Published in final edited form as:
Biochemistry. 2008 June 24; 47(25): 6685–6694. doi:10.1021/bi800422s.

Analysis of Methionine/Selenomethionine Oxidation and Methionine Sulfoxide Reductase Function Using Methionine-Rich Proteins and Antibodies against Their Oxidized Forms†

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Abstract

Methionine (Met) residues are present in most proteins. However, this sulfur-containing amino acid is highly susceptible to oxidation. In cells, the resulting Met sulfoxides are reduced back to Met by stereospecific reductases MsrA and MsrB. Reversible Met oxidation occurs even in the absence of stress, is elevated during aging and disease, but is notoriously difficult to monitor. In this work, we computationally identified natural Met-rich proteins (MRPs) and characterized three such proteins containing 21–33% Met residues. Oxidation of multiple Met residues in MRPs with H_2O_2 and reduction of Met sulfoxides with MsrA/MsrB dramatically influenced the mobility of these proteins on polyacrylamide gels and could be monitored by simple SDS–PAGE. We further prepared antibodies enriched for reduced and Met sulfoxide forms of these proteins and used them to monitor Met oxidation and reduction by immunoblot assays. We describe applications of these reagents for the analysis of MsrA and MsrB functions, as well as the development of the assay for high-throughput analysis of their activities. We also show that all Met sulfoxide residues in an MRP can be reduced by MsrA and MsrB. Furthermore, we prepared a selenomethionine form of an MRP and found that selenomethionine selenoxide residues can be efficiently reduced nonenzymatically by glutathione and other thiol compounds. Selenomethionine selenoxide residues were not recognized by antibodies specific for the Met sulfoxide form of an MRP. These findings, reagents, assays, and approaches should facilitate research and applications in the area of Met sulfoxide reduction, oxidative stress, and aging.

Methionine (Met) is one of 20 common amino acids in proteins and is an important metabolite at the junction of methylation and transsulfuration pathways (1). However, this sulfur-containing amino acid is susceptible to oxidation by reactive oxygen species (ROS), especially under conditions of oxidative stress (2). The product of Met oxidation is Met sulfoxide (MetO), [1] which exists in the form of two diastereomers, methionine *S*-sulfoxide (Met-SO) and

†This study was supported by NIH Grant AG021518 (to V.N.G.) and the Intramural Research Program of the NIH, NCI, Center for Cancer Research (to D.L.H.). The Nebraska Redox Biology Center is supported by NIH Grant RR017675.

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[1]Abbreviations: MRP, methionine-rich protein; Msr, methionine sulfoxide reductase; DTT, dithiothreitol; MetO, methionine sulfoxide; Met-SO, methionine *S*-sulfoxide; Met-RO, methionine *R*-sulfoxide; KLH, keyhole limpet hemoglobin; SeMet, selenomethionine.

methionine *R*-sulfoxide (Met-RO) (3). Met oxidation may affect protein structure and function, and MetO levels are known to increase under stress, in disease, and during aging (2,4–6).

To counteract Met oxidation in proteins, most organisms utilize methionine sulfoxide reductases (Msrs). Two distinct enzyme families, MsrA and MsrB, exist, and members of these families reduce Met-SO and Met-RO, respectively (7–9). Although a number of studies examined the structure and function of these enzymes (10–15), progress in the field has been limited by the nonavailability of sensitive assays for these enzymes and methods of identifying MsrA and MsrB targets. Most of the proteins containing Met-SO and Met-RO have been identified on an individual basis, and which proteins are most susceptible to Met oxidation in cells and tissues is not known.

The first MetO reduction assay was developed by Brot, Weissbach, and collaborators (16) on the basis of the observation that MsrA can reduce *N*-acetyl-MetO to *N*-acetyl-Met. These authors used an isotope-labeled *N*-acetyl[^{3}H]MetO as the substrate and employed ethyl acetate extraction to assay the resulting radioactive *N*-acetyl-Met. However, disadvantages of this method include the use of radioactivity, incomplete extraction of the reaction product, and its contamination with the substrate. The authors also described an assay for detection of free MetO reduction activity (16). In this method, [^{3}H]MetO is reduced by Msrs, the reaction mixture fractionated by TLC, and the Met spot visualized by ninhydrin treatment and extracted for quantification of radioactivity.

Another method that is commonly used employs dabsylated MetO as the substrate (17), and the product, dabsylated Met, is detected in a HPLC procedure by monitoring the absorbance at 436 nm. This method has high accuracy and sensitivity, but it requires a HPLC system and significant expertise in the procedure and substrate preparation and is time-consuming. An enzyme-coupled method, which relies on the detection of the absorbance change of NADPH at 340 nm, is also used (18). In this method, the reduced state of MsrA or MsrB is regenerated during the reaction with thioredoxin, which in turn is reduced by NADPH-dependent thioredoxin reductase. The detection limit of this assay is lower than that of the HPLC assay; however, it can be used with both free and dabsylated MetO forms of the substrate.

The availability of simpler, user-friendly, cost-effective, and high-throughput methods to monitor Met oxidation and reduction and assay MsrA and MsrB activities has the potential to facilitate research in this area. Such procedures may help in functional characterization of Msrs, their mechanisms of regulation and cellular targets and in establishing roles of these processes in physiological and patho-physiological states as well as during aging. In this work, we describe the use of Met-rich proteins (MRPs) and antibodies specific for their oxidized forms as tools for examining MsrA- and MsrB-dependent processes. We also describe selenomethionine (SeMet) forms of MRPs and their applications.

MATERIALS AND METHODS

Materials

Genomic DNA of *Nostoc* sp. (catalog no. 29133D-5), *Shewanella oneidensis* MR1 (catalog no. 700550D), and *Legionella pneumophila* (catalog no. 33152D-5) was obtained from ATCC (Manassas, VA). *Saccharomyces cerevisiae* strain BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) was used for isolation of yeast genomic DNA. *Escherichia coli* Novablue cells (Novagen, La Jolla, CA) were used for DNA manipulation, and BL21(DE3) (Invitrogen, Carlsbad, CA) and Rossettagami cells were used for protein expression. Restriction enzymes were from Fermentas (Glen Burnie, MD) and PCR reagents from Invitrogen, and Talon polyhistidine purification resin was from Clontech (Mountain View, CA). The mouse line

containing the knockout of the selenocysteine tRNA$^{[Ser]Sec}$ gene and the corresponding wild-type mouse line were described previously (19).

Computational Identification of Met-Rich Proteins

An in-house Perl script was developed to search for proteins with a high level of Met and a length more than 40 residues. The NCBI nonredundant protein database was searched with the script, and proteins identified were further grouped on the basis of their Met content, i.e., <20%, 20–29%, 30–40%, and >40% Met.

Cloning, Expression, and Purification of Proteins

Select genes encoding MRPs and an Msr with MsrA and MsrB domains (MsrBA) were PCR-amplified from the corresponding genomic DNA using primers listed below (restriction sites underlined): CTR1a, 5′-AAACATATGGAAGGTATGAATATGGGTAGC-3′; CTR1b, 5′-AAGCGGCCGCGTTTTTATATGTGGGAGTC-3′; Tther1, 5′-AAACATATGACTAGAGGAATGATGCATCC-3′; Tther2, 5′-AAGCGGCCGCAGAAAGCATCATTTCATTC-3′; Fe_Nos-F, 5′-AAACATATGATGATGAATGAAACCATGACTGCCG-3′; Fe_Nos-R, 5′-AAGCGGCCGCCATCATCTGCATACTTCTG-3′; Legp-1, 5′-AAACATATGTCTTCTGGTTCTCAAATG-3′; Legp-2, 5′-AAGCGGCCGCTTTCATCATGGGACATCC-3′; MsrBA1, 5′-AAACATATGAACAAACTGACTGATTTTGAACGC- 3′; and MsrBA2, 5′-AAGCGGCCGCTTGCAGTTCGGCAA-ATAATG-3′.

PCR products were digested with *Nde*I and *Not*I and cloned into the pET21b vector. Expression constructs for mouse MsrA and MsrB2 were previously described by our laboratory (15,20). The plasmids were transformed into *E. coli* BL21(DE3) cells. Protein synthesis was induced for 4 h at 30 °C when OD_{600} reached 0.6–0.8 by adding IPTG to a final concentration of 0.25 mM. Cells were pelleted by centrifugation at 5000 rpm for 5 min, washed with PBS, and stored at −70 °C until they were used.

