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Cloning and characterization of antioxidant enzyme methionine sulfoxide-*S*-reductase from *Caenorhabditis elegans*

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Abstract

Methionine (Met) residues in proteins are susceptible to oxidation. The resulting methionine sulfoxide can be reduced back to methionine by methionine sulfoxide-*S*-reductase (MsrA). The MsrA gene, isolated from *Caenorhabditis elegans*, was cloned and expressed in *Escherichia coli*. The resultant enzyme was able to revert both free Met and Met in proteins in the presence of either NADPH or dithiothreitol (DTT). However, approximately seven times higher enzyme activity was observed in the presence of DTT than of NADPH. The enzyme had an absolute specificity for the reduction of L-methionine-*S*-sulfoxide but no specificity for the *R* isomer. K_m and k_{cat} values for the enzyme were ~1.18 mM and 3.64 min^{-1}, respectively. Other kinetics properties of the enzyme were also evaluated.

Keywords: Catalytic efficiency; *Caenorhabditis elegans*; MsrA; Methionine sulfoxide; Methionine-*S*-sulfoxide reductase

Increased levels of protein oxidation have been implicated in the etiology of a panoply of pathologies, most notably arthritis, Alzheimer's disease, multiple sclerosis, cardiovascular dysfunction, neurodegenerative diseases, and aging [1–8]. Oxidatively damaged proteins have been demonstrated to increase as a function of age. On the other hand, life has developed various repair systems, which can sometimes restore the catalytic activities of proteins. Among the major components of the system are various antioxidant enzymes, including superoxide dismutase, catalase, and methionine sulfoxide reductase (Msr),[2] which are involved in the amelioration of the deleterious effects of ROS [9,10]. Many recent studies have demonstrated that those proteins are also involved in the delay or prevention of aging and senescence related to oxidative damage, as well as the delays in the progression of various diseases [11–13].

Unlike other antioxidant enzymes, Msr can convert damaged products (i.e., methionine sulfoxide in proteins or free methionine sulfoxide) back to their original states (i.e., methionine in proteins or free methionine, respectively) [14]. This ability of the enzyme has been hypothesized to play an important role in the protection of cells against oxidative damages via two mechanisms. The first of these mechanisms involves the repair of important proteins. The oxidation of the important Met residues in essential proteins tends to be a lethal event, unless a mechanism exists which can repair the damage inflicted on these proteins. The second mechanism is a sink for some form of ROS which involves the use of methionine residues in proteins and free methionine. The cyclic

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[2] *Abbreviations used:* Msr, methionine sulfoxide reductase; MsrB, methionine sulfoxide reductase B; MsrA, methionine sulfoxide reductase A.

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oxidation and reduction of methionine residues in proteins and free methionine with ROS and Msr, respectively, results in an effective reduction in cellular ROS levels. Thus, this mechanism plays an important role in maintaining the integrity and/or activity of a multiplicity of proteins and other biomolecules [14,15]. Although another form of the enzyme, methionine sulfoxide reductase B (MsrB), has been reported in recent studies to reduce methionine-*R*-sulfoxide to methionine, methionine sulfoxide reductase A (MsrA) is a principle enzyme, which can reduce methionine-*S*-sulfoxide back to methionine in the presence of reduced thioredoxin or dithiothreitol, and has been observed to exert a pronounced effect on the reduction of the isomers to support the survival of an organism. MsrA is also called methionine sulfoxide-*S*-reductase [16–18].

Many forms of ROS are known to oxidize methionine residues in proteins, creating a mixture of the *R*- and *S*-isomers of methionine sulfoxide. This study was undertaken to characterize the MsrA enzyme isolated from *Caenorhabditis elegans*. MsrA catalyzes the thioredoxin-dependent reduction of the S-isomer of methionine sulfoxide to methionine. This enzyme is of singular importance in metabolism, as it provides a mechanism by which the oxidized methionine residues in proteins can be repaired [14]. This attempt to characterize the MsrA enzyme in *C. elegans* could pave the way for further investigations of this important enzyme. Because of its small size, rapid life cycle, ease of laboratory cultivation and genetic manipulation, and its susceptibility to inbreeding and cross-breeding, the soil nematode *C. elegans* is widely used as a model organism for studies in animal development and animal behavior.

Materials and methods

Cloning and expression

The PCR amplification was carried out to obtain the full length of the MsrA cDNA, using the Nematode Lambda cDNA Library of *C. elegans* (Stratagene, La Jolla, CA, USA). The following primers were used: the sense, 5′-GGTATTGAGGGTCGCATGGCTTATTTG GAGCGTG-3′, and the antisense, 5′-AGAGGAGA GTTAGAGCCTTTAGCATGACAGTTTC-3′. The amplified fragment was 656 bp in size, including additional sequences at both ends of the main cDNA for purpose of directional insertion. The fragment was inserted into pET-30Xa/LIC (Novagen, Madison, WI, USA) according to the manufacturer's recommendations. The nucleotide sequence of the cloned insert was conformed by sequencing at least twice in each direction. Then, *Escherichia coli* BL21(DE3) was transformed with the cloned plasmid. Subsequently, the MsrA protein was expressed in BL21(DE3) cells and isolated as follows: cells were grown in Luria–Bertani broth medium (BD Diagnostic Systems, Sparks, MD, USA) with 50 μg/ml kanamycin at 37 °C to $A_{600} = 0.45$, incubated with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h at 37 °C, harvested by centrifugation at 8000*g* for 25 min at 4 °C, and stored at −72 °C for 24 h. To purify the MsrA protein, cells were resuspended at 4 °C in B-PER bacterial protein extraction reagent (Pierce Biotechnology, Rockford, IL, USA) and vortexed vigorously for 10 min. Insoluble material was removed by centrifugation at 14,000*g* for 30 min and the supernatant was kept at 4 °C until use. The expression system has a low yield of the enzyme but the yield was enough for the enzyme assay below.

MsrA purification

The supernatant was loaded onto either 1 or 5 ml HiTrap affinity columns (Amersham Biosciences, Piscataway, NJ, USA) which were chelated with nickel and equilibrated with 50 mM Tris–Cl buffer (pH 8.0) at 4 °C containing 0.5 M NaCl and 100 mM imidazole. Then, the MsrA protein was eluted with 50 mM Tris–Cl buffer (pH 8.0) at 4 °C containing 0.5 M NaCl and 500 mM imidazole. The eluent was dialyzed in a Slide-A-Lyzer Dialysis Product (10,000 MWCO, Pierce Biotechnology) against 50 mM Tris–Cl buffer (pH 8.0) containing 0.1 mM EDTA at 25 °C for 2 h and then 4 °C for 12 h. The dialyzed protein was loaded onto a Mono-Q anion exchange column (Amersham Biosciences) equilibrated with 50 mM Tris–Cl buffer (pH 8.0) at 4 °C, and then eluted at a flowrate of 1 ml/min, with a 10-column volume gradient from 0 to 1 M NaCl using a Waters 625 LC system (Waters, Milford, MA, USA). Fractions corresponding to MsrA, as determined by SDS–PAGE analysis and assay, were pooled. After dialysis overnight against 2 L of 20 mM Tris–Cl buffer (pH 7.4), the protein was concentrated with a centrifugal filter device (10,000 MWCO, Millipore, Billerica, MA, USA). Protein concentrations were determined by the Bradford method, using bovine serum albumin (Sigma Chemical, St. Louis, MO, USA) as a standard. The MsrA protein was stored at −20 °C until needed.

Substrates preparation

The different diastereomers of methionine sulfoxide were prepared as described by Moskovitz et al. [19]. In brief, 0.3 g of L-methionine-*R*,*S*-sulfoxide (Sigma Chemical) was dissolved in 2 ml of distilled water in a boiling water bath. After slight cooling, a solution of picric acid in methanol (0.5 g/4 ml) was added and, after further cooling, the resultant precipitate was collected on cellulose paper, washed with methanol, and redissolved in 20 ml of distilled water. In order to liberate the sulfoxide, amylamine was added until, pH 8.0, was reached. L-Methionine-*S*-sulfoxide was reprecipitated by the