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A Sulfenic Acid Enzyme Intermediate Is Involved in the Catalytic Mechanism of Peptide Methionine Sulfoxide Reductase from *Escherichia coli**

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Methionine oxidation into methionine sulfoxide is known to be involved in many pathologies and to exert regulatory effects on proteins. This oxidation can be reversed by a ubiquitous monomeric enzyme, the peptide methionine sulfoxide reductase (MsrA), whose activity *in vivo* requires the thioredoxin-regenerating system. The proposed chemical mechanism of *Escherichia coli* MsrA involves three Cys residues (positions 51, 198, and 206). A fourth Cys (position 86) is not important for catalysis. In the absence of a reducing system, 2 mol of methionine are formed per mole of enzyme for wild type and Cys-86 → Ser mutant MsrA, whereas only 1 mol is formed for mutants in which either Cys-198 or Cys-206 is mutated. Reduction of methionine sulfoxide is shown to proceed through the formation of a sulfenic acid intermediate. This intermediate has been characterized by chemical probes and mass spectrometry analyses. Together, the results support a three-step chemical mechanism *in vivo*: 1) Cys-51 attacks the sulfur atom of the sulfoxide substrate leading, via a rearrangement, to the formation of a sulfenic acid intermediate on Cys-51 and release of 1 mol of methionine/mol of enzyme; 2) the sulfenic acid is then reduced via a double displacement mechanism involving formation of a disulfide bond between Cys-51 and Cys-198, followed by formation of a disulfide bond between Cys-198 and Cys-206, which liberates Cys-51, and 3) the disulfide bond between Cys-198 and Cys-206 is reduced by thioredoxin-dependent recycling system process.

Aerobic metabolism produces a great number of activated oxygen species. These species can react with various targets including proteins. In particular, methionine residues can be oxidized into methionine sulfoxide (MetSO).¹ Such modifications can alter the biological properties of the targeted proteins (1). For instance, this likely is the case for the α -proteinase inhibitor whose oxidation of a methionine residue decreases its

affinity relative to its protease target (2) and also for calmodulin whose methionine oxidation leads to a decrease in the efficiency of activation of plasma membrane (3). On the other hand the fact that methionine modifications can be also restricted to only surface-exposed residues was interpreted as a way to protect cells against the action of reactive oxygen species (4). *In vivo* a ubiquitous enzyme named peptide methionine sulfoxide reductase (MsrA) exists, which reduces both free and protein bound MetSO (5, 6). The fact that the null mutants of both *Escherichia coli* and yeast showed increased sensitivity against oxidative damage and that overexpression of MsrA gave higher resistance to hydrogen treatment supports an essential role of MsrA in cell viability (7, 8). Thus the important biological role attributed to MsrA *in vivo* justifies a study of the chemical mechanism of the reduction of MetSO by MsrA. The fact that MsrA activity necessitates a thioredoxin recycling system (9–11) suggested a cysteine residue in the chemical catalysis. Recently, two groups have shown that mutating the invariant cysteine located in the conserved signature Gly-Cys-Phe-Trp resulted in total loss of enzyme activity (12, 13). Moreover Lowther *et al.* (13) presented convincing evidence of involvement of intra-thiol-disulfide exchanges in the catalytic mechanism. Based on their data they formulated a reaction mechanism requiring formation of a covalent tetracoordinate intermediate via a nucleophilic attack by the thiolate of the essential cysteine followed by breakdown of the intermediate by means of two thiol-disulfide exchanges, which leads to release of a methionine and a water molecule. In this mechanism, methionine release only occurs if the disulfide exchange is operative. In the present study we show that in fact the nucleophilic attack of the essential cysteine on MetSO leads to formation of a sulfenic acid enzyme intermediate with a concomitant release of methionine. Return of the active site to a reduced state is achieved *in vivo* via intra-disulfide exchange reactions involving two other cysteines and then by a thioredoxin-dependent recycling process.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis, Production, and Purification of Wild Type and Mutant E. coli MsrAs—The *E. coli* strain used for wild type and mutant MsrAs production was DH5 α (supE44, Δ lacU169 (ϕ 80 lacZ Δ M15), hsdR17, recA1, endA1, gyrA96, thi-1, relA1) transformed with a plasmid construction containing the *msrA* gene under the control of the lac promoter. Site-directed mutageneses were performed using the QuikChange site-directed mutagenesis kit (Stratagene).