To purify MRPs and Msrs, cell pellets were dissolved, sonicated in 50 mM Tris-HCl (pH 7.5) containing 300 mM NaCl, 15 mM imidazole, and 0.5 mM PMSF, and centrifuged at 10000 rpm for 30 min, and the supernatants were applied onto Talon resin columns pre-equilibrated with washing buffer [50 mM Tris-HCl (pH 7.5), 300 mM NaCl, and 15 mM imidazole]. The columns were washed with 10 column volumes of washing buffer, and bound proteins were eluted with elution buffer [50 mM Tris-HCl (pH 7.5), 300 mM NaCl, and 300 mM imidazole]. Eluted proteins were analyzed by SDS–PAGE, pooled, and dialyzed against PBS. Identities of proteins were confirmed by tandem MS sequencing. Three MRPs could be expressed, where a *Tetrahymena* MRP was not. It should be noted that the cloned *Tetrahymena* sequence did not have TAA and TAG codons which encode glutamine in this organism but serve as stop codons in most other organisms, including *E. coli*. Thus, the lack of expression of the *Tetrahymena* hypothetical protein in *E. coli* was not due to the presence of these codons.

To express a selenomethionine-containing MRP, the plasmid harboring the gene encoding a putative ferredoxin from *Nostoc* sp. was transformed into Met auxotroph *E. coli* strain B834 (DE3) (Novagen). Protein expression was carried out in M9 minimal medium (without Met and Cys) supplemented with SeMet.

Preparation of CTR1-N under Denaturing Conditions

Cells were sonicated in 50 mM Tris-HCl (pH 7.5). After centrifugation, supernatant was removed, and the resulting inclusion bodies were mixed with 6 M guanidine chloride in 50 mM Tris-HCl (pH 7.5). Supernatant was collected by centrifugation and applied onto a Talon

resin column pre-equilibrated with 50 mM Tris-HCl (pH 7.5) containing 6 M guanidine chloride. After being washed with the same buffer, the protein was eluted with 50 mM Tris-HCl (pH 7.5), 400 mM NaCl, 300 imidazole, and 6 M guanidine chloride. Guanidine chloride was removed from the purified protein by dialysis against PBS.

Oxidation of Met-Rich Proteins by H_2O_2

Purified MRPs were subjected to controlled oxidation by various concentrations of H_2O_2 and at different pHs. Oxidation was monitored by the mobility of proteins on SDS–PAGE gels. To verify that the shift in mobility was due to Met oxidation, the oxidized proteins were subjected to reduction with various Msrs in the presence of 20 mM DTT. We also tested lower concentrations of DTT and found that as little as 2.5 mM DTT was sufficient for full reduction of an MRP. Protein masses in different oxidation states (as isolated, oxidized, and oxidized and then reduced) were determined by ESI-MS (see the procedures below). To test whether the oxidation procedure resulted in oxidation of Cys residues, we subjected proteins in each state to alkylation with 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS) in 50 mM Tris-HCl (pH 7.5), and the resulting proteins were analyzed by SDS–PAGE. MRPs oxidized with 5 mM H_2O_2 for 12–16 h were used to immunize rabbits generating polyclonal antibodies using standard protocols at Covance Immunoservice Inc. (Denver, PA).

LC–MS Analysis

Samples were analyzed with a LC–MS/MS system [Qstar XL (ABS)] mass spectrometer using ESI source. The analysis was performed by loading 200 μL of the protein sample into a 2 mm × 20 mm preconcentration loop filled with perfusion material POROS 10 R2 (PerSeptive Biosystems). The salt was removed from the protein sample by passing 2 mL of 0.25% formic acid through the preconcentration loop. After being desalted, the intact protein was directed to a Micro-Tech Scientific C_{18} column [1.0 (inside diameter) × 50 mm (length), 5 μm (particle size)]. A Shimadzu (SCL-10A) HPLC (high-performance liquid chromatography) system was used for gradient elution with a flow rate of 100 μL/min at ambient temperature. Analytes of interest were eluted from the column by using gradient elution of 0.3% formic acid in H_2O (solvent A) and 0.3% formic acid in acetonitrile (solvent B). The percentage of solvent B was gradually increased from 10 to 90% with a linear gradient over a time period of 10 min followed by washing and an equilibration step. The data were acquired and processed using Analyst QS version 1.1. Data were acquired in TOF (time-of-flight) positive ion mode, and the mass range of 700–1100 amu (atomic mass units) was scanned in 6 s. The ion-spray voltage was set to 5500 V, the nebulizer gas (GS1) to 35 (arbitrary units), the auxiliary heater gas (GS2) to 0 (arbitrary units), the curtain gas (Cur) to 35 (arbitrary units), the source temperature to 150 °C, the skimmer declustering potential (DP) value to 40 V, and the ring focusing potential (FP) value to 230 V. The total run time for each sample was 20 min. Molecular masses of proteins were generated from several multiply charged peaks using the Bayesian Protein Reconstruct option in Bioanalyst Extensions version 1.1.5.

Nano-LC–MS/MS Analysis

Proteins were digested with trypsin at a 50:1 ratio (protein:trypsin) in a water bath at 37 °C overnight. The digested samples were injected, using a FAMOS autosampler, onto a LC Packings C18 PepMap100 column (75 μm × 15 cm, 3 μm). Tryptic peptides were then separated using a gradient consisting of 0.3% formic acid in H_2O as mobile phase A and 0.3% formic acid in acetonitrile as mobile phase B. The gradient started at 10% B, followed by a linear increase to 15% B over 10 min and then by a linear increase to 40% B over 30 min and to 80% B over the following 10 min. The column was then washed with 80% B for 10 min before being restored to the initial condition. A flow rate of 130 nL/min was generated using Ultimate LC Packing Systems. The MS/MS analysis of tryptic peptides was performed using

a quadrupole time-of-flight mass spectrometer (Q-Star XL, Applied Biosystems Inc.), equipped with a nano-ESI source. The parent ions for tryptic peptides were scanned in positive ion mode from 260 to 1250 amu for 1 s. Information-dependent acquisition (IDA) was performed using Analyst QS version 1.1. The IDA criteria were set to perform collisionally activated dissociation (CAD) fragmentation (MS/MS) on all parent ions containing two to five charge states with an area count of >60 cps (counts per second). Daughter ions generated from the fragmentation were accumulated for 4 s and scanned from 50 to 1900 amu. Resolution of masses for this experiment was ~13000, and mass accuracy was ~5 ppm. The observed masses for peptides were both verified by mass accuracy and MS/MS fragmentation pattern. The parent ion mass of the tryptic peptides, its daughter ion, and the modifications were searched by an in-house MASCOT search engine.

Enrichment of Polyclonal Antibodies Specific for Oxidized MRPs

The antisera were first purified on a protein G-coupled Sepharose column, and the antibodies were further fractionated on a column containing reduced MRP, the oxidized form of which was used for immunization. In this procedure, 1–2 mg of reduced MRP was coupled to NHS-activated Sepharose (GE Life Science, Piscataway, NJ) according to the manufacturer's protocol. Diluted antibodies were incubated with the protein-coupled Sepharose overnight at 4 °C to facilitate binding of the antibodies specific for the reduced form of MRP. The unbound fraction was collected and the column washed, and the bound antibodies were eluted with 0.2 M glycine hydrochloride (pH 2.5). We found that the unbound fraction was enriched for the antibodies specific for oxidized MRP.

Production of the Monoclonal Antibody against MetO

We conjugated MetO and Met sulfone onto succinylated keyhole limpet hemoglobin (KLH) (Sigma, St. Louis, MO) at different hapten valence and used it to generate monoclonal antibodies in mice. Immunization, selection of hybridomas, and ascite fluid production procedures were conducted at a GMP-certified facility of Bioland Ltd. (Cheonan, South Korea). The screening of fusion hybridoma using MetO-conjugated ovalbumin revealed two positive clones. However, Western blot analysis of the antibodies purified from the ascite fluids of these two clones on oxidized Met-rich proteins did not result in the specific signal for MetO.

Detection of Msr Activity via an ELISA

Oxidized FeNos at different concentrations was coated onto enzyme immunoassay (EIA) 96-well microplates (Sigma, St. Louis, MO) in carbonate buffer, pH 9.6 (150 mM sodium carbonate, 350 mM sodium bicarbonate, 30 mM sodium azide). After coating, the plates were washed 5 times with washing buffer (PBS, pH 7.4, 0.02% Tween 20). The unbound areas were blocked with 1% bovine serum albumin in PBS, 0.1% sodium azide, either overnight at 4 °C or for 2 h at room temperature. Substrate-coated plates were used in enzyme-catalyzed reactions, and Msrs in PBS containing 5 mM DTT were added into each well and allowed to react for 1 h at room temperature or 30 min at 37 °C. The liquid phase was removed, and the plates were washed 5 times with the same washing buffer. The plates were incubated with the enriched antibodies specific for oxidized FeNos at different dilution factors for 2 h at room temperature or at 4 °C overnight. After washing, plates were incubated with antirabbit IgG conjugated with phosphatase. Finally, phosphatase substrates (Sigma, St Louis, MO) were added, and optical density was measured in a microplate reader at 450 nm.

