Increased Catalytic Efficiency following Gene Fusion of Bifunctional Methionine Sulfoxide Reductase Enzymes from *Shewanella oneidensis*[†]

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ABSTRACT: Methionine sulfoxide reductase enzymes MsrA and MsrB have complementary stereospecificities that reduce the *S* and *R* stereoisomers of methionine sulfoxide (MetSO), respectively, and together function as critical antioxidant enzymes. In some pathogenic and metal-reducing bacteria, these genes are fused to form a bifunctional methionine sulfoxide reductase (i.e., MsrBA) enzyme. To investigate how gene fusion affects the substrate specificity and catalytic activities of Msr, we have cloned and expressed the MsrBA enzyme from *Shewanella oneidensis*, a metal-reducing bacterium and fish pathogen. For comparison, we also cloned and expressed the wild-type MsrA enzyme from *S. oneidensis* and a genetically engineered MsrB protein. MsrBA is able to completely reduce (i.e., repair) MetSO in the calcium regulatory protein calmodulin (CaM), while only partial repair is observed using both MsrA and MsrB enzymes together at 25 °C. A restoration of the normal protein fold is observed co-incident with the repair of MetSO in oxidized CaM (CaM$_{ox}$ by MsrBA, as monitored by time-dependent increases in the anisotropy associated with the rigidly bound multiuse affinity probe 4′,5′-bis(1,3,2-dithioarsolan-2-yl)fluorescein (FlAsH). Underlying the efficient repair of MetSO in CaM$_{ox}$ is the coordinate activity of the two catalytic domains in the MsrBA fusion protein, which results in a 1 order of magnitude rate enhancement in comparison to those of the individual MsrA or MsrB enzyme alone. The coordinate binding of both domains of MsrBA permits the full repair of all MetSO in CaM$_{ox}$. The common expression of Msr fusion proteins in bacterial pathogens is consistent with an important role for this enzyme activity in the maintenance of protein function necessary for bacterial survival under highly oxidizing conditions associated with pathogenesis or bioremediation.

Methionines are highly susceptible to oxidation to their corresponding methionine sulfoxides (MetSO)[1] by a range of commonly generated reactive oxygen species (ROS), such as hydrogen peroxide, singlet oxygen, or peroxynitrite (*1−3*). Methionine sulfoxide reductase (Msr) enzymes recognize MetSO within unfolded sequences of proteins and bind with high affinity (i.e., $K_d = 70 \pm 10$ nM) prior to the reduction of MetSO to restore the native Met structure (*4−6*). In addition, distinct Msr enzymes are critical to the maintenance of cellular Met pools (*7*), oxidation of which can downregulate the initiation of protein synthesis.

Two different enzyme classes of Msr, i.e., MsrA and MsrB, are expressed in virtually all organisms (*8*, *9*). While these enzymes have little structural homology, the catalytic sites of MsrA and MsrB possess a mirror image symmetry that promotes the selective reduction of *S* and *R* diastereomers of MetSO (*10−12*). High-affinity binding and reduction of free MetSO by either MsrA or MsrB are rapid ($k_{ob} > 50$ s^{-1}), with subsequent steps involving re-formation of the reduced catalytic center, which limits steady-state rates of catalysis (*13*, *14*). However substantial amounts of MetSO are present in a range of different healthy mouse tissues (i.e., >4% of all Met) (*2*), suggesting that Msr enzymes are insufficient for maintaining cellular Met in the fully reduced state. The incomplete repair of MetSO in proteins has been linked to the substrate requirements of Msr enzymes, which do not recognize the majority of MetSO within folded protein structures (*5*). Rather, sensitive proteins whose structures are disrupted upon oxidation of Met are the major substrates of Msr enzymes, and repair of these regulatory proteins has the potential to modulate cellular metabolism in a redox-dependent manner that is regulated by the NADPH-dependent reduction of thioredoxin by thioredoxin reductase (*15*).

Msr enzymes are critical to bacterial survival in high-oxidative stress environments and permit the colonization of host organisms by a range of pathogens, including *Erwinia chrysanthemi*, *Escherichia coli*, *Helicobacter pylori*, *Mycobacterium smegmatis*, *Mycobacterium tuberculosis*, *Mycoplasma genitlium*, and *Neisseria gonorrhoeae* (*11*, *16−21*).

[1] Abbreviations: β-ME, 2-mercaptoethanol; CaM, calmodulin; CaM$_{ox}$, CaM in which all nine Met residues are oxidized to MetSO; ESI-MS, electrospray ionization mass spectrometry; FlAsH-EDT$_2$, 4′,5′-bis(1,3,2-dithioarsolan-2-yl)fluorescein; MetSO, methionine sulfoxide(s); MS, mass spectrometry; Msr, methionine sulfoxide reductase; ROS, reactive oxygen species; TCEP, triscarboxyethylphosphine.

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MsrB_NEISS  GSHMAATYKKPSDAELKRTL390  TEEQYQVTQN400  SATEYAFSHE410  YDHLFKPGIY420  VDVVSGEPLF430  SSADKYDSGC440  GWPSFTRPID450
MsrBA_MR1   ---------------MNKL      TDFERYVIEE     KGTERPFSGQ     YYQHDAKGVY     LCKKCHTPLY     RSEDKFNAHC     GWPAFDDEIP
MsrA_MR1    --------------------     ----------     ----------     ----------     ----------     ----------     ----------
MsrA_E coli --------------------     ----------     ----------     ----------     ----------     ----------     ----------

MsrB_NEISS  AKSVTEHDDF460  SFNMRRTEVR470  SRAADSHLGH480  VFPDGPRDKG490  GLRYCINGAS500  LKFIPLEQMD510  AAGYGALKGE520  VK
MsrBA_MR1   G-AVSRHID-     -ADGRRTEIV     CSQCGGHLGH     VFEGEFLTPK     NIRHCVNSVS     MLFKAQDEAE     DIAPKHAFAT     FGAG
MsrA_MR1    ----------     ----------     ----------     ----------     ----------     ----------     -----MALAT     FGAG
MsrA_E coli ----------     ----------     ----SLFDKK6    HLVSPADALP16   GRNTPMPVAT26   LHAVNGHSMT36   NVPDGMEIAI46   FAMG50