For purification of wild type and mutant MsrAs, cells were harvested by centrifugation, resuspended in minimal volume of buffer A (50 mM Tris-HCl, 2 mM EDTA, pH 8) containing 20 mM dithiothreitol (DTT), and sonicated. The MsrA was then precipitated at 45% ammonium sulfate saturation. The contaminating proteins were removed by apply-

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¹ The abbreviations used are: MetSO, methionine sulfoxide; MsrA, methionine sulfoxide reductase; DTT, dithiothreitol; DTNB, 5,5'-dithiobis(2-nitro)benzoate; TNB⁻, thionitrobenzoate; dimedone, 5,5-dimethyl-1,3-cyclohexanedione.

ing the enzymatic solution onto exclusion size chromatography on ACA 54 gel (IBF) at pH 8 (buffer A). Purified fractions were then pooled and applied to a Q-Sepharose column equilibrated with buffer A, followed by a linear gradient of KCl (0–0.4 M) using a fast protein liquid chromatography system (Amersham Pharmacia Biotech). The MsrA was eluted at 250 mM KCl. At this stage, wild type and mutant MsrAs were pure as checked by electrophoresis on 12% SDS-polyacrylamide gel (14) followed by Coomassie Brilliant Blue R-250 staining. Briefly, 10 μg of enzyme was mixed with sample loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 0.01% bromophenol blue) with and without DTT (100 mM), and samples were analyzed without heating.

Purified enzymes were stored at -20°C in the presence of 20 mM DTT and 45% ammonium sulfate. Under these conditions, the enzymes were stable for several weeks. Their molar concentrations were determined spectrophotometrically, using a molar absorptivity at 280 nm of $34632\text{ M}^{-1}\text{ cm}^{-1}$, deduced from the method of Scopes (15), for wild type and mutant MsrAs. Native molecular mass determination was performed by gel filtration using a Superose 12 H.R. (10×30) fast protein liquid chromatography column (Amersham Pharmacia Biotech) equilibrated with buffer A containing 150 mM KCl, at a flow rate of 0.2 ml/min. For calibration, the following molecular mass standards (Bio-Rad) were used: vitamin B12 (1.35 kDa), myoglobin (horse, 17.5 kDa), ovalbumin (chicken, 44 kDa), γ -globulin (beef, 158 kDa), and tyroglobulin (670 kDa). The void and total volumes of the column, 6.9 and 18.2 ml, respectively, were determined with potassium bichromate and blue dextran dyes to enable calculation of the distribution coefficient K_{AV} .

Mild Oxidation of C86S/C198S/C206S Mutant MsrA—The triple mutant C86S/C198S/C206S was incubated with 1 equivalent of H_2O_2 at room temperature for 30 min in buffer A. The evolution of the oxidation was followed by quantification of the remaining Cys-51 content with 5, 5'-dithiobis(2-nitro)benzoate (DTNB).

Quantification of the Free Cysteine Content with 5,5'-Dithiobis(2-nitro)benzoate—Cysteine content of protein samples was determined using DTNB under nondenaturing and denaturing conditions, either after treatment or no treatment with 10 mM MetSO but without the addition of any exogenous reducing system. Progress curves of thionitrobenzoate (TNB^-) production for wild type and mutant enzymes were recorded at 412 nm in buffer A for nondenaturing conditions. For denaturing conditions, SDS was added to a final concentration of 10% and enzyme solution was heated 10 min at 25 or 70°C . Enzyme concentrations were 7.35 and 14.7 μM , and DTNB concentration was 300 μM . The amount of TNB^- formed was calculated using an extinction coefficient at 412 nm of $13600\text{ M}^{-1}\text{ cm}^{-1}$.

