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Reaction Mechanism, Evolutionary Analysis, and Role of Zinc in *Drosophila* Methionine-*R*-sulfoxide Reductase*

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Methionine residues in proteins are susceptible to oxidation, and the resulting methionine sulfoxides can be reduced back to methionines by methionine-*S*-sulfoxide reductase (MsrA) and methionine-*R*-sulfoxide reductase (MsrB). Herein, we have identified two MsrB families that differ by the presence of zinc. Evolutionary analyses suggested that the zinc-containing MsrB proteins are prototype enzymes and that the metal was lost in certain MsrB proteins later in evolution. Zinc-containing *Drosophila* MsrB was further characterized. The enzyme was found to employ a catalytic Cys^{124} thiolate, which directly interacted with methionine sulfoxide, resulting in methionine and a Cys^{124} sulfenic acid intermediate. A subsequent reaction of this intermediate with Cys^{69} generated an intramolecular disulfide. Dithiothreitol could reduce either the sulfenic acid or the disulfide, but the disulfide was a preferred substrate for thioredoxin, a natural electron donor. Interestingly, the C69S mutant could complement MsrA/MsrB deficiency in yeast, and the corresponding natural form of mouse MsrB was active with thioredoxin. These data indicate that MsrB proteins employ alternative mechanisms for sulfenic acid reduction. Four other conserved cysteines in *Drosophila* MsrB (Cys^{51}, Cys^{54}, Cys^{101}, and Cys^{104}) were found to coordinate structural zinc. Mutation of any one or a combination of these residues resulted in complete loss of metal and catalytic activity, demonstrating an essential role of zinc in *Drosophila* MsrB. In contrast, two conserved histidines were important for thioredoxin-dependent activity, but were not involved in zinc binding. A *Drosophila* MsrA gene was also cloned, and the recombinant enzyme was found to be metal-free and specific for methionine *S*-sulfoxide and to employ a similar sulfenic acid/disulfide mechanism.

The side chains of the sulfur-containing amino acid residues (cysteine and methionine) are susceptible to oxidation by reactive oxygen species (1). Such modifications may change protein function, modulate its activity, or result in a signaling event. Cellular thiol-dependent antioxidant systems, primarily thioredoxin and glutathione systems, are involved in maintaining the reduced state of cysteines and methionines.

The product of methionine oxidation is a diastereomeric mixture of methionine *S*-sulfoxide and methionine *R*-sulfoxide (2, 3). One of the enzymes that can reduce methionine sulfoxides, methionine-sulfoxide reductase (MsrA), has been known for several decades, but further studies revealed that its activity is restricted to methionine *S*-sulfoxides (3–7). Recent identification of a new methionine-sulfoxide reductase (MsrB; also called SelR) with specificity for methionine *R*-sulfoxides provided an explanation of how cells cope with racemic methionine oxidation (8–10).

Although MsrA has been well characterized structurally and functionally (4), little information on MsrB is available. Interesting aspects of MsrB function include the presence of zinc in mammalian and fruit fly MsrB proteins and the presence of selenocysteine in selenoprotein R, which is one of the mammalian MsrB proteins (8). In functionally characterized selenoproteins, selenium is located at enzyme active sites and is involved in redox reactions (11, 12), suggesting that selenocysteine in selenoprotein R and corresponding cysteines in other MsrB homologs are directly involved in catalysis (8).

The reaction mechanism of MsrA has been characterized in great detail. The enzyme employs a reactive Cys thiolate that attacks a sulfoxide (13, 14), with the formation of methionine and a Cys-based sulfenic acid intermediate (15). The short-lived sulfenic acid is then reduced by cysteines located in the C-terminal portion of the enzyme through a thiol/disulfide exchange mechanism (13, 15). The resulting C-terminal disulfide is a substrate for thioredoxin, a natural electron donor for methionine sulfoxide reduction. Crystal structures of bacterial and bovine MsrA proteins were solved, revealing an $\alpha\beta$-structural fold not found in other proteins (16, 17). MsrA has structural similarity to thioredoxin with regard to the location of a central β-sheet, surrounded by α-helixes, and to the presence of a catalytic cysteine at the N terminus of an α-helix dipole (18). The structure of MsrB is not known; but the enzyme is predicted to be a β-rich protein, and it was suggested that MsrA and MsrB independently evolved their stereo-specific methionine-sulfoxide reductase functions (8).

In this work, we identified two classes of MsrB proteins that differ by the presence of zinc. Evolutionary analyses suggested that the metal-containing form of MsrB is the prototype MsrB. To characterize the role of zinc and the reaction mechanism of the prototype enzyme, we identified zinc-binding and catalytic residues in *Drosophila* MsrB. We also report the cloning and characterization of fruit fly MsrA.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis, Expression, and Purification of Wild-type and Mutant Drosophila MsrB Proteins—The expression construct pET28_MsrB was obtained by cloning the *Drosophila* MsrB cDNA into the pET28a(+) vector (Novagen) designed for expression of N-terminal His_6-tagged proteins. *Drosophila* MsrB mutants were generated with a QuikChange site-directed mutagenesis kit (Stratagene), and all constructs were confirmed by nucleotide sequencing. Wild-type and mutant MsrB proteins were expressed in *Escherichia coli* BL21(DE3) cells and isolated as follows. Cells were grown in LB medium with 50 mg/liter

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF541958.

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kanamycin at 37 °C to A_{600} = 0.5, incubated with 1 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 37 °C, harvested by centrifugation at 5000 rpm for 15 min at 4 °C, and stored at −80 °C. To purify MsrB proteins, cells were resuspended at 4 °C in 50 mM sodium phosphate (pH 7.0) containing 300 mM NaCl and 1 mM phenylmethylsulfonyl fluoride and treated by sonication. Insoluble material was removed by centrifugation, and the supernatant was applied to a 2-ml Talon metal affinity column (CLONTECH) equilibrated with the extraction buffer. Proteins were eluted with an imidazole elution buffer, analyzed for purity by SDS-PAGE, and stored at −80 °C until used.

Determination of Methionine-sulfoxide Reductase Activity—The ability of wild-type *Drosophila* MsrB to reduce protein methionine *R*-sulfoxides was determined in reaction between dabsylated methionine *R*-sulfoxide and 10 mM DTT[1] as described previously (8). Briefly, the reaction mixture (100 μl) contained 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 30 mM KCl, 20 mM DTT, 200 μM dabsylated methionine *RS*-sulfoxide (or individual sulfoxides), and 5 μg of MsrB. The reaction mixture was incubated at 37 °C for 1 h, and the reaction was stopped by adding 200 μl of acetonitrile. After centrifugation, 20 μl of the supernatant was applied to an ODS C_{18} column (Vydac) pre-equilibrated with 0.14 M sodium acetate, 0.5 ml/liter triethylamine (pH 6.1), and 30% acetonitrile. The column was developed using a linear gradient (from 30 to 70%) of acetonitrile in the equilibration buffer, and the dabsyl derivatives were monitored by absorbance at 436 nm. Using this assay, the specific activity of wild-type MsrB was 64.3 nmol/min/mg of protein. For K_m determination, the concentrations of methionine *R*-sulfoxide and methionine *RS*-sulfoxide varied from 0.1 to 8.3 mM.

To analyze thioredoxin-dependent methionine sulfoxide reduction, the reaction mixture consisted of 50 mM Tris HCl (pH 7.4), 0.2 mM NADPH, 78 μg/ml *E. coli* thioredoxin, 3 ng/ml human thioredoxin reductase, 3 μM dabsylated methionine *R*-, *S*-, or *RS*-sulfoxide, and 5 μg of MsrB. The reaction mixture was incubated for 30 min, and the reaction was stopped by addition of 200 μl of acetonitrile. 50 μl was injected and analyzed on a reversed-phase HPLC column essentially as described above. The specific activity of wild-type MsrB was 494 pmol/min/mg in this assay. These assays were also used to characterize *Drosophila* MsrA and mouse MsrB activities.