MsrBA_MR1   CFWCVEAIFK     SLKGVLNVRS     GYSGGEASDA     DYKSVCSGQT     GHAEVVHIEF     DPEQIAFTTL     LQVLFESHDP
MsrA_MR1    CFWGVEYFFR     QVNGVTNATC     GYMGGNNEVT     TYREVKKGKT     GHAEVVQVEF     DPTIVSYDEL     LEVFWKNHNP
MsrA_E coli CFWGVERLFW60   QLPGVYSTAA70   GYTGGYTPNP80   TYREVCSGDT90   GHAEAVRIVY100  DPSVISYEQL110  LQVFWENHDP120

MsrBA_MR1   TTLNRQGNDK     GPQYRSVVFA     HDATQVDEAN     A-MIAK--LT     EARVFDAPIV     TQVVAFESFY     PAEAYHNDYF
MsrA_MR1    TTLNMQGGDI     GTQYRSTIFF     HDREQKAAAE     ASKLAF--AR     SGRWGLRHIV     TEIVPLQQFH     VAEEYHQNYI
MsrA_Ecoli  AQGMRQGNDH130  GTQYRSAIYP140  LTPEQDAAAR150  ASLERFQAAM160  LAADDDRHIT170  TEIANATPFV180  YAEDDHQQYL190

MsrBA_MR1   ALHGEQPYCQI    VVRPKVEKIR     ALFAELQV
MsrA_MR1    DKNN-LPSCHL    EY--------     --------
MsrA_Ecoli  HKNP-YGYCGI200 GGIGVCLPPE210  A-------
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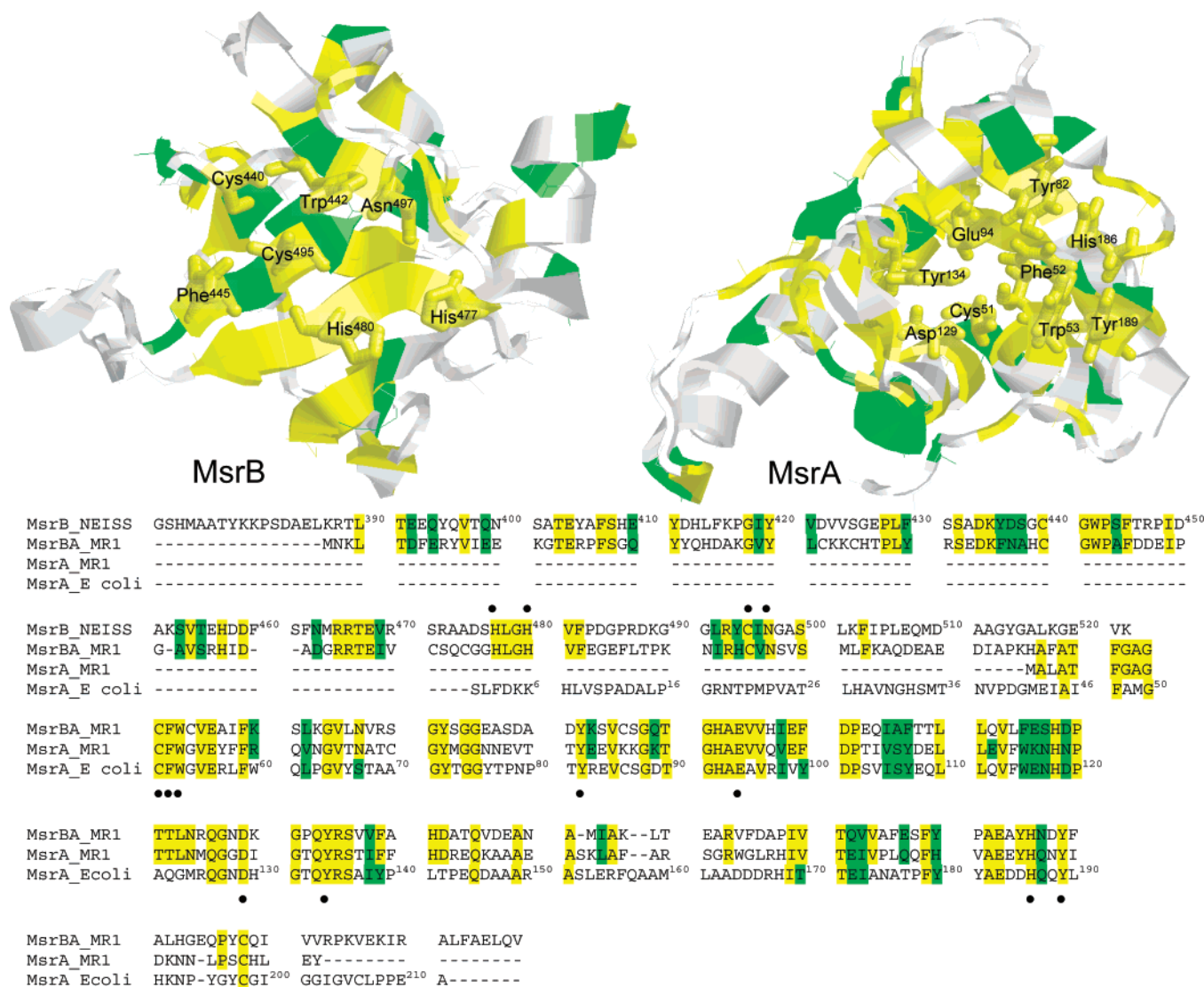
FIGURE 1: Conserved active site sequences of Msr proteins. Tertiary structures (top) and sequences (bottom) of Msr proteins highlighting identical (yellow) and conserved (green) amino acids. Side chains implicated in catalysis or substrate recognition are highlighted (*12*, *49*, *50*). Structures correspond to MsrB (left) or MsrA (right). Sequences compare amino acids between MsrB from *N. gonorrhoeae* and MsrA from *E. coli* with *S. oneidensis* MR-1 proteins MsrA (SO2337) and MsrBA (SO2588), as determined using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). Structures correspond to PDB entry 2gt3 for MsrA from *E. coli* and PDB entry 1l1d for MsrB from *N. gonorrhoeae* (*12*, *51*, *52*) and were drawn using RASMOL (*53*).

