Identification and Characterization of a Putative Active Site for Peptide Methionine Sulfoxide Reductase (MsrA) and Its Substrate Stereospecificity*

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Peptide methionine sulfoxide reductases (MsrA) from many different organisms share a consensus amino acid sequence (GCFWG) that could play an important role in their active site. Site-directed single substitution of each of these amino acids except glycines in the yeast MsrA resulted in total loss of enzyme activity. Nevertheless, all the recombinant MsrA mutants and native proteins had a very similar circular dichroism spectrum. The demonstration that either treatment with iodoacetamide or replacement of the motif cysteine with serine leads to inactivation of the enzyme underscores the singular importance of cysteine residues in the activity of MsrA. The recombinant yeast MsrA was used for general characterization of the enzyme. Its $K_m$ value was similar to the bovine MsrA and appreciably lower than the $K_m$ of the bacterial enzyme. Also, it was shown that the enzymatic activity increased dramatically with increasing ionic strength. The recombinant yeast MsrA activity and the reduction activity of free methionine sulfoxide(s) were stereoselective toward the L-methionine S-sulfoxide and S-methyl p-tolyl sulfoxide. It was established that a methionine auxotroph yeast strain could grow on either form of L-methionine sulfoxide.

Methionine residues of proteins are particularly susceptible to oxidation by almost all forms or reactive oxygen. However, unlike most other forms of oxidative damage, the oxidation of methionine residues to methionine sulfoxide (MetO) derivatives is readily repaired by the action of methionine sulfoxide reductase (MsrA), which catalyzes the thioredoxin-dependent reduction of MetO back to methionine (Reaction 1).

\[
\text{MetO + Th(TH}_2\text{)} \rightarrow \text{Met + Th(S-S)}
\]

**REACTION 1**

Significantly, selective oxidation of methionine residues of some enzymes leads to complete loss or alteration of their catalytic activity, whereas oxidation of methionine residues of other proteins is restricted to surface-exposed residues whose oxidation has little or no effect on biological function. This has led to the propositions (a) that interconversion of methionine residues between oxidized and reduced forms may serve as an important mechanism for the regulation of some enzyme activities, analogous to that obtained by phosphorylation/dephosphorylation cycles (1) or (b) that the cyclic oxidation and reduction of surface-exposed methionine residues of other proteins may serve as a first line of antioxidant defense against reactive oxygen-mediated damage (2). The ability of MsrA to reduce free MetO as well as protein-bound MetO leads to the possibility that the enzyme may be involved in salvation of free methionine from free MetO. This role of MsrA could have other partners since it has been shown that both *Escherichia coli* and *Saccharomyces cerevisiae* possess an activity that can specifically reduce free MetO and cannot reduce protein-bound MetO or any methyl sulfoxide group (3, 4). Null mutants of msrA in bacteria and yeast exhibited lower survival rates under oxidative stress conditions than their parent strains. Moreover, overexpression of the *msrA* cDNA from bovine in human T lymphocytes (Molt-4) and *S. cerevisiae* resulted in a much higher survival rate under oxidative stress condition in comparison to their control cells (5, 6). In addition, the null msrA mutant in yeast was shown to accumulate free and protein-bound MetO when the cells were exposed to hydrogen peroxide or 2,2'-azobis(2-aminopropane) dihydrochloride (4). These results demonstrated for the first time that MsrA can reduce free and protein-bound methionine sulfoxide to methionine in *vivo* and that accumulation of oxidized form of methionine could lead to physiological malfunctions and to enhanced cell death. It therefore seems reasonable that tissues containing high levels of MsrA are sensitive targets to oxidative stress (for example, in macrophages (7), neutrophils (8), kidney, and pigment epithelial cells of the retina (7)). We show here that amino acid substitutions in a highly conserved region of the MsrA leads to inactivation of MsrA activity, suggesting that this region embodies the active catalytic site. We show further that the enzyme possesses a β-strand, revealed by circular dichroism spectroscopy, and that the β-strand content is unaffected by mutations in the conserved motif, consistent with the conclusion that these mutations do not lead to major alterations in the secondary structure. In addition, we have examined the kinetics and stereospecificity of peptide MsrA and free methionine sulfoxide reductase (FMsr) from several sources (bacteria, yeast) and have examined the possibility that there is competition between MsrA-catalyzed reduction of free forms of MetO stereoisomer.

