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Repair of oxidized proteins: Identification of a new methionine sulfoxide reductase

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GGCAGACAGAC) as primers. The PCR product was inserted in plasmid pET21a between the *EcoRI* and *HindIII* sites. Plasmid pBAD24MsrB was obtained by cloning the *msrB* coding region into pBAD24 plasmid. The *msrB* coding region was amplified by PCR, using chromosomal DNA from *E. coli* strain MG1655 as a template and oligonucleotides pFMrB1 (CTGATAGAAATTCATATGGCTAATAAACCTTCGGC) and pBMrB3 (TACTATTCTAAGCTTGGATCCTCAACCGT-TGATTCTTCGCCG) as primers. The PCR product was inserted in pBAD24 plasmid between the *EcoRI* and *HindIII* sites. All plasmid constructs were verified by DNA sequencing.

Expression and Purification of Proteins—The C-terminal His-tagged form of MsrA was purified. *E. coli* strain BL21(DE3) cells containing the plasmids pET21MsrA plasmid grown at 37 °C in Luria Bertani containing 100 µg/ml ampicillin. Cells were grown until an A_{600} value of 0.6, then 1 mM isopropyl-1-thio- β -D-galactopyranoside was added, and growth was continued for another 2 h. Cells were harvested by centrifugation and the pellet stored at -80 °C. The thawed pellet (2 g) was resuspended in 8 ml of buffer M (25 mM Hepes, pH 7.5, 10% (v/v) glycerol) containing 15 mM β -mercaptoethanol and 0.3 M KCl. Resuspended cells were broken by a single pass through a chilled French pressure cell at 6 tons. The resulting crude extract was centrifuged at $30,000 \times g$ for 30 min at 4 °C. The supernatant was loaded on a 5-ml Hi-trap column (Amersham Biosciences) charged with nickel and equilibrated with buffer M plus 0.1 M KCl. Proteins were eluted by a 13-ml gradient from 0.05 to 0.5 M imidazole, and the fractions were analyzed by SDS-polyacrylamide gel electrophoresis. The MsrA-containing fractions were pooled, brought to 0.1 M KCl, and loaded on a 0.5 \times 5 cm MonoQ column (Amersham Biosciences) equilibrated with buffer M plus 0.1 M KCl and 5 mM dithiothreitol. MsrA was eluted with a 7-ml gradient from 0.1 to 0.5 M KCl. Several fractions containing MsrA that were >98% pure as estimated by SDS-PAGE were aliquoted and stored at -80 °C. Typical yields were 20 mg per liter of culture. Protein concentrations were determined by the Bradford method using the Bio-Rad Protein assay kit.

MsrB protein was overproduced in DH5 α cells containing the plasmid pBAD24MsrB. Cells were grown at 37 °C until an A_{600} value of 0.6 in 4 liters of Luria Bertani broth containing 100 µg/ml ampicillin. Expression was induced by adding 0.2% arabinose and growth was continued for another 3 h. Cells were harvested by centrifugation, and the pellet (12 g) was resuspended in 48 ml of buffer A (50 mM Tris, pH 7.5, 10% (v/v) glycerol, 5 mM dithiothreitol). Resuspended cells were broken by a single pass through an ice-chilled French pressure cell at 5.5 tons. The crude extract was centrifuged at $30,000 \times g$ for 30 min at 4 °C. The supernatant was precipitated with 0.1% (v/v) polyethylenimine and centrifuged at $15,000 \times g$ at 4 °C for 20 min. The resulting supernatant was adjusted to 50 mM KCl and applied on a 30-ml Q-Sepharose (Sigma) column (column XK 16/20 Amersham Biosciences) equilibrated with buffer A plus 0.05 M KCl. A 150-ml gradient, running from 0.05 to 0.5 M KCl, was used for elution. Fractions containing MsrB were detected by immunoblot using anti-MsrB antibodies. The MsrB-containing fractions were pooled and concentrated by ultrafiltration on ultrafree biomax-5K (Millipore). Concentrated proteins were run on a gel filtration column (Superdex 75 HR 10/30 Amersham Biosciences) equilibrated with buffer M supplemented with 50 mM KCl, 5 mM dithiothreitol. MsrB protein was eluted at a molecular mass of 15,000 Da. Purity was estimated to be greater than 98% on SDS-PAGE Coomassie Blue staining. Mass spectrometry on the sample confirmed that MsrB was pure. Calibration of the gel filtration column indicated that the size of MsrB was 15,000 Da as predicted from amino acid sequence. Typical yields were 6 mg per liter of culture. Protein concentration was determined by the Bradford method using the Bio-Rad Protein assay kit. Calmodulin VU1, a recombinant calmodulin able to activate all calmodulin targets was used (17). VU1 was expressed and purified by column chromatography as previously described (18). Purity of the protein was checked by SDS-PAGE and electrospray mass spectrometry.