Dabsyl derivatives of L-methionine and L-methionine *RS*-sulfoxide and individual sulfoxides were prepared as described by Minetti *et al.* (19). Briefly, amino acids were dissolved in 40 μl of $NaHCO_3$ buffer, and 80 μl of freshly prepared dabsyl chloride solution in acetonitrile was added to the mixture. The mixture was incubated at 70 °C for 15 min and spotted on a TLC plate for the separation of dabsyl derivatives from the excess of underivatized substrates. The plates were developed for 2 h with 1-butanol/acetic acid/water (60:12:25). The region containing dabsyl derivatives was scrapped and extracted with ethanol.

The different diastereomers of methionine sulfoxide were prepared by the method of Lavine (20). Briefly, 0.3 g of L-methionine *RS*-sulfoxide was dissolved in 2 ml of water in a boiling water bath. After slight cooling, a solution of picric acid in methanol (0.5 g (wet weight)/4 ml) was added; and after further cooling, the precipitate was collected on cellulose paper, washed with methanol, and redissolved in 20 ml of water. To liberate the sulfoxide, amylamine was added to pH ~8.0. L-Methionine *S*-sulfoxide was reprecipitated by addition of 200 ml of acetone and collected by centrifugation. The pellet was washed twice with acetone and dried under vacuum. The original filtrate was evaporated under vacuum and redissolved in 20 ml of water and 80 μl of amylamine, and 200 ml of acetone was added. The L-methionine *R*-sulfoxide pellet was collected by centrifugation, washed twice with acetone, and dried.

Expression of the Drosophila MsrB Gene in Yeast—The *Drosophila* MsrB gene and its C69S mutant were amplified by PCR using the pET28_MsrB construct as template. Recovered fragments were cloned into the *Sma*I site of pBluescript II KS (Stratagene) to create pBS_{DM}MsrB and pBS_{DM}MsrBC69S. *Bam*HI and *Xho*I fragments containing MsrB genes were moved to the yeast high copy expression vector p425 (kindly provided by Dr. Gary Merrill, Oregon State University) to create $p425_{DM}$MsrB and $p425_{DM}$MsrBC69S. Mutant yeast cells lacking both MsrA and MsrB genes, a methionine auxotroph strain (8), were transformed, and transformants were selected for leucine prototrophy. Cells containing plasmid only or plasmids with the inserts were grown in supplemented minimal medium (yeast nitrogen base (YNB)) and tested for H_2O_2 sensitivity and methionine sulfoxide utilization.

Zinc Content and Catalytic Activity of Drosophila MsrB Incubated with Metal Chelators—MsrB was incubated at 25 °C with 5 mM EDTA and 50 mM HEPES (pH 6.2 or 8.0) or with 20 mM CDTA in the same buffer at pH 8.0, followed by removal of the complexed metal using Microcon microconcentrators and analyses of zinc content and catalytic activity.

Expression Analyses of Drosophila MsrB—Rabbit polyclonal antibodies were raised against recombinant wild-type *Drosophila* MsrB and subsequently purified using the antibody purification kit from Pierce. 1-week-old and 1-month-old *Drosophila* flies were kindly provided by Hadise Kabil and Dr. Lawrence Harshman (University of Nebraska, Lincoln, NE). Total *Drosophila* protein extracts as well as separate body and head protein extracts were separated by SDS-PAGE and electrotransferred onto a polyvinylidene difluoride membrane (Invitrogen). Membranes were incubated with the anti-*Drosophila* MsrB antibody at a dilution of 1:750. The blots were developed using the ECL detection system (Amersham Biosciences).

Drosophila MsrA—Drosophila MsrA cDNA sequence was determined by sequencing *Drosophila* expressed sequence tag clones (Research Genetics) containing the MsrA open reading frame. The expression construct pET21_MSR was obtained by cloning the MsrA gene into the pET21 vector designed for expression of C-terminal His_6-tagged proteins. The sequences of the primers were as follows: primer 1, CGTCACAACATATGTCTCTGACTATTACTTCCAG; and primer 2, GTCGACAAGCTTGCAGTAGAGACCCTGGCC. The PCR product was inserted into pET21a(+) using the *Nde*I and *Hin*dIII sites and verified by sequencing. C-terminal His-tagged *Drosophila* MsrA was purified from *E. coli* strain BL21(DE3) as described above for MsrB. In some preparations, the C-terminal His tag was removed using a thrombin kit (Novagen), and cleavage efficiency was monitored by SDS-PAGE. The specific activity of the enzyme in the DTT assay was 214 nmol/min/mg.

Mass Spectrometry—Wild-type and mutant MsrB and wild-type MsrA proteins were analyzed by mass spectrometry in the native state and after the treatments indicated. The sulfenic acid intermediate was detected by analyzing the modified protein obtained by reaction of an active-site thiolate with 5,5-dimethyl-1,3-cyclohexanedione (dimedone). Modification reactions were performed in 50 mM Tris HCl (pH 7.4) using aliquots of 20 μM enzymes. Methionine sulfoxide was added to a final concentration of 15 mM, and the mixture was incubated for 10 min at room temperature, followed by addition of dimedone at a concentration of 10 mM and finally by incubation of the mixture overnight in the dark at room temperature. Alkylation studies were performed by incubating the enzyme with either DTT or methionine sulfoxide or, in the absence of the these two, with iodoacetate at a concentration of 1 mM for 30 min at room temperature. To remove unreacted iodoacetate, DTT was added to a final concentration of 5 mM, and the mixture was incubated for 30 min. The number of cysteines alkylated was monitored by measuring masses of treated and untreated samples.

All mass spectrometric measurements were performed on a Q-Tof hybrid quadrupole time-of-flight mass spectrometer (Micromass, Manchester, UK) using electrospray ionization. The instrument was operated in the positive ion mode over a mass range of 700–1600 Da. Mass calibration was performed by direct infusion (20 μl/min) of a 1 pmol/μl solution of horse heart myoglobin (Sigma) in 1:1 acetonitrile/water containing 0.1% formic acid. Samples were diluted by a factor of 10 with a 10% acetonitrile aqueous solution with 0.1% formic acid. The pH of the solution was adjusted to 3–4 by addition of formic acid. 50-μl aliquots of the samples were injected onto a reversed-phase protein-trapping C_4 column. The column was washed with 2× 500 μl of a 10% acetonitrile aqueous solution in 0.1% formic acid. The proteins were eluted from the trapping column at 20 μl/min using a 25-min gradient of water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). The gradient was developed using the following program: 0 min, 10% solvent B; 10 min, 20% solvent B; 13 min, 60% solvent B; 18 min, 60% solvent B; 20 min, 95% solvent B; 22 min, 95% solvent B; and 25 min, 10% solvent B. The column was equilibrated with 10% solvent B for 20 min between injections. The data were processed using MaxEnt software (Micromass) to obtain the reconstructed mass spectra of the proteins from the multiple charge state raw data.

Other Methods—Recombinant mouse MsrB in which cysteine was present in place of the natural selenocysteine was prepared as described (8). Wild-type *Drosophila* MsrA and MsrB and various MsrB mutants (~0.5 mg of each protein) were analyzed for the presence of 20 biologically relevant metals using inductively coupled argon plasma at the Chemical Analysis Laboratory of the University of Georgia. In parallel, control samples containing corresponding buffers were analyzed. No metals were detected in control samples. Sequence analyses

[1] The abbreviations used are: DTT, dithiothreitol; HPLC, high pressure liquid chromatography; CDTA, *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid.