Determination of MsrA Activity—The ability of wild type and mutant MsrAs to reduce free MetSO was assayed by using D,L-MetSO as a substrate and either 10 mM DTT or a thioredoxin-regenerating system (10 μM *Chlamydomonas reinhardtii* thioredoxin, 0.64 μM *Arabidopsis thaliana* thioredoxin reductase, and 0.2 mM NADPH). The reaction mixture contained 10 mM MetSO in buffer A and various concentrations (1–20 μM) of pure wild type or mutant MsrAs. Thioredoxin from *C. reinhardtii* and thioredoxin reductase from *A. thaliana* were prepared in the laboratory of Dr. J. P. Jacquot following experimental procedures already published (16, 17). The *E. coli* recycling system was provided by Dr. J. P. Jacquot.

Initial rate measurements were carried out by following at 25°C the appearance of free methionine measured by high pressure liquid chromatography. For that, aliquots of the reaction mixture were removed at different times of incubation up to 2.5 min, and reaction was stopped by addition of trifluoroacetic acid to a final concentration of 1%. A 100- μl sample of these aliquots was injected onto a 25-cm Sephasil C18 reverse phase column (Amersham Pharmacia Biotech) on an ÄKTA explorer system (Amersham Pharmacia Biotech) equilibrated with H_2O /trifluoroacetic acid 0.1% buffer in the presence of 10% acetonitrile. Met was eluted isocratically after MetSO as monitored by peak integration at 215 nm (1 nmol of Met gives 2.66 area units).

Stoichiometry of Met Formation in the Absence of Regenerating System—The reaction mixture containing 10 mM MetSO and 100–500 μM of enzyme was incubated at 25°C for 10 min in 50 mM Tris-HCl, 2 mM EDTA, pH 8 buffer. Then, 100 μl of the reaction mixture was injected onto a Sephasil C18 column for Met quantification as described above.

Biochemical Characterization of the Sulfenic Acid Intermediate—Sulfenic acid intermediate was characterized using TNB^- under nondenaturing conditions. TNB^- was prepared by reducing the corresponding disulfide using the procedure of Silver (18). Progress curves of TNB^- disappearance for wild type and mutant enzymes were recorded at 412 nm in buffer A. Enzyme concentrations were 7.35 and 14.7 μM , and TNB^- concentration was 60 μM . The amount of TNB^- consumed was calculated using a molar absorptivity at 412 nm of $13600\text{ M}^{-1}\text{ cm}^{-1}$.

Electrospray Mass Spectrometry Analyses of Wild type and Mutant E. coli MsrAs—Analyses were performed for wild type and mutant MsrAs, either after treatment or without treatment with MetSO and/or 5,5-dimethyl-1,3-cyclohexanedione (dimedone). All the modification reactions were performed in buffer A in the presence of 20 μM of enzyme. 10 mM of MetSO was added, and the mixture was incubated for 10 min. The reactions with dimedone were carried out by addition of 200 μM dimedone before or after reaction with MetSO.

Proteins to be subjected to mass spectrometry analysis were purified and desalted on an anionic exchange column (Resource Q; Amersham Pharmacia Biotech) equilibrated in 0.05 M ammonium bicarbonate, pH 7.7, and eluted with a linear gradient (0–0.4 M) of ammonium bicarbonate, pH 7.7. A major peak contaminated by a minor peak was observed. This latter peak corresponded to higher order oligomers formed most likely because of the high concentration of the protein samples. Only the major fraction, which corresponded to the monomeric form, was collected and analyzed by mass spectrometry. Mass spectrometric measurements were performed on a VG-Bio Q triple quadrupole mass spectrometer (Micromass), upgraded so that the electrospray ionization source has Quattro II performances.

In all this work, ions were produced in the positive ion mode and were detected at the exit of the first analyzer for measurements in denaturing conditions and at the exit of the second analyzer for analysis in nondenaturing conditions. Mass data were recorded either from the denaturated protein or from the protein prepared under near physiological conditions.