Western Blotting

Samples were electrophoresed on 10% Bis-Tris gels, transferred onto PVDF membranes, and immunoblotted with enriched antibodies specific for oxidized forms of MRPs. To determine enrichment efficiency, membranes probed with the enriched antibodies were stripped with

"Restore" buffer (Pierce, Rockford, IL) and blotted with the nonenriched antibodies. Immunoblot signals were visualized by using ECL detection systems (GE Healthcare or Sigma).

RESULTS

Identification of Met-Rich Proteins

The NCBI nonredundant protein database was searched with an in-house Perl script to identify proteins with a high content of Met residues. The identified MRP sequences were analyzed for the occurrence of protein domains and homology to proteins with known function. Proteins with Met-rich repeats were filtered out. We selected sequences that were conserved (this step removed spurious predictions and erroneous annotations). Finally, to select for candidate soluble proteins with maximal Met content, we chose sequences with significant secondary structure as well as smaller proteins (and in one case an N-terminal extracellular sequence of a membrane protein). The list of MRPs selected for further characterization is given in Table 1. These proteins had 21–52% Met content, which is 14–35 times higher than that in an average protein. The sequence of an MRP most extensively examined in this study, a protein of unknown function from *Nostoc* sp. annotated as putative ferredoxin (FeNos) (33% Met content), is shown in Figure 1A.

Preparation of Recombinant MRPs

The selected MRPs included three hypothetical proteins and a segment of yeast copper transporter CTR1 (CTR1-N) (Table 1). Three MRPs could be expressed in *E. coli* in the form of His-tagged proteins and purified to homogeneity (Figure 1B), whereas the *Tetrahymena* hypothetical protein could not be expressed in *E. coli* cells. CTR1-N was present in inclusion bodies and was purified after solubilization with urea. The proteins from *Nostoc* sp. (i.e., FeNos) and *Legionella* (hypothetical protein; further designated as LegP) were expressed as soluble proteins and were purified using standard procedures. Migration of purified MRPs on gels agreed with their predicted molecular masses, except that FeNos migrated slightly faster than would be predicted from its sequence.

Preparation of Met Sulfoxidized Forms of MRPs

The three purified MRPs were subjected to oxidation by H_2O_2. Interestingly, H_2O_2-treated proteins exhibited mobilities on reducing SDS–PAGE gels decreased compared to those of proteins that were not oxidized, with the difference in mobility between reduced and oxidized proteins well exceeding that expected if all Met residues were oxidized (Figure 2A–C). We further examined FeNos in greater detail. The change in its mobility on SDS–PAGE gels upon oxidation was mostly (or exclusively) due to formation of MetO as its migration could be restored (partially or completely depending on conditions used) when the oxidized protein was treated with a recombinant *S. oneidensis* Msr composed of MsrA and MsrB domains (further designated as MsrBA) in the presence of DTT (Figure 2A, D). Treatment with DTT alone did not affect the mobility of FeNos. The oxidized FeNos, when treated with either mouse MsrA or MsrB (in the presence of DTT), migrated halfway between reduced and oxidized proteins (Figure 2D, lanes 7 and 8). When the oxidized FeNos was treated with both MsrA and MsrB, the mobility of the original, reduced protein was restored (Figure 2D, lanes 4–6). These observations indicate an approximately equimolar ratio of *S* and *R* forms of MetO exists in the oxidized FeNos. The data also show that each of the two diastereomers of MetO can be completely or almost completely reduced by the corresponding stereospecific Msrs.

The shifts in FeNos migration on SDS–PAGE gels agreed with direct ESI-MS analyses (Figure 3). The initial protein had an observed mass of 15034 ± 3 Da (predicted mass of the His-tagged FeNos was 15089 Da). FeNos treated with 1 mM H_2O_2 exhibited a pattern characteristic of

Met oxidation as peaks differed by 16 Da. A small peak with the lowest mass was the remaining reduced protein, whereas other peaks corresponded to the addition of one to seven oxygen atoms. Oxidation of FeNos with 2 mM H_2O_2 resulted in a similar profile, but it was shifted toward higher mass and corresponded to 4–13 MetO residues. At 10 mM H_2O_2, FeNos exhibited a broad peak with a maximum at 15562 Da. The right side of this peak corresponded to FeNos having all its Met residues oxidized. When this oxidized form was treated with MsrBA in the presence of DTT, FeNos returned to the less oxidized form. Interestingly, a small amount of the original protein with a mass of 15034 Da was detected as well as peaks with only one to six oxygen atoms added to the original MRP mass. This experiment indicated that Met is the major target of H_2O_2 in FeNos, that this treatment produced MetO, and that MsrBA is capable of reducing most of the MetO residues in this protein. These data also verified a disproportionately large decrease in the mobility of FeNos on SDS–PAGE gels upon oxidation, a property which provides a useful tool for examining oxidation and reduction of MRPs in an Msr-dependent manner.

It should be noted that, in addition to Met, FeNos had 15 Cys residues which could also be susceptible to oxidation. Thus, we examined the contribution of Cys oxidation to the decreased mobility of H_2O_2-treated FeNos. First, FeNos was treated with 20 mM DTT, and the Cys residues were alkylated with AMS. This compound adds a mass of ~500 Da to each modified Cys. The data showed that the fully alkylated FeNos (Figure 2A, lane 6) exhibited reduced mobility upon treatment with H_2O_2 (Figure 2A, lane 7), indicating that the mobility shift caused by H_2O_2 is independent of Cys oxidation. Second, we found that AMS treatment could further increase the mass of the oxidized FeNos, suggesting availability of Cys for AMS modification following Met oxidation and excluding significant participation of Cys residues in the altered mobility of FeNos in H_2O_2-treated samples (Figure 2A, lanes 8–10).

Preparation of Antibodies Specific for Met and MetO Forms of MRPs

We prepared rabbit polyclonal antibodies against all three H_2O_2-treated MRPs (i.e., FeNos, LegP, and CTR1-N). To enrich proteins with antibodies specific for reduced and MetO forms of proteins, we coupled MRPs in their reduced forms onto Sepharose resins and incubated them with the corresponding antisera. The initial antibodies and unbound fractions were then tested in immunoblot assays. Panels A and B of Figure 4 show the data for FeNos. The non-enriched antibodies recognized the reduced protein slightly better than the oxidized form (Figure 4A, top panel, lane 1), and treatment with MsrBA for 90 (lane 3) or 180 min (lane 5) did not significantly change the signal. Oxidation of FeNos with H_2O_2 slightly decreased the magnitude of the immunoblot signal (Figure 4A, top panel, lanes 2, 4, and 6), but the protein could still be detected. However, the antibodies enriched for the oxidized FeNos recognized the H_2O_2-treated protein much better than the reduced protein (Figure 4A, bottom panel, lane 2). Moreover, treatment with MsrBA reduced the intensity of the signal (Figure 4A, bottom panel, lanes 4 and 6), indicating specificity for MetO in FeNos. Another example of the use of antibodies specific for reduced and oxidized MRPs is shown in Figure 4B. Mobility of oxidized FeNos on SDS–PAGE could be increased by treatment with recombinant mouse MsrA, mouse MsrB, or both, and the magnitude of the corresponding immunoblot signal with the antibodies specific for oxidized FeNos also decreased (Figure 4B, bottom panel).

The antibodies against oxidized LegP recognized its reduced and oxidized forms almost equally (Figure 4C, top panel). After enrichment (using the same approach described above), the antibodies recognized the oxidized form better (Figure 4C, bottom panel, lane 1). This signal was less pronounced (was lighter) when the oxidized LegP was treated with Msrs (Figure 4C, bottom panel, lanes 2 and 3) and was lost following the treatment with both MsrA and MrsB (Figure 4C, lane 4). The immune response to CTR1-N upon immunization in rabbits was weak, and the resulting antibodies were not further analyzed.

Semiquantitative Determination of Msr Activity Using Oxidized MRPs as Substrates

We took advantage of the altered mobility of oxidized MRPs to develop a semiquantitative assay of Msr activities. We incubated the oxidized FeNos with liver extracts of wild-type mouse and a knockout mouse in which the selenocysteine tRNA gene was specifically excised in the liver (19). In the knockout mouse, selenocysteine-containing MsrB1 is not expressed, which leads to a reduced total Msr activity. Indeed, Msr activity of the knockout liver extract assayed with dabsylated MetO was only 10% of that of the liver extract from the wild-type mouse (data not shown). Figure 5C shows Western blot analyses of oxidized FeNos incubated with the liver extracts for various periods of time. With the antibodies enriched for oxidized FeNos, the magnitude of the signal decreased after incubation for just 30 min with wild-type liver extract (lanes 2–4, bottom panel), showing a good sensitivity of the assay, whereas it did not change significantly when the sample was treated with the liver knockout extract (lanes 6–8, bottom panel). With the non-enriched antibodies, the signal did not change upon treatment. In addition, mobility of FeNos on the gel was increased during treatment with the wild-type liver extract, indicative of MetO reduction in FeNos by liver Msrs. Thus, the antibodies enriched for oxidized FeNos could also be used to analyze Msr activity in biological samples.

