HHS Public Access

Author manuscript

Free Radic Biol Med. Author manuscript; available in PMC 2020 August 06.

Published in final edited form as:

Free Radic Biol Med. 2019 February 01; 131: 154–161. doi:10.1016/j.freeradbiomed.2018.12.001.

Drosophila methionine sulfoxide reductase A (MSRA) lacks methionine oxidase activity

Sreya Tarafdar, **Geumsoo Kim**, **Rodney L. Levine***

Laboratory of Biochemistry, National Heart, Lung, and Blood Institute, Bethesda, MD 20892, United States

Abstract

Mouse, human, and *E. coli* methionine sulfoxide reductase A (MSRA) stereospecifically catalyze both the reduction of S-methionine sulfoxide to methionine and the oxidation of methionine to S-methionine sulfoxide. Calmodulin has 9 methionine residues, but only Met77 is oxidized by MSRA, and this is completely reversed when MSRA operates in the reductase direction. Given the powerful genetic tools available for *Drosophila*, we selected this model organism to identify the *in vivo* calmodulin targets regulated by redox modulation of Met77. The active site sequences of mammalian and *Drosophila* MSRA are identical, and both contain two cysteine residues in their carboxy terminal domains. We produced recombinant *Drosophila* MSRA and studied its biochemical and biophysical properties. The enzyme is active as a methionine sulfoxide reductase, but it cannot function as a methionine oxidase. The first step in the mammalian oxidase reaction is formation of a sulfenic acid at the active site, and the second step is the reaction of the sulfenic acid with a carboxy terminal domain cysteine to form a disulfide bond. The third step regenerates the active site through a disulfide exchange reaction with a second carboxy terminal domain cysteine. *Drosophila* MSRA carries out the first and second steps, but it cannot regenerate the active site in the third step. Thus, unlike the *E. coli* and mammalian enzymes, *Drosophila* MSRA catalyzes only the reduction of methionine sulfoxide and not the oxidation of methionine.

Keywords

Drosophila; Methionine sulfoxide reductase; Methionine oxidase; Calmodulin

1. Introduction

Methionine residues in proteins are subject to oxidation to methionine sulfoxide (MetO), particularly if they are solvent exposed [1]. Endogenously or exogenously produced reactive nitrogen and oxygen species can mediate the oxidation. *In vitro*, hypochlorous acid, a major halogenated oxidant generated by leukocytes, reacts rapidly with methionine at physiological pH [2,3]. Hydrogen peroxide is not particularly reactive, although the rate can be accelerated by the bicarbonate/ carbon dioxide present *in vivo* [4]. MetO is reduced back to methionine by the methionine sulfoxide reductases, thioredoxin-dependent enzymes that are virtually universal among aerobic organisms [5,6]. Oxidation of methionine to MetO

*Corresponding author: rlevine@nih.gov (R.L. Levine).

introduces a chiral center at the sulfur atom so there are two epimers of MetO, R-MetO and S-MetO [7]. While generation of MetO is facile and can occur non-enzymatically, the sulfoxide cannot be reduced back to Met by incubation with high concentrations of physiological reducing agents such as NADH or glutathione nor by dithiothreitol or phosphines. *In vivo*, reversal of the oxidation is mediated by the methionine sulfoxide reductases (MSR). The MSRA class of reductases specifically reduces S-MetO, but not R-MetO. Conversely, the MSRB class reduces R-MetO, but not S-MetO. Recycling by the reductases allows the methionine residue to react again with oxidizing species, creating a system with catalytic efficiency in scavenging reactive species [8]. Fig. 1 shows the catalytic cycles.

The mechanism of the MSRA reductase reaction has been established in detail [9]. From microorganisms to mammals, the sequence of the active site is highly conserved, Gly-Cys-Phe-Trp-Gly, and it usually is located in the amino terminal domain of the protein, which is the case for *E. coli*, mammals, and *Drosophila* (Fig. 2). The cysteine residue has a low pK_a that facilitates its oxidation to the sulfenic acid by MetO. In general, thioredoxin reduces the sulfenic acid back to cysteine, although the pathway for reduction varies among species. In some organisms, thioredoxin reacts directly with the sulfenic acid at the active site. In many other organisms, one or two additional cysteine residues situated in the carboxy terminal domain participate in the reduction. These cysteines are termed “recycling” or “resolving cysteines”. *E. coli* and mammals have two recycling cysteines spaced about 9 residues apart. In the mouse or human, the active site cysteine sulfenic acid (Cys72 in the mouse) reacts with the first recycling cysteine (Cys218) to form a disulfide bond. These disulfide-linked residues undergo an exchange with the second recycling cysteine (Cys227) leading to formation of a disulfide between Cys218 and Cys227 and reduction of the active site Cys72 back to its thiol form. Thioredoxin reduces the carboxy terminal domain disulfide, fully regenerating the active form of the reductase. *Drosophila* also has 2 cysteine residues in its carboxyl terminal domain, separated by 13 residues (Fig. 2). Thus, the *Drosophila* MSRA may be catalytically and functionally similar to mammalian MSRA, but an experimental test is essential.

The *in vivo* importance of the reductases in protecting against oxidative stress is well established. Knocking out the enzyme caused increased susceptibility to oxidative stress in mice [10], yeast [11], and bacteria [12–15]. Conversely, overexpressing MSRA conferred increased resistance to oxidative stress in *Drosophila* [16], *Saccharomyces* [17], *Arabidopsis* [18], PC-12 cells [19], human T cells [17], in micro-glial-mediated neuroinflammation [20], and in *Helicobacter pylori* in which MSRA reduces MetO in the bacterial catalase [15,21]. Notably, overexpression of bovine MSRA in *Drosophila* almost doubled the lifespan of the flies [16], and this lifespan extension has been replicated in an independent laboratory using *Drosophila* MSRA [22].

Thus, methionine oxidation is a reversible covalent modification analogous to phosphorylation and dephosphorylation. Cyclic oxidation and reduction of methionine residues might therefore also function as a regulatory or signaling mechanism [23,24]. If oxidation were to occur non-enzymatically, the products would then be a mixture of the R and S epimers. Reversal would then require coordinated action of MSRA and MSRB which

is not an attractive regulatory mechanism. Enzymatic oxidation would likely be stereospecific and thus require coupling to only one reductase to complete the regulatory cycle.

The Terman and Gladyshev laboratories have identified an intriguing example of regulation by methionine oxidation [25–28]. These investigators established that the oxidation and reduction of a specific methionine in actin is enzymatically mediated and is specific for the R-epimer of MetO. Oxidation of actin is catalyzed by a family of NADPH oxidoreductases, the MICALs. Oxidation caused severing of actin filaments and decreased polymerization. MSRB1 reduces the methionine sulfoxide, restoring the capacity of actin to polymerize.