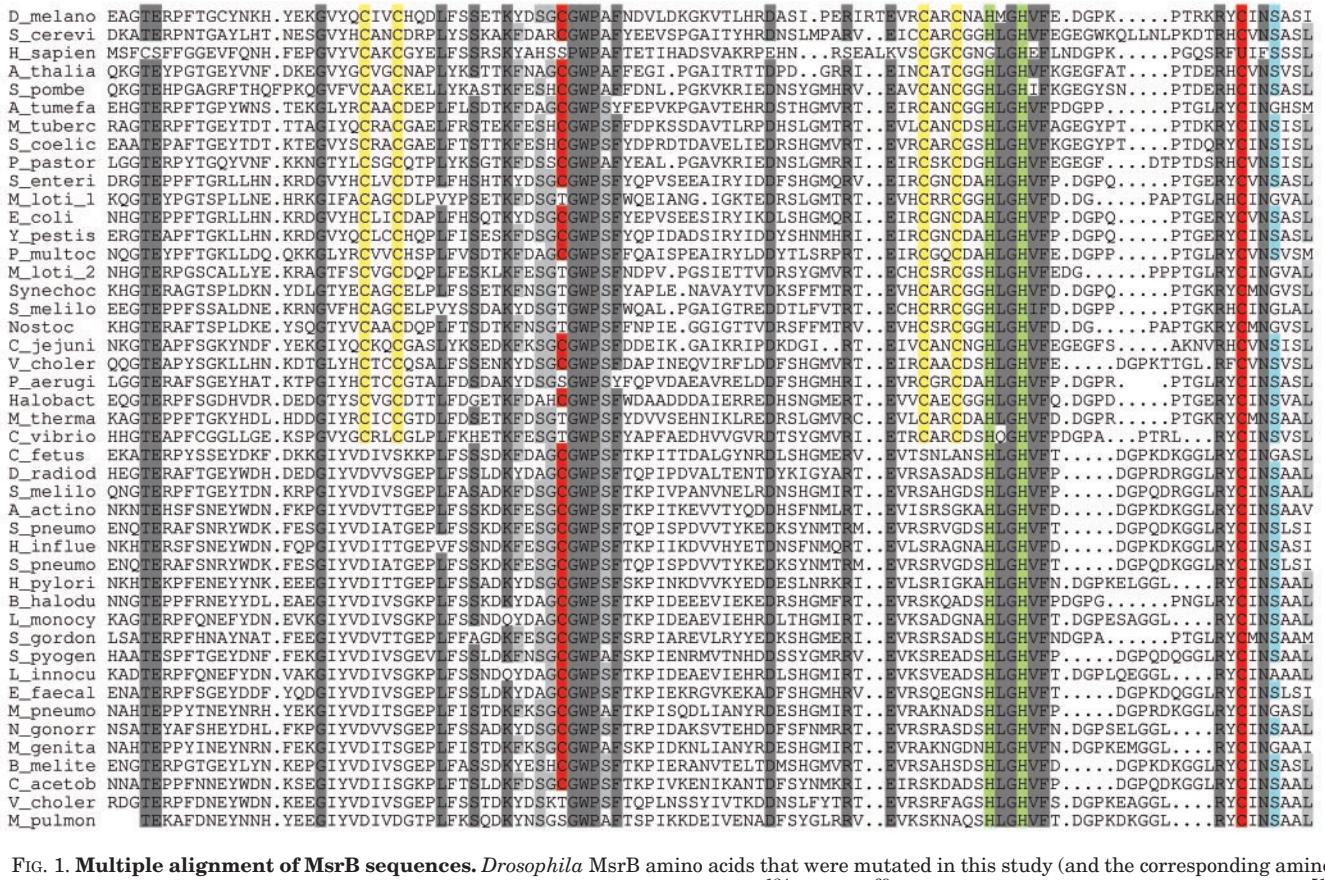

FIG. 1. **Multiple alignment of MsrB sequences.** *Drosophila* MsrB amino acids that were mutated in this study (and the corresponding amino acids in other MsrB proteins) are indicated as follows: the catalytic cysteines (Cys^{124} and Cys^{69}) in *red*, the zinc-coordinating cysteines (Cys^{51}, Cys^{54}, Cys^{101}, and Cys^{104}) in *yellow*, the conserved histidines (His^{107} and His^{110}) in *green*, and the conserved serine (Ser^{127}) in *blue*. Other conserved residues are highlighted in *gray*. *U* in human MsrB indicates selenocysteine. Only conserved regions of MsrB sequences are shown, whereas nonconserved N- and C-terminal regions are not included in the alignment. GenBank™/EBI Data Bank accession numbers are as follows: *Drosophila melanogaster*, 17944415; *S. cerevisiae*, 6319816; *Homo sapiens*, 7305478; *Arabidopsis thaliana*, 4115939; *Schizosaccharomyces pombe*, 19112148; *Agrobacterium tumefaciens*, 15888246; *Mycobacterium tuberculosis*, 15609811; *Streptomyces coelicolor*, 7481112; *Pichia pastoris*, 13235615; *Salmonella enterica*, 16760604; *Mesorhizobium loti*, 13474036; *E. coli*, 15802192; *Yersinia pestis*, 16122390; *Pasteurella multocida*, 15602788; *Mesorhizobium loti*, 13473382; *Synechocystis* sp., 16329386; *Sinorhizobium meliloti*, 15964194; *Nostoc* sp., 17231393; *Campylobacter jejuni*, 15792437; *V. cholerae*, 15642000; *Pseudomonas aeruginosa*, 15598023; *Halobacterium* sp., 15790420; *Methanothermobacter thermautotrophicus*, 15678738; *Caulobacter vibrioides*, 16126422; *Campylobacter fetus*, 14547126; *Deinococcus radiodurans*, 15806395; *Sinorhizobium meliloti*, 16263495; *Actinobacillus actinomycetemcomitans*, 12963881; *Streptococcus pneumoniae*, 15902621; *Hemophilus influenzae*, 16273361; *S. pneumoniae*, 15900561; *Helicobacter pylori*, 3252887; *Bacillus haloduraus*, 10174030; *Listeria monocytogenes*, 16803899; *Streptococcus gordonii*, 7108561; *Streptococcus pyogenes*, 15675047; *Listeria innocua*, 16801039; *Enterococcus faecalis*, 5457308; *Mycoplasma pneumoniae*, 13508401; *Neisseria gonorrhoeae*, 19526685; *Mycoplasma genitalium*, 12045307; *Brucella melitensis*, 17989164; *Clostridium acetobutylicum*, 15894828; *V. cholerae*, 15601373; and *M. pulmonis*, 14089695.

were performed using BLAST programs. Multiple alignments and phylogenetic trees were either generated with the ClustalW and GCG programs or uploaded from Clusters of Orthologous Groups and Pfam.

RESULTS

Prediction of a Putative Catalytic Residue—To identify potential catalytic and metal-binding residues, we analyzed conserved regions in MsrB sequences. Because mouse and *Drosophila* MsrB proteins catalyze a redox reaction and are zinc-containing proteins (8), we were particularly interested in cysteine residues that could be involved in catalysis as well as in cysteine and histidine residues that could participate in zinc coordination. Multiple alignment of MsrB sequences (Fig. 1) revealed only a single cysteine that is conserved in all family members. This cysteine is located in the C-terminal portion of the protein and is replaced with selenocysteine in mammalian MsrB (selenoprotein R). The presence of selenocysteine is generally indicative of a catalytic residue that is involved in a redox process (11, 12). Moreover, the conserved cysteine/selenocysteine residue was independently implicated in catalysis by bioinformatics analyses that searched for simple conserved redox motifs located within certain secondary structure patterns.[2] In this case, a C*XX*S redox motif (*i.e.* a cysteine separated from a serine by two amino acids) was identified. Thus, analyses of MsrB sequences predicted that the conserved C-terminal cysteine is the catalytic residue that directly attacks methionine sulfoxide. In addition, the serine located two residues downstream from this cysteine could be assisting the catalytic cysteine in methionine sulfoxide reduction and/or thiol-dependent regeneration of the catalytic thiolate.