To utilize the antibodies specific for oxidized MRPs for high-throughput Msr analyses, we tested the use of these reagents for detection of Msr activity using an ELISA. With the antibodies enriched for sulfoxidized FeNos, we observed a decrease in optical density in oxidized FeNos upon treatment with Msrs (Figure 6A), and the decrease was dependent on the amount of enzyme used (Figure 6B). This figure also suggests that 2 μg of MrsBA was sufficient for reduction of all reducible MetO in the substrate; a further increase in the amount of enzyme did not result in further substrate reduction. To examine the reliability of ELISA, we conducted a heat stability study with MsrBA. After incubation at 60 °C, the remaining activity was measured with ELISA as well as with a standard HPLC method. As shown in panels C and D of Figure 6, the activity data from these assays were in agreement. Thus, the ELISA method can be adapted for high-throughput analyses of Msr activities. Small kinetic differences between the two methods were due to the fact that substrate was in excess in the HPLC method whereas it was limiting in ELISA.

Selenomethionine-Rich FeNos (SeMet-FeNos) for Analysis of Msr Function

We expressed FeNos as a SeMet-rich protein in the *E. coli* B834 host strain. Since the recombinant FeNos contains 38 Met residues, SeMet-FeNos was expected to be 1786 Da heavier than its Met form. However, the purified SeMet-FeNos migrated like the Met form. Purified FeNos and SeMet-FeNos were oxidized in parallel with 5 mM H_2O_2 and analyzed by reducing SDS–PAGE. The mobility of FeNos changed substantially after it was treated with 5 mM H_2O_2 (Figure 7A, lane 2), whereas the mobility of SeMet-FeNos was changed only slightly (Figure 7A, lanes 4 and 5). It should be noted that free Met selenoxide can be reduced by GSH (21), but the redox properties of SeMet residues are not known. Since β-ME was used in the sample buffer during SDS–PAGE, the oxidized form of SeMet-FeNos may have been reduced by β-ME.

To confirm the identity of SeMet-FeNos, we subjected the two proteins to intact mass analysis and MS/MS. This preparation of FeNos had a mass of 15037 Da, whereas SeMet-FeNos exhibited a mass of 16819 Da (Figure 7B), which is 1782 Da heavier than FeNos. These data suggest that all Met residues in FeNos were replaced with SeMet. Figure 7C shows an isotopic distribution pattern of a C-terminal SeMet-FeNos peptide containing three SeMet residues. This particular mass envelope had four charges as the peaks were separated on average by 0.25 Da, and the isotopic distribution of this mass envelope was consistent with the complex isotopic abundance of Se. In addition, we observed this peptide in different oxidation states (see the

inset of Figure 7C) with the corresponding forms of the peptide containing one, two, or three SeMet selenoxide residues.

To determine if SeMet selenoxide residues can be reduced by thiol compounds, which had been found with free methionine selenoxide (21), we subjected SeMet-FeNos to oxidation by H_2O_2 and subsequent reduction by thiol-containing compounds. Figure 8A shows SeMet-FeNos before and after oxidation. The oxidized form of SeMet-FeNos exhibited a decreased mobility (Figure 8A, lane 2). When treated with DTT (Figure 8A, lane 3) or GSH (Figure 8A, lane 4), SeMet-FeNos migrated as the reduced protein, indicating that these compounds could efficiently reduce SeMet selenoxide residues.

Finally, we found that, although the initial antibodies raised against oxidized FeNos could recognize SeMet-FeNos in immunoblot assays, the antibodies enriched for oxidized FeNos recognized neither reduced nor oxidized forms of SeMet-FeNos (Figure 8C). These data suggested that the signal provided by the antibodies enriched for oxidized FeNos was mostly due to MetO and that SeMet selenoxide residues were not recognized by these antibodies.

DISCUSSION

The lack of reagents for gel-based and immunoblot analyses of Met oxidation and reductive repair, for functions and activities of Msrs and for identities of targets of these enzymes, has been a major limitation in assessing the biological significance of this pathway and its role in disease and aging. In this work, we developed and utilized reagents that can assist in addressing this deficiency. Although the Msr system plays important roles in protein regulation and repair (22–24), there has been no systematic characterization of Msr targets. While a list of currently known proteins that reversibly form MetO residues is extensive (25), those that are targeted for oxidation under physiological conditions and that are the most important Msr targets in vivo or in vitro are not known. We devised a strategy in which MRPs, proteins with Met content exceeding 20%, were used as tools to examine Met oxidation and reduction using simple SDS–PAGE analyses. Development of antibodies enriched for oxidized and reduced forms of MRPs provided additional critical reagents, which could independently monitor Met oxidation.

In the past few years, several research groups reported the potential of Msrs in extending life span in yeast, fruit flies, and mammals (22–24). To regulate the activity of Msrs, researchers overexpressed or knocked out the corresponding genes. However, under physiological conditions, a more useful approach is often to modulate (inhibit or activate) activities of specific Msrs. Current Msr assays do not allow high-throughput screening for such compounds. Using MRPs and MRP antibodies, we describe a simple method for routine detection of Msr activity with an option to scale it up for high-throughput assays.

In an initial effort to produce antibodies specific for MetO, we conjugated MetO and Met sulfone onto various carrier proteins, including bovine serum albumin, ovalbumin, and succinylated KLH. The resulting immunogens were used to immunize rabbits to generate polyclonal antibodies and mice for monoclonal antibodies. However, to date these efforts have not been successful. Although we identified two hybridoma clones from mice immunized with MetO-conjugated succinylated KLH that cross-reacted with MetO-conjugated ovalbumin, these antibodies did not react with oxidized MRPs.

We hypothesized that reduced and oxidized MRPs will have significant structural differences and be amenable to generation of antibodies with a higher affinity for MetO. To test the hypothesis, we first identified MRPs computationally and then examined four such proteins. Two proteins could be obtained in soluble form (FeNos and LegP), and one (CTR1-N) was purified from inclusion bodies. Interestingly, upon oxidation with H_2O_2, MRPs migrated more slowly on SDS–PAGE gels, and this shift was attributed to Met oxidation. Mass spectrometry

analyses also showed the mass shifts characteristic of Met oxidation. Thus, the altered mobility of oxidized MRPs of SDS–PAGE gels was due to oxidation of Met residues, and this property could be used for gel-based analyses of their Met redox state. Moreover, we found that treatment of an oxidized MRP with MsrA or MsrB resulted in the protein migrating halfway between reduced and oxidized MRP, and treatment with both enzymes (or a fusion protein composed of MsrA and MsrB domains) restored MRP mobility to that of the reduced protein. Thus, this MRP system also proved useful for gel-based assays of Msr activities, including activities of these enzymes in biological samples. Using these tools, we further found that direct oxidation of Met residues with H_2O_2 resulted in an approximately 1:1 mixture of methionine *R*-sulfoxide and methionine *S*-sulfoxide and that MsrA and MsrB could reduce all or almost all MetO residues in target proteins.

Upon injection of oxidized MRPs into rabbits, oxidized FeNos and LegP gave strong immunological responses. The antisera obtained were first purified on a protein G-coupled Sepharose column and then enriched by removal of antibodies specific for the reduced protein. This procedure produced antibodies specific for MetO forms of FeNos and LegP. The enriched antibodies reacted strongly with sulfoxidized MRPs, whereas the signal was weak when oxidized MRPs were reduced by Msrs. To further explore the applicability of these antibodies, we developed an activity assay for Msrs using ELISA. This assay should be useful for high-throughout analysis of their activities, and inhibitor and activator screens.

Our data show that Msr activities can be monitored by either the changed mobility of MRPs on SDS–PAGE or the use of MRP antibodies. Perhaps the SDS–PAGE procedure is currently more straightforward, but ultimately, the use of antibodies specific for MetO forms of MRPs should provide both better sensitivity and the ability to monitor protein oxidation in in vivo settings.