In the case of MSRA, which is specific for the S-epimer, no separate methionine oxidase has been described. However, mammalian MSRA itself is a bifunctional enzyme, capable of mediating both the reduction of S-MetO to Met and the oxidation of Met to S-MetO [29]. A simple mechanism for regulating whether the enzyme functions as a reductase or as an oxidase has been proposed [29]. A number of proteins are substrates for the MSRA oxidase *in vitro*, including actin, α_1-antitrypsin, glutamine synthetase, peroxiredoxin 6, and calmodulin [30]. The oxidation of calmodulin is particularly intriguing because calmodulin has 9 methionine residues, all of which are susceptible to oxidation by hydrogen peroxide to yield a mixture of the R and S epimers. However, MSRA stereospecifically oxidizes only Met77 to S-MetO. Because the oxidation is stereospecific, MSRA can completely reverse the oxidation when operating in the reductase direction.

There are hundreds of known targets of calmodulin [31], but it is not known if any are regulated through reversible oxidation of Met residues in calmodulin. We reasoned that identification of these targets could be facilitated by switching our studies from mice to *Drosophila*, where powerful genetic techniques could be utilized. The active site of *Drosophila* MSRA is identical to that in the mouse, and it has two cysteine residues in its carboxy terminal domain, presumed to be the recycling cysteines. As a first step in this project, we cloned, expressed, purified, and characterized *Drosophila* MSRA. We show here that the enzyme is active in the reductase direction, but unexpectedly, it cannot function as an oxidase. We report investigations that elucidate why it is not an oxidase.

2. Materials and methods

2.1. Reagents and recombinant proteins

L-Methionine Sulfoxide (MetO) (Cat # M1126) and iodoacetamide (Cat # I6125) were obtained from Sigma-Aldrich. MetO-hexapeptide [Pro-Met(O)-Ala-Ile-Lys-Lys or PM(O)AIKK] and Met-hexapeptide [Pro-Met-Ala-Ile-Lys-Lys or PMAIKK] were synthesized by American Peptide (Sunnyvale, CA). Dithiothreitol was Pierce # 20291 and acrylamide was Affymetrix # 32800, purchased from Thermo Fisher Scientific. Hydrogen peroxide, 30%, was a product of Acros, product # UN2014. Sequencing grade chymotrypsin (V106A) and sequencing grade trypsin (with reductively methylated lysine residues and treated with tosyl phenylalanyl chloromethyl ketone, V5111) were purchased from Promega. Restriction digestion enzymes and the Quick Ligase enzyme used for cloning purposes were obtained from New England Biolabs.

Drosophila Eip71CD, which is *msrA*, was PCR amplified from the *Drosophila* Gene Gold library obtained from the *Drosophila* Genomics Resource Center (Bloomington, IN). The cDNA clone for *Eip71CD* from the Gold collection was found to harbor a lysine residue at position 96 instead of threonine as is found in wild type *Drosophila*. Thus, Lys-96 was mutated to threonine by site directed mutagenesis with the QuikChange® II XL kit from Stratagene. The kit was also used to create MSRA mutant C232S/C246S. Using *Eco*RI, *Nde*I, and *Kpn*I sites, wild-type full-length *Drosophila msrA* or *cam* (calmodulin, also from the *Drosophila* Gene Gold library) were cloned into the pET17b expression vector downstream of the isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible T7*lac* promoter.

Recombinant non-myristoylated mouse MSRA without the mitochondrial targeting sequence was prepared and purified as described [32]. Recombinant *Drosophila* calmodulin, *Drosophila* MSRA, and chimeric MSRA were produced in BL21(DE3) *E. coli* grown at 37°. When the cultures expressing *Drosophila* calmodulin reached an OD_{600} of 0.6, they were induced with 500 μM IPTG and grown for another 4 h. Cells expressing *Drosophila* MSRA were initially grown at 37°. When they reached an OD_{600} of 0.6, the temperature was lowered to 25°. They were induced with 300 μM IPTG and grown overnight.

Each step in the purification was carried out at 4°, except for HPLC column chromatography which was conducted at room temperature. Cells were lysed using a French press (Thermo Spectronic) in buffer A (50 mM Na_2HPO_4, pH 7.4, 1 mM diethylenetriaminepentaacetic acid [DTPA], 1 mM phenylmethylsulfonyl fluoride, protease inhibitor cocktail (1:100 dilution of Calbiochem # 539134), and benzonase nuclease (EMD Millipore # 70746), diluted 1:1000 to a final concentration of 25 U/ml lysate). After centrifugation for 30 min at 27,000*g*, the supernatant was made 1% in streptomycin sulfate and rocked for 30 min to precipitate nucleic acids. After centrifugation again for 30 min at 27,000*g*, the supernatant was brought to 80% saturation in ammonium sulfate and rocked for 30 min. After centrifugation for another 30 min at 27,000*g*, the pellet was redissolved in and then dialyzed against buffer B (50 mM Na_2HPO_4, pH 7.4, 1 mM DTPA) at 4°.

Using an Agilent 1100/1200 HPLC, protein solutions were loaded onto a DEAE anion-exchange column (TSK-GEL DEAE-5PW, TOSOH Bioscience, LLC # 07574; 21.5 mm × 15 cm; particle size: 13μ) equilibrated with buffer B. Proteins were eluted by a gradient of 3%/min buffer C (50 mM Na_2HPO_4, pH 7.4, 1 mM DTPA, 1 M NaCl) at a flow rate of 3 ml/min. Eluted fractions were subjected to SDS-PAGE followed by Coomassie Brilliant Blue staining. Fractions containing protein were pooled and dialyzed against buffer B. The sample was concentrated to 3 ml with a 10 kDa cutoff Amicon Ultra-15 centrifugal filter (Millipore # UFC901024), after which solid ammonium sulfate was added with stirring to a final concentration of 1 M. The solution was then loaded onto a phenyl column (TSKgel Phenyl-5PW, TOSOH Bioscience, LLC # 07656; 21.5 mm × 15 cm; 13μ particle size) equilibrated with buffer D (50 mM Na_2HPO_4 buffer, pH 7.4, 1 mM DTPA, 1 M ammonium sulfate). Proteins were eluted by a gradient of 3%/min of buffer D to buffer B with a flow rate of 3 ml/min. Fractions were analyzed by SDS-PAGE with Coomassie Brilliant Blue staining. Fractions containing purified protein were pooled and dialyzed against buffer B. The calmodulin content of the fractions was quantitated from the fluorescence of the Coomassie Blue bands after SDS gel electrophoresis, using BSA as a standard [33]. MSRA

solutions were quantitated by their absorbance at 280 nm, using ε_M = 31,860 as calculated by GPMAW version 11 (Lighthouse Data, Odense, Denmark).

Purity of the protein preparations was determined from the areas of peaks in their HPLC chromatograms monitored at 210 nm. For calmodulin it was 91%, for wild-type MSRA 88%, and for MSRA C232S/C246S mutant 83%. Proteins were stored at −80°.