**EXPERIMENTAL PROCEDURES**

Site-directed Mutagenesis of MsrA—All constructs for the expression and isolation of mutated yeast MsrA proteins were made using the pQE30 vector (Qiagen), harboring the yeast MsrA gene, as a template (4). To create an amino acid substitution in the recombinant MsrA, we used the Stratagene kit for mutagenesis (QuickChange site-directed mutagenesis). The following sets of primers were used, separately, for the amplification of the expression vector pQE30 harboring each mu-
tation for a single amino acid (the changes from the native sequence are given in bold letters).

Set 1: A 5’ sense primer
where Cys26 was mutated to give Ser (5'-CACCATTACATGGTGGAGACTTTGGGTACAGAACAGCAGACGAGGTTTACAGAAC) and its compatible reverse complement primer.

Set 2: A 5’ sense primer where Phe26 was mutated to give His (5’-CACATTACATGGTGGATGTCACTTGGGGTACAGAAC) and its compatible reverse complement primer.

Set 3: A 5’ sense primer where Gly26 was mutated to give Ala (5’-CACATTACATGGTGGATGTTTTTGGGTTTCAGAACAGACGAGGTTTACAGAAC) and its compatible reverse complement primer.

Set 4: A 5’ sense primer where Trp26 was mutated to give Ala (5’-CACATTACATGGTGGATGTTTTTGGGTTTCAGAACAGACGAGGTTTACAGAAC) and its compatible reverse complement primer.

Set 5: A 5’ sense primer where Gly26 was mutated to give Ala (5’-CACATTACATGGTGGATGTTTTTGGGTTTCAGAACAGACGAGGTTTACAGAAC) and its compatible reverse complement primer.

Every set of primers was added separately for the polymerase chain reaction using the pQE30 harboring the mrsa gene as a template. Polymerase chain reaction was performed for one cycle of 30 s at 94 °C, followed by 30 cycles of 30 s at 94 °C, 60 s at 55 °C, and 12 min at 68 °C. The polymerase chain reaction products were treated with DpnI. The DpnI was used to digest the parental DNA template and select for the desired DNA construct, and existence of the construct was performed for each plasmid construct, and existence of the desired mutation in each case was verified. The 6-His-tagged recombinants of the various MarsA mutants were made by overexpressing them in E. coli and purified to homogeneity by adsorption on nickel nitrilotriacetic acid resin, as described previously (4).

Circular Dichroism (CD) Spectrum of Native and Mutants of Yeast MsrA Protein—CD spectra were performed with a J-710 spectrometer using 0.2-mm water-jacketed cylindrical cells. The temperature of cells was controlled by an external programmable water bath (Neolab RTE-110). Spectra were corrected for the solvent CD signal. CD spectra were normalized for protein concentration using the value of 116.2 for the enzyme mean residue molecular weight. For determining secondary structure content L-Met-(−) sulfoxide was analyzed by HPLC chromatography. The reaction mixture (30 ml) was added to reprecipitate the free sulfoxide. The resulted material was dialyzed against buffer A at 4 °C. The protein solution was then applied to a sizing column Ultrogel AcA 54 (Amersham Pharmacia Biotech) and fractionated using a gradient starting at 20 ml buffer A containing 1M ammonium sulfate and ending at buffer A.

Partial Purification of FMsr from E. coli—The screening for the FMsr was performed by using the TLC assay for the conversion of free methionine (L-[3H]MetO) to methionine, as described above. Activity measurements using either DTT or the thioredoxin system gave 34,000 pmol/min/Met formed when dabsyl-MetO was used as a substrate, 4,200 pmol/min/Met formed when L-[3H]MetO was used as a substrate, and 4 nmol of NADPH oxidized/min when Me_SO was used as a substrate under the conditions described under “Experimental Procedures.”

The activities of yeast MsrAs, mutants, and native recombinant proteins were assayed with various substrates. Each of the MsrA proteins (2.5 μg) was incubated either with dabsyl-MetO, L-[3H]MetO, or Me_SO, as previously described (“Experimental Procedures”). 100% activity represents 43,000 pmol/min/Met formed when dabsyl-MetO was used as a substrate, 4,200 pmol/min/Met formed when L-[3H]MetO was used as a substrate, and 4 nmol of NADPH oxidized/min when Me_SO was used as a substrate under the conditions described under “Experimental Procedures.”