In Vitro Oxidation of Calmodulin—Prior to oxidation, calmodulin was decalcified. One to 10 mg of lyophilized CaM was dissolved in water and precipitated with 3.3% trichloroacetic acid. The pellet was suspended in a minimal volume of Tris 1 M, pH9, water added to 1 ml of trichloroacetic acid precipitation was repeated three times, and, the last time, calmodulin-containing pellet was suspended in Hepes 50 mM, pH 7.5. Decalcified calmodulin (100 µM in Hepes 50 mM, pH 7.5) was treated with 50 mM H₂O₂ for 4 h at room temperature. H₂O₂ was removed by gel filtration through G25 Sephadex. Calmodulin was then concentrated by ultrafiltration on ultrafree biomax-5K (Millipore).

Methionine Sulfoxide Reductase Activity Assay—Methionine sulfoxide reductase activity was assayed in 50 mM Tris buffer, pH 7.5, containing the substrate CaMox or MetSO, thioredoxin (5 µM), thioredoxin

reductase (87 nM), and NADPH (that was used either at 200 or 400 µM). Reducing equivalents required for MetSO reduction were given by NADPH through the thioredoxin/thioredoxin reductase system (19). Assays were carried out at 37 °C in a final volume of 400 µl. All of the components were mixed together before adding the enzyme. The amount of NADPH oxidized was determined by measuring the absorbance at 340 nm. A unit of activity was defined as 1 nmol of NADPH oxidized per min. The rate of NADPH oxidation was linear with respect to enzyme concentration.

Mass Spectrometry Analysis—Sample preparation was performed as follows. 30 µM CaMox was incubated at 37 °C in the presence of 1 µM of MsrA, MsrB, or both in 50 mM Tris-HCl (pH 7.5) containing thioredoxin (5 µM), thioredoxin reductase (87 nM) and NADPH (400 µM). The reaction was stopped by loading the sample onto a Sephadex G-25 column equilibrated with NH₄⁺ acetate (pH 6.2). The eluted protein was subsequently lyophilized. Mass spectrometry measurements were made using a Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (Bruker Daltonics, Billerica, MA) equipped with a shielded 9.4T superconducting magnet (Magnex Scientific Ltd, Abingdon, Oxon, United Kingdom) a cylindrical “infinity” ion cyclotron resonance cell with a diameter of 0.06 m and an external electrospray source (Analytica of Branford, Branford, CT) (20). Carbon dioxide heated to 200 °C was used as the drying gas in the electrospray source. The background pressure in the ion cyclotron resonance cell was typically below 2×10^{-10} millibars. Calmodulin samples were prepared at a concentration of 30 µM in 50:50 water:acetonitrile 1% formic acid and injected into the mass spectrometer using a syringe pump, at a rate of 60 µl h⁻¹.

Construction of a *msrB::aphA-3* Mutant—Plasmid pMsrB was obtained as follows. The *msrB* coding region was amplified from chromosomal DNA from *E. coli* strain MG1655 using oligonucleotides 5' coding pFMrB2 containing an *EcoRI* site (5'-ACTGATCATGAATTCGAAGCTTTGTAGTGAATAAAAGGTTG-3') and 3'-complementary pBMrB3 containing a *SphI* site (5'-TAGCTCAGCATGCAACTCAGATCACAAT-TACGC-3'). Note that pFMrB2 and pBMrB3 oligonucleotides are found at 300 nt upstream and 560 nt downstream of the *msrB* coding region. The resulting PCR product was cloned into pUC18 plasmid, previously cut with *EcoRI* and *SphI* enzymes, yielding pMsrB01. The *msrB* gene was interrupted by insertion of an *aphA-3* cassette (Kan^R) (21) to generate a non-polar mutation as follows. The pMsrB01 plasmid was digested with *AgeI*, and the extremities blunted with the Klenow fragment of DNA polymerase I. The *aphA-3* cassette was obtained after *SmaI* digestion of pUC18K plasmid and inserted at the *AgeI* linearized pMsrB01 plasmid, yielding pMsrB02 plasmid. Note that the *AgeI* site is located at nt 210 downstream the ATG start codon. The pMsrB02 plasmid was then linearized by using *SphI* and *EcoRI* restriction enzymes and the resulting linear fragment was electroporated into *E. coli* KM354 (*recJ*) strain carrying the pTP223 plasmid (*bet gam exo*) (22). Kan^R clones were selected and checked for Amp^S phenotype. PCR was then used to check that recombination had taken place at the *msrB* locus. Last, the mutation was transferred into the *E. coli* MG1655 wild type strain by transduction with P1 phage, and the resulting mutant strain was called BE017.