Chimeric MSRA constructs contained residues Mouse$_{1–198}$-*Drosphila*$_{233–246}$ and *Drosophila*$_{1–232}$-Mouse$_{199–213}$. The PCR amplified genes were cloned into the pET28b vector using the *Nde*I and *Bam*HI sites and transformed into BL21(DE3) cells with a kanamycin resistance marker. The expression vector pET28b has an N-terminal 6xHis tag followed by a thrombin protease cleavage site upstream of the first residue of the MSRA chimera. Transformed cells were induced overnight with 300 μM IPTG at 25 °C when the O.D$_{600}$ = 0.6. The Mouse-*Drosophila* chimera was produced as a soluble protein while the *Drosophila*-Mouse chimera was in inclusion bodies. Inclusion bodies were extracted as described [34], and the protein was solubilized with 6M guanidine hydrochloride.

Both chimeric proteins were purified by metal-chelate affinity chromatography, with 6M guanidine hydrochloride included in the buffer for the *Drosophila*-Mouse chimera. The fractions containing purified *Drosophila*-Mouse chimera were subjected to refolding at a protein concentration of 0.08 mg/ml by dialysis against 25 mM Na_2HPO_4, 1 mM DTPA, 10 mM DTT, pH 7.67. Much of the refolded protein was insoluble, but sufficient soluble protein was recovered to support the reported experiments.

2.2. Assays

Amino acid analysis of Met was performed as described [35] except that the column was a Zorbax Eclipse AAA (#966400–902) fitted with a guard column (Agilent #820950–931), and the gradient program was from Agilent [36]. Reductase activity was determined with 2 different substrates, free MetO and a synthetic peptide, PM(O)AIKK. For determination of enzyme kinetics, care was taken to assure that substrate consumption was ≤ 15%. Wild type MSRA (2.8 μM) was incubated with free MetO (0.25–10 mM) for 30 min or with MetO-hexapeptide (0.25–5 mM) for 6 min. Assays with the C232S/C246S mutant were performed at a protein concentration of 4 μM with MetO-hexapeptide at 0.1–0.5 mM. The incubations were performed at 25° in 50 mM Na_2HPO_4, pH 7.4, 1 mM DTPA, and 10 mM dithiothreitol. To determine the stoichiometry of the reaction mechanism, dithiothreitol was omitted, MSRA concentration was 20 μM, MetO concentration was 1 mM, and amino acid analyses were performed in triplicate on 2 different days. All reactions were stopped by the addition of acetic acid to a final concentration of 0.5%. The Met-hexapeptide was measured by HPLC-mass spectrometry [37]. K_m and V_{max} were obtained by plotting the product formation versus substrate concentration and fitting the curve to a hyperbola with Prism version 7 (GraphPad Software, La Jolla, CA).

Oxidase activity was also measured with two substrates, the Met containing form of the hexapeptide, PMAIKK, and *Drosophila* calmodulin. Incubations were performed at 25° in 50 mM Na_2HPO_4, pH 7.4, with 10 mM MetO as the oxidizing substrate. The hexapeptide concentration was 25–500 μM and that of wild-type *Drosophila* MSRA was 3.7 μM. After

30 min the reaction was stopped by making the solution 0.5% in trifluoroacetic acid. Assays with the C232S/C246S mutant were performed at a protein concentration of 4 μM with hexapeptide at 0.1 mM.

When *Drosophila* calmodulin was used as an oxidase substrate, its concentration was 20 μM, *Drosophila* MSRA was 4 μM, and mouse MSRA was 5 μM. Incubations were at 25° in 50 mM Na_2HPO_4, pH 7.4, with 0.5 mM calcium chloride and 10 mM MetO. After 1 h, reactions were stopped by adding trifluoroacetic acid to a final concentration of 0.1%. Oxidized hexapeptide or oxidized calmodulin were measured by HPLC-mass spectrometry [30].

The initial step in both the reductase and oxidase reactions is the oxidation of the active site cysteine thiol to the sulfenic acid which is a reactive intermediate. It can be trapped by reaction with dimedone [38]. *Drosophila* MSRA, 5.6 μM, was incubated with 1.45 mM dimedone in 20 mM Na_2HPO_4, pH 7.4, at 25° for 10 min 10 mM MetO was then added and incubation continued for 1 h. The mass of the MSRA was measured by HPLC-mass spectrometry as described below. Formation of a covalent adduct with dimedone increases the mass by 138 Da.

2.3. HPLC-mass spectrometry

Protein separations and mass determinations were performed on a Zorbax 300 Å StableBond C18 MicroBore column (1.0 × 50 mm, 3.5 μm particle size, Agilent #865630–902) with an Agilent 1200 series high pressure liquid chromatography system equipped with an autosampler set to 4° and a column compartment set to 30°. The initial solvent was water/0.05% trifluoracetic acid and proteins were eluted by a gradient of 2%/min acetonitrile/0.05% trifluoroacetic acid with a flow rate of 20 μL/min. For separation of native and oxidatively modified calmodulin in the oxidase assay, the gradient was 0.5%/min from 30% to 50%. Effluent from the column was mixed in a tee with 20 μL/min neat acetic acid just prior to the electrospray needle to displace the bound trifluoroacetic acid and generate internal standards [39,40]. Peptide separation and sequencing was carried out as for proteins except that the gradient was 1%/min from 0% to 45%.

Electrospray mass spectrometry was performed on an Agilent Model 6520 accurate mass quadrupole-time of flight instrument. Positive electrospray ionization spectra were obtained in the mass range of 100–2500 *m/z*. The drying gas temperature was 350° with a flow rate of 10 L/min and a nebulizer pressure of 2 bar. The voltages were capillary 3500 V, fragmentor 235 V, skimmer 65 V, and octopole 1 750 V. MS/MS fragmentation used a collision energy of 30 V with a data collection range of 20–2000 *m/z*. Mass spectra were analyzed using Agilent software, MassHunter version B.05. Predicted MS/MS spectra were generated by GPMAW and matched to the experimentally obtained spectra. The sequences were independently confirmed by *de novo* sequencing with PEAKS version 7.0 (Bioinformatics Solutions, Waterloo, ON).

2.4. Peptide mapping for cysteine status by double alkylation

In this procedure, *Drosophila* MSRA was first alkylated with iodoacetamide to label cysteines with a free thiol. Then disulfide bonds were reduced and the newly reduced thiols

were labeled with acrylamide. Those cysteines that had a free thiol have a mass increase of 57.1 Da while those that were in disulfide linkage have an increase of 71.0 Da, allowing calculation of the oxidized fraction of each cysteine containing peptide.