Bifunctional Msr enzymes, which reduce both *S* and *R* diastereomers of MetSO, are present in many pathogenic and metal-reducing bacteria (including *Haemophilus influenze*, *H. pylori*, *Vibrio cholerae*, *Neisseria meningitidis*, *Bacillus anthracis*, and *Shewanella oneidensis*) and have arisen through gene fusion to create both MsrAB or MsrBA variants (*8*). These fusion proteins are commonly located on the outer surface of the inner membrane in the periplasm, permitting the efficient redox coupling of cytosolic NADPH for maintenence of these enzymes in a reduced state necessary for the repair of oxidized proteins (*11*, *22*). It remains unclear whether the formation of bifunctional Msr enzymes through gene fusion has any additional functional significance that may, for example, be related to the catalytic ability to simultaneously bind multiple MetSO within an oxidized protein.

To assess possible improvements in the ability of bifunctional Msr enzymes to reduce MetSO in oxidized proteins, we have cloned and expressed the two genes encoding MsrBA and MsrA from *S. oneidensis* (Figure 1). This soil microbe encodes 42 multiheme cytochromes, which underlie a diverse metabolism that allows growth using solid metal oxides as terminal electron acceptors, resulting in a considerable functional sensitivity to reactive oxygen species (ROS) (*23*, *24*). For comparison, we have constructed and expressed the MsrB encoding region of MsrBA. The abilities of MsrBA, MsrA, and MsrB to repair protein-bound MetSO were assessed using the calcium regulatory protein calmodulin (CaM) that has previously been used as a protein substrate to assess the ability of mammalian forms of both MsrA and MsrB to recognize MetSO in proteins (*6*, *25*). Furthermore, CaM can play an important role in promoting bacterial pathogenesis, suggesting the physiological relevance of its oxidation (*26*). We find that the bifunctional MsrBA enzyme is able to fully reduce all *S* and *R* stereoisomers of MetSO in CaM$_{ox}$. In contrast, a mixture of the isolated MsrA and MsrB proteins is unable to catalyze the complete repair of all MetSO, consistent with the inability of Msr enzymes to recognize MetSO within folded proteins (*5*). Accompanying reduction of MetSO in CaM$_{ox}$ by MsrBA is the

restoration of the overall protein fold of CaM, which is not observed using either MsrA or MsrB alone. These results suggest an important role for bifunctional Msr enzymes in the maintenance of protein functions under conditions of acute oxidative stress.

EXPERIMENTAL PROCEDURES

Materials. Thioredoxin, thioredoxin reductase, L-methionine sulfoxide (L-MetSO), 2-mercaptoethanol (β-ME), triscarboxyethylphosphine-HCl (TCEP), and β-nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Sigma Chemical Co. (St. Louis, MO). 4′,5′-Bis(1,3,2-dithioarsolan-2-yl)fluorescein (FlAsH-EDT$_2$) was synthesized as previously described (*27*, *28*). All other chemicals were of reagent grade.

Cloning and Expression of Msr Isoforms. Two putative Msr genes, i.e., MsrA (SO2337) and MsrBA (SO2588) (Figure 1), identified in the *S. oneidensis* MR-1 genome (*29*), contained 480 and 903 bp, respectively, and their DNA fragments were PCR amplified with MsrA-F (5′-CAC-CARGGCGTTAGCAACTTTCGGT-3′) and MsrA-R (5′-GTACTCGAGCTGACAACTTGGTAA-3′) primers and MsrBA-F (5′-CACCATGGACAAACTGACTGATTTTGAA-3′) and MsrBA-R (5′-AACTTGCAGTTCGGCAAATAAATGC-3′) primers, respectively. For comparison, the MsrB region of the MsrBA gene containing the first 128 amino acids in the sequence was PCR amplified using the following forward and reverse primers, i.e., MSRB#2-F (5′-CACCATGAA-CAAACTGACTGATTTTGA-3′) and MSRB#2R (5′-AG-CATGTTTTGGGGCAATAT-3′). This sequence encodes the entire MsrB gene based on homology with other MsrB sequences in a range of microbes, including *B. anthracis*, *E. coli*, *M. tuberculosis*, *Salmonella typhimurium*, *V. cholerae*, and *Yersinia pestis*. The amplified DNA fragments were cloned into pENTR/SD/D-TOPO vector (Invitrogen) to contain a V5-His tag sequence (KGGRADPAFLYKVVIN-SKLEGKPIPNPLLGLDSTRTGHHHHHH) engineered at the C-terminus of the expressed proteins and transformed into *E. coli* strain TOP10. Positive clones were screened, and all DNA sequences were verified by DNA sequencing (Amplicon Express, Pullman, WA).

For overexpression of Msr isoforms in *E. coli*, individual plasmids were cloned into pETDEST 42 (Invitrogen catalog no. 12276-010) using LR Clonase (Invitrogen catalog no. 11791-019) and transformed into *E. coli* strain BL21Star (DE3). One liter of innoculated culture was grown at 37 °C to an optical density of 0.4 at 600 nm. Isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1.0 mM, and growth was continued overnight at 30 °C. Bacteria were harvested by centrifugation, and following resuspension in 10 mM Tris-HCl (pH 8.0), 300 mM NaCl, and 10 mM imidazole, cells were lysed by three passes through a French pressure cell (Glenmills Inc., Clifton, NJ). Lysates were first clarified by centrifugation at 5000 rpm for 15 min, and the resulting supernatant was then centrifuged at 15 000 rpm for an additional 30 min. The clear supernatant was loaded onto a cobalt column (Talon Metal Affinity Resins; BD Biosciences-Clontech, Palo Alto, CA), and following 5 volumes of washing buffer [10 mM Tris-HCl (pH 8.0), 300 mM NaCl, and 20 mM imidazole]. MsrBA, MsrA, and MsrB recombinant proteins were eluted

with 5 mL of elution buffer [10 mM Tris-HCl (pH 8.0), 300 mM NaCl, and 300 mM imidazole]. The N-terminal His-Patch thioredoxin sequence from the MsrB recombinant protein was enzymatically digested using EnterokinaseMax (EKMax; Invitrogen catalog no. 190-01). Msr protein concentrations were determined using the Coomassie Plus protein assay (Pierce Inc., Rockford, IL).