Cadmium Sensitivity—Cultures of *E. coli* were grown overnight in M9 medium. Cells were then pelleted and resuspended in fresh M9 to an A_{600} of 0.01. Growth was followed by measuring the A_{600} . During the exponential growth phase (A_{600} of 0.6), cultures were split into two subcultures, one of which received cadmium (final concentration 22 µM).

RESULTS

MsrB Is Highly Conserved among Living Organisms—In six of the available genome sequences, *msrA*-encoded protein is fused to an additional domain of ~150 residues in size and of unknown function. This additional domain was found to occur in all available genomic sequences, including eubacteria, animals, plants, humans, and some archaea, most often as a single gene product. This domain will be referred to as MsrB. The level of conservation within the MsrB family is quite high since, for instance, human and *E. coli* sequences share around 25% identity.

MsrB Does Not Interact with MsrA—The fusion between MsrA and MsrB in some genomes was recently used as a basis to predict physical interactions between the two proteins (23). We investigated this issue by using *E. coli* MsrB and MsrA proteins as models. Both proteins were purified (see “Experi-

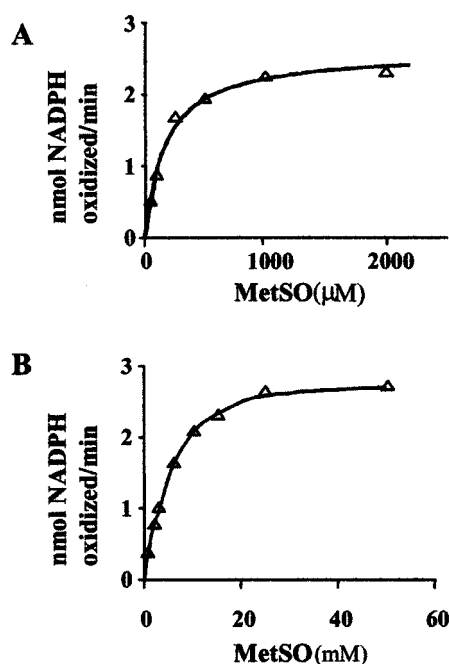


FIG. 1. Characterization of MsrB and MsrA activity with MetSO as a substrate. MsrA and MsrB activities were assayed at various concentrations in MetSO. Reactions mixtures included studied enzymes, NADPH (200 μM), thioredoxin (5 μM), and thioredoxin reductase (87 nM). Curve fitting and K_m calculation were done using Sigma Prism software. *A*, reaction carried out with MsrA (0.3 μM). The calculated turn-over number was 20 min^{-1} . *B*, reaction carried out with MsrB (10 μM). The calculated turn-over number was 0.6 min^{-1} .

mental Procedures”) mixed, and run on a gel filtration column. Despite use of various running conditions, MsrA and MsrB always eluted at their respective apparent molecular weight (data not shown). Likewise, interaction between MsrA and MsrB failed to be revealed by either cross-linking or yeast two-hybrid assays (data not shown). Taken together, these data rendered a physical interaction between MsrA and MsrB highly unlikely.

MsrB Is a Sulfoxide Reductase—Another possibility to account for the fusion of MsrA and MsrB was that they are functionally related. Therefore, we tested whether MsrB had sulfoxide reductase activity. MetSO and dimethyl sulfoxide (Me_2SO) were used as substrates as well as methionine. The thioredoxin recycling assay, where reducing equivalents are provided by NADPH through the thioredoxin/thioredoxin reductase system, was used (19). Reductase activity was determined by following spectrophotometrically the oxidation of NADPH. NADPH oxidation was observed when MetSO (2.8 nmol NADPH oxidized/min) or Me_2SO (5.7 nmol NADPH oxidized/min) were used as substrates. In contrast, no NADPH oxidation was observed with methionine as a substrate. This indicated that MsrB has a sulfoxide reductase activity.