MSRA, 40 μg, was incubated with 10 mM MetO in 25 mM Na_2HPO_4, pH 8.0 containing 1 mM DTPA in a total volume of 50 μL for 30 min at 25°. The solution was then made 6 M in guanidine hydrochloride by adding 1 mg solid guanidine hydrochloride for each μL. The solution was made 10 mM in iodoacetamide and incubated in the dark for 15 min at 25°. It was then brought to 10% in ice-cold trichloroacetic acid, incubated on ice for 5–10 min, and centrifuged for 6 min at 16,000*g*. The supernatant was discarded and the pellet containing protein, residual trichloroacetic acid, and precipitated guanidine was extracted twice with 0.5 ml ice-cold ethanol:ethyl acetate (1:1 v/v), centrifuging 6 min at 16,000*g* after each extraction. After air drying, the protein was redissolved in 25 mM Na_2HPO_4, pH 8.0, 1 mM DTPA and made 6 M in guanidine and 10 mM in dithiothreitol. After incubation for 30 min at 25°, acrylamide was added to a final concentration of 50 mM and incubated for 45 min at 25°. The trichloroacetic acid precipitation and ethanol:ethyl acetate extraction were repeated as above. After drying, the sample was redissolved in 40 μL 25 mM Na_2HPO_4, 1 mM DTPA, pH 8.0 and subjected to chymotryptic or tryptic digestion overnight at 37° with a ratio of protease:MSRA of 1:20. The reaction was stopped by making the solution 0.1% in trifluoroacetic acid. Peptides were sequenced by HPLC-mass spectrometry.

2.5. Differential scanning calorimetry

Differential scanning calorimetry measurements were performed using a VP-DSC calorimeter (MicroCal, Northampton, MA) as described [41]. Proteins were scanned from 17° to 55° at a rate of 1°/min at a pressure of 1.9 bar. Instrument baselines were measured prior to sample runs by scanning with buffer (10 mM Na_2HPO_4, pH 7.4, 50 mM NaCl) in both reference and sample cells. Mouse MSRA and *Drosophila* MSRA were reduced with DTT just prior to scanning, and their concentration was 0.4 μM. MetO oxidized MSRA was scanned at a concentration of 5–6 μM. Scans were corrected for instrument baselines. Excess heat capacity (Cp) was expressed as cal/°C. Data conversion and analysis were performed with Origin software (OriginLab Corporation, Northampton, MA.)

3. Results

3.1. Heat stability of Drosophila MSRA

In the laboratory, *D. melanogaster* is usually bred and maintained at 18–25°, and flies experience heat shock at 37° [42]. In our initial attempt to produce recombinant MSRA in *E. coli* at 37°, we found that all of the protein was in inclusion bodies. Soluble protein was then produced by growing the *E. coli* at 25°. Given these observations, we compared the heat stability of the *Drosophila* and mouse MSRA by differential scanning calorimetry (Fig. 3). The reduced *Drosophila* MSRA begins to unfold at 37° and has a melting temperature (T_m) of 43°, while the T_m of the mouse MSRA is 49°. The unfolding is irreversible. Given this heat stability pattern, enzymatic assays of *Drosophila* MSRA were performed at 25°. The difference in stability of the two species' MSRA was eliminated when they were oxidized by MetO. The T_m of both MSRA is 48°.

3.2. Drosophila MSRA has methionine sulfoxide reductase activity

The mass of the *Drosophila* protein determined by mass spectrometry was 27,567.4 Da, in good agreement with the mass of 27,566.9 Da calculated from its sequence with the initiating methionine removed. The enzyme could reduce both free MetO and MetO in peptide linkage (Fig. 4). The kinetic parameters of the *Drosophila* and mouse MSRA are shown in Table 1. With either free MetO or the hexapeptide PM(O)AIKK as substrate, the V_{max} for the *Drosophila* enzyme is considerably less than that of the mouse enzyme. The K_m is similar. Even if one corrects for the temperature difference of the assays with a typical Q_{10} effect of 2, the *Drosophila* MSRA still has a much lower catalytic efficiency than the mammalian enzyme.

3.3. Drosophila MSRA lacks methionine oxidase activity

As noted in the introduction, as with human and mouse MSRA, *Drosophila* MSRA has two cysteine residues in its carboxy terminal domain and was thus expected to have methionine oxidase activity. Mouse MSRA effects methionine oxidation on a number of substrates, including the hexapeptide PMAIKK and calcium-bound calmodulin. We used these two substrates to compare the methionine oxidase activity of mouse and fly MSRA. The mouse enzyme oxidized both the peptide and calcium-bound *Drosophila* calmodulin, but the *Drosophila* enzyme did not (Fig. 5). However, the *Drosophila* enzyme did react with the oxidizing agent, MetO, demonstrated by a 2 Da mass decrease when incubated with one MetO [29] (Fig. 6A). We also confirmed the stoichiometry of reaction by measuring the product, Met, by amino acid analysis. We found that 0.86 ± 0.01 mol Met was produced per mol MSRA.

In our standard oxidase assay, MetO is used as the oxidizing substrate. We also performed the oxidase assay with hydrogen peroxide at 10–1000 μM concentrations and as with MetO, no methionine oxidase activity was observed and the enzyme underwent a 2 Da mass decrease when the peroxide was at least 250 μM.

We considered the possibility that only one of the two cysteine residues in the carboxy terminal domain participated in the recycling reaction, as is the case for some MSR [9]. If that were to occur, the recycling cysteine would form a disulfide with the sulfenic cysteine at the active site, preventing generation of another sulfenic cysteine that acts as the methionine oxidase [9,29]. *In vivo*, the disulfide is reduced by thioredoxin or a similar molecule. In such reductases, preventing disulfide formation by mutating all cysteines except the active site cysteine endows the enzyme with methionine oxidase activity (Kim G and Levine RL, unpublished). We therefore mutated both Cys232 and Cys246 to Ser in fly MSRA. The protein was active as a reductase but still lacked methionine oxidase activity. However, when the C232S/C246S mutant was incubated with MetO, we observed that the mass of the MSRA still decreased by 2 Da (Fig. 6A), and it increased by 2 Da when incubated with dithiothreitol. These are identical to the mass changes observed in mouse MSRA in which all but the active site cysteine residues were mutated [29]. The 2 Da decrease in the mouse protein was shown to be due to formation of a sulfenylamide bond between the active site sulfenic acid and the tryptophan in the highly conserved active site (GCFWG). We confirmed that a sulfenic acid also formed in the *Drosophila* MSRA by trapping of a

dimedone adduct. Based on the mechanism established for the mouse MSRA, we conclude that sulfenylamide formation *via* the sulfenic acid also occurs in the *Drosophila* MSRA.

3.4. Cys232 does not function as a resolving cysteine

We considered this explanation: Wild-type fly MSRA reacts with MetO to form an active site sulfenic acid and then one of the carboxy terminal domain cysteine residues rapidly reacts with the sulfenic acid to form a disulfide bond. However, the second carboxy terminal domain cysteine is unable to access that disulfide in order to mediate the disulfide exchange that occurs in the mammalian MSRA. Thus, the active site cysteine is not regenerated to react with a second molecule of MetO, as is required for the MSRA to mediate methionine oxidation [29]. This model predicts that one of the two putative recycling cysteine residues would be in disulfide linkage while the other would remain as a free thiol. The model is testable because disulfide linked cysteine residues cannot react with alkylating agents such as iodoacetamide or acrylamide, while cysteines with a free thiol do react. Reaction with iodoacetamide increases the mass of the residue by 57.0 Da while reaction with acrylamide increases the mass by 71.1 Da, a difference easily detected by mass spectrometry analysis. One can therefore label the free cysteine residues with iodoacetamide without treating the protein with a reducing agent. After this first derivatization, treatment with dithiothreitol reduces the disulfide bonds, allowing them to react with acrylamide. Carrying out this sequential alkylation tags all free cysteine residues with a 57.0 Da tag and all cysteines that were in disulfide linkage with a 71.1 Da tag [43].