Expression and Purification of CaM. DNA encoding a wild-type and a mutant CaM containing four cysteines engineered in helix A (at Glu^6, Glu^7, Ala^{10}, and Glu^{11}) (i.e., C4-CaM) was cloned into the pET-15b (Novagen) expression vector and expressed in *E. coli* strain BL21(DE3), as previously described (*30*). CaM was purified by chromatography on phenyl-Sepharose CL-4B (Pharmacia, Piscataway, NJ) (*31*), and its protein concentration was measured using a micro BCA assay reagent kit (Pierce), using desalted wild-type CaM as the standard ($\epsilon_{277} = 3029$ M^{-1} cm^{-1}) (*31*).

Oxidation of Calmodulin. Following incubation with H$_2$O$_2$ (100 mM) in 10 mM Tris-HCl (pH 7.5) for 24 h at 25 °C, all nine methionines in CaM (60 μM) were oxidized to their corresponding methionine sulfoxides (*25*, *32*). The concentration of H$_2$O$_2$ was determined by using the published extinction coefficient [$\epsilon_{240} = 39.4$ M^{-1} cm^{-1} (*33*)]. The reaction was stopped by exhaustive dialysis at 4 °C.

FlAsH-Labeled CaM. C4-CaM (50 μM) was incubated in 50 mM HEPES (pH 7.5), 140 mM KCl, 1 mM β-ME, and 1 mM TCEP for 1 h to reduce any disulfide bonds prior to the addition of FlAsH-EDT$_2$ (50 μM); essentially complete labeling required 2 h at room temperature. FlAsH-labeled CaM was separated from unbound FlAsH-EDT$_2$ using a Sephadex G25 size exclusion column (*34*).

Fluorescence Measurements. Rotational mobility (anisotropy) of FlAsH-labeled CaM was measured using a SPEX (Edison, NJ) FluoroMax-2 fluorometer, where the anisotropy (*r*) was calculated as the ratio of fluorescence intensities (*I*) with the polarizers in the vertical (v) or horizontal (h) position:

$$r = (I_{vv} - gI_{vh})/(I_{vv} + 2gI_{vh}) \qquad (1)$$

where $g = I_{hv}/I_{hh}$.

Enzymatic Activities of Msr. Unless otherwise indicated, the reductase activity of Msr isoforms was measured in 10 mM Tris-HCl buffer (pH 7.5), NADPH (400 μM), thioredoxin (50 μM), and thioredoxin reductase (2 μM) following the decrease in the absorbance of NADPH at 340 nm ($\epsilon_{340} = 6220$ M^{-1} cm^{-1}) at 24 °C, essentially as previously described (*6*, *35*, *36*).

Mass Spectrometric Analysis. Whole protein ESI spectra were acquired on an AUTOSPEC-Q mass spectrometer equipped with Mark III ESI source, essentially as previously described (*5*).

RESULTS

Conserved Structures of Msr Protein from Shewanella. We assessed the ability of the two Msr proteins encoded in the genome of *S. oneidensis* MR-1 (i.e., MsrBA and MsrA) to repair oxidized CaM (CaM$_{ox}$), in which all nine methionines were oxidized to their methionine sulfoxides. Together, these proteins function to reduce methionine sulfoxides in *Shewanella* proteins. This maintains the ability of this oxidatively sensitive organism to contribute to the bioreme-

diation of soils through its diverse metabolism that permits the reduction of solid metal oxides as terminal electron acceptors (*24*). The C-terminal sequence of MsrBA (SO2588) is homologous to the protein sequence of MsrA (SO2337), with 61% overall positive sequence homology determined using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). The active site and substrate binding recognition sequences in MsrA are absolutely conserved (Figure 1), permitting a comparison between these two enzyme activities to determine the functional implications of the MsrB fusion protein. As there is no homologous MsrB protein encoded in the *Shewanella* genome, we have cloned and expressed the N-terminal sequence of MsrBA, the sequence of which is essentially identical within the active site region and has a 50% overall positive sequence homology to the MsrB protein sequence in *N. gonorrhoeae* (Figure 1).

Enhanced Repair by MsrBA Reduces All Nine MetSO in CaM_{ox}. Taking advantage of large shifts in the mobility of CaM following Met oxidation using SDS−PAGE, we assessed the ability of MsrBA in the presence of the artificial electron donor DTT to repair MetSO in fully oxidized CaM (CaM_{ox}), where authentic standards of unoxidized or fully oxidized CaM with apparent molecular masses of 17.9 and 20.6 kDa were used for comparison. Upon incubation of CaM_{ox} with MsrBA, there is a time-dependent increase in the mobility of CaM on SDS−PAGE, which approaches that of unoxidized CaM after incubation for 4 h (Figure 2). These results suggest that MsrBA is able to fully repair the majority of the MetSO in CaM_{ox}. In comparison, much smaller changes in CaM mobility are apparent upon incubation of CaM_{ox} with MsrA over the same time course, suggesting a diminished capacity of MsrA to recognize and repair MetSO in CaM_{ox}.

Additional resolution regarding the abilities of Msr enzymes to recognize and reduce MetSO in proteins was obtained using intact protein mass spectrometry to assess the extent of Met oxidation in oxidized CaM (CaM_{ox}). CaM_{ox} has previously been used to assess the ability of both MsrA and MsrB to repair oxidized proteins due to the presence of nine surface-exposed methionines (*6, 25*). Prior to oxidation, the average mass of CaM was 16 706.7 Da (Figure 2A), which is consistent with the theoretical mass of 16 706.4 Da. A dehydration artifact associated with the loss of water in the ion trap results in the appearance of a lower-molecular mass feature at 16 688.4 Da (*37*). For these experiments, Met residues in CaM were quantitatively oxidized to their corresponding MetSO using hydrogen peroxide, resulting in a 143.3 Da shift in the average mass of CaM to 16 850.0 Da which is indicative of the quantitative oxidation of all nine methionines (i.e., 9 Met × 16 Da/oxygen = 144 Da) (Figure 2B).