Identification of Two MsrB Forms—Further analysis of the MsrB alignment (Fig. 1) revealed that the majority of sequences have four additional conserved Cys residues that are organized in two C*XX*C motifs (two cysteines separated by two amino acid residues). We designated the MsrB proteins containing these four cysteines as Form 1 MsrB proteins. Interestingly, the four Cys residues are either all present or all absent in MsrB proteins. Phylogenetic analyses suggested that MsrB sequences lacking the two C*XX*C motifs form a single evolutionary group, which we designated as Form 2 MsrB proteins. We found that Form 1 MsrB proteins are distantly

[2] Fomenko, D. E., and Gladyshev, V. N., *Protein Sci.*, in press.

TABLE I
Catalytic activities and zinc content of various Drosophila MsrB forms
Activities are expressed as a percentage of wild-type MsrB activity. NA, no activity.

Enzyme	DTT assay	Thioredoxin assay	Zinc
	%	%	*eq*
Wild-type	100	100	0.82
C69S	82	10	0.80
C124S	NA	NA	0.78
C51G	NA	NA	0.07
C54G	NA	NA	0.06
C101S	NA	NA	0.00
C104S	NA	NA	0.00
C51G/C54S	NA	NA	0.06
C101G/C104S	NA	NA	0.06
C51G/C54S/C101G/C104S	NA	NA	0.05
H107G	81	NA	0.88
H110G	80	NA	0.79
S127G	85	NA	0.61

related to each other and are present in various bacteria and in all MsrB-containing Archaea and eukaryotes. In contrast, Form 2 MsrB proteins are more closely related to each other and are mostly present in pathogenic bacteria. Based on these analyses, we suggest that Form 1 MsrB proteins are prototype enzymes and that Form 2 enzymes evolved from Form 1 enzymes through the loss of four Cys residues (*i.e.* two conserved C*XX*C motifs).

Prediction of Other Functional Residues in MsrB—Further analysis of Form 1 and Form 2 MsrB proteins revealed that almost all Form 2 sequences conserve a second cysteine that is located in the middle of MsrB sequences. In contrast, this cysteine is absent in approximately half of Form 1 MsrB proteins, which have serine or threonine in its place.

In addition to participation in the reaction mechanism, the six cysteines described above could potentially be involved in zinc coordination. Zinc is bound in proteins almost exclusively through cysteines and histidines (21). Because the C-terminal cysteine was predicted to be a catalytic residue and the second cysteine was only conserved in Form 2 enzymes, the remaining four Cys residues (two C*XX*C motifs) were attractive candidates for being metal-binding residues. In addition, both Form 1 and Form 2 MsrB proteins have a pair of conserved histidines that are located upstream of the predicted catalytic cysteine. These are other candidate zinc-coordinating residues.

To summarize analyses of MsrB sequences, we identified six cysteines, two histidines, and one serine that could be involved in catalysis and/or zinc coordination. These nine residues were selected as targets for mutagenesis, followed by analyses of catalytic activities, catalytic intermediates, and metal content of mutant proteins.

Form 1 Drosophila MsrB Functions as Methionine-R-sulfoxide Reductase in Vivo and in Vitro—We selected *Drosophila* MsrB, one of the prototype (Form 1) enzymes. In this protein, the predicted catalytic cysteine is Cys^{124}, the conserved serine is Ser^{127}, the two C*XX*C motifs are Cys^{51}/Cys^{54} and Cys^{101}/Cys^{104}, and the conserved histidines are His^{107} and His^{110}. Moreover, *Drosophila* MsrB is one of the Form 1 proteins that conserves a Form 2 cysteine, Cys^{69}, which allowed us to better test the role of this residue in catalysis. The purified fruit fly enzyme was found to function as methionine-*R*-sulfoxide reductase in both DTT- and thioredoxin-based assays (Table I). The K_m for methionine *R*-sulfoxide is 2.1 mM, and that for mixed methionine *RS*-sulfoxide is 5.8 mM. Because methionine *S*-sulfoxide is not a substrate for MsrB (8), the K_m for the mixed sulfoxide is larger and approximately reflects the proportion of methionine *R*-sulfoxide in the mixed sulfoxide.

We also found that *Drosophila* MsrB was functional *in vivo*.

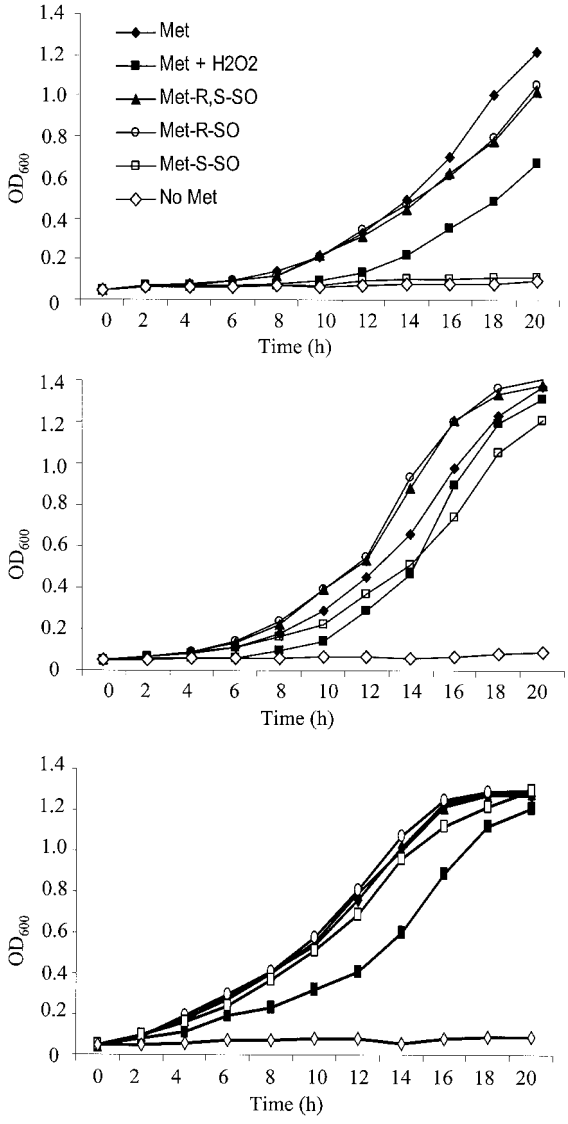

FIG. 2. **Expression of *Drosophila* MsrB compensates for methionine-sulfoxide reductase deficiency in yeast.** Shown is the growth of the yeast strain lacking the MsrA and MsrB genes (*A*) and the corresponding strains expressing wild-type (*B*) and C69S mutant (*C*) *Drosophila* MsrB proteins in the presence of 0.14 mM methionine, 0.14 mM methionine *R*-sulfoxide (*Met-R-SO*), 0.14 mM methionine *S*-sulfoxide (*Met-S-SO*), 0.14 mM methionine *RS*-sulfoxide (*Met-R,S-SO*), or 0.5 mM hydrogen peroxide and 0.14 mM methionine. Cells maintained in the absence of methionine and methionine sulfoxide were used a control. Cell growth was assayed spectrophotometrically by absorbance at 600 nm.