### Table I

| Amino acid substitution substrates | Dabsyl-MetO | Me_SO | L-[3H]MetO |
|-----------------------------------|------------|------|------------|
| Native MsrA                       | 100        | 100  | 100        |
| Gly98 → Ser                       | 37         | 40   | 39         |
| Cys26 → Ser                       | 0          | 0    | 0          |
| Phe26 → Ala                       | 0          | 0    | 0          |
| Phe26 → His                       | 0          | 0    | 0          |
| Trp26 → Ala                       | 0          | 0    | 0          |
| Gly26 → Ala                       | 17         | 19   | 18         |

### Determination of MsrA Activity—The ability of methionine sulfoxide reductase to reduce free methionine sulfoxide was assayed by using L-[3H]MetO as substrate, as described by Brot et al. (10) or by using L-[35S]MetO, prepared as described below. The reaction mixture (30 μl) contained 20 mM dithiothreitol (DTT), 25 mM Tris-HCl (pH 7.4), various concentrations of L-[3H]MetO, L-[35S]MetO or L-[35S]-Me2SO and pure yeast MsrA protein or cell extract. After incubation at 37 °C for 30 or 60 min, the reaction mixtures containing radiolabeled substrates were analyzed by thin-layer chromatography (TLC). The reaction mixture was stopped by adding 0.33 mM MetO and methionine, and conversions of L-[3H]MetO, L-[35S]MetO or L-[35S]-Me2SO to L-[3H]Met or L-[35S]Met were analyzed by thin-layer chromatography on a silica gel plate using the solvent consisting of n-butanol:acetic acid:water (60:12:25). After ninhydrin treatment of the plate, the spot that corresponded to the migration of Met was extracted by water, and the radioactivity was measured. Reaction mixtures containing nonradioactive substrates were analyzed by HPLC chromatography. The reaction mixtures were passed through microconcentrators (Microcon 3, Amicon), and the flow-through was collected and derivatized by OPA and then injected onto a 15-cm C18 column (Apex, Jones Chromatography, Denver, CO) for free amino acid analysis, as described previously (11).

The reduction of protein-bound methionine sulfoxide by MsrA was assayed using dabsyl-MetO as described above. A unit of activity was defined as the change in absorbency at 340 nm at room temperature (3).

Partial Purification of FMsr from E. coli—The screening for the FMsr was performed by using the TLC assay for the conversion of free methionine (L-[3H]MetO) to methionine, as described above. Activity measurements using either DTT or the thioredoxin system gave 34,000 pmol/min/Met formed when dabsyl-MetO was used as a substrate, 4,200 pmol/min/Met formed when L-[3H]MetO was used as a substrate, and 4 nmol of NADPH oxidized/min when Me_SO was used as a substrate under the conditions described under “Experimental Procedures.”

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| Native MsrA                       | 100        | 100  | 100        |
| Gly98 → Ser                       | 37         | 40   | 39         |
| Cys26 → Ser                       | 0          | 0    | 0          |
| Phe26 → Ala                       | 0          | 0    | 0          |
| Phe26 → His                       | 0          | 0    | 0          |
| Trp26 → Ala                       | 0          | 0    | 0          |
| Gly26 → Ala                       | 17         | 19   | 18         |
However, mutation of either Gly 24 or Gly 28 resulted in only partial (60–80%) loss of MsrA activity. Although mutations in these amino acids caused alteration of MsrA activities, the Cys25 mutant or the native protein (Fig. 1). These small differences could not account for the total loss of MsrA activity in its N terminus (14). We chose the yeast MsrA (4) to serve as the model for our mutagenesis study and checked the ability of each of its different mutants to reduce free and protein-bound MetO and Me₂SO. As shown in Table I, mutations of motifs Cys²⁵, Phe²⁶, or Trp²⁷ led to complete loss of the ability of MsrA to catalyze reduction of either MetO, dabsyl-MetO, or Me₂SO. The recombinant enzymes from different species were determined using dabsyl-MetO as substrate. It was found that the recombinant enzymes from S. cervisiae and Bos taurus had a similar K_m value (44 and 33 μM, respectively), whereas the E. coli MsrA had a higher K_m value (120 μM). Among the various conditions under which the enzymatic activity of MsrA was tested, it was found that a high ionic strength increased the specific activity of the MsrA enzyme in a linear manner (Fig. 3). NaCl, KCl, NaF, and Na₂SO₄ had a similar effect on the enzyme activity, whereas LiCl and NaBr inactivate the enzyme. It is known that iodoacetamide is able to alkylate thiol groups of Cys residues in proteins. As shown in Fig. 2, the MsrA activity was inhibited by iodoacetamide, which implies that Cys residues have been modified.