Upon incubation for 12 h with MsrBA in the presence of 15 mM DTT, all nine MetSO in CaM are reduced to form methionine (Figure 2F), and the resulting mass spectrum is virtually identical to that of unoxidized CaM (Figure 2A). In comparison, a distribution of CaM oxiforms remains that contains up to eight MetSO per protein following incubation of CaM_{ox} with an equimolar mixture of MsrA and MsrB for 12 h (Figure 2E). The retention of large numbers of MetSO in CaM following repair by both MsrA and MsrB is consistent with prior results indicating that the restoration
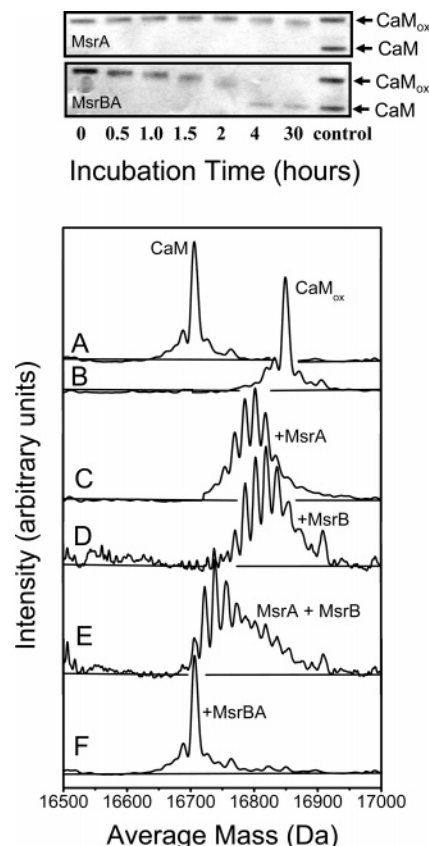

FIGURE 2: Complete repair of CaM_{ox} by MsrBA. (Top) SDS−PAGE (14% Tris-glycine) gel showing time-dependent mobility changes of CaM_{ox} in the presence of either MsrA or MsrBA. (Bottom) Intact protein ESI-MS spectra of unoxidized CaM (A) and oxidized CaM prior to (B) and following incubation for 12 h with MsrA (C), MsrB (D), an equimolar mixture of MsrA and MsrB (E), and MsrBA (F). Experimental conditions involved CaM (10 μM) incubated with indicated Msr isoforms (1.0 μM) of either MsrBA, MsrA, or MsrB in 10 mM MOPS (pH 7.5), 50 mM KCl, and 15 mM DTT at 25 °C. No MetSO reduction is observed in the absence of added DTT, which acts to reduce the active site of Msr enzymes to permit reduction of MetSO.

of the protein fold prevents further repair of MetSO (*5, 25*). Thus, while the complementary specificities of MsrA and MsrB for repair of the *S* and *R* stereoisomers of MetSO result in additional repair in comparison to either enzyme alone (Figure 2C,D), the fusion of these enzyme activities to form the bifunctional MsrBA results in a striking enhancement in the extent of CaM_{ox} repair.

Our results demonstrate an enhanced ability to reduce *S*- and *R*-MetSOs in oxidized proteins by the MsrBA fusion protein in comparison to either MsrA or MsrB enzymes. These latter results are consistent with the observation that the oxidation of Met[145] disrupts the tertiary structure of CaM, resulting in the ability of Msr enzymes to recognize MetSO within disordered sequences (*5, 15, 38*). Reduction of this conformationally sensitive site in CaM_{ox} by either MsrA or MsrB will result in a restoration of the protein fold and will weaken the ability of Msr enzymes to recognize and repair other MetSO in the refolded protein. Thus, depending on the order in which individual MetSO are reduced in CaM_{ox}, the protein will assume its native fold and essentially trap remaining MetSO within the folded structure. In contrast, MsrBA is able to bind and fully repair all MetSO in CaM_{ox}, suggesting that the simultaneous binding of both domains facilitates protein repair.
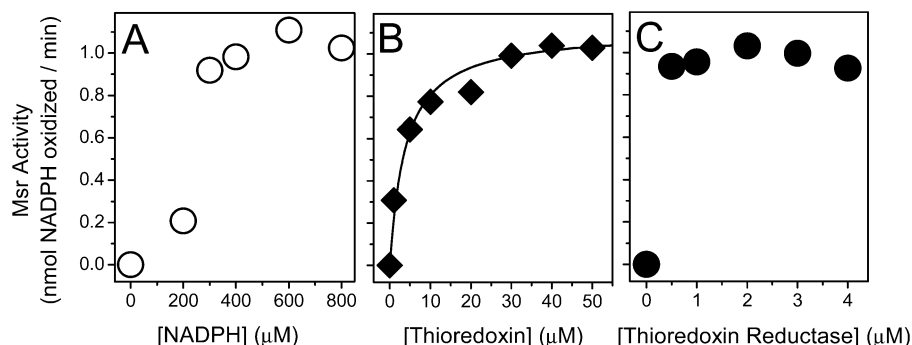
FIGURE 3: Substrate dependence of MsrBA-mediated repair of CaM$_{ox}$. MsrBA-dependent rates of repair of CaM$_{ox}$ as a function of NADPH (A), thioredoxin (B), or thioredoxin reductase (C). Experimental conditions included MsrBA (0.5 μM) and CaM$_{ox}$ (30 μM) in 10 mM Tris-HCl (pH 7.5) containing indicated substrate concentrations and saturating concentrations of NADPH (400 μM) (B and C), thioredoxin (50 μM) (A and C), or thioredoxin reductase (2 μM) (A and B) at 24 °C. The line in panel B represents a nonlinear least-squares fit to the Michaelis−Menten rate equation, where $K_M = 3.3 \pm 0.8$ μM.

Redox-Dependent Modulation of MsrBA Function. The repair of free MetSO involves the reduction of the active site dithiol, which under cellular conditions is mediated by reduced thioredoxin whose concentration is maintained by the NADPH-dependent activity of thioredoxin reductase (*39*). To determine whether changes in cellular concentrations of NADPH and reduced thioredoxin affect rates of MsrBA activity, we measured the substrate dependence of repair as a function of NADPH and thioredoxin concentrations. An equimolar stoichiometry of thioredoxin reductase relative to MsrBA is sufficient to maintain optimal function (Figure 3C), indicating a high-affinity binding interaction that promotes the efficient reduction in the catalytic site of MsrBA. Half-points of maximal catalytic activity occur at 240 $\pm$ 10 μM NADPH and 3.3 $\pm$ 0.8 μM thioredoxin (Figure 3A,B), which are near physiological levels of NADPH and thioredoxin in bacteria (*40−43*). These latter results indicate that the action of MsrBA is under redox control such that reduced rates of MetSO repair will occur under conditions of oxidative stress and associated reductions in the levels of NADPH and reduced thioredoxin.