Comparison of MsrA and MsrB Activity on MetSO—To compare the efficiencies of MsrA and MsrB in reducing free MetSO, we undertook steady-state kinetics analysis of these enzymes. Under our assay conditions, MsrB and MsrA exhibited Michaelis-Menten kinetics (Fig. 1). K_m values were 170 μM and 6.7 mM for MsrA and MsrB, respectively. The value found with MsrA is in good agreement with the previously published value of 120 μM (24). Turn over numbers were 20 min^{-1} and 0.6 min^{-1} for MsrA and MsrB, respectively. Catalytic efficiency (k_{cat}/K_m) of MsrA was found to be 1000-fold greater than that of MsrB reflecting the much lower ability of MsrB to reduce free MetSO. These results indicated that MsrB is much less efficient at reducing free MetSO than MsrA.

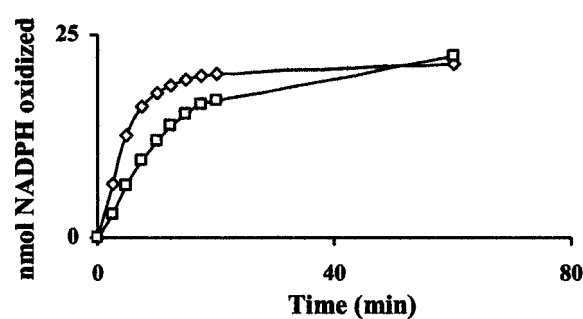


FIG. 2. Use of CaMox as a substrate by MsrA or MsrB. Reactions mixtures included NADPH (200 μM), thioredoxin (5 μM), thioredoxin reductase (87 nM), and CaMox (30 μM). Reactions were carried out with 1 μM MsrA (\diamond), 1 μM MsrB (\square).

Comparison of MsrA and MsrB Activity on Oxidized Calmodulin (CaMox)—To know whether MsrB could act on peptide-bound MetSO, CaMox was used as a substrate since repair of CaMox by MsrA has been well documented (9). Kinetic studies showed that activities of MsrA and MsrB, using CaMox as a substrate, were similar (Fig. 2). MsrA exhibited higher initial rate of NADPH oxidation, e.g. 2 nmol of NADPH oxidized/min, than MsrB, e.g. 1.3 nmol of NADPH oxidized/min (Fig. 2). However, the total amount of NADPH oxidized by either MsrA or MsrB was identical. This analysis showed that MsrB can also act on MetSO residues within oxidized proteins.

Analysis of CaMox Repair by MsrB Using Mass Spectrometry—The repair of CaMox by MsrB was further studied by mass spectrometry. Calmodulin was first decalcified and subsequently oxidized *in vitro*, and the resulting product analyzed by mass electrospray coupled to Fourier Transform ion cyclotron resonance (FTICR) (Fig. 3A). The major species (45%) was CaMox containing 8 MetSO (there are eight methionines in the CaM used). Additional abundant species containing 6 and 7 MetSO were also detected, amounting to 24 and 31%, respectively. No other species were detected indicating that only MetSO were generated. Incubation of CaMox with MsrA resulted in reduction of several, but not all of the MetSO residues (Fig. 3B) The most abundant population contained 3 MetSO (41%) though oxiforms containing 1 to 4 MetSO residues were present (Fig. 3B). Such a partial repair of CaMox by MsrA is consistent with previous work by others (9). Incubation of CaMox with MsrB led to a pattern similar to that observed with MsrA (Fig. 3C). Oxiforms containing from 1 to 4 MetSO were found, with the 3 MetSO-containing species being the most abundant (40%). It is noteworthy that the 1 MetSO-containing oxiform population accounted for 12% of the population repaired by MsrB while it was in very low abundance (4%) when MsrA was used as a reductase. Incubation of CaMox with both MsrA and MsrB yielded three populations. The first exhibited a mass that corresponded to the expected value for fully reduced CaM. This species accounted for 60% of the population. A second species, the mass of which corresponded to reduced CaM with one calcium ion bound, represented about 32% of the population. We could also observe a very low abundance species, the mass of which corresponded to that expected for a reduced calmodulin with two calcium ions bound (6%). It is likely that calcium found in these species came from trace amounts present in instruments or the preparations of MsrA, MsrB, or thioredoxin reductase.