RESULTS

Site-directed Mutagenesis in a Putative Active Site of MsrA—
The conserved motif of the amino acids GCFWG in the N terminus of all known MsrAs (except for the Neisseria gonorrhoeae MsrA) prompted us to check if a single substitution of each of these amino acid would lead to inactivation of enzyme activity without causing a major change in protein structure. The MsrA from N. gonorrhoeae (denoted as Pilb) is different from the rest of the other MsrAs by its molecular mass (58 kDa versus 20–26 kDa) and the fact that it has a thioredoxin motif in its N terminus (14). We chose the yeast MsrA (4) to serve as the model for our mutagenesis study and checked the ability of each of its different mutants to reduce free and protein-bound MetO and Me₂SO. As shown in Table I, mutations of motifs Cys²⁵, Phe²⁶, or Trp²⁷ led to complete loss of the ability of MsrA to catalyze reduction of either MetO, dabsyl-MetO, or Me₂SO. However, mutation of either Gly²⁴ or Gly²⁶ resulted in only partial (60–80%) loss of MsrA activity. Although mutations in these amino acids caused alteration of MsrA activities, the corresponding recombinant proteins behaved as the native MsrA recombinant protein in regard to their level of expression in E. coli and their solubility in the E. coli extracts.

The CD spectrum of the native and mutant forms of yeast MsrA exhibited similar patterns that were slightly altered in the case of the Trp²⁷ and Phe²⁶ mutants in comparison to the Cys²⁵ mutant or the native protein (Fig. 1). These small differences could not account for the total loss of MsrA activity in these mutants. Under denaturing conditions (50 °C) the CD spectra of the proteins were the same and totally different than under non-denaturating conditions (20 °C) (see “Discussion”). Far-UV CD spectra of the MsrA protein at pH 7.5 indicated that the native protein has –11% α-helix, –65% β-strand, and ~22% random structures, and ~2% turns at 20 °C (Fig. 1). Since Cys²⁵ was found to be essential for MsrA activity (Table I), we examined the ability of iodoacetamide to...
we compared the abilities of L-Met, L-Met-Dabsyl-MetO to methionine in the absence of MsrA activity. The yeast MsrA enzyme has been shown to equally reduce both (S)-sulfoxide and (S)-Methyl p-Tolyl Sulfoxide—

The yeast MsrA enzyme represents NaBr; the closed circle represents NaF; the open triangle represents KCl; closed squares represent NaCl; the open square represents LiCl.

**TABLE II**

**Survival rates of methionine auxotroph yeast cells growing on either L-Met-(S)-sulfoxide or L-Met-(R)-sulfoxide**

| Medium                      | Number of colony-forming units (40 μl of cells after 1:10^4 dilution) |
|-----------------------------|-------------------------------------------------------------------------|
| Synthetic medium + Met      | 0                                                                        |
| Synthetic medium + Met-His  | 164                                                                     |
| Synthetic medium + Met-(S)-sulfoxide | 116                                                                  |
| Synthetic medium + Met-(R)-sulfoxide | 177                                                                  |

were less effective. None of the salts promoted reduction of the Dab-sulfoxide-MetO in the absence of MsrA.