We expressed this protein in the *Saccharomyces cerevisiae* double mutant strain lacking the MsrA and MsrB genes. The growth of this double mutant methionine auxotroph strain was inhibited when methionine sulfoxides replaced methionine in the growth medium, and the strain was also extremely sensitive to hydrogen peroxide (Fig. 2*A*) (8). In contrast, the same mutant expressing wild-type *Drosophila* MsrB grew better on methionine *S*-, methionine *R*-, or methionine *RS*-sulfoxide, and the protein protected cells from hydrogen peroxide (Fig. 2*B*). Thus, *Drosophila* MsrB could compensate for deficiency in methionine-sulfoxide reductases and provide antioxidant defense in yeast. Interestingly, overexpression of MsrB allowed yeast cells to grow on both methionine *R*-sulfoxide and methionine *S*-sulfoxide, suggesting the presence of an additional component(s), such as racemase (epimerase), that is involved in methionine sulfoxide reduction.

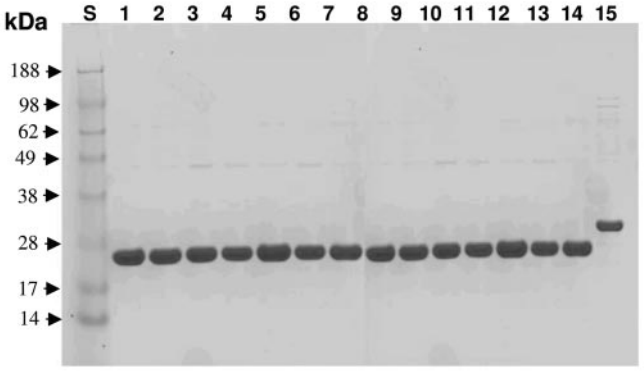

FIG. 3. **SDS-PAGE analysis of recombinant *Drosophila* MsrB and MsrA.** Proteins were prepared as described under "Experimental Procedures." *Lane S*, protein standards (molecular masses in kDa are given on the left); *lane 1*, wild-type MsrB; *lane 2*, C69S MsrB; *lane 3*, C124S MsrB; *lane 4*, C69S/C124S MsrB; *lane 5*, C51G/C54S MsrB; *lane 6*, C101G/C104S MsrB; *lane 7*, C51G/C54S/C101G/C104S MsrB; *lane 8*, C51G MsrB; *lane 9*, C54G MsrB; *lane 10*, C101G MsrB; *lane 11*, C104G MsrB; *lane 12*, S127G MsrB; *lane 13*, H107G MsrB; *lane 14*, H110G MsrB; *lane 15*, wild-type MsrA. Proteins were stained with Coomassie Blue. Approximately 5 μg of each protein was loaded on the gel.

Through site-directed mutagenesis, we developed 13 MsrB mutants lacking one or more of the nine residues that were predicted to be involved in the redox reaction and/or metal coordination. The wild-type and mutant proteins were purified to near homogeneity (Fig. 3) and characterized with respect to catalytic activities and metal content (Table I, Fig. 4).

Cys^{124} Is the Catalytic Residue in Drosophila MsrB—As predicted, mutation of Cys^{124} to serine or lysine resulted in inactive enzymes as assayed by DTT- and thioredoxin-based assays (Table I). In contrast, the zinc content of the mutants was only partially reduced, suggesting that Cys^{124} is not directly involved in zinc binding. The C69S mutation also had little effect on the zinc content. Thus, Cys^{69} is not a residue-coordinating metal. However, activity assays of this mutant revealed a more complex picture: DTT-dependent reduction of methionine sulfoxide was not significantly changed, but the thioredoxin-dependent activity, although clearly detectable, was dramatically reduced (Table I). Thus, it seemed possible that Cys^{69} was involved in the thioredoxin-dependent regeneration of Cys^{124}. Consistent with our prediction of the involvement of Ser^{127} in catalysis, the Ser^{127} mutant was inactive in thioredoxin-dependent assays. However, it had significant activity with DTT and a nearly full content of zinc. Ser^{127} could potentially stabilize the catalytic thiolate or sulfenic acid intermediate or may have additional functions.

Metal-binding Residues—To identify residues that coordinate zinc, we separately mutated two conserved histidines and four conserved Form 1 cysteines. Histidine mutants exhibited activity in DTT assays, but not in thioredoxin assays, and zinc was present in these proteins in significant amounts (Table I). In contrast, mutation of any of the four cysteines or various combinations of these residues resulted in complete loss of zinc. The data suggest that the four Form 1 cysteines, but not the conserved histidines, are directly involved in zinc binding. Mutation of metal-coordinating Cys residues also resulted in complete loss of activity (either DTT- or thioredoxin-dependent), suggesting that zinc is essential for Form 1 MsrB function.

Attempts to remove zinc by dialyzing MsrB in the presence of EDTA at two different pH values failed to extract significant amounts of the metal from the enzyme. A stronger chelating agent (CDTA) was also insufficient in zinc removal. However, changes in metal content observed in these experiments correlated with changes in enzyme activity (Table II). It appears

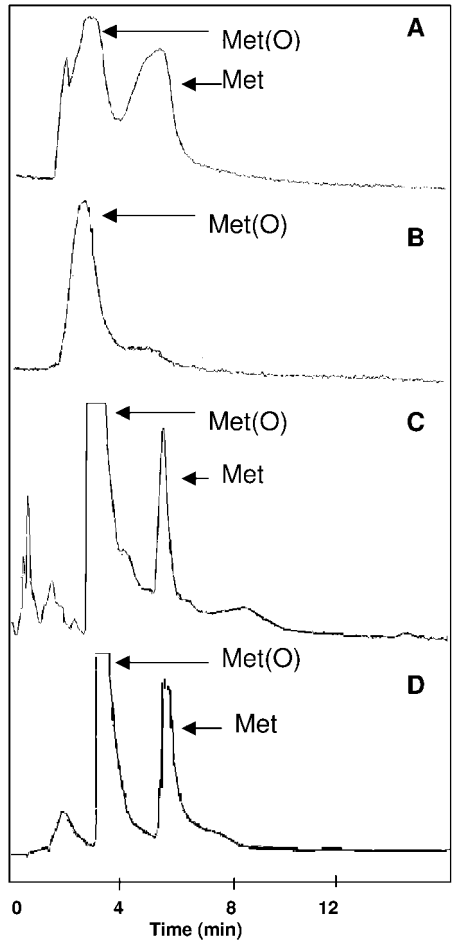

FIG. 4. **Substrate specificity of *Drosophila* MsrA and MsrB and mouse MsrB.** *A*, DTT-dependent reduction of dabsylated methionine *S*-sulfoxide by *Drosophila* MsrA; *B*, DTT-dependent reduction of dabsylated methionine *R*-sulfoxide by *Drosophila* MsrA; *C*, thioredoxin-dependent reduction of dabsylated methionine *R*-sulfoxide by *Drosophila* MsrB; *D*, thioredoxin-dependent reduction of dabsylated methionine *R*-sulfoxide by mouse MsrB. HPLC detection of dabsylated methionine (*Met*) and methionine sulfoxides (*Met(O)*) is shown, and the locations of these compounds are indicated by *arrows*. Enzymes were assayed as described under "Experimental Procedures."

TABLE II
Zinc content and catalytic activity of Drosophilla MsrB incubated with metal chelators

Treatment	Zinc	Activity
	(eq)	*% of control*
MsrB	0.80	100
MsrB + EDTA (pH 6.2)	0.74	94
MsrB + EDTA (pH 8.0)	0.71	89
MsrB + CDTA (pH 8.0)	0.60	62

that zinc in MsrB is tightly bound and is not readily accessible to metal chelators. Addition of Zn^{2+} or Cd^{2+} to purified *Drosophila* MsrB did not result in significant increases in activity.