Additive Effects of MsrA and MsrB—The fact that full repair of CaMox required the presence of both MsrA and MsrB suggested that the two enzymes possess complementary properties. To investigate this issue, we submitted CaMox to sequential repair by MsrA and MsrB. CaMox was incubated with MsrB in the presence of both NADPH and the couple thiore-

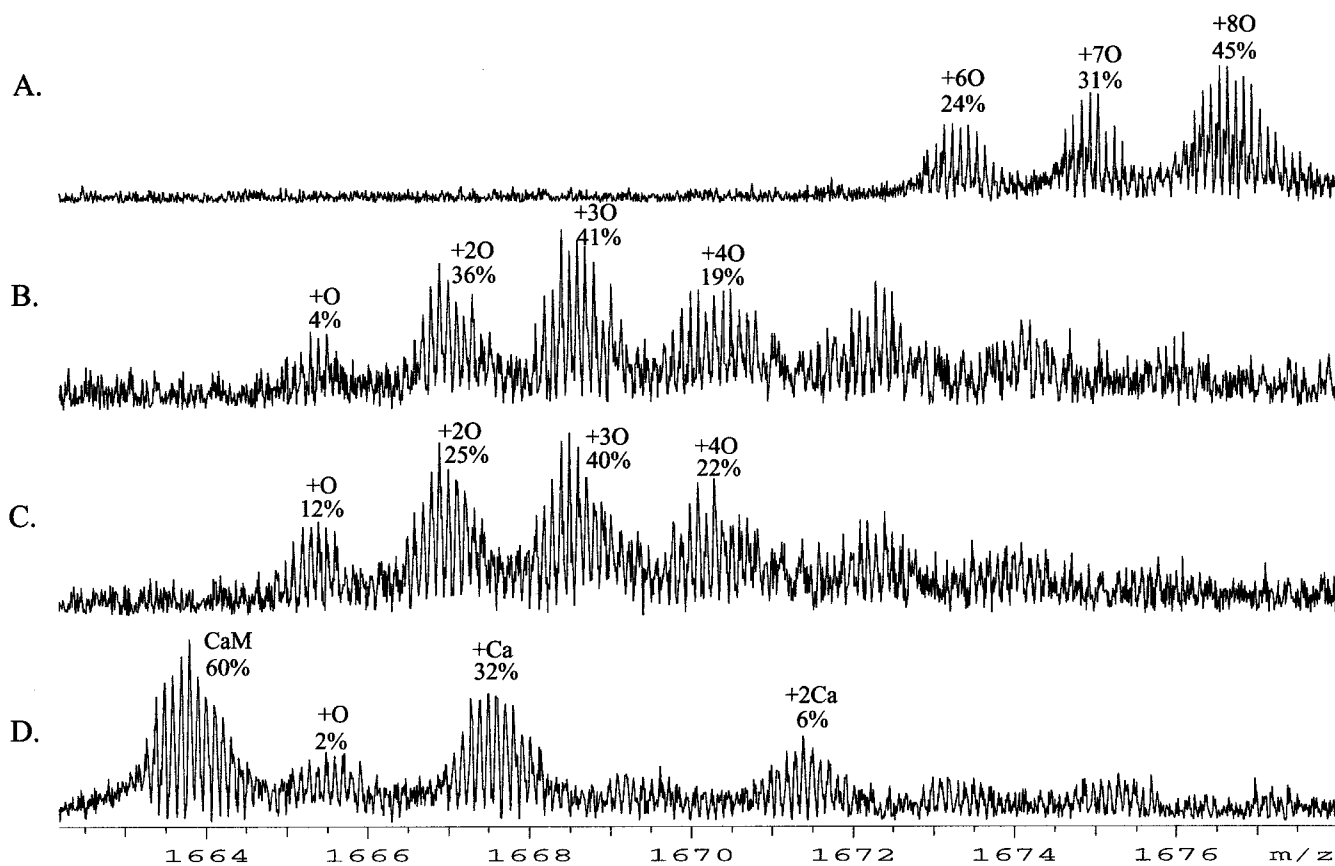


FIG. 3. **CaMox is fully repaired by the combined action of both MsrA and MsrB.** CaM was first decalcified and subsequently oxidized. FTICR spectra of CaMox (A), CaMox repaired by MsrA (B), CaMox repaired by MsrB (C), and CaMox repaired by both MsrA and MsrB (D). Spectra shown are for the 10+ charge state. Percentages indicate proportion of species seen in spectrum, based on peak intensity summed for all charge states.

doxin/thioredoxin reductase, and the reaction left to proceed until NADPH oxidation stopped (Fig. 4A). Arrest of the reaction could be due to limitation of either a chemical required for the reaction or substrate. Subsequent addition in the same mixture of a new batch of CaMox allowed the reaction to resume (Fig. 4A). In contrast, addition of new enzyme did not allow the reaction to resume (Fig. 4A). Taken together, these experiments indicate that the reaction had previously ceased because of substrate limitation. In a new experiment, we left the MsrB-catalyzed reaction to proceed until it reached substrate limitation and then added MsrA. We observed that NADPH oxidation resumed (Fig. 4A). The converse experiment was also performed. CaMox was first reduced by MsrA, the reaction again left to proceed until apparent completion, *i.e.* substrate limitation, and MsrB was subsequently added to the mixture. Again, we observed that the consumption of NADPH resumed upon addition of the second enzyme (Fig. 4B).