For measurements in denaturing conditions, MsrA samples were diluted to 10 μM in a 1:1 water-acetonitrile mixture (v/v) containing 1% formic acid. Samples were introduced into the ion source via a 10- μl syringe loading injector, and spectra were acquired from m/z 500 to 2000. This provided good accuracy and sensitivity of the electrospray ionization/mass spectrometry measurements. To investigate the oligomerization state or the metal binding properties of MsrAs, the proteins were mass analyzed in nondenaturing conditions. Therefore, samples were diluted to 10 μM in ammonium acetate buffer (50 mM, pH 7). The samples were continuously infused into the ion source at a flow rate of 5 $\mu\text{l}/\text{min}$, and mass data were recorded from m/z 1000 to 6000.

RESULTS

Biochemical and Enzymatic Properties of Wild-Type and Mutant E. coli MsrAs

MsrA-encoded proteins were overexpressed in *E. coli* strain. Over 30% of the soluble proteins in the supernatant were MsrA. The molecular mass of 23185 ± 1 Da determined by mass spectrometry for the wild type was in good agreement with the 23,185 Da predicted from the MsrA DNA sequence (19), assuming the cleavage of the N-terminal Met residue. The elution profile of MsrAs on gel filtration under native conditions was in agreement with a monomeric state. Mass spectrometry analysis under nondenaturing conditions revealed the presence of the MsrAs in their monomeric state (data not shown). Comparison of the mass obtained under denaturing and nondenaturing conditions indicates the absence of tightly bound metal ion.

DTNB reagent revealed four cysteines under denaturing conditions. This result was expected from the MsrA DNA sequence that indicates four cysteines at positions 51, 86, 198, and 206.² Under native conditions, all four cysteines were also reactive.

To evaluate their role in the catalysis, the four cysteines were mutated into Ser, individually or in a series of permutations (Table I). The mutation of the invariant Cys-51 abolished all activity, whereas no significant effect was observed for the C86S mutant. This latter result excluded any role of Cys-86 in the catalysis. Site-directed mutations of Cys-198 or Cys-206 led to mutants, which presented significant activity in the DTT-based assay. The double mutants C86S/C198S and C86S/C206S showed activities higher than those of the corresponding single mutants C198S and C206S. These unexpected results remain to be explained but are not related to formation of

² The numbering of the amino acids is based on the *E. coli* MsrA proteic sequence, i.e. without the N-terminal Met residue.

TABLE I
The enzymatic activity of wild type and Cys to Ser mutants
of *E. coli* MsrA

The activities of wild-type and mutant MsrAs were assayed with MetSO and DTT or thioredoxin-regenerating system as described under "Experimental Procedures." Enzyme concentration was varied from 1 to 20 μM for wild type and mutants, and reaction was followed up to 2.5 min. Activities of 100% were assigned to 48.2 and 53.4 μmol of Met formed/min/ μmol of enzyme when DTT or thioredoxin-regenerating systems were used, respectively. Each value represented the average of three independent measurements of at least two enzyme concentrations (relative S.D. range, 5–10%). An activity of 96 μmol of Met formed/min/ μmol of enzyme for the wild type was determined with the thioredoxin-regenerating system of *E. coli* under the same conditions. ND, not detectable with the conditions used (<0.2%).

Enzyme	Activity	
	With DTT	With thioredoxin system
	%	
Wild type	100	100
C51S	ND ^a	ND ^a
C86S	90.4	86.1
C198S	13.1	ND
C206S	25.4	ND
C86S/C198S	41.9	ND
C86S/C206S	41.1	ND
C86S/C198S/C206S	27.7	ND

^a Increasing the enzyme concentration up to 100 μM and the reaction time up to 25 min for the C51S mutant with DTT or thioredoxin-regenerating system gave an activity that was 0.05% of the wild type enzyme. This low activity probably reflects the copurification of the endogenous *E. coli* MsrA from the DH5 α strain used for mutant MsrA production.