Because zinc-coordinating cysteines are not present in Form 2 enzymes, our findings also suggest that Form 2 MsrB proteins do not contain metals. Taking into account evolutionary analyses, it appears that Form 2 enzymes evolved from Form 1 MsrB proteins through the loss of metal. Interestingly, two of the zinc-coordinating cysteines are replaced in Form 2 MsrB proteins by aspartate and serine. Side chains of these residues could potentially interact with each other via hydrogen bonds.

MsrB Employs a Catalytic Sulfenic Acid Intermediate—To

TABLE III
Mass spectrometric analysis of Drosophila MsrB and MsrA

All proteins analyzed lacked the N-terminal methionine. ND, not determined. His-tagged proteins were analyzed except where indicated for wild-type MsrB.

Enzyme	Predicted mass	Observed Mass	Mass after addition of DTT	Mass after addition of methionine sulfoxide and dimedone	Change in mass after addition of methionine sulfoxide and dimedone
	Da	*Da*	*Da*	*Da*	*Da*
Wild-type MsrB	19,439.7	19,439.6	19,439.6	19,577.0	137.4
Wild-type MsrB without Gly and His tag	17,631.7	17,628.9	17,630.6	17,768.9[a], 17,628.9[b]	+138 −2
Wild-type MsrB without His tag	17,688.8	17,686.0	17,687.9	ND	ND
C69S MsrB	19,423.7	19,421.1	19,422.4	19,560.8	+138
C124S MsrB	19,423.7	19,421.9	19,422.6	19,421.8	−1
C69S/C124S MsrB	19,407.6	19,406.2	19,406.6	ND	ND
C124K MsrB	19,464.8	19,465.5	19,465.6	ND	ND
C51G/C54S MsrB	19,377.6	19,376.0	19,376.7	ND	ND
C101G/C104S MsrB	19,377.6	19,375.2	19,376.7	ND	ND
C51G/C54S/C101G/C104S MsrB	19,315.5	19,313.4	19,314.8	ND	ND
Wild-type MsrA	29,088.1	29,085.0	29,087.0	29,236.0[a] 29,084.9[b]	+151 −2

[a] Minor species.
[b] Major species.

further characterize the reaction mechanism of MsrB, mass spectroscopic methods were employed. Because the sulfenic acid intermediate was previously found to be involved in the reaction mechanism of MsrA (15), we tested the presence of this intermediate in various MsrB mutants. Cysteine sulfenic acids can react with dimedone, resulting in modified proteins whose masses increase by 138 Da. Dimedone was previously used to establish the presence of sulfenic acid intermediates in alkylhydroperoxide reductase (22), S100A8 (23), and other proteins (24) and was also used to identify sulfenic acid in bacterial MsrA (15).

Methionine sulfoxide-treated, dimedone-reacted, wild-type *Drosophila* MsrB had a small peak with a +138-Da mass shift, suggesting the presence of the modified sulfenic acid (Table III). However, the major fraction of the enzyme was converted by methionine sulfoxide to an oxidized form, suggesting that the sulfenic acid intermediate is not stable and is likely rearranged into a different form of oxidized cysteine. Mutation of Cys^{124} prevented the formation of the +138-Da species (Table III), whereas the C69S mutation resulted in complete conversion of the protein into the dimedone-modified form (Fig. 5 and Table III). Thus, the data suggest that sulfenic acid is initially formed on Cys^{124} and subsequently rearranged into a Cys^{69}–Cys^{124} disulfide bond. In the Cys^{69} mutant, the disulfide bond could not be formed; therefore, the Cys^{124} sulfenic acid could be quantitatively modified with dimedone.

Drosophila MsrB Forms an Intramolecular Disulfide Bond—The formation of the disulfide bond between Cys^{69} and Cys^{124} was further characterized by modifying wild-type and mutant proteins with iodoacetic acid, followed by mass spectrometric analyses. Wild-type *Drosophila* MsrB had seven cysteines, and Cys^{69} and Cys^{124} mutants each had six cysteines. Alkylation of one cysteine with iodoacetic acid was expected to result in a 58-Da increase in protein mass. Reduced wild-type MsrB incubated with iodoacetic acid occurred as a mixture of protein species that had one to seven alkylated cysteines (Table IV). Thus, all seven cysteines in this protein could be modified with iodoacetate. In contrast, when the protein was oxidized with methionine sulfoxide, only five cysteines were accessible for alkylation (Table IV). The C69S mutant had six residues that could be alkylated; but in the presence of methionine sulfoxide; only five residues were modified with iodoacetate. These data are consistent with the formation of a disulfide bond involving Cys^{69} in wild-type MsrB. However, in the C69S mutant, methionine sulfoxide-dependent oxidation of the catalytic Cys^{124} to the sulfenic acid intermediate presumably made this residue inaccessible for alkylation.

To determine whether the Cys^{69}–Cys^{124} disulfide bond is intra- or intermolecular, we compared the thioredoxin-dependent catalytic activity of the C69S mutant with that of the mixture of Cys^{69} and Cys^{124} mutants. If the disulfide was intermolecular, addition of the Cys^{124} mutant to the reaction mixture containing the Cys^{69} mutant was expected to increase the catalytic activity. However, no changes in the activity of the Cys^{69} mutant were detected (data not shown), suggesting that the disulfide bond is intramolecular.

Alternative Mechanisms of Sulfenic Acid Reduction—The presence of both sulfenic acid (although in small amounts) and disulfide intermediates in wild-type fruit fly MsrB raised questions of possible alternative routes for sulfenic acid reduction. Multiple alignment of MsrB sequences suggested that most Form 2 MsrB proteins can employ residues corresponding to Cys^{69} for disulfide bond formation (Fig. 1). However, the majority of Form 1 MsrB proteins have Thr or Ser in place of Cys^{69}, so the formation of the disulfide bond is not possible. To test whether enzymes that lack the cysteine corresponding to Cys^{69} in the fruit fly enzyme are active in thioredoxin assays, we examined the activities of mouse MsrB, which contains Ser in this position. The enzyme was active in both thioredoxin (Fig. 4) and DTT (8) assays. Thus, it appears that thioredoxin and DTT can reduce either the sulfenic acid or disulfide intermediates of MsrB, but that disulfide is a better substrate in the presence of thioredoxin as an electron donor.

To directly test whether Cys^{69} is essential for enzyme function *in vivo*, we expressed the C69S mutant of *Drosophila* MsrB in the yeast MsrA/MsrB double mutant strain and compared the growth of this strain with that of cells expressing wild-type MsrB under various conditions. No significant differences in growth characteristics were observed (Fig. 2*C*), suggesting that Cys^{69} is not essential and that the alternative route of direct sulfenic acid reduction provides sufficient enzyme activity *in vivo*.

The alternative routes of sulfenic acid reduction were also evident from analyses of MsrB sequences. Indeed, disulfide bonding was not possible in the Form 2 enzymes encoded in *Mycoplasma pulmonis* and *Vibrio cholerae* genomes because