We also described the use of these reagents to assess redox properties of SeMet residues. We found that SeMet residues can be easily oxidized with hydrogen peroxide and reduced with low-molecular mass thiols. This property should be useful for researchers that utilize SeMet forms of proteins, e.g., for solving the phase problem in X-ray crystallography. Thus, SeMet forms of proteins could be simply treated with DTT or GSH to completely reduce any SeMet selenoxides formed in these proteins upon protein expression, isolation, or handling. Interestingly, MRPs and SeMet MRPs could also be distinguished with our antibodies enriched for oxidized MRPs, as the antibodies did not react with the SeMet selenoxide forms of MRPs. This finding suggests a strong specificity for MetO residues. In the future, it should also be possible to develop antibodies specific for SeMet selenoxide forms of MRPs. The use of reagents, assays, and approaches described in this study should facilitate research and applications in the area of Met sulfoxide reduction, oxidative stress, and aging.

Acknowledgments

We thank Drs. Mark Wilson and Joseph Barycki for help with expression of SeMet MRPs.

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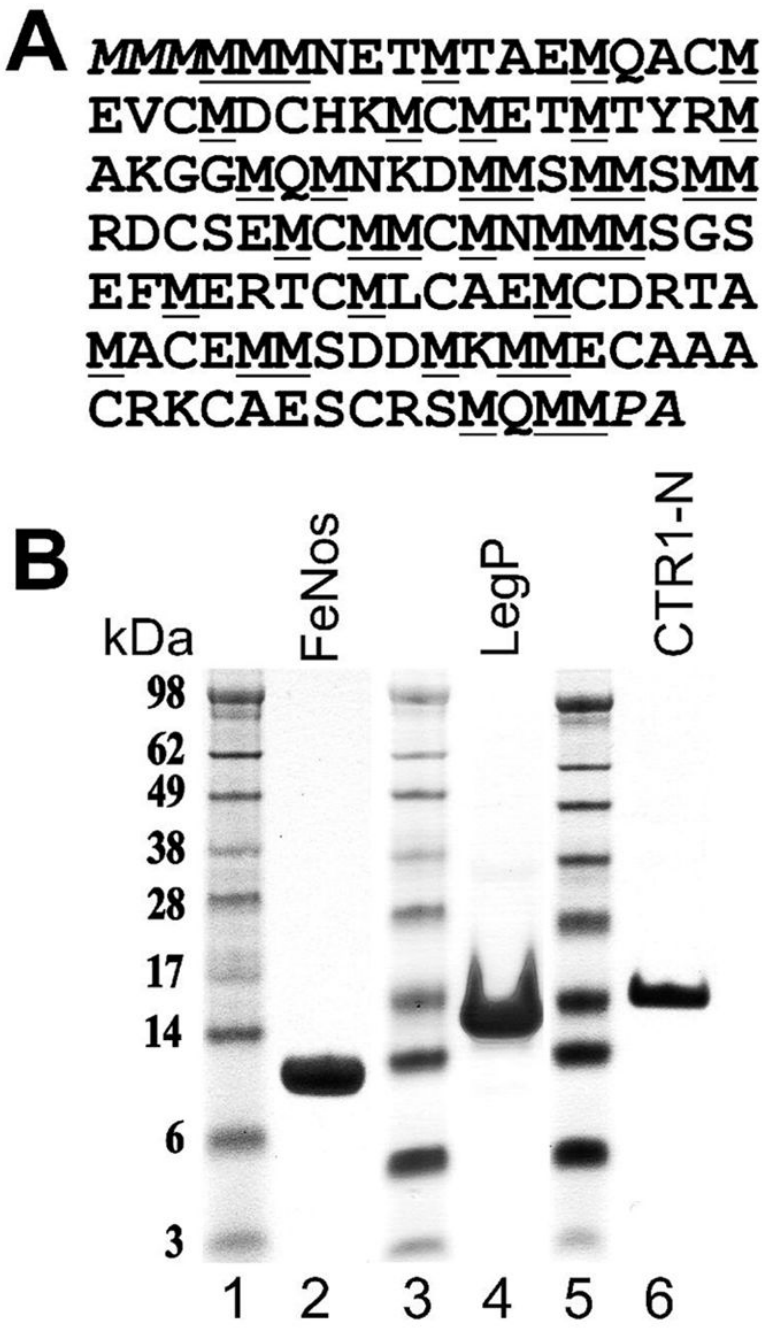

Figure 1.

Methionine-rich proteins (MRPs). (A) Amino acid sequence of FeNos, an MRP from *Nostoc* sp. Methionine (M) residues are underlined. Residues shown in italics were encoded in the FeNos gene, but were not included in the expression construct. (B) SDS–PAGE analysis of protein markers (lanes 1, 3, and 5) and purified recombinant FeNos (lane 2), LegP (lane 4), and CTR1-N (lane 6). Note that the 15 kDa FeNos migrates as a 10 kDa protein. Proteins were electrophoresed on a 10% SDS–PAGE gel and stained with Coomassie blue.

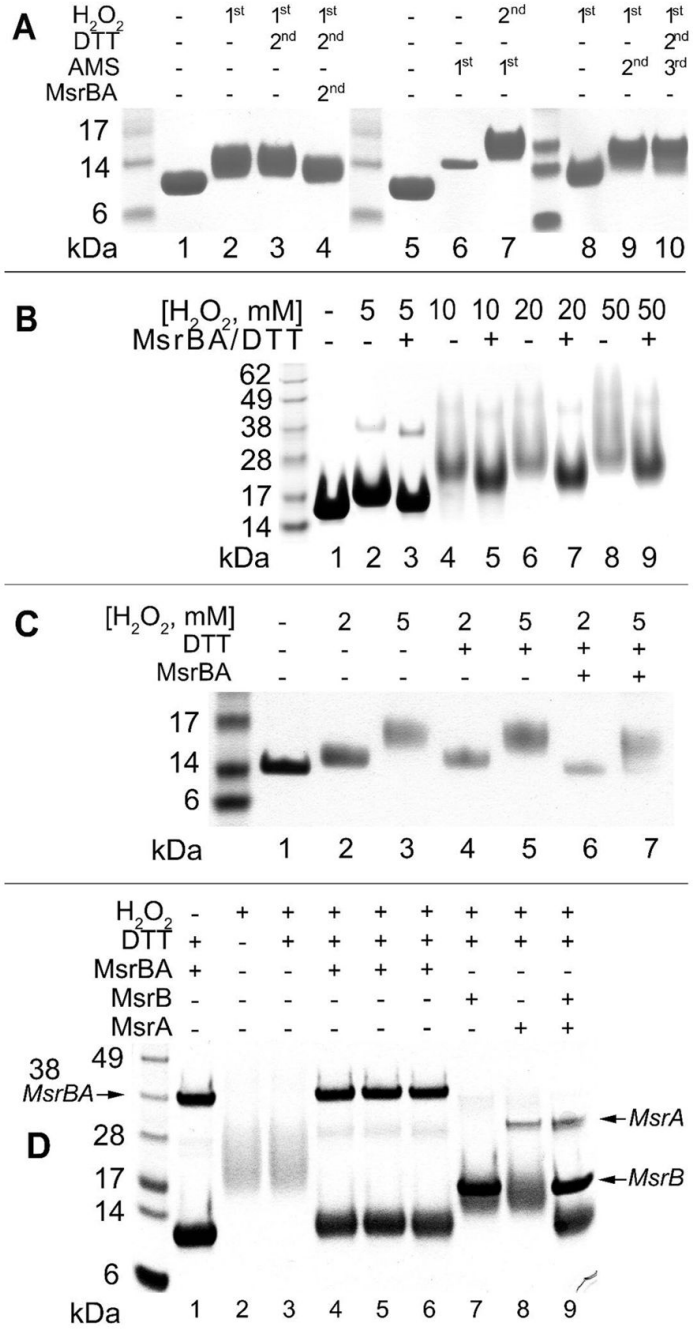

Figure 2.