Restoration of the Protein Fold upon Repair of CaM$_{ox}$ by MsrBA. The oxidation of Met[145] in CaM induces a loss of function and results in global structural changes that disrupt the secondary structure and uncouple the opposing domains of CaM, resulting in the nonproductive binding between CaM$_{ox}$ and some target proteins (*15, 32, 38, 44, 45*). This structural change results in the uncoupling between the opposing domains of CaM, which is normally associated with the calcium-dependent activation of CaM. Following binding of the fluorophore FlAsH, the oxidant-induced disruption of the protein fold of CaM can be readily monitored in real time as a reduction in the steady-state anisotropy (*30, 44*). Similar oxidant-induced changes in protein structure are observed using SDS−PAGE, where oxidation of all nine Met residues results in a substantial reduction in the electrophoretic mobility (Figure 4).

To measure alterations in the protein fold of CaM, we have employed the same FlAsH-labeled CaM mutant that we have previously used to assess oxidant-induced changes in the conformation of CaM (*30, 44*). Upon incubation of equimolar amounts of CaM$_{ox}$ with MsrBA in the presence of saturating amounts of NADPH, thioredoxin, and thioredoxin reductase, there is a time-dependent increase in the fluorescence anisotropy of FlAsH-labeled CaM, which after approximately
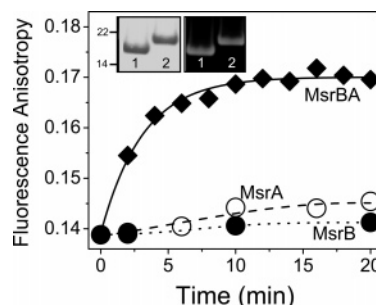

FIGURE 4: Restoration of the CaM$_{ox}$ protein fold upon repair of Met(SO) by MsrBA. Steady-state anisotropies for FlAsH-labeled CaM$_{ox}$ (1.0 μM) in the presence of MsrBA ($\blacklozenge$), MsrA ($\bigcirc$), and MsrB ($\bullet$). The reaction mixture consists of NADPH (400 μM), thioredoxin (50 μM), thioredoxin reductase (2 μM), and the indicated isoform of Msr (1.0 μM) in 50 mM HEPES (pH 7.5), 140 mM KCl, and 0.2 mM CaCl$_2$ in a total volume of 2 mL. Initial rates associated with MsrBA-dependent increases in anisotropy are $(5.9 \pm 1.1) \times 10^{-3}$, $(0.53 \pm 0.12) \times 10^{-3}$, and $(0.19 \pm 0.02) \times 10^{-3}$ min^{-1} for MsrBA, MsrA, and MsrB, respectively. Excitation was at 500 nm, and emitted light was measured at 530 nm; slit widths were set at 5 nm. The inset shows the electrophoretic mobility determined via SDS−PAGE of FlAsH-labeled CaM (10 μg) prior to (lane 1) and following oxidation of all nine methionines (lane 2) using a 14% Tris-glycine gel visualized by fluorescence detection (right) or following Coomassie blue staining (left).

10 min increases from 0.139 $\pm$ 0.001 to a value that approaches that of unoxidized CaM (i.e., 0.170 $\pm$ 0.001) (Figure 4). These measurements demonstrate the full restoration of the protein fold associated with the repair of oxidized calmodulin by MsrBA over a time scale that is relevant to cellular repair processes. In comparison, much smaller increases in fluorescence anisotropy are observed following the incubation of either MsrA or MsrB with FlAsH-labeled CaM, indicating that the separate enzyme activities are unable to efficiently bind and reduce MetSO in oxidized calmodulin. Only MsrBA has the ability to recognize and repair MetSO within oxidized proteins to restore the protein fold associated with native function. In comparison, limited refolding occurs using either MsrA or MsrB enzymes alone.

Enhanced Rates of CaM$_{ox}$ Repair by MsrBA. The enhanced catalytic efficiency of MsrBA in comparison to either MsrA or MsrB alone toward CaM$_{ox}$ is apparent using an enzyme-linked assay involving measurements of decreases in the absorbance of NADPH to measure initial rates of catalysis (Figure 5B). In the presence of saturating amounts of all
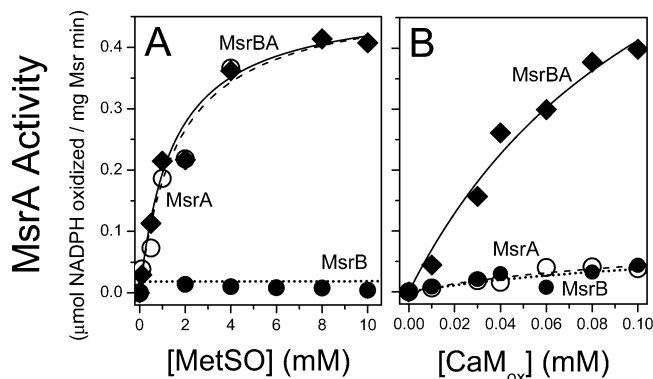
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FIGURE 5: Preferential recognition and repair of CaM$_{ox}$. Catalytic activities of Msr isoforms (0.5 μM) against L-MetSO (A) and CaM$_{ox}$ (B) for MsrBA (◆, solid lines), MsrA (○, dashed lines), and MsrB (●, dotted lines). Experimental conditions involved 10 mM Tris-HCl (pH 7.5), NADPH (400 μM), thioredoxin (50 μM), and thioredoxin reductase (2 μM) in a total volume of 0.2 mL at 24 °C. Lines represent fits to the Michaelis−Menten rate equation (see Table 1).

substrates and 100 μM CaM$_{ox}$ (in which all nine Met residues are oxidized to their corresponding sulfoxides), MsrBA exhibits the highest initial rate (39.2 mol of NADPH oxidized per mole of MsrBA per minute) relative to that of MsrA and MsrB (3.3 and 1.9 mol of NADPH oxidized per mole of Msr per minute, respectively). These results indicate that fused protein MsrBA results in significantly increased activity in comparison to the individual MsrA or MsrB enzymes.