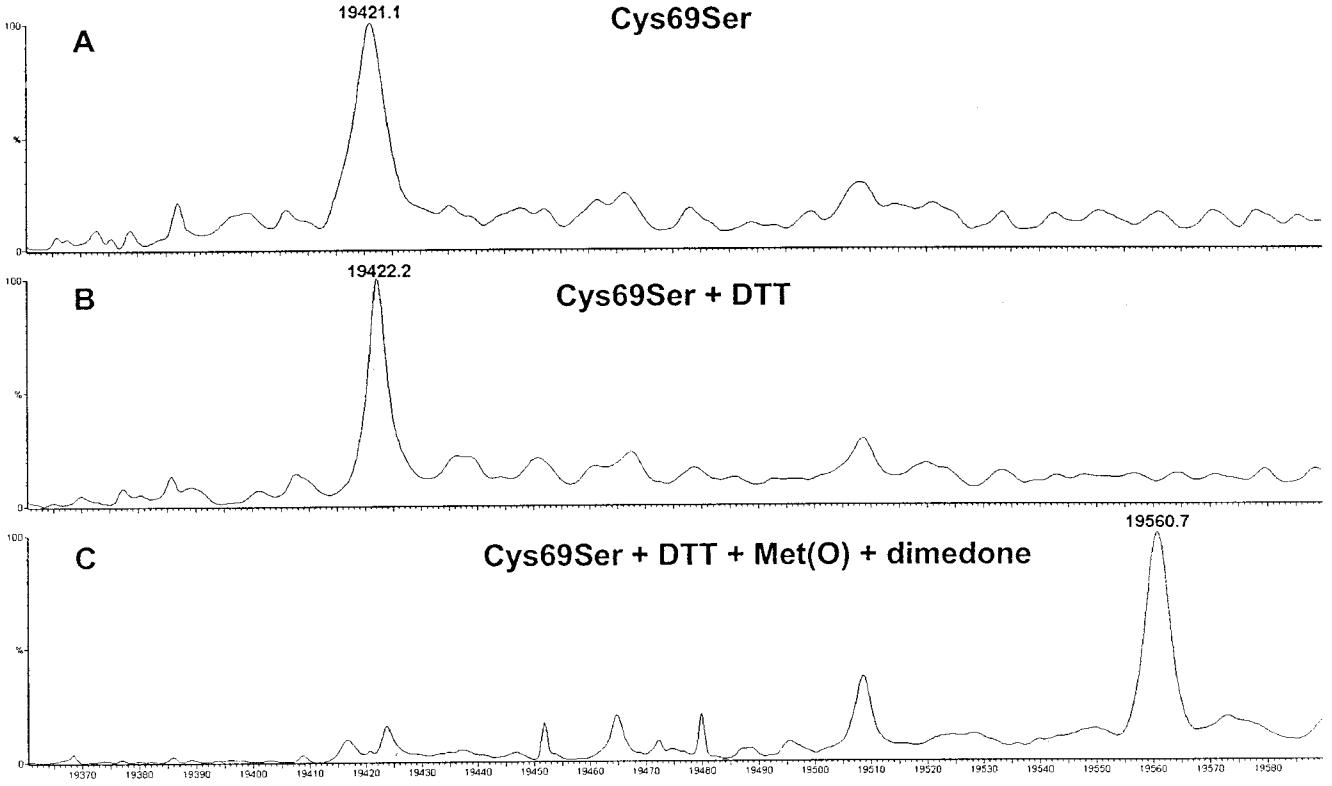

FIG. 5. **Mass spectrometric analysis of the C69S mutant of *Drosophila* MsrB.** Shown are the mass spectra of the native (*A*) and DTT-reduced (*B*) mutant proteins and the mutant protein that was treated with DTT, methionine sulfoxide (*Met(O)*), and dimedone (*C*). Samples were prepared as described under "Experimental Procedures." The mass increase of 138 Da in the dimedone-treated sample matches that of the expected dimedone-modified sulfenic acid intermediate.

TABLE IV

Mass spectrometric analysis of cysteine alkylation in wild-type and C69S MsrB proteins

Numbers in parentheses show mass changes relative to unalkylated proteins or to species with the preceding lower number of alkylated cysteines. Accumulated mass changes for each experiment are shown under "Change in mass" columns. The His tag was removed with thrombin in wild-type MsrB that was used in these experiments, whereas the His tag was present in the C69S mutant. IAA, iodoacetic acid; Met(O), methionine sulfoxide.

Enzyme	No Treatment	DTT → IAA → DTT	Change in mass	IAA → DTT	Change in mass	Met(O) → IAA → DTT	Change in mass
	Da	*Da*	*Da*	*Da*	*Da*	*Da*	*Da*
Wild-type MsrB	17,628.9	17,746.9 (118.0)	118	17,681.71 (52.8)	52.8	17,686.34 (57.4)	57.4
		17,803.8 (56.8)	174.8	17,745.40 (63.7)	116.5	17,744.84 (58.5)	115.9
		17,862.0 (58.2)	233	17,802.12 (56.7)	173.2	17,860.97 (116.1)	232.1
		17,919.7 (57.7)	290.8	17,861.60 (59.5)	232.7	17,919.20 (58.2)	290.3
		17,977.1 (57.4)	348.2	17,919.69 (58.1)	290.8		
				17,977.95 (58.3)	349.0		
				18,037.94 (60.0)	409.0		
			6 Cys		**7 Cys**		**5 Cys**
C69S	19,420.5	19,539.10 (118.6)	113.4	19,476.40 (55.9)	55.9	19,476.70 (56.2)	56.2
		19,596.10(57.0)	175.7	19,539.10 (62.7)	118.6	19,536.40 (59.7)	115.9
		19,656.10(60.0)	235.2	19,595.80 (56.7)	175.3	19,594.90 (58.7)	174.4
		19,712.50(56.4)	292.0	19,652.80 (57.0)	232.3	19,652.80 (57.9)	232.3
				19,712.20(59.4)	291.7	18,710.40 (57.6)	289.9
				19,772.20(60.3)	352.0		
			5 Cys		**6 Cys**		**5 Cys**

these enzymes have only a single Cys residue. Likewise, several Form 1 enzymes (*e.g.* those encoded in *Synechocystis* and *Mesorhizobium loti* genomes) have only a single cysteine besides the zinc-coordinating cysteines.

Expression of MsrB in Drosophila Tissues—Polyclonal antibodies were developed against recombinant fruit fly MsrB. Using these antibodies, we detected MsrB in *Drosophila* head and body (Fig. 6). Recent reports indicated that MsrA activity is decreased in old rats (25) and that the disruption of the MsrA gene in mice shortens their life span (26). Moreover, overexpression of MsrA in *Drosophila* increases the life span by ~40%

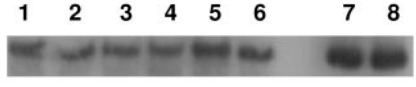

FIG. 6. **Expression of *Drosophila* MsrB.** *Lane 1*, protein extract from 1-week-old flies; *lane 2*, protein extract from 1-month-old flies; *lane 3*, body extracts from 1-week-old flies; *lane 4*, body extracts from 1-month-old flies; *lane 5*, heads from 1-week-old flies; *lane 6*, heads from 1-month-old flies; *lanes 7* and *8*, purified recombinant *Drosophila* MsrB. MsrB was detected in immunoblot assays using anti-MsrB antibodies. 5 μg of protein extract was loaded on each lane.

FIG. 7. **Multiple alignment of MsrA sequences.** GenBank™/EBI Data Bank accession numbers are as follows: *Drosophila* (this work), X04024_Dros (previously reported *Drosophila* MsrA sequence X04024); human, NP_036463; bovine, U37150; *E. coli*, NP_313224; and *Halobacterium*, AAG19555. Identical residues are highlighted in *gray*.

(27). To test whether expression of MsrB is affected during aging, we analyzed the levels of this protein in young (1 week) and old (1 month) flies. We found no significant changes in MsrB expression (Fig. 6).

Characterization of Drosophila MsrA—Analysis of a previously reported *Drosophila* MsrA sequence revealed that this protein contains only a single cysteine residue that corresponds to the catalytic cysteine in bovine and *E. coli* enzymes (Fig. 7). The latter two proteins are known to employ the thiol/disulfide exchange mechanism for reduction of the sulfenic intermediate using a pair of cysteines located in the C-terminal portion of the protein (4). The lack of these cysteines in the predicted fruit fly MsrA sequence raised the possibility of direct reduction of the sulfenic acid intermediate in this enzyme by thioredoxin (18).

To test this possibility, we cloned *Drosophila* MsrA and found that its sequence differs from that previously determined and that the enzyme has two Cys residues in the C-terminal region (Fig. 7). Thus, these cysteines could possibly be involved in thiol/disulfide exchange reactions, similar to bovine and *E. coli* enzymes.

