-Ac³H]Met(O), 0.26 μg of purified MsrA protein, 25 mM Tris-HCl (pH 7.4), and 15 mM dithiothreitol (DTT) in a final volume of 30 μl. The reactions were carried at 37°C for 60 min. The amount of *N*-Ac³H]Met formed was determined as described (18). Activity of 100% represents 398 pmol of *N*-Ac³H]Met formed. The thioredoxin system contained 180 pmol of thioredoxin, 3 μg of thioredoxin reductase, and 50 nmol of NADPH; 15 mM 2-mercaptoethanol was added where indicated. In the case of oxidized α-1-PI, 100% activity is equal to 75% reactivation of the completely inactivated α-1-PI when tested for its ability to inhibit porcine neutrophil elastase (2).

Table 2. Relative substrate specificity of bovine MsrA

Substrate	Activity, %
<i>L</i> -Met(O)	100
<i>D</i> -Met(O)	97
<i>N</i> -AcMet(O)	91
Tetramethylene sulfoxide	98
<i>S</i> -(-)-Methyl <i>p</i> -tolyl sulfoxide	102
Dimethyl sulfoxide	81
Diphenyl sulfoxide	4
<i>L</i> -Ethionine sulfoxide	62

The assay mixture contained in a final volume of 0.5 ml 50 mM Tris-HCl (pH 7.4), 2.4 μg of MsrA, 1.5 μg of thioredoxin reductase, 2.5 nmol of thioredoxin, 50 nmol of NADPH, and 3 μM of substrate. The reactions were carried at room temperature, and 100% activity represents 3.3 nmol of NADPH oxidized per min under these conditions.

purified by Fukazawa *et al.* (14). We had reported earlier (3) that, in *E. coli*, two enzymes could be separated that reduced Met(O). One (EC 1.8.4.5) was able to reduce free *L*-Met(O), whereas the second (EC 1.8.4.6) could reduce Met(O) in peptide linkage but did not reduce free *L*-Met(O). Since the eukaryotic recombinant MsrA could reduce free *L*-Met(O), we reinvestigated the substrate specificity of recombinant *E. coli* MsrA (21). In contrast to our previous results, the *E. coli* recombinant enzyme was also able to reduce free *L*-Met(O) in addition to Met(O) in peptide linkage, as well as other substrates (Table 2). The activity of the *E. coli* MsrA toward free Met(O) was also confirmed *in vivo*. In an *E. coli* *msrA* mutant (6), the activity toward *N*-AcMet(O) was completely absent, but the activity towards free *L*-Met(O) was about 60% lower than the parental strain. Therefore, it appears that in *E. coli* there are at least two enzymes that can reduce free *L*-Met(O) residues. One has a broad specificity and can use [in addition to free *L*-Met(O)] *D*-Met(O), *N*-blocked Met(O) derivatives, including Met(O) in proteins, and other nonmethionine methyl or ethyl sulfoxide compounds. This enzyme has been referred to as "peptide-Met(O) reductase" (MsrA). Preliminary results indicate that the other enzyme uses only free *L*-Met(O) as substrate but not Met(O) in peptide linkage or other alkyl sulfoxide compounds such as those listed in Table 2 (unpublished results).

msrA mRNA is highly expressed in rat kidney (Fig. 5) as well as in human kidney (data not shown), but it is also expressed to a lesser extent in other rat tissues. In agreement with the data obtained from the mRNA levels, the highest MsrA activity was observed in the kidney. The reason for its high

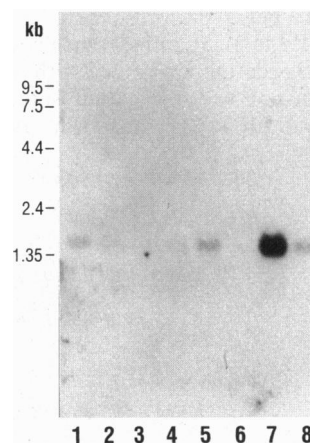


FIG. 5. mRNA blot analysis of the bovine *msrA* mRNA in various rat tissues. Rat multiple tissue blot (2 μg of mRNA in each lane) was used for the analysis. Lanes: 1, heart; 2, brain; 3, spleen; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, testis. The mRNA was hybridized with a [³²P]dCTP-labeled *msrA* DNA probe as described in text.

expression in kidney remains unclear. Preliminary results with immunocytochemical staining for MsrA in a variety of rat tissues has shown that the protein appears to be localized in the kidney medulla and in retinal pigmented epithelial cells and is most prominent in large neurons as well as in granular neurons of the dentate and cerebellum (data not shown). The retinal pigmented epithelial cells are of interest since they are phagocytic in nature and produce H₂O₂ (23).

The broad substrate specificity of MsrA leaves open the question of its normal function. It may have a dual function in being able to detoxify foreign methyl sulfoxide compounds as well as to reduce Met(O) residues in proteins. The latter may be its physiological and most important function in protecting against oxidative damage. The elucidation of the role of mammalian MsrA *in vivo* may require gene "knock-out" experiments in mice. The phenotype of such a "knock-out" could provide information about the possible involvement of the *msrA* gene in protecting against oxidative stress. Recently, we have shown that an *E. coli msrA* mutant was more sensitive to H₂O₂ than the parental strain (6). These results suggest that methionine in proteins might be a primary target for H₂O₂ oxidation, since growth inhibition of the *E. coli msrA* mutant by H₂O₂ was specifically reversed by transformation of these cells with the wild-type *msrA* gene (6). Furthermore, the expression of the enzyme is elevated in late logarithmic and stationary stages of growth in bacteria (6) and yeast (unpublished results), where there may be a high rate of protein damage without sufficient new protein synthesis to replace the damaged proteins.

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