**Stereospecificity of MsrA toward the Reduction of Free L-Met-(S)-sulfoxide and (S)-Methyl p-Tolyl Sulfoxide**

The MsrA protein has been shown to equally reduce both d and l forms of free MetO (3). However, the enzyme exhibits high specificity in its ability to reduce the R and S forms of MetO. As shown in Table III, MsrA reduces L-Met-(S)-sulfoxide but not L-Met-(R)-sulfoxide. This was verified also by comparing the ability of MsrA to reduce the R and S forms of methyl p-tolyl sulfoxide. When yeast MsrA (2.5 μg) was incubated with 3 μM of either S-methyl p-tolyl sulfoxide or R-methyl p-tolyl sulfoxide as described under “Experimental Procedures”, the S form was reduced at a rate of 4.0 nmol/min, but the R form was not reduced at all. Furthermore, it was found that the ability of MsrA to reduce 35S-labeled free Met-(S)-sulfoxide was unaffected by the presence of an 18-fold excess of nonradioabeled free Met-(R)-sulfoxide. It is therefore evident that Met-(R)-sulfoxide is not a substrate for MsrA nor can it compete with Met-(S)-sulfoxide for binding to the catalytic site.

To investigate the possibility that another in vivo biological system might be able to utilize L-Met-(R)-sulfoxide for methionine biosynthesis, we compared the abilities of L-Met, L-Met-(S)-sulfoxide, and L-Met-(R)-sulfoxide to support growth of a methionine auxotroph strain of yeast. As summarized in Table II, all three substrates were able to support growth of the methionine auxotroph. Thus, although Met-(R)-sulfoxide cannot be reduced to methionine by MsrA, yeast must possess an alternative route by which the R isomer can be converted to methionine. Yeast and other organisms contain two MetO reductases, one (MsrA) that can reduce both free and protein-bound MetO, and another (FMsr) that is specific for free MetO. Therefore the ability of the methionine auxotroph to grow on Met-(R)-sulfoxide might reflect a lack of stereospecificity of the FMsr enzyme. To test this possibility we examined the ability of crude extracts of mnrA null mutants of E. coli and yeast (i.e., extracts of strains that contains FMsr but no MsrA) to reduce the R and S isomers of 35S-labeled MetO. As shown in Table III, extracts of both null mutants exhibited the same stereospecificity as did MsrA. They both reduced the S isomer but not the R isomer. It was further shown that partially purified FMsr preparations of the E. coli enzyme exhibited the same ste-

**TABLE III**

**Stereospecific reduction of L-Met-(S)-sulfoxide by FMsr from E. coli and S. cervisiae**

| E. coli protein extracts                  | Specific activity | +L-Met-(S)-O | +L-Met-(R)-O |
|------------------------------------------|------------------|--------------|--------------|
| Wild-type parent strain                  | 12.0             | 0.0          |
| msrA null mutant strain                  | 3.3              | 0.0          |
| Partly purified FMsr (SK8779)            | 495.0            | 0.0          |
| S. cervisiae protein extracts             |                  |              |
| Wild-type parent strain                  | 15.3             | 0.0          |
| mnrA null mutant strain (H9)             | 4.0              | 0.0          |
| mnrA::URA3 (H8ΔmrsA::URA3)               |                  |              |
DISCUSSION

In this study we describe the identification of a region in the Msra protein (GCFWG) that is shared with 100% conservation by all known Msra proteins, except for the one from N. gonorrhoeae (Pilb), which is about double the size of other Msras and contains a thioredoxin like motif in its N-terminal region. Site-directed mutagenesis of each of the amino acids in this region resulted in either total loss of Msra activity (when residues Cys, Phe, and Trp were mutated) or caused the enzyme to be less active (when either of the two Gly residues were mutated) (Table I). None of the mutations had a significant effect on the secondary structure of the Msra, as judged by CD and far UV spectroscopy, which could account for the total loss of their Msra activity. It was noted that in each of the Trp27 and Phe26 mutants, an aromatic amino acid residue was replaced with a non-aromatic residue. This may cause a conformational strain on the polypeptide backbone, as reflected by the slightly altered CD spectra of these mutant proteins. For comparison, the Cys25 substitution showed exactly the same CD spectra as the native protein, since in this case there was no aromatic amino acid involved. This possible explanation was suggested by Fasman (15). Overall, it seems to us that the shift that was observed with regard to the Trp27 and Phe26 mutants is not due to denaturation of the proteins nor to small conformational differences, which would lead to a total loss of activity (Fig. 1). The crystal structure of the Msra protein is not yet known, but results of preliminary studies with crystals of the yeast Msra confirms the presence of a high level of $\beta$-strand configuration, consistent with our CD results. The core of the consensus motif (CFW) appears to be the most important from the standpoint of Msra activity.