To further assess the specificity of these bacterial Msr enzymes with respect to the ability to recognize and reduce either free or protein-bound MetSO, we compared their substrate dependencies using both L-MetSO and CaM$_{ox}$ (Figure 5). Maximal catalytic rates (i.e., k_{cat}) against CaM$_{ox}$ are approximately 20-fold higher for MsrBA in comparison to that of either MsrA or MsrB alone, indicating that the fusion of these enzyme activities results in large rate enhancements (Figure 5B and Table 1). Similar increases in the catalytic efficiency (k_{cat}/K_M) of MsrBA are apparent, which are more than 10-fold larger than that of either MsrA or MsrB. These results indicate that the ability of MsrBA to maintain the MetSO within protein substrates in an accessible partially unfolded state results in an increase in the overall catalytic rates of repair.

In comparison to the large rate enhancements observed for MsrBA against CaM$_{ox}$ relative to values observed for MsrA or MsrB, the catalytic rates for MsrBA and MsrA are very similar when MetSO is used as a substrate (Figure 5A). This insensitivity of catalytic rates of repair of free MetSO for MsrBA and MsrA activities is consistent with earlier data using MsrAB isolated from *Neisseria*, where the steady-state activities against MetSO were also observed to be unaffected by gene fusion (*46*). In contrast, minimal rates of repair are observed for the genetic construct encoding the MsrB domain, consistent with prior measurements indicating that the turnover number of MsrA is more than 30-fold that of MsrB for free MetSO using physiological substrates (i.e., NADPH, thioredoxin, and thioredoxin reductase) (*6*).

The similar catalytic efficiencies of MsrBA and MsrA against free MetSO indicate that increases in the catalytic efficiencies of association with fusion of both enzyme activities in bifunctional MsrBA enymes against CaM$_{ox}$ require the simultaneous binding of both domains to the

protein-bound MetSO substrates. These results are consistent with earlier reports indicating a cooperative binding between mammalian MsrA enzymes and highly oxidized CaM (*5*), which suggested that induced structural changes within CaM$_{ox}$ upon binding one domain of MsrBA enhance the binding of the other domain.

DISCUSSION

Using both intact protein mass spectrometry and fluorescence spectroscopy to assess reduction of MetSO and restoration of the structural coupling between the opposing domains of CaM$_{ox}$, respectively, we have demonstrated that the fusion of complementary enzyme activities associated with the repair of *R* and *S* stereoisomers of MetSO in MsrBA results in the full repair of all MetSO within CaM$_{ox}$ and the restoration of the native fold (Figures 2 and 4). MsrBA acts to stabilize an unfolded state through the coordinated binding of either MsrA or MsrB domains to CaM$_{ox}$ (Figure 6), permitting the full reduction of all MetSO in CaM$_{ox}$ within 10 min (Figure 4), a time scale that is relevant to bacterial host colonization. A consequence of the stabilization of the unfolded state is an increased catalytic rate due to the increased local concentration of MetSO within unstructured sequences, which permit access of the oxidized side chain of MetSO to the Msr active site for repair (*5*). In comparison, an incomplete repair of either *S*- or *R*-MetSO in CaM$_{ox}$ is observed for the individual MsrA or MsrB enzymes, where the rebinding of the Msr enzyme following dissociation competes with protein refolding and the associated retention of MetSO and reductions in catalytic rates. These latter results are in agreement with prior measurements for homologous Msr proteins from bovine sources under these experimental conditions (*6, 25*), indicating that the inability of mixtures of MsrA and MsrB enzymes to fully reduce MetSO in comparison to MsrBA is not the result of sequence differences between these homologous enzymes. Likewise, limited restoration of the overall structure of CaM$_{ox}$ occurs using either MsrA or MsrB enzymes alone, indicating that the separate enzyme activities are unable to efficiently bind and reduce all MetSO within CaM$_{ox}$. These measurements demonstrate that the enhanced ability of the MsrBA fusion protein to reduce MetSO in oxidized proteins in comparison to the individual MsrA or MsrB enzymes arises as a result of the coordinate action of the MsrA and MsrB elements that maintain CaM$_{ox}$ in a unfolded state necessary for the recognition and repair of MetSO.

The retention of *S*-MetSO in CaM$_{ox}$ following enzymatic repair by MsrA is consistent with the spatial requirements around the active site cysteine, as steric limitations prevent the catalytic sulfur atom from interacting with MetSO residues within helices (*5*). Thus, upon reduction of a critical MetSO in CaM$_{ox}$ (i.e., Met[145]), the restoration of the protein fold results in the retention of MetSO residues within secondary structures that are inaccessible to the active sites of Msr enzymes (*5, 25, 32, 38*). Substantial amounts of both *S*- and *R*-MetSO are retained following the simultaneous incubation of MsrB and MsrA with CaM$_{ox}$, where a stochastic distribution of CaM oxiforms that retain up to eight MetSO per CaM is observed (Figure 2E). The simultaneous binding of both catalytic domains of MsrBA prevents the refolding of CaM$_{ox}$ (irrespective of the reduction of Met-SO[145]) and promotes the complete repair of all MetSO in

Table 1: Catalytic Rates of MetSO Repair by Msr Enzymes

enzyme	substrate	V_{max}^a (μmol of NADPH mg^{-1} min^{-1})	K_m^a (mM)	k_{cat}^b (s^{-1})	k_{cat}/K_m^c (M^{-1} s^{-1})
MsrBA	MetSO	0.49 ± 0.03	1.7 ± 0.4	0.31 ± 0.02	180 ± 40
	CaM$_{ox}$	0.9 ± 0.2	0.12 ± 0.03^d	0.6 ± 0.1	5000 ± 2000
MsrA	MetSO	0.5 ± 0.1	2.0 ± 0.8	0.19 ± 0.04	100 ± 40
	CaM$_{ox}$	0.07 ± 0.02	0.07 ± 0.05^d	0.027 ± 0.007	400 ± 300
MsrB	MetSO	ND^e	ND^e	ND^e	ND^e
	CaM$_{ox}$	0.05 ± 0.01	0.05 ± 0.02^d	0.01 ± 0.01	200 ± 200