Mobility of oxidized and reduced MRPs on SDS–PAGE gels. Proteins were electrophoresed on 10% SDS–PAGE gels and stained with Coomassie blue. Lanes with protein standards are not numbered. (A) FeNos as isolated (lanes 1 and 5), FeNos oxidized with 5 mM H_2O_2 at pH 5.0 for 12 h (lanes 2 and 8), DTT-treated oxidized FeNos (lane 3), oxidized FeNos further treated with MsrBA for 2 h [lane 4; unless otherwise noted, all treatments with enzymes were at a 1:20 (w/w) enzyme:substrate ratio, at 37 °C for 2 h], AMS-alkylated FeNos (lane 6), oxidized alkylated FeNos (lane 7), FeNos oxidized after alkylation (lane 9), and oxidized FeNos treated with DTT and then alkylated (lane 10). (B) LegP (lane 1), LegP with the indicated H_2O_2 concentrations (lanes 2, 4, 6, and 8), and oxidized LegP treated with MsrBA in the

presence of 20 mM DTT (lanes 3, 5, 7, and 9). (C) CTR1-N (lane 1), CTR1-N oxidized with the indicated concentrations of H_2O_2 (lanes 2 and 3) and then treated with 20 mM DTT (lanes 4 and 5) or MsrBA and 20 mM DTT (lanes 6 and 7). (D) FeNos treated with MsrBA and DTT (lane 1), FeNos oxidized with 10 mM H_2O_2 at pH 7.0 (lane 2) for 12 h, H_2O_2 removed by dialysis, and the protein treated for 2 h at 37 °C with 20 mM DTT (lane 3), 4 μM MsrBA and 20 mM DTT (lanes 4–6), 4 μM MsrB and 20 mM DTT (lane 7), 1 μM MsrA and 20 mM DTT (lane 8), or both enzymes and 20 mM DTT (lane 9). Migration of MsrA, MsrB, and MsrBA is indicated by arrows. The other major lower band (see lanes 1, 4–6, and 9) is the reduced FeNos. Upon oxidation (lane 2 and 3) or oxidation following a partial reduction (lanes 7 and 8), this protein migrates more slowly.

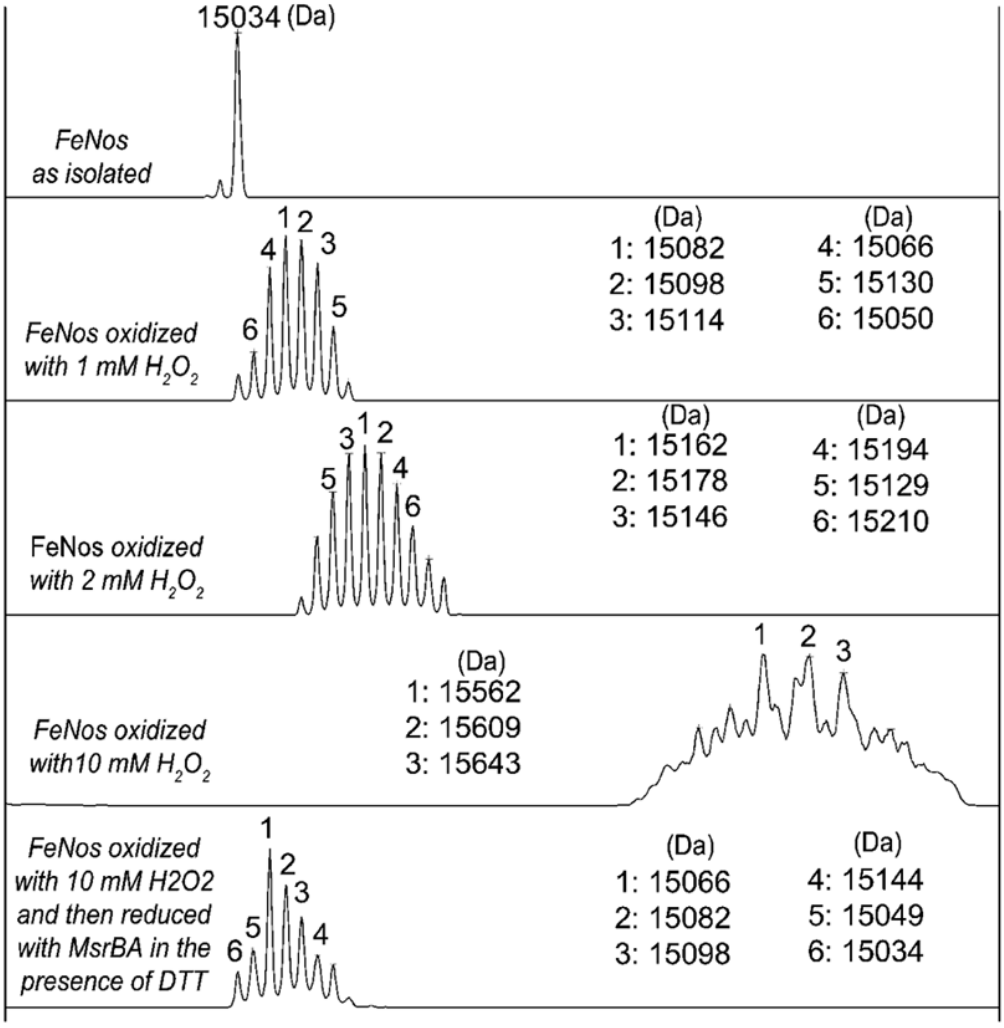

FIGURE 3.

Mass spectrometry analysis of FeNos. Analysis of isolated (top panel), oxidized (three center panels), and oxidized and then reduced FeNos (bottom panel) by ESI-MS. Treatment conditions are indicated on the left side of each spectrum. Reduction reactions were conducted at 37 °C for 2 h. Major peaks are numbered, and their masses (in daltons) are shown to the right (of left) of the spectra.

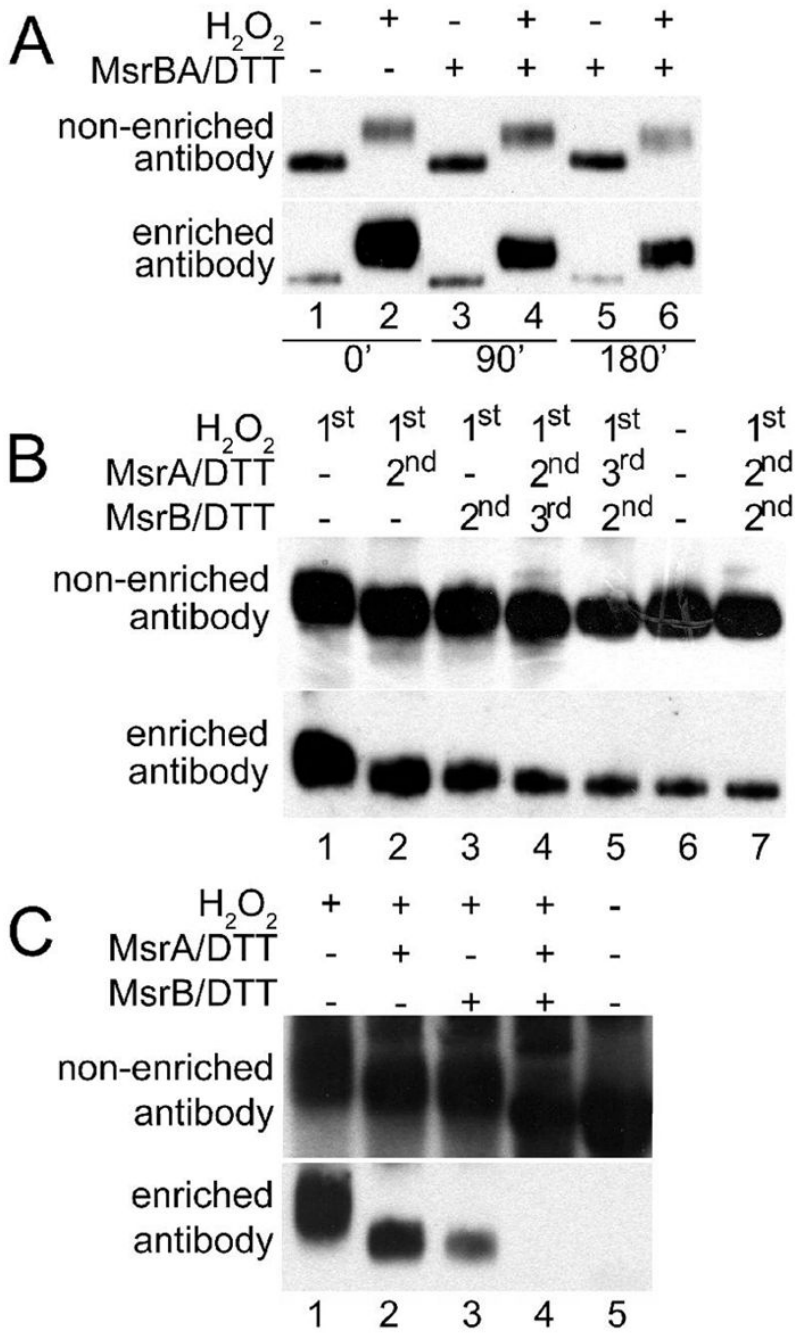

FIGURE 4.