To further characterize *Drosophila msrA*, we generated a recombinant enzyme (Fig. 3). Mass spectrometric analysis revealed that the mass of the affinity-purified protein was consistent with the protein lacking the N-terminal methionine and having a disulfide bond. In the presence of DTT, the mass increased by ~2 Da, consistent with the reduction of the disulfide bond. Subsequent treatment with methionine sulfoxide and dimedone resulted in a 151-Da increase in mass. The fact that MsrA was modified with dimedone was consistent with the presence of a sulfenic acid. However, the modification was expected to give a +138-Da shift. The nature of the additional +13-Da mass change is not known.

We further characterized the substrate specificity of *Drosophila* MsrA in parallel with that of the fruit fly and mouse MsrB proteins. In contrast to MsrB, which shows strict specificity for methionine *R*-sulfoxides (8), MsrA could reduce only methionine *S*-sulfoxides (Fig. 4). This observation is consistent with the previously reported stereospecific activities of bovine and *E. coli* MsrA proteins (2, 3, 14). Finally, recombinant fruit fly MsrA was assayed for the presence of metals. In contrast to MsrB, neither zinc nor other biologically relevant metals were present in the enzyme.

DISCUSSION

Characterization of the metal-binding properties and reaction mechanisms of *Drosophila* MsrB and MsrA revealed that these functionally related but structurally distinct proteins share a common reaction mechanism. Both proteins employ a sulfenic acid intermediate, followed by the formation of the disulfide, which can be subsequently reduced with either DTT or thioredoxin. However, in contrast to MsrA, the evolutionary divergence of MsrB proteins resulted in two major families that differ with regard to the mechanism of sulfenic acid reduction and the requirement for structural zinc.

The prototype Form 1 MsrB proteins coordinate structural zinc, which we found to be essential for protein function. Zinc is coordinated in these enzymes by four conserved cysteines, and mutation of any of these residues resulted in complete loss of activity and metal. In addition to Form 1 MsrB proteins, which can be found in bacteria, Archaea, and eukaryotes, certain bacteria evolved Form 2 MsrB proteins that lack structural zinc, yet retain methionine sulfoxide reduction function. An additional difference between Form 1 and Form 2 MsrB proteins is the mechanism of sulfenic acid reduction. Whereas Form 2 enzymes, similar to MsrA, employ thiol/disulfide exchange processes involving intramolecular disulfide bonds, many Form 1 MsrB proteins lack the cysteine that forms the disulfide bond with the catalytic cysteine.

In our work, we concentrated on characterization of the Form 1 enzyme *Drosophila* MsrB. Although it is a zinc-containing protein, it has a disulfide-bonding Cys^{69}, which we found to be involved in thioredoxin-dependent reduction. Thus, the use of the fruit fly enzyme helped us to study both MsrB families. We also tested a Form 1 mouse MsrB, which contains serine in place of Cys^{69} in the fruit fly enzyme, and found that the enzyme was active in thioredoxin-dependent assays.

While preparing this work for publication, a study was published that characterized the substrate specificity and reaction mechanism of *Neisseria* MsrB, a Form 2 enzyme (28). A similar procedure was used to detect a sulfenic acid intermediate; however, upon treatment of the enzyme with methionine sulfoxide and dimedone, an increase of 154 Da instead of 138 Da was obtained. The data were interpreted as indicating the presence of a dimedone-trapped sulfenic acid and an unknown protein-based sulfoxide. With the exact +138-Da theoretical mass increase for *Drosophila* MsrB, our data support the con-

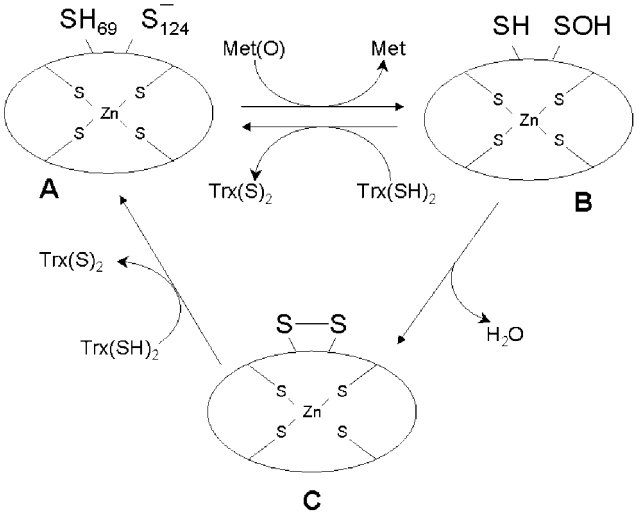

FIG. 8. **MsrB-catalyzed methionine *R*-sulfoxide reduction.** In the initial stage (*A*), Cys^{69} and the catalytic Cys^{124} of *Drosophila* MsrB are in the reduced state, and the latter residue is likely ionized. Attack of the Cys^{124} thiolate on methionine *R*-sulfoxide (*Met(O)*) results in methionine and the Cys^{124} sulfenic acid intermediate (*B*). This intermediate can be directly reduced by DTT and, to some extent, by thioredoxin (*Trx(SH)$_2$*), resulting in the formation of oxidized thioredoxin and the initial reduced MsrB. However, the most efficient way for regeneration of the Cys^{124} thiolate is reaction of sulfenic acid with Cys^{69}, generating a Cys^{69}–Cys^{124} disulfide bond (*C*), followed by thioredoxin-dependent reduction of the disulfide. In contrast, the direct reduction of sulfenic acid is the preferred route for some MsrB proteins that lack cysteine in the position corresponding to Cys^{69} in *Drosophila* MsrB.

clusion that Form 2 (like Form 1) MsrB proteins work through the sulfenic acid intermediate.

Our current view on the mechanism of methionine sulfoxide reduction by MsrB is shown in Fig. 8. Central to the MsrB-catalyzed reaction is the Cys^{124} thiolate (or the corresponding selenolate in selenoprotein R), which directly attacks methionine *R*-sulfoxide. The intermediate in this reaction is rearranged to the Cys^{124} sulfenic acid, with the release of methionine. Subsequently, sulfenic acid can be directly reduced by thioredoxin; and in many Form 1 MsrB proteins, this appears to be the major mechanism of Cys^{124} thiolate regeneration. In proteins that contain a cysteine corresponding to Cys^{69} in *Drosophila* MsrB, a disulfide bond is formed between Cys^{69} and Cys^{124}, generating a better substrate for thioredoxin. Except for alternative sulfenic acid reduction, this mechanism resembles that of MsrA, suggesting that active sites of MsrA and MsrB independently evolved to accommodate similar chemistry, but different stereochemistry of methionine sulfoxide reduction.

The role of zinc in this process appears to be structural. Cysteine-coordinated zinc often serves this role by stabilizing protein structure (21). Such function appears to be unnecessary in Form 2 MsrB proteins, which presumably led to the loss of metal binding properties in these proteins while conserving methionine-*R*-sulfoxide reductase function.

Recent studies reported that overexpression of bovine MsrA in fruit flies results in a significant increase in their life span (27), whereas disruption of the MsrA gene in mice decreases their life span (26). As a first step toward characterizing the role of MsrB in aging, we determined expression of this protein in young and old flies, but no significant differences in MsrB levels were observed. Further studies may be needed to address the biological significance of methionine *R*-sulfoxide reduction by MsrB.

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Addendum—While our paper was under review, a report was published on the crystal structure of Form 2 bacterial MsrB (29). The structure is consistent with the conclusions of our study.

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Reaction Mechanism, Evolutionary Analysis, and Role of Zinc in *Drosophila* Methionine- *R*-sulfoxide Reductase

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