^{a} Data presented were obtained by fitting the experimental data to the Michaelis−Menten equation $V = V_{max}[S]/K_m + [S]$, where [S] is the concentration of substrate, K_m is the Michaelis constant, and V_{max} is the velocity when the reaction approaches a maximum. Reaction conditions are described in the legend of Figure 5. ^{b} $k_{cat} = V_{max} \times$ molecular weight = turnover number. ^{c} Errors were propagated. ^{d} Values are apparent and do not take into account the presence of multiple MetSO in oxidized CaM. ^{e} Not determined.
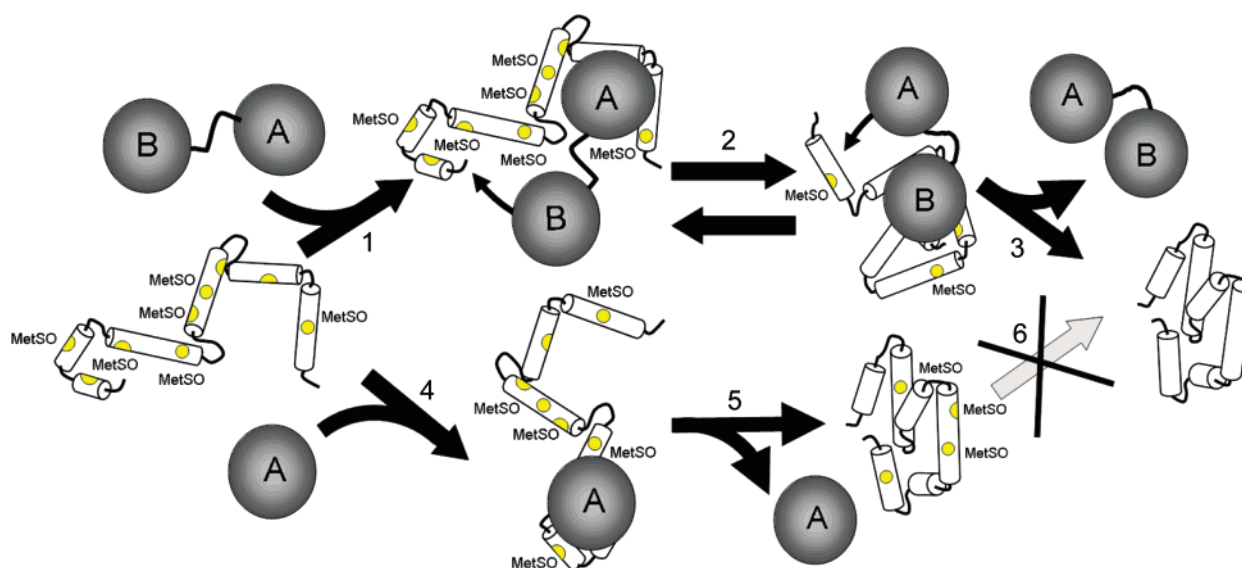

FIGURE 6: Coordinated binding at multiple MetSO in CaM$_{ox}$ by MsrBA facilitates repair. Depiction of the proposed role of the MsrBA fusion protein (gray connected circles denoted A and B) in binding and stabilizing the unfolded state of oxidized CaM (white cylinders) (step 1), permitting reduction of all nine MetSO (yellow) to their native Met structure through the coordinate binding of both active sites A and B in MsrBA (top) at multiple MetSO sites (step 2), where diffusional steps are minimized due to the role of the complemetary protein domain in anchoring MsrBA to the oxidized protein. Underlying the capacity of MsrBA to fully repair CaM$_{ox}$ is an ability to stabilize the oxidized and partially unfolded CaM to promote recognition of exposed MetSO by the Msr enzymes (Figure 4). The ability to maintain the MetSO within protein substrates in an accessible partially unfolded state results in increased catalytic rates of repair (Figure 5). Following MetSO reduction, MsrBA dissociates, releasing the fully repaired protein (step 3). In contrast, while binding of MsrA (bottom) or MsrB (not depicted) to fully oxidized CaM (step 4) readily occurs, neither MsrA nor MsrB alone can fully repair all *S*- or *R*-MetSO in CaM$_{ox}$ under these experimental conditions, where there is substantial protein refolding at 25 °C (step 5) that competes with the ability of the individual Msr enzymes to recognize and bind MetSO in CaM$_{ox}$ (step 5) (*5, 6, 25, 32*). Following protein refolding, there is no additional reduction of MetSO (step 6 is blocked), resulting in the retention of MetSO as occurs during biological aging (*2*).

oxidized proteins (i.e., CaM$_{ox}$) (Figure 2F). These results indicate that the simultaneous binding of both active sites in MsrBA keeps CaM$_{ox}$ in a partially unfolded state that exposes MetSO within conformationally disordered sequences for Msr enzyme recognition and repair (*5*) (Figure 6). Consistent with the latter suggestion that the inability of individual MsrA or MsrB enzymes to fully repair MetSO in CaM$_{ox}$ is due to protein refolding, prior results have demonstrated the full repair of all MetSO by MsrA and MsrB upon disruption of the structure of CaM$_{ox}$ at higher temperatures (*47*). In total, these results indicate that the simultaneous binding of both domains in MsrBA contributes to the ability of this bifunctional Msr enzyme to efficiently and completely reduce MetSO in CaM$_{ox}$. This enhanced repair activity against oxidized proteins represents an advantage under acute conditions of oxidative stress associated with microbial colonization, when high levels of protein oxidation are likely to occur (*11, 16, 48*).

Prior measurements have established the physiological importance of the correct localization of MsrAB to the outer membrane in both *N. gonorrhoeae* and *H. pylori*, whose periplasmic localization promotes colonization under conditions of oxidative stress (*11, 16*). Coupling MsrAB to a thioredoxin domain (i.e., NT) at the N-terminus of MsrAB in *Neisseria* efficiently reduces the active site in both catalytic domains in the absence of added thioredoxin (*22*). Cytosolic reducing potential (i.e., NADPH) is coupled to the MsrAB systems through the integral membrane protein DspD, which promotes the reduction of the active sites in both MsrA and MsrB domains of MsrAB (*22*). These results suggested that enzyme fusion in bifunctional Msr enzymes functions primarily as an organizing mechanism to effectively couple the cytosolic reducing equivalents to active MsrA and MsrB sites in the periplasm. Consistent with this suggestion, gene fusion results in no change in the catalytic repair of free MetSO for MsrAB in comparison to MsrA and MsrB alone

(*46*). Our results suggest that gene fusion of MsrA and MsrB to form MsrBA in *Shewanella* plays an additional role in promoting the rapid and complete repair of MetSO in oxidized proteins, which is expected to promote bacterial colonization in oxidizing environments.

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