We performed the double alkylation on control and on the −2 Da form generated by incubation with MetO. The double alkylated MSRA had a measured mass of 27,766.6 Da which is 199.3 Da higher than the unmodified protein that had mass 27,567.3 Da. This difference establishes that two cysteine residues were in disulfide linkage and one was free (calculated increase = 199.2 Da). The proteins were then digested with either chymotrypsin or trypsin and the peptides mapped by HPLC-mass spectrometry with sequencing by MS/MS analysis. With chymotryptic cleavage, the peptide containing the active site Cys48 spans residues 45–49. It would have a monoisotopic mass of 570.193 Da if derivatized by iodoacetamide and 584.209 Da if derivatized by acrylamide. The observed mass was 584.209 Da. MS/MS sequencing confirmed that this was the active site peptide derivatized by acrylamide (GMG-pam-C-F). The tryptic peptide containing Cys232 and Cys246 spans residues 230–246. If derivatized by one iodoacetamide and one acrylamide, its mass would be 2223.90 Da. The observed mass was 2223.90 Da. MS/MS sequencing demonstrated that Cys232 was derivatized by iodoacetamide and Cys246 by acrylamide (Table 2). In summary, the active site Cys48 and carboxy-terminal Cys246 had been derivatized by acrylamide and were thus in disulfide linkage. The other cysteine in the carboxy terminal domain, Cys232, was present as the free thiol.

3.5. Characterization of chimeric MSRA

The substantial differences in the carboxy-terminal domain of the mouse and *Drosophila* proteins suggest that they are the basis for the lack of oxidase activity in the fly enzyme. To test this suggestion, we created chimeric proteins in which the last 14 or 15 residues following the first putative cysteine in the carboxy-termini were swapped between the mouse

and *Drosophila* MSRA. We expected that the chimera containing the amino domain from mouse and the carboxy-terminus from *Drosophila* would lack oxidase activity while the chimera containing the amino domain from *Drosophila* and the carboxy-terminus from mouse would possess oxidase activity. However, as shown in Table 3, the opposite was observed. Swapping the mouse carboxy-terminus into the *Drosophila* MSRA did not confer oxidase activity. Swapping the *Drosophila* carboxy-terminus into the mouse MSRA did not cause significant loss of reductase or oxidase activity.

4. Discussion

Our primary conclusion from these studies is that wild-type *Drosophila* MSRA lacks methionine oxidase activity because Cys232 cannot function as a recycling cysteine. As a result, the active site cysteine is trapped in disulfide linkage with the C-terminal Cys246. One might expect that if the trapping were prevented, oxidase activity would be enabled. *In vivo*, trapping could be prevented by a protein-protein interaction in which a regulatory protein bound to the carboxy terminal domain of MSRA and blocked Cys246 from reacting with the active site cysteine sulfenic acid [29]. Whether this would enable oxidase activity was tested experimentally by mutating the two carboxy terminal Cys to Ser. As reported above, the Cys232S/Cys246S MSRA was active as a reductase and formed a sulfenylamide when incubated with MetO. However, unlike the single-Cys containing mutant of mouse MSRA, the fly mutant still did not exhibit oxidase activity.

The active sites of the two MSRA are located in their amino domains and are identical. The carboxy-terminal domain sequences with their putative resolving cysteines are rather different. However, chimeric constructs showed that the *Drosophila* carboxy-terminus supported oxidase activity when swapped into the mouse MSRA while swapping the mouse carboxy-terminus into *Drosophila* MSRA did not confer oxidase activity. The structural basis for oxidase activity does not lie in the carboxy-terminus. The basis for oxidase activity may be elucidated when a solution or crystal structure of *Drosophila* MSRA is obtained.

Acknowledgments

We are grateful to Nasser Rusan for introducing us to the genetics and methodology of *Drosophila*. We thank the National Heart, Lung, and Blood Institute's Biophysics Core and its director, Grzegorz Piszczek, for the thermal denaturation determinations. The National Heart, Lung, and Blood Institute's Biochemistry Core performed the amino acid analysis. This study was supported by the Intramural Research Program of the National Heart, Lung, and Blood Institute.

Abbreviations:

DTPA	diethylenetriaminepentaacetic acid
MetO	methionine sulfoxide
MSR	methionine sulfoxide reductase

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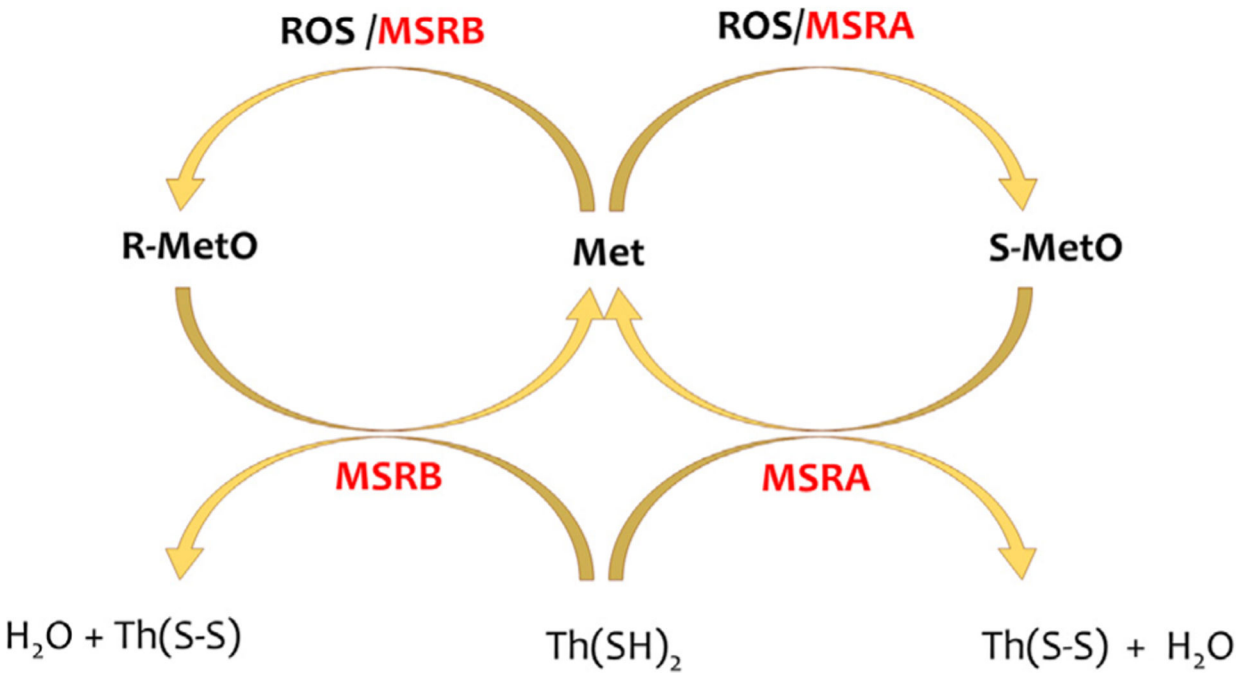

Fig. 1.