intermolecular disulfide bonds (see "Intermolecular Disulfide Bonds Formed upon MetSO Treatment"). In contrast, no turnover activity was observed in thioredoxin-based assay with *C. reinhardtii* thioredoxin and *A. thaliana* thioredoxin reductase. Similar results were obtained with *E. coli* thioredoxin recycling system (data not shown). Thus, this excluded any bias in interpreting the results because of the use of heterologous thiol recycling system. The triple mutant C86S/C198S/C206S was also shown to be only active with DTT. Moreover, the triple mutant oxidized on Cys-51 by one equivalent of H_2O_2 showed catalytic properties similar to those of the nontreated mutant (data not shown).

To further investigate the catalytic mechanism, the free thiol content of each mutant was determined with DTNB before and after incubation with MetSO but in the absence of reducing agent. Under native and denaturing conditions, the results were similar (Table II). This result indicates that loss of free thiol in native proteins is not the consequence of lack of accessibility. In particular, about three thiol groups for wild type and C86S mutant were lost upon treatment with MetSO, whereas none and about one and two thiol groups were lost for C51S, C198S, and C206S mutants, respectively. The situation for the double mutants C86S/C198S and C86S/C206S was less clear because of lack of reproducibility of the measurement of the thiol content. The reason of this inaccuracy remains to be explained. It is also important to note that the triple mutant C86S/C198S/C206S revealed no free thiol. The same results were obtained for this triple mutant treated with a stoichiometric amount of H_2O_2 .

Stoichiometry of Methionine Formation in the Absence of Reductant

2 mol of methionine were formed for wild type and C86S mutant, whereas only 1 mol was found for mutants in which Cys-198 or Cys-206 or both were mutated (Table III). In contrast no methionine was formed for the C51S mutant.

Characterization of the Sulfenic Acid Intermediate

Biochemical Characterization—A way to biochemically characterize a sulfenic acid intermediate is to use TNB^- . Except for reacting on disulfide bonds, TNB^- is a specific reagent for sulfenic acid derivatives provided that the oxidized Cys is accessible. Action of TNB^- was performed only on wild type and the triple mutant C86S/C198S/C206S. These enzymes were first incubated in the presence of MetSO, and then excess of TNB^- was added. A decrease of the absorbance at 410 nm to 1 equivalent of TNB^- /mole of triple mutant was observed. A similar result was obtained when the triple mutant was treated with a stoichiometric amount of H_2O_2 . In contrast no change in absorbance was observed with the triple mutant in the absence of MetSO treatment. This strongly suggested that the loss of absorbance at 410 nm was due to formation of a mixed disulfide bond between Cys-51 and TNB^- with a concomitant release of 1 mol of H_2O . Moreover, the fact that only an absorbance decrease at 410 nm was observed excluded a regeneration of free Cys-51 via an attack of a second molecule of TNB^- on the TNB^- moiety of the mixed disulfide bond. For the wild type, a decrease of the absorbance at 410 nm was first observed but rapidly followed by an increase that led to a final constant absorbance at 410 nm (curves not shown). This suggested that formation of the mixed disulfide bond between Cys-51 and TNB^- was followed by formation of another disulfide bond involving Cys-51 and another Cys via an intramolecular or intermolecular attack with concomitant release of TNB^- . The nature of the Cys involved remains to be defined.

Electrospray Mass Spectrometry Analyses—Determinations of the molecular mass of wild type, C51S and C86S mutants, and C86S/C198S/C206S triple mutant were performed before and after treatment with MetSO but without thiol regenerating system. As shown in Table IV, a mass increase of about 16 Da was observed upon MetSO treatment for all tested MsrAs except for the C51S mutant, which showed no variation in mass. However, it should be pointed out that in cases in which a variation in mass was observed a mixture of two almost equivalent populations was characterized, one with a mass increase of about 16 Da and a second one without mass increase. In contrast, treatment with MetSO followed by incubation with dimedone, which is specific for sulfenic acid (20, 21), led to a homogeneous population with a mass increase of about 140 Da except for the C51S mutant, which showed no mass increase, whereas no mass increase was observed upon treatment with only dimedone (data not shown). All these results proved that reaction with MetSO was necessary for dimedone modification and strongly suggested the formation of a sulfenic acid on Cys-51. Similarly a mass increase of about 140 Da was observed upon dimedone treatment when C86S/C198S/C206S triple mutant was first treated with a stoichiometric amount of H_2O_2 (data not shown).