MsrB Contributes to Resistance of *E. coli* Against Cadmium—A strain of *E. coli* lacking a functional copy of *msrB* was constructed by reverse genetics (see “Experimental Procedures”). No defect in growth rate or colony morphology was observed when grown in LB medium. The expression of an ortholog of *msrB* has been reported to be induced by cadmium in *Enterococcus faecalis* (25). Therefore, we investigated whether *msrB* had any relationship with cadmium resistance in *E. coli*. While wild type *E. coli* MG1655 strain grew well in the presence of cadmium, MG1655 *msrB* strain stopped growing (Fig. 5). We should note that in some experiments, cadmium sensitivity was less dramatic. We have no explanation for this but suspect changes in trace elements concentration in the growth medium. In any case, throughout over 10 experi-

ments carried out with simultaneously growing pair of isogenic wild type and mutant, this later always exhibited increased sensitivity to cadmium.

DISCUSSION

In this study, we identified a new methionine sulfoxide reductase, the structural gene of which is conserved throughout almost all free-living organisms with the exception of a few archaeobacteria. The function of this enzyme is to repair proteins that have been damaged by exposure to oxidative agents.

MsrB was found to act on free MetSO and Me₂SO. This argues for MsrB being specific for the -SO functional group. MsrB was also very efficient in reducing MetSO residues in peptides. To demonstrate this, we used calmodulin as a model substrate, since this later had been extensively used for probing the reductase activity of MsrA (for reviews see Refs. 4, 26). Comparison of MsrA and MsrB activities on CaMox suggested that they reduced CaMox with similar efficiencies. Electrospray mass spectrometry coupled to FTICR was then used to characterize the products of MsrA and/or MsrB acting on CaMox. It should be remarked that the analysis of such protein mixtures is quite complex since species with similar molecular masses can overlap (there is only a 6-Da differences between a double oxidation and a one Ca²⁺ adduct), generating poorly resolved peaks and inaccuracies in mass assignment. The high accuracy (less than 10 ppm) and resolution of FTICR measurements allowed us to characterize oxiforms of calmodulin recovered after treatment with either reductase. A first set of experiments, in which MsrA and MsrB were added separately to CaMox, revealed a similar pattern of products. Neither one was able to fully reduce CaMox, and, in both cases, the most popu-

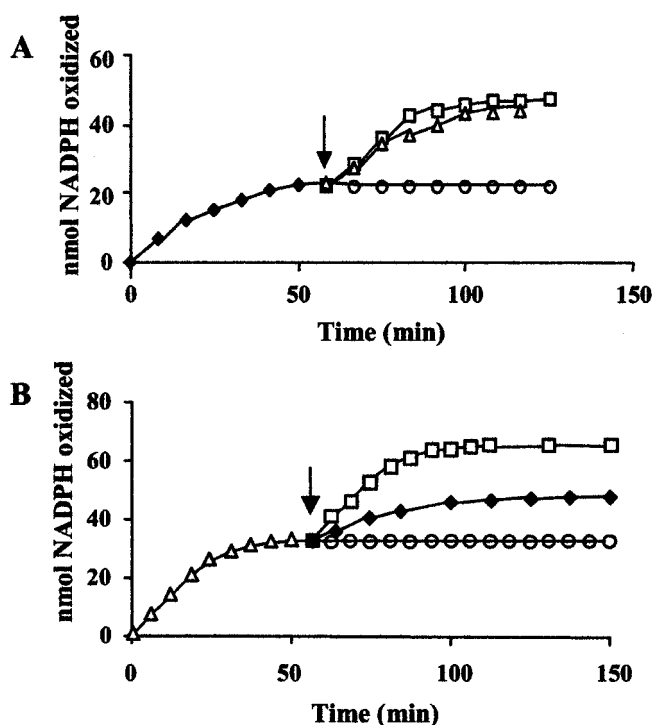


FIG. 4. Sequential action of MsrA and MsrB on CaMox as a substrate. Reactions mixtures included NADPH (400 μM), thioredoxin (5 μM), thioredoxin reductase (87 nM), and CaMox (30 μM). A, reaction was carried out first with 1 μM of MsrB (◆) until completion, at which point (indicated by an arrow), CaMox (30 μM) (□), 1 μM MsrB (○), or 1 μM MsrA (△) was added. B, reaction was carried out first with 1 μM of MsrA (△) until completion, at which point, CaMox (30 μM) (□), 1 μM MsrA (○), or 1 μM MsrB (◆) was added.

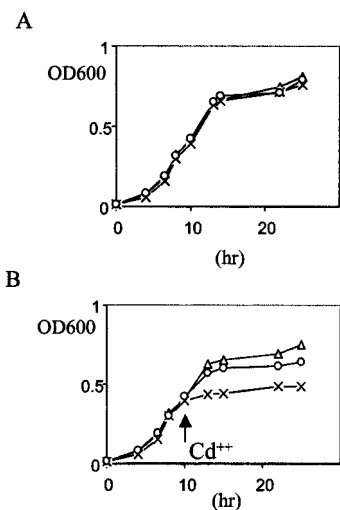


FIG. 5. *msrB* is required for cadmium resistance of *E. coli*. A, growth of *E. coli* MG1655 (wild type) (△), BE017 pUC18 (*msrB::aphA3*) (X) and BE017 pMsrB strains (○). Strains were grown in M9 medium. A_{600} values were recorded. B, at the time indicated by an arrow, cadmium was added to the cultures at a final concentration of 22 μM. Results of a typical experiment are shown.

lated oxiform contained 3 MetSO out of 8 initially present. Repair of CaMox by MsrA was extensively studied by Squier and collaborators (9, 26). Overall our results are consistent with those reported by these authors although some differences appeared in the population size of each oxiform. These apparent discrepancies could be accounted for by differences in sample preparation or the origin of the calmodulin used. In particular, these authors used calcium-loaded CaMox while we

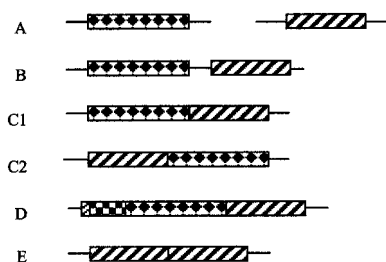


FIG. 6. Schematic representation of *msrA* and *msrB* genetic organization in selected genomes. A, *E. coli*: *msrA* (P27110) and *msrB* (P39903) genes are located at 4439.80 kb and 1860.50 kb on the chromosome, respectively. B, *B. subtilis*: *msrA* (P54154) and *msrB* (P54155) open reading frames are separated by 131 nucleotides. C1, *H. pylori*: *msrA* and *msrB* are fused in this order (025011). C2, *Treponema pallidum*: *msrB* and *msrA* are used in this order (083641). D, *Neisseria gonorrhoeae*: *msrA* and *msrB* are fused to each other and to *dsbE* encoding disulfide oxidoreductase E (P14930). E, *A. thaliana*: two *msrB* domains are fused (Q9ZS93). Note that additional copies of *msrA* and *msrB* are to be found in other regions of the *A. thaliana* genome. Filled diamonds, *msrA* domain. Diagonal lines, *msrB* domain. Checkered squares, *dsbE* domain. Wavy lines, signal sequence.

used decalcified calmodulin. Analysis of the effect of calcium on oxidation and subsequent MsrA/B-mediated repair is under way using calorimetric methods.

Full repair of CaMox is an as yet undocumented phenomenon. This was achieved when MsrA and MsrB were added simultaneously. To explain that MsrA was unable to fully repair CaMox, Squier and collaborators put forward the hypothesis that MsrA repairs MetSO residues that are located in hydrophobic regions of CaMox, *i.e.* those residues that are buried in the native structure. This led support to the model that MsrA acts upon unfolded forms (9). As a consequence, we might explain the full repair of CaMox by postulating that MsrB acts upon solvent-exposed MetSO. However, recent results revealed that MsrA exhibits diastereoselectivity and acts selectively on L-Met-S-SO in CaMox (27, 28). Hence, another possibility is that MsrB repairs selectively L-Met-R-SO. CaMox would then contain a mixture of both diastereoisomers that would require both types of methionine sulfoxide reductase to be fully reduced. The hypothesis of substrate specificity received additional experimental support by submitting CaMox to sequential action of MsrA and MsrB. Indeed, we observed that CaMox that had previously been reduced by MsrA remained a *bona fide* substrate for MsrB and vice versa. The simplest interpretation is that each Msr targets different MetSO diastereoisomers within CaMox. Recently, this hypothesis received additional support using purified L-Met-R-SO and L-Met-S-SO and MsrB-purified protein.²