Reversible oxidation and reduction of methionine mediated by reactive oxygen species (ROS) or the MSR. Methionine oxidation by reactive oxygen species produces a mixture of two epimers, S-MetO and R-MetO. These can be reduced by two distinct, stereospecific enzymes, MSRA and MSRB. MSRA from some species such as mouse and human is bifunctional and capable of stereospecific reduction of S-MetO and oxidation of Met to S-MetO. Recently, human MSRB3 was shown to be bifunctional, capable of stereospecific reduction of R-MetO and oxidation of Met to R-MetO [44]. The oxidized forms of both MSRA and MSRB are reduced by the thioredoxin system (Th).

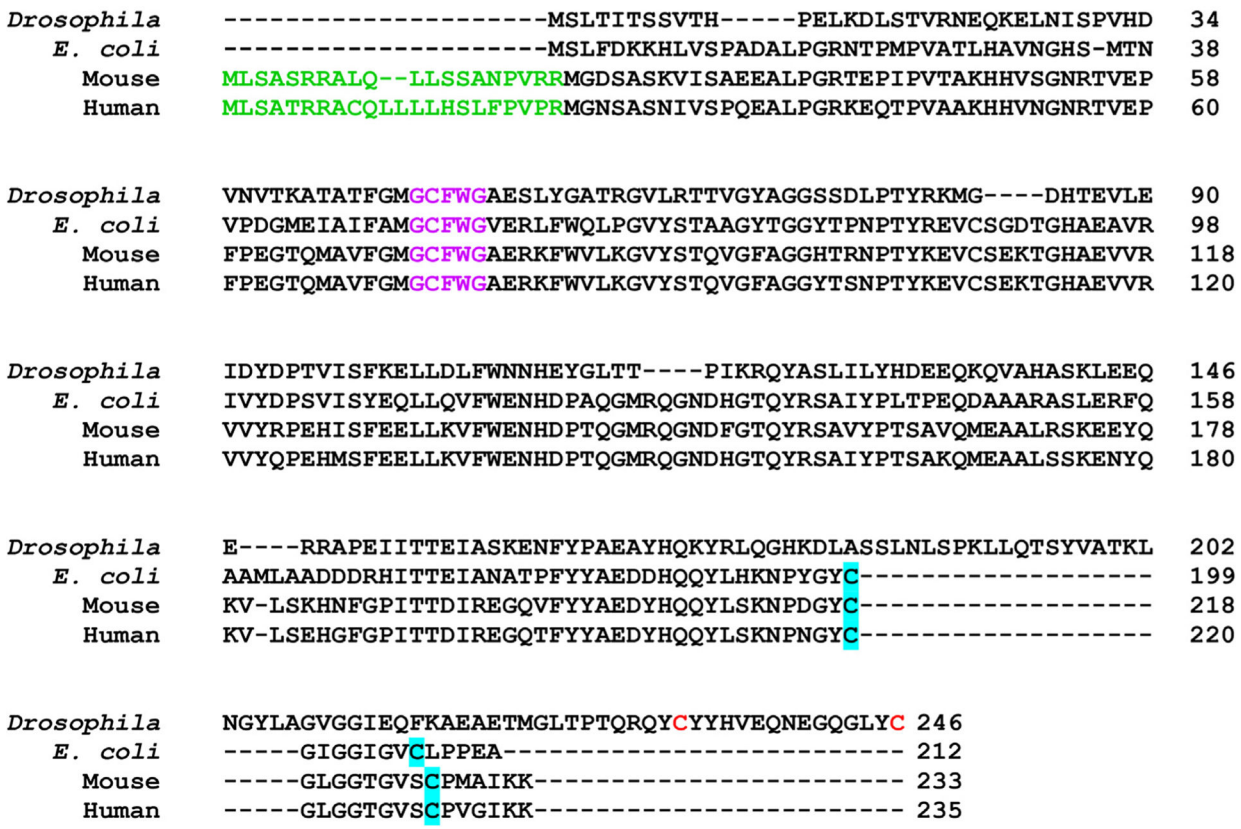

Fig. 2.

Alignment of *Drosophila, E. coli*, mouse, and human MSRA amino acid sequences. The active site sequence, GCFWG is colored magenta. The resolving cysteines in the carboxy terminal domains of *E. coli*, mouse, and human are colored cyan. The putative resolving cysteines in *Drosophila* are colored red. The mitochondrial targeting sequence of mouse and human MSRA is colored green. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