Intermolecular Disulfide Bonds Formed upon MetSO Treatment

SDS/polyacrylamide gel electrophoresis analysis of wild type and mutant MsrAs incubated at high concentration with MetSO, in the absence of reductant and without heating, indicated formation of oligomers via intermolecular disulfide bonds, except for the C51S single mutant and the C86S/C198S/C206S triple mutant (data not shown). This emphasized the instability of the sulfenic acid derivative and the high accessibility of the cysteine residues in *E. coli* MsrA. Moreover the percentage of oligomers, which remained in all cases small, was shown to decrease with decreasing protein concentration. A similar situation was observed for C86S/C198S/C206S triple mutant treated with a stoichiometric amount of H_2O_2 . Because

TABLE II
Free sulfhydryl content of wild type and Cys to Ser mutants of *E. coli* MsrA

The values indicated represent the average of three independent measurements of at least two enzyme concentrations (relative S.D. range, 10%). Cysteine content was determined spectrophotometrically using DTNB under nondenaturing and denaturing conditions (10% SDS), in the absence or in the presence of 10 mM MetSO without any regenerating system as previously described ("Experimental Procedures").

Enzyme	No. of Cys	No. of Cys							
		Nondenaturing conditions				Denaturing conditions			
		Without MetSO	With MetSO	Decrease in free thiols ^a		Without MetSO	With MetSO	Decrease in free thiols ^a	
		Calculated from the data	Theoretical ^b			Calculated from the data	Theoretical ^b		
		<i>mol Cys/mol enzyme</i>							
Wild type	4	3.8	1	2.8	3	4.2	0.8	3.4	3
C51S	3	2.8	2.7	0.1	0	2.7	2.8	-0.1	0
C86S	3	2.7	0	2.7	3	3.0	0	3.0	3
C198S	3	2.6	1.6	1.0	1	2.7	1.5	1.2	1
C206S	3	2.6	0.9	1.7	2	2.6	1.0	1.6	2
C86S/C198S	2	1.7	0-0.6 ^c	1.1-1.7	1	1.5	0-0.5 ^c	1.0-1.5	1
C86S/C206S	2	1.7	0-0.6 ^c	1.1-1.7	2	1.9	0-0.4 ^c	1.5-1.9	2
C86S/C198S/C206S	1	0.8	0	0.8	1	0.8	0	0.8	1

^a The difference in the number of free cysteine thiols upon treatment with D,L-MetSO versus no treatment.

^b Based on the chemical mechanism proposed in Fig. 1.

^c The experimental results were not reproducible and thus did not allow distinguishing between 1 or 2 free thiols.

TABLE III

Stoichiometry of Met formed in the absence of regenerating system

The values indicated represent the average of three independent measurements of at least two enzyme concentrations (relative S.D. range, 10%). Quantity of Met formed was determined by high pressure liquid chromatography after reaction with 10 mM MetSO without any regenerating system as described under "Experimental Procedures."

Enzyme	<i>mol Met/mol enzyme</i>
Wild type	2.1
C51S	0
C86S	1.9
C198S	0.9
C206S	1.0
C86S/C198S	1.0
C86S/C206S	1.0
C86S/C198S/C206S	0.9

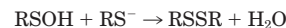
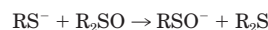
the concentration of MsrA in *E. coli* is low, it is unlikely that formation of oligomers *in vivo* plays a role in the reduction of the sulfenic acid intermediate via formation of intermolecular disulfide bonds.