Despite being functionally related, MsrA and MsrB appear not to share a recent origin since comparison of their amino acid sequences failed to reveal any overall similarity. Moreover, 1D NMR analysis of *E. coli* MsrB³ as well as CD spectra of the *Mycoplasma genitalium* *msrB* ortholog (MG448) suggested that MsrB is unstructured (29). This contrasts with MsrA that is well structured, as recently shown by the resolution of the three-dimensional structures by x-ray crystallography (5, 6). Of potential interest, however, is a motif reading CGWP(S/A)F that is present in MsrB sequences. This motif is reminiscent of the signature motif CGFWG, containing the Cys catalytic residue in MsrA (7). Ongoing studies aim at testing the role of the CGWP(S/A)F motif in MsrB activity.

Analysis of *msrA* and *msrB* genes distribution in all sequenced genomes revealed a great diversity of genetic organi-

² H. Weissbach, personal communication.

³ P. Gans, unpublished material.

zations (Fig. 6). In some genomes, *msrA* and *msrB* are located in different positions (e.g. *E. coli*). In some genomes, they are fused such as to encode a bifunctional MsrA-MsrB polypeptide (e.g. *Helicobacter pylori*). In *Bacillus subtilis*, the intermediate situation is to be found since *msrA* and *msrB* genes lie adjacent one to the other, most probably in an operon structure. In *Neisseria*, they are fused with the *dsbE* ortholog that encodes a disulfide oxidoreductase. Intriguingly, in this later case, the three-domain protein possess a signal sequence, suggesting that it acts in an extracytoplasmic compartment. Another type of organization is found in *Arabidopsis thaliana*, in which there are multiple *msrA* and *msrB*, with one gene predicted to encode a polypeptide containing two MsrB domains in tandem. Another remarkable case is provided by human where MsrB was called SelX, a selenocysteine-containing protein (30). Characterization of these diverse MsrB orthologs will be of interest in revealing the biological importance of these diverse genetic arrangements. It would, for instance, be of interest to know whether increased efficiency in repair was gained by the fusion of *msrA* and *msrB* or by the tandem duplication of *msrB* as found in plants.

Phenotypic analysis of an *E. coli* strain lacking a functional copy of *msrB* revealed its importance in cadmium resistance. Cadmium is a potent carcinogenic and damages cells in several ways, among which is catalysis of AOS production. Hypersensitivity of *E. coli msrB* to cadmium is consistent with the finding that expression of the *E. faecalis msrB* ortholog (and not *msrA* as misquoted by the authors) is induced in the presence of cadmium (25). In this context, it is important to remember that *msrA* mutation confers increased sensitivity to oxidative stress in both *E. coli* and *Erwinia chrysanthemi* (13, 14). These phenotypic analyses together with the biochemical features of MsrA and MsrB suggest that these methionine sulfoxide reductases have an important function in protecting cells from oxidative damages. Furthermore, a crucial role for *msrB* in cell physiology was recently advanced by a systematic alteration of *Mycobacterium* open reading frame that identified the *msrB* ortholog as an essential gene (31).

Although most proteins contain solvent-exposed methionine that are potential targets for oxidation, one can expect that only a subset of cell proteins will be fully inactivated by methionine oxidation. Hence, importance of the Msr repair pathway might be appreciated by identifying those proteins that contain structural and/or functionally important Met residues. Alternatively, insight might be provided by proteomic approaches aimed at describing proteins networks. A systematic search for protein/protein interactions by the yeast two-hybrid screen was recently carried out in *H. pylori* (32). Interestingly, the *H. pylori* bi-functional MsrA/MsrB protein (Fig. 6) was found to interact with ClpX, a chaperone that assists folding of abnormal proteins or associates with the ClpP protease for degradation of those misfolded proteins. Hence, a possibility is that MsrA/B repairs oxidized ClpX. Alternatively, MsrA/B and ClpX might form a complex such that a given oxidized protein might either get repaired by MsrA/B or be directed to ClpP-mediated degradation. Our ongoing studies aim at identifying *in vivo*

substrates of MsrA and MsrB to define the repair pathway of MetSO-containing proteins.

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