The observation that Msra activity is inhibited by iodoacetamide is consistent with the proposition that cysteine sulfhydryl groups are important for catalytic activity. This together with the consideration that Cys25 is the only Cys residue conserved in Msra protein from several sources and the fact that substitution of serine for Cys25 leads to inactivation of the enzyme suggests that alkylation of Cys25 is mainly responsible for the inactivation of Msra activity by iodoacetamide and invites speculation that Cys25 may participate in the transfer of electrons from thioredoxin/thioredoxin reductase system to MetO. Among other possibilities, it seems plausible that the reduction of MetO occurs by way of Reactions 2–6 as follows.

\[
\begin{align*}
&MetO + ESH \rightarrow Met + ESOH \\
&ESOH + Th(SH)_{2} \rightarrow ES-SThSH + H_{2}O \\
&ES-SThSH \rightarrow ESH + Th(S-S) \\
&NADPH + H^{+} + Th(S-S) \rightarrow NADP^{+} + Th(SH)_{2} \\
&\text{Sum: NADPH + H^{+} + MetO} \rightarrow \text{NADP^{+} + Met}
\end{align*}
\]

Reactions 2–6

in which ESH and ESOH refer to the reduced and sulfenic acid derivatives of Cys25 in Msra, and Th(SH)$_{2}$ and Th(S-S) refer to the fully reduced and oxidized forms of thioredoxin, respectively.

DTT could replace thioredoxin in the in vitro system. However, this mechanism does not account for the fact that Phe26 and Trp27 are important players in the catalytic action of Msra. Perhaps their proximity to the active cysteine moiety confers upon it unusual catalytic properties (viz. a favorable redox potential), but a direct role of these residues in the transfer of electrons is not excluded.

The demonstration that the $K_{m}$ value for peptide-linked MetO for Msra from bacteria is 3-fold higher than in eukaryotes is interesting, but its physiological relevance, if any, is not obvious. It is well known that the cellular regulation of critical enzymes in metabolism often involves alterations in the $K_{m}$ values for substrates in response to interactions of the enzymes with allosteric effectors. The possibility that cellular regulation of Msra is subject to regulation of $K_{v}$ values for MetO should be considered.

The finding that activity of Msra is greatly increased by high ionic strength (Fig. 3) may have physiological implications. The level of Msra is highest in the kidney medulla (3, 7) where the loop of Henle resides and is known to reach high levels of osmolality (in humans the maximum is 1400 mosmol/liter and in some rodents is even higher (16). Thus the enhanced Msra activity in high ionic strength may reflect the need to operate maximally in the kidney where reduction of MetO might serve an important salvage function for the recovery of methionine from oxidatively damaged proteins following their degradation by proteolytic enzymes that discriminate between native and oxidized proteins (2, 17, 18, 19).

The demonstration that yeast Msra is stereospecific for Met(-S)-sulfoxide (Table IV and Ref. 20) is true also for the bovine enzyme (21). To the contrary it was reported that the dabsyl-Met(-R)-sulfoxide is a better substrate for Msra in extracts of polymorphonuclear leukocytes (22). The reason for this difference in stereospecificity is not evident. It is significant that FMr, the enzyme that is specific for free MetO (cannot reduce protein-bound MetO), is also stereospecific. Like Msra from bacteria, yeast, and bovine, FMmr is able to reduce the S isomer but not the R isomer of free MetO (Table III). This discounts the possibility that regeneration of methionine from protein-bound R-MetO could involve proteolytic degradation of the protein to release free R-MetO, followed by its reduction by FMmr. Nevertheless, the further observation that a methionine auxotroph of yeast is able to grow on both R and S forms of MetO (Table II) shows that in vivo, yeast are endowed with the ability to convert both R and S isomers of MetO to methionine. These results highlight the need for further studies to determine the mechanism by which Met(-R)-sulfoxide is converted to methionine (i.e. does it reflect the presence of a racemase able to
interconvert the $R$ and $S$ forms, or does it involve a more complicated mechanism?)

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