DISCUSSION

MsrA from *E. coli* contains four cysteines at position 51, 86, 198, and 206. As already pointed out (12, 13), the fact that MsrA activity requires DTT or thioredoxin recycling systems supports the involvement of at least one essential cysteine in the catalytic mechanism. Recently, Lowther *et al.* (13) have confirmed the essential role of the Cys-72 from the bovine enzyme (equivalent to *E. coli* Cys-51 MsrA) and also the involvement of two other cysteines. These cysteines, namely Cys-218 and Cys-227, which are equivalent to *E. coli* Cys-198 and Cys-206, were postulated to be involved in a thiol-disulfide exchange implying a thioredoxin-dependent recycling process. Considering all their data, Lowther *et al.* (13) proposed a catalytic mechanism with formation of a trigonal-bipyramidal intermediate between Cys-72 and MetSO. A nucleophilic attack by Cys-218 on the intermediate would then release 1 mol of water and 1 mol of Met per mol of enzyme and form a disulfide bond between Cys-72 and Cys-218. Finally, return of the active site to a reduced state would be achieved by thiol exchange via Cys-227 and thioredoxin/thioredoxin reductase recycling system. In this mechanism, Met is only released after the Cys-218 nucleophilic attack.

Our results clearly proved that in the absence of Cys-198 or Cys-206 or both, 1 mol of Met was formed per mol of *E. coli* MsrA in the absence of reducing system. This shows that the

attack on the sulfur atom of the MetSO by Cys-51 was accompanied by the release of Met (see the mechanism proposed in Fig. 1). This cannot be explained by the mechanism proposed by Lowther *et al.* (13). In contrast our data are in accord with the alternative mechanism also proposed by Wallace and Mahon (22) for reactions of thiols with sulfoxides. This alternative mechanism involves three steps: 1) the formation of a sulfenate ion; 2) the protonation of the sulfenate ion; and 3) the formation of a mixed disulfide with release of H₂O via a nucleophilic attack of a second thiolate molecule, as in the following reactions.



REACTIONS 1-3

Thus to prove the formation of a sulfenic intermediate, it is necessary to prevent the third step from occurring. This is exactly the situation with the triple mutant with only Cys-51 retained. Four kinds of data support the formation of a sulfenic enzyme intermediate upon MetSO treatment: 1) the thiol group of Cys-51 is not free; 2) a majority of the enzyme population (~60%) has an increased mass that was compatible with the presence of a sulfenic acid; 3) the dimedone reagent, which is specific for sulfenic acid derivatives (20, 21), forms almost 100% of a covalent adduct as proven by mass spectrometry analysis; and 4) TNB⁻ reacts with a stoichiometry of 1. Moreover, the fact that the activity was not affected by the absence of oxygen (data not shown), shows that the oxygen atom of the sulfenic acid intermediate probably comes from the sulfoxide of the substrate.

The absence of another Cys in the triple mutant prevented any possible recycling of Cys-51 sulfenic acid into free thiol via intra-disulfide bond formation. This was not the case for the wild type. In fact, 2 mol of Met were shown to be formed per mole of wild type enzyme in the absence of reductant. Mass spectrometry analyses also showed a mass increase compatible with the presence of a sulfenic acid for at least 40% of the enzyme population and about 100% formation of a covalent adduct with dimedone. Mutations of Cys-198 or Cys-206 or both abolished any turnover but only with the thioredoxin recycling system. Together, these results support the involvement of both cysteines in the recycling of the activity. More-

TABLE IV
Electrospray mass spectrometry analyses

Molecular masses of wild-type and mutant MsrAs were determined by electrospray mass analysis without any modification or after reaction with 10 mM MetSO and/or 200 μ M dimedone as described under "Experimental Procedures." Molecular mass and difference in mass are expressed in daltons. No change in mass was observed for the mutant C51S after treatment with MetSO and/or dimedone (data not shown).