Western blot analyses of MRPs with antibodies enriched for their oxidized forms. (A) FeNos was analyzed with antibodies enriched for oxidized FeNos (bottom panel) and with non-enriched antibodies (top panel). The time of reduction (in minutes) is indicated below the panel. (B) FeNos was analyzed with antibodies against oxidized FeNos (bottom panel) and with non-enriched antibodies (top panel). 1st, 2nd, and 3rd indicate the order in which the corresponding treatments were made. (C) LegP was analyzed with antibodies enriched for oxidized LegP (bottom panel) or with non-enriched antibodies (top panel). Two micrograms of MRPs, either treated or not treated with H_2O_2 and Msrs as indicated above the panels, was loaded onto each lane.

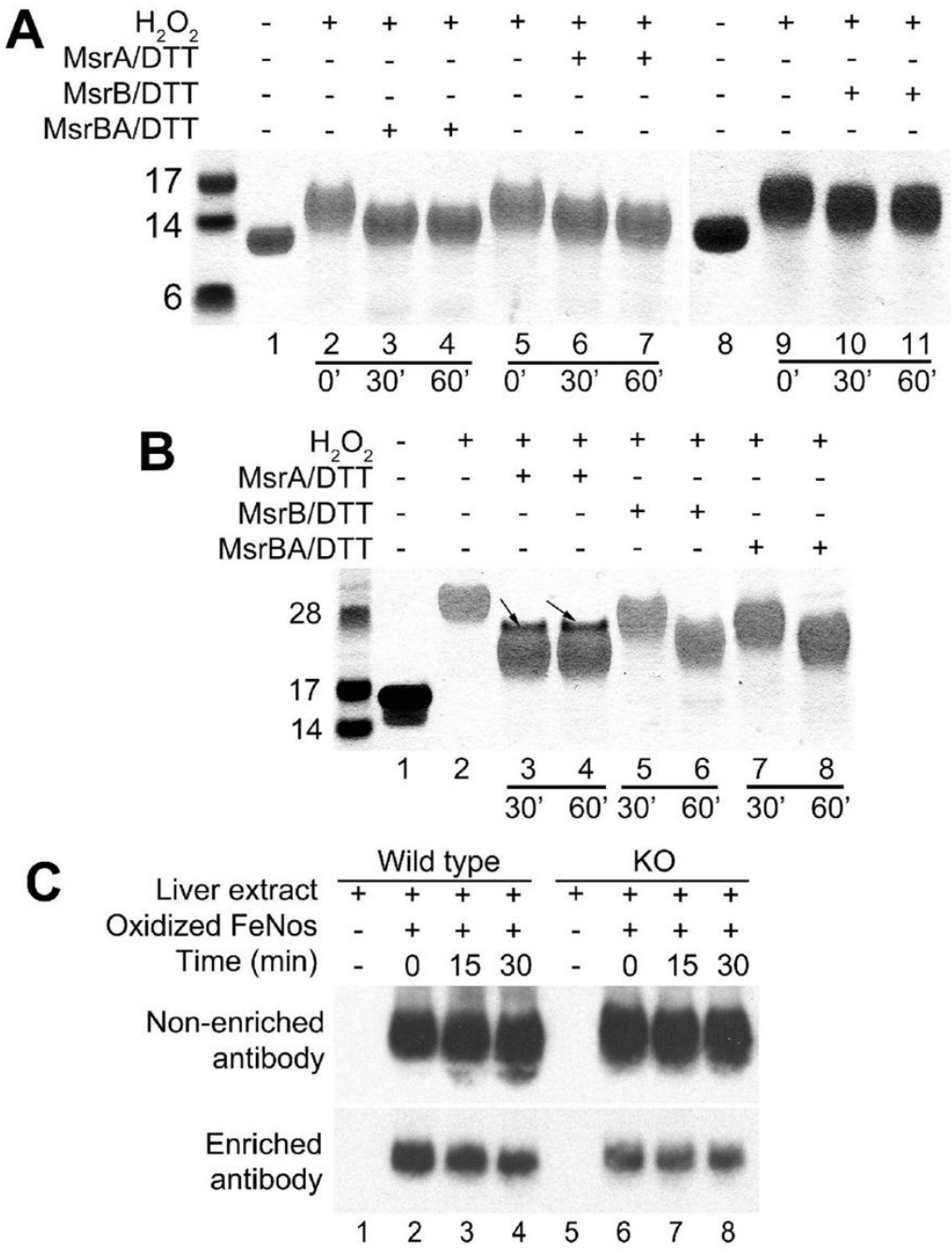

Figure 5.

Qualitative detection of Msr activities on SDS–PAGE gels using oxidized MRPs as substrates. (A) FeNos was oxidized with 5 mM H_2O_2 for 12 h at room temperature, dialyzed against PBS, and subjected to treatment with Msrs at a ratio of 1:20 (enzyme:substrate, w/w) for the indicated time periods. (B) LegP was oxidized with 5 mM H_2O_2 for 12 h at room temperature, dialyzed against PBS, and treated with Msrs and DTT at a ratio of 1:20 (enzyme:substrate, w/w) for the indicated time periods. Arrows indicate the mouse MsrA used in the reduction. (C) Analysis of methionine sulfoxide reductase activity in liver extracts. Three micrograms of oxidized FeNos were incubated with 15 μg of liver extract from wild-type (Wild type) or selenocysteine tRNA knockout (KO) mice at 37 °C for the indicated time periods. Samples were analyzed by

Western blotting with antibodies enriched for oxidized FeNos (bottom panel) and with non-enriched antibodies (top panel).

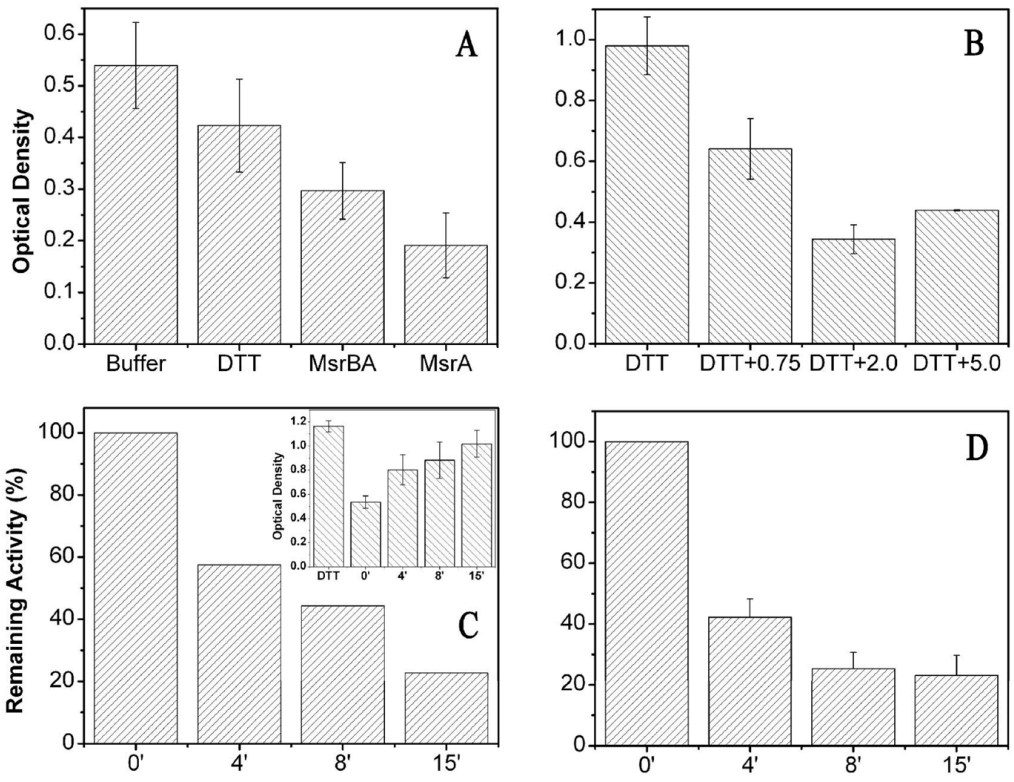

Figure 6.

Analysis of Msr activities by ELISA using oxidized FeNos and the antibodies enriched for oxidized FeNos. (A) An EIA plate was coated with 10 μg/mL oxidized FeNos and then treated with 0.75 μg of either MsrBA or MsrA per well (in 50 μL) for 1 h at room temperature. Controls included DTT treatment and addition of buffer, and blank assays included plates coated with BSA alone. After being washed, the plate was analyzed with antibodies enriched for oxidized FeNos. (B) An EIA plate was coated with 20 μg/mL oxidized FeNos and then treated with DTT and the indicated amount of MsrBA (in micrograms per well) for 1 h at room temperature. After being washed, the plate was assayed as described for panel A. (C and D) Analysis of the heat stability of MsrBA by an ELISA (C) and a HPLC procedure using dabsylated MetO (D). In this experiment, MsrBA (1 mg/mL) was incubated at 60 °C for the indicated time periods and chilled on ice, and activity remaining in the sample was assayed with an ELISA (C) and a HPLC procedure as described previously (22) (D). The inset in panel C shows changes in optical density in the ELISA following heat treatment. The left column shows absorption of the substrate treated with 2 mM DTT, and the remaining columns indicate absorption of the substrate treated with 2 mM DTT and the enzyme after heating for the indicated periods of time.

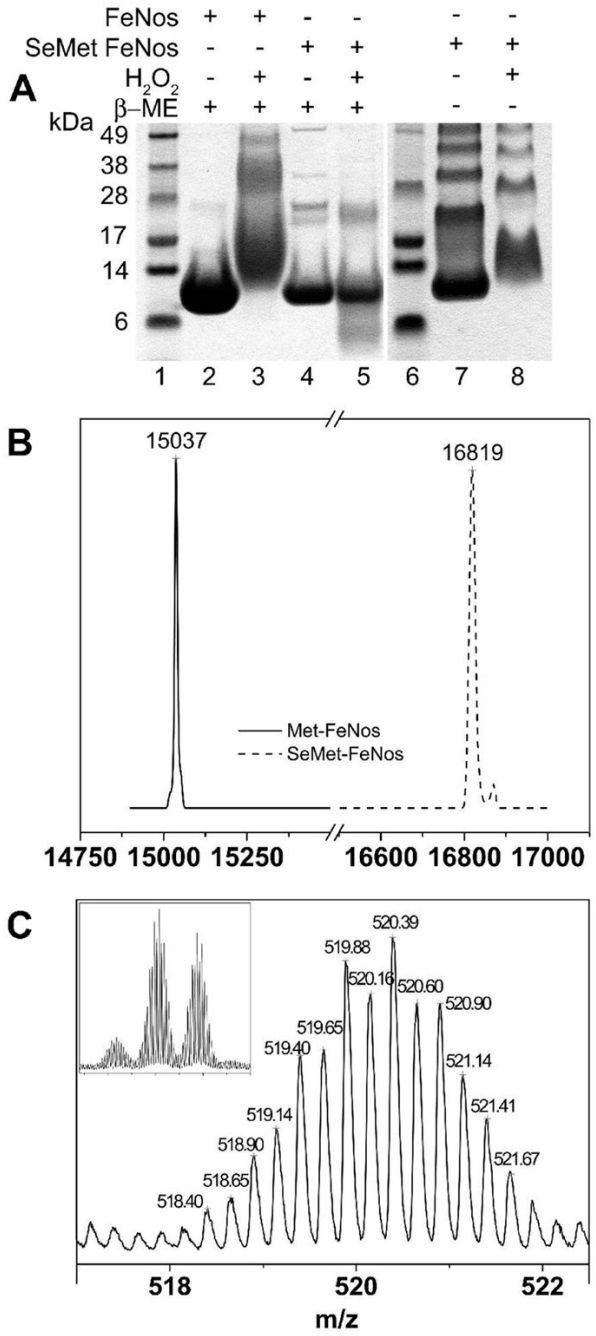

Figure 7.

SeMet-FeNos. (A) SDS–PAGE analysis of FeNos (lanes 2 and 3) and SeMet-FeNos (lanes 4, 5, 7, and 8). FeNos was purified from cells grown in LB medium and SeMet-FeNos from cells grown in M9 minimal medium supplemented with SeMet. Lanes 1 and 6 show molecular mass standards. Other treatment conditions are indicated above lanes (SDS–PAGE sample buffer did not contain reductants). (B) Intact mass of FeNos and SeMet-FeNos determined by ESI-MS. (C) An *m*/*z* profile of a C-terminal peptide containing three SeMet residues. Masses of quadruply charged ions are shown above peaks and correspond to a complex selenium isotopic distribution from three Se atoms. The inset shows a profile of three ions with oxidation of one, two, or three SeMet residues. Each of these three broad peaks is composed of narrow peaks

due to Se isotope distribution. Panel C shows the peak at the center of the inset, in which two SeMet residues were oxidized.

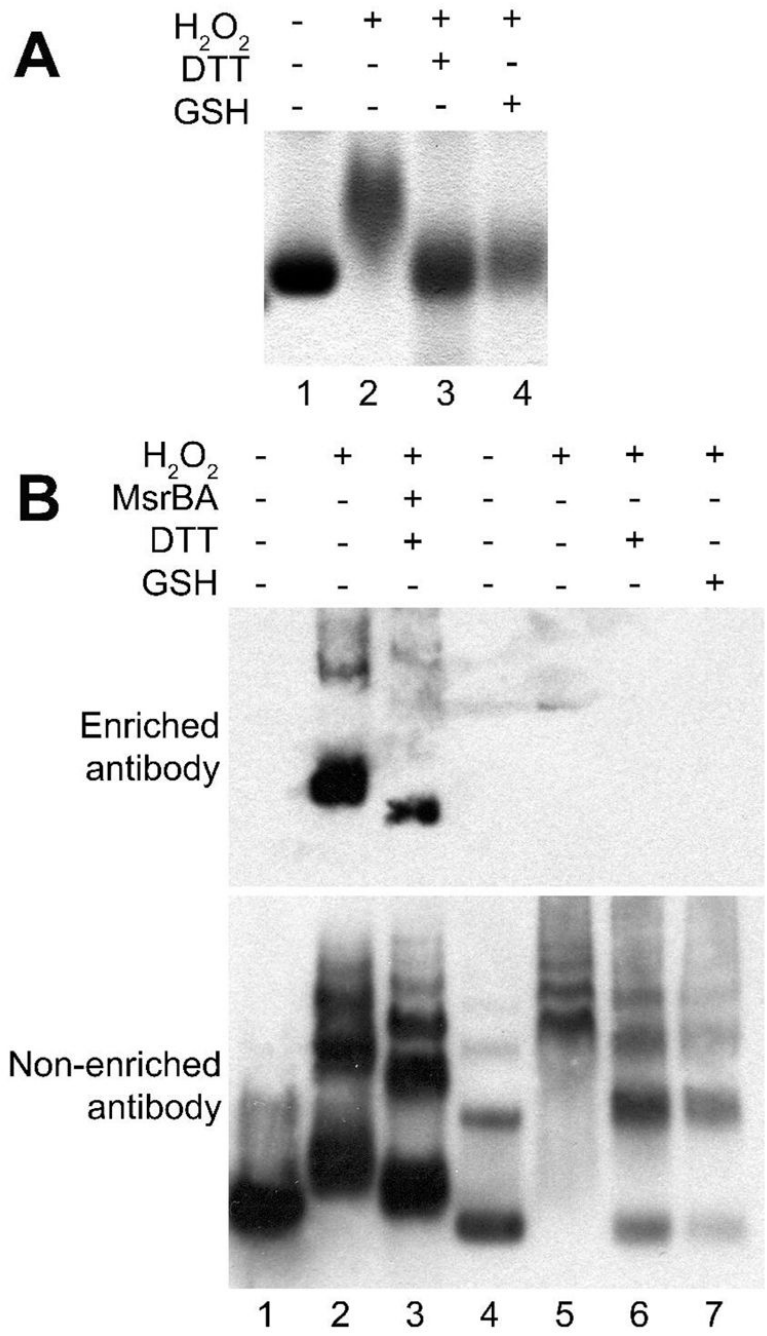

Figure 8.

Redox properties of SeMet-FeNos. (A) SeMet-FeNos (lane 1) and SeMet-FeNos oxidized with 1 mM H_2O_2 (lane 2) and then reduced with 5 mM DTT (lane 3) or 5 mM GSH (lane 4). (B) Immunoblot analysis of FeNos (lanes 1–3) and SeMet-FeNos (lanes 4–7) with antibodies enriched for the oxidized form of FeNos (top panel) and nonenriched antibodies (bottom panel): FeNos (lane 1), FeNos oxidized with 5 mM H_2O_2 (lane 2) and then reduced with MsrBA in the presence of DTT (lane 3), as-isolated SeMet-FeNos (lane 4), and SeMet-FeNos oxidized with 1 mM H_2O_2 (lane 5) and then reduced with 5 mM DTT (lane 6) or 5 mM GSH (lane 7).

Table 1

MRPs Selected for Further Experimental Analysis following Computational Searches for Proteins with High Met Contents

protein	species	no. of Met residues	% Met	accession number
CTR1 N-terminal domain (CTR1-N)	*Saccharomyces cerevisiae*	30	22	NP_015449
TTHERM_00938910	*Tetrahymena thermophila* SB210	142	52	EAR83403
putative ferredoxin (FeNos)	*Nostoc* sp. PCC 73102	41	33	ZP_00345849
hypothetical protein (LegP)	*Legionella pneumophila*	35	29	YP_095088