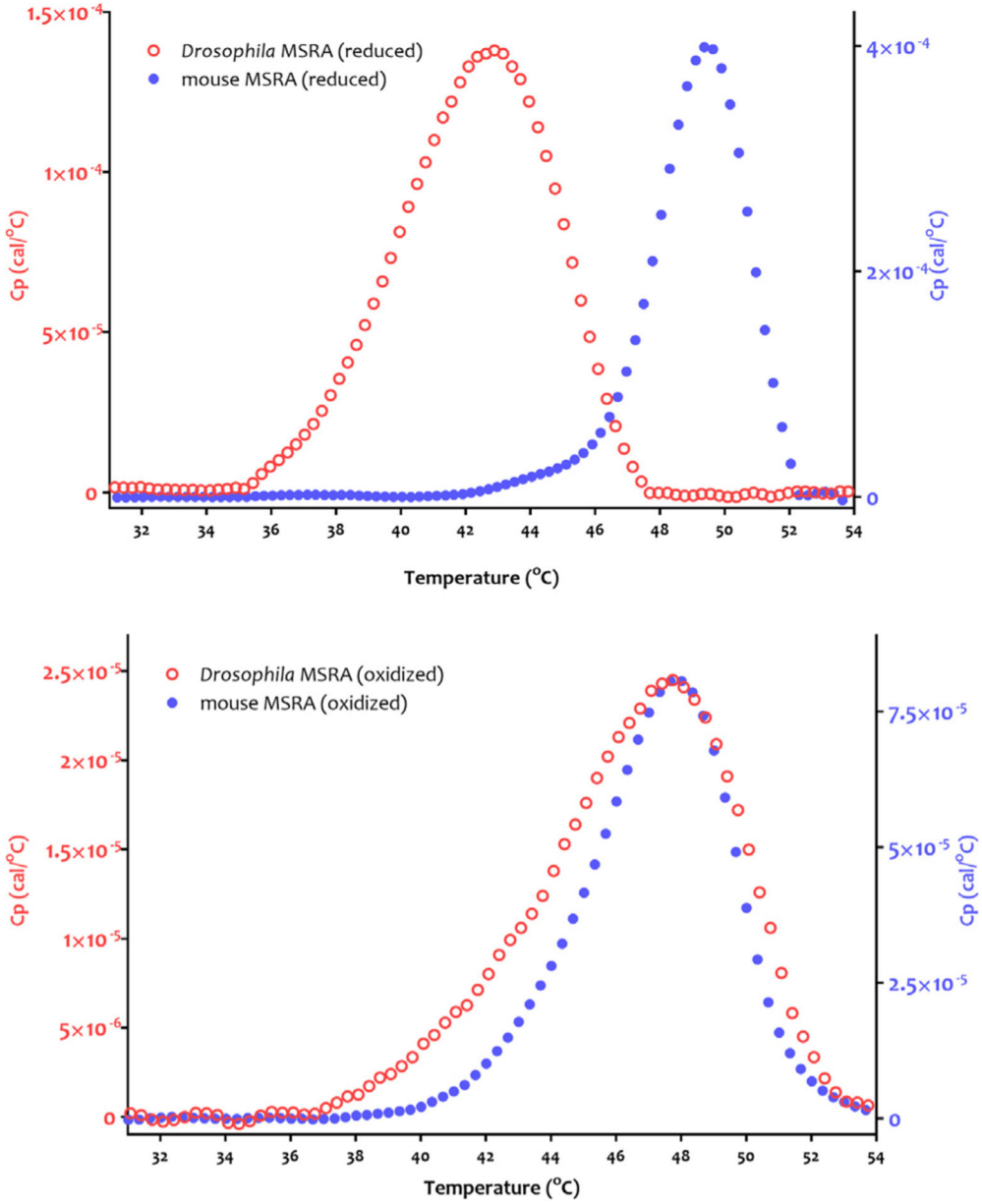

Fig. 3.

Thermal denaturation curves of *Drosophila* and mouse MSRA from differential scanning calorimetry. The excess heat capacity of *Drosophila* (○ ○ ○) and mouse (• • •) MSRA are plotted on the left and right y-axes respectively. The upper panel shows the reduced forms, and the lower panel shows the forms oxidized by MetO.

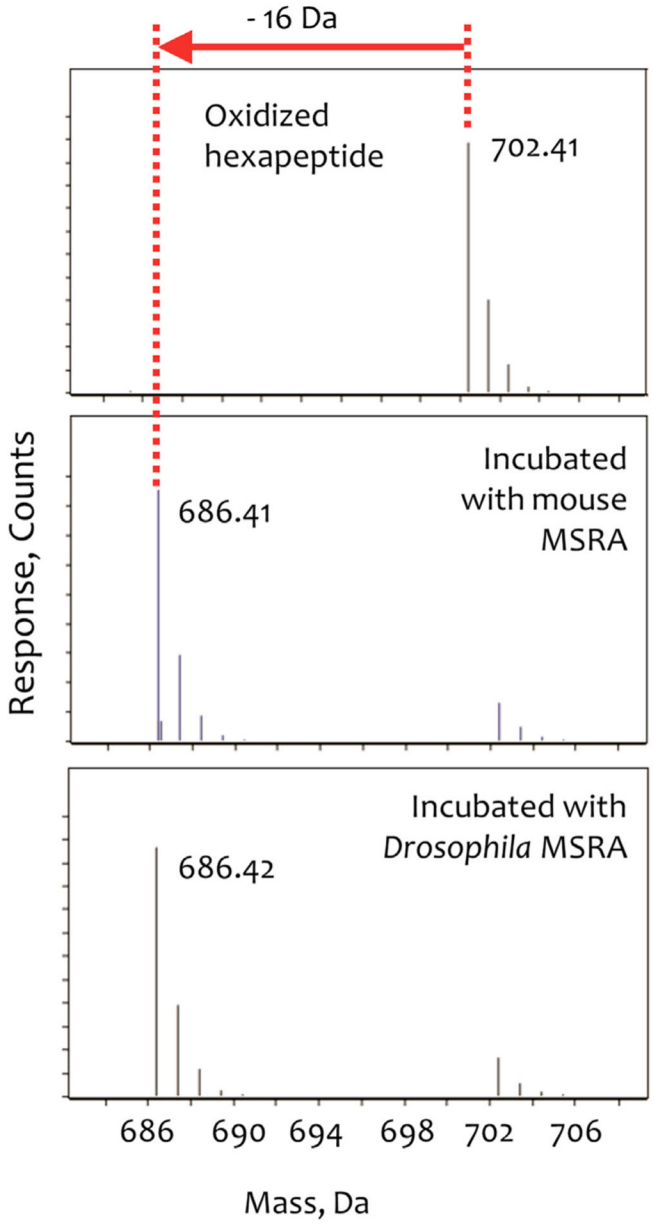

Fig. 4.

Mass spectra demonstrating reduction of the MetO hexapeptide by both *Drosophila* and mouse MSRA. Its calculated monoisotopic mass is 702.41 Da while that of the reduced peptide is 686.41 Da. The masses shown in the panels are those measured by the mass spectrometer. The untreated hexapeptide with a calculated mass of 702.41 Da is shown in the upper panel. The middle and lower panels are the spectra of peptide incubated with mouse or *Drosophila* MSRA.

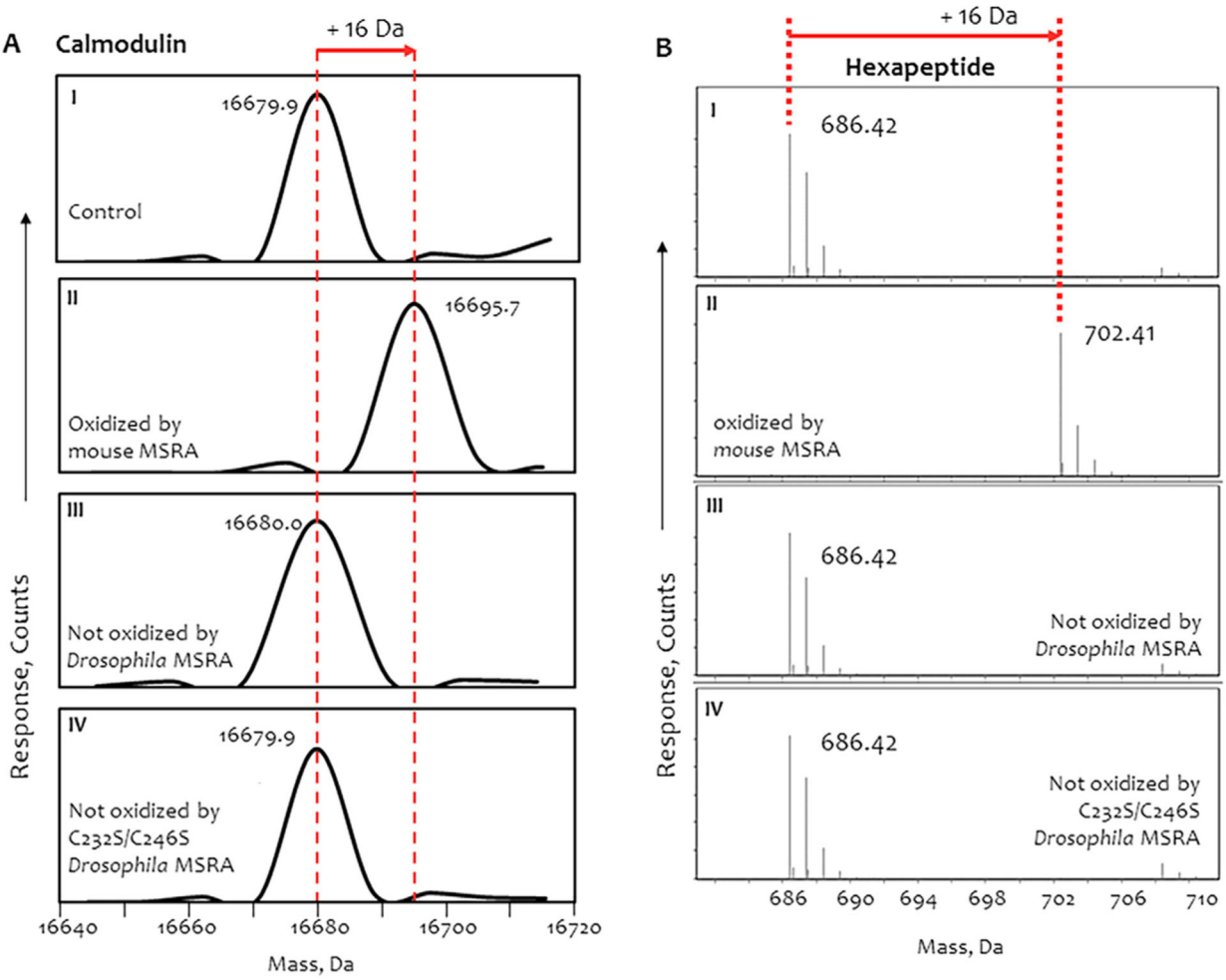

Fig. 5.

Activity of mouse or *Drosophila* MSRA as oxidases. (A) *Drosophila* calmodulin as substrate (B) Met-hexapeptide as substrate. The deconvoluted mass spectrum of the untreated controls are in the top panels. The measured calmodulin masses are the average mass while the hexapeptide masses are monoisotopic.

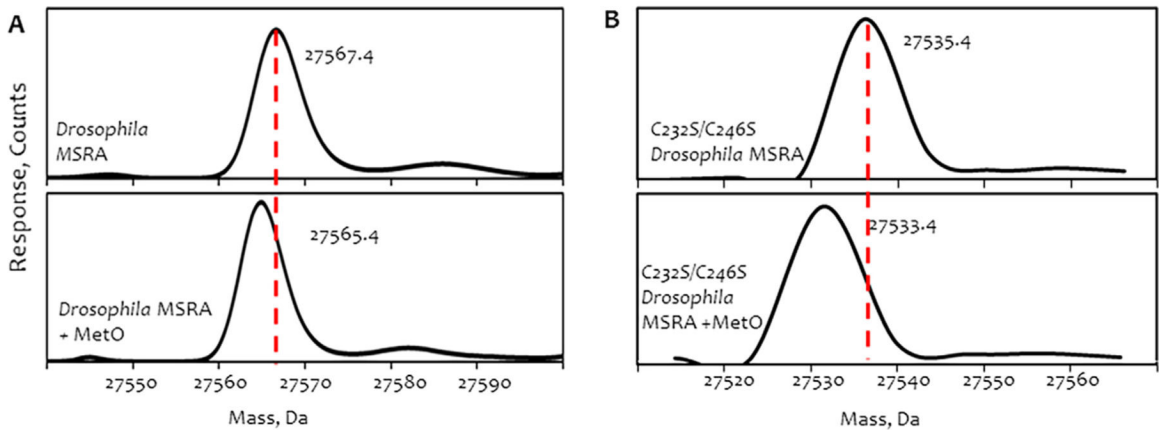

Fig. 6.

Incubation with MetO induces covalent modifications in *Drosophila* MSRA as demonstrated by mass changes. (A) wild type *Drosophila* MSRA, (B) C232S/C246S *Drosophila* MSRA.

Table 1.

Kinetic parameters of *Drosophila* and mouse MSRA.

	Drosophila		Mouse	
Substrate	**K_m (mM)**	**V_{max} (μmol min^{-1} mg^{-1})**	**K_m (mM)**	**V_{max} (μmol min^{-1} mg^{-1})**
MetO	3.6	0.15	1.0	5.0
PM(O)AIKK	0.53	0.16	3.1	6.2

The mouse parameters are from [32].

Table 2.

Sequencing of the carboxy terminal tryptic peptide by mass spectrometry.

	b-ions			y-ions		
Residue	**Calculated**		**Observed**	**Calculated**		**Observed**
Gln230	b1	129.07	129.07	-		
Tyr231	b2	292.13	292.13	Y16	2096.8	ND
cam-Cys232	b3	452.16	452.16	Y15	1933.8	ND
Tyr233	b4	615.22	ND	yl4	1773.8	ND
Tyr234	b5	778.29	ND	yl3	1610.7	ND
His235	b6	915.35	915.34	yl2	1447.6	ND
Val236	b7	1014.4	ND	y11	1310.6	ND
Glu237	b8	1143.5	1143.4	y10	1211.5	ND
Gln238	b9	1271.5	1271.5	y9	1082.4	ND
Asn239	b10	1385.6	1385.5	y8	954.4	ND
Glu240	b11	1514.6	ND	y7	840.36	ND
Gly241	bl2	1571.6	ND	y6	711.31	ND
Gln242	bl3	1699.7	ND	y5	654.29	654.31
Gly243	bl4	1756.7	ND	y4	526.23	536.24
Leu244	bl5	1869.8	ND	y3	469.21	469.21
Tyr245	bl6	2032.8	ND	y2	356.13	356.13
pam-Cys246	-	-		y1	193.06	193.06

The expected mass of each b and y ion was calculated by GPMAW and compared to the observed mass, demonstrating that Cys232 was derivatized by iodoacetamide and Cys246 by acrylamide. The error in mass measurement is ≤ 10 ppm. ND, not detected. cam-Cys is carboxyamido-Cys from derivatization by iodoacetamide and pam-Cys is proprionamide-Cys from derivatization by acrylamide.

Table 3.

Enzyme Activities in Native and Chimeric MSRA.

Protein	Residues from mouse	Residues from *Drosophila*	Reductase activity (% of Wild-Type Mouse)	Oxidase activity (% of Wild-Type Mouse)
Wild-Type Mouse	1–213	None	100	100
Mouse-*Drosophila*	1–198	233–246	99 ± 1	82 ± 13
Drosophila-Mouse	199–213	1–232	37 ± 2	0

Enzyme activities were measured with oxidized or reduced hexapeptides in 2–3 separate experiments. Activities were normalized to 100% for the wild-type mouse. The values are means ± 1 standard deviation.