Enzyme	Before modification ^a		After modification			
	Theoretical mass	Measured mass	With MetSO		With MetSO and dimedone	
			Measured mass	Mass difference ^b	Measured mass	Mass difference ^b
Wild type	23185	23185 \pm 1	23196 \pm 1 ^c	+11 (\pm 2)	23322 \pm 2	+137 (\pm 3)
C86S	23169	23169 \pm 1	23182 \pm 2 ^c	+13 (\pm 3)	23306 \pm 1	+137 (\pm 2)
C86S/C198S/C206S	23137	23137 \pm 1	23154 \pm 5 ^c	+17 (\pm 6)	23277 \pm 4	+140 (\pm 5)

^a The same results were observed after reaction with 200 μ M dimedone (data not shown).

^b Mass differences measured by electrospray ionization/mass spectrometry correspond to the differences between the mass of the protein before and after treatment.

^c A second peak, having the molecular mass of the native enzyme, was also observed in these experiments.

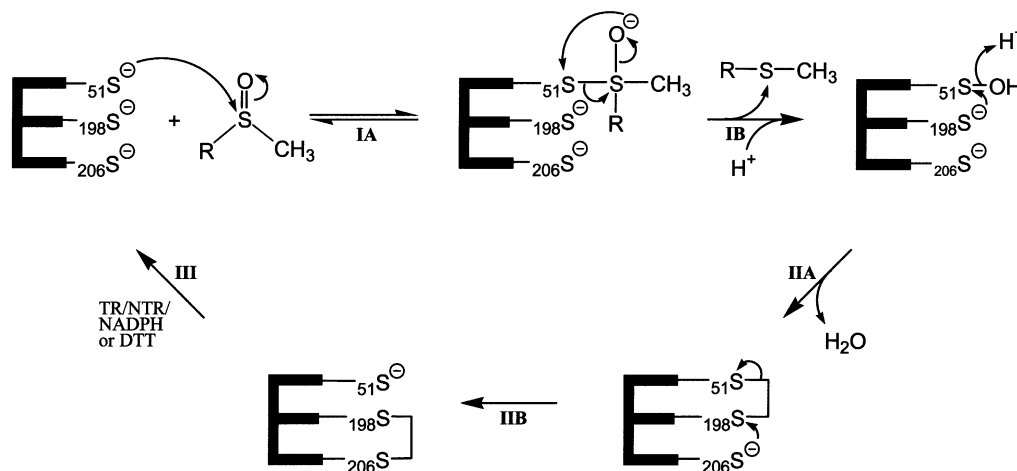


FIG. 1. **Proposed reaction mechanism for *E. coli* MsrA catalysis.** Attack of Cys-51 on the sulfur atom of the substrate sulfoxide leads to the formation of a tetrahedral intermediate (*step IA*), rearrangement of which leads to the formation of a sulfenate ion, the release of a molecule of Met and the protonation of the sulfenate ion (*step IB*). Attack of Cys-198 on the sulfur atom of the sulfenic acid intermediate leads to the formation of a transient disulfide bond between Cys-51 and Cys-198 and the release of a molecule of water, facilitated by acid catalysis (*step IIA*). Return of the active site to a fully reduced state proceeds by two thiol-disulfide exchanges via Cys-206 (*step IIB*) and either DTT or a thioredoxin regenerating system (*steps III*). RSOCH₃ and RSCH₃ represent MetSO and Met, respectively. TR, thioredoxin reductase; NTR, NADPH thioredoxin reductase.

over, these results and those showing that in the absence of reductant about three thiol groups were lost for the wild type, whereas about one and two thiol groups were lost for C198S and C206S mutants respectively, supported the following additional conclusions: 1) Cys-198 was probably the only attacking thiolate on the Cys-51 sulfenic intermediate; 2) Cys-206 can also form a disulfide bond but probably only with Cys-198 via a nucleophilic attack on the Cys-51/Cys-198 disulfide bond; 3) the Cys-198/Cys-206 disulfide bond is reduced by the thioredoxin recycling system while the transient Cys-51/Cys-198 disulfide bond cannot be reduced; 4) the Cys-51 sulfenic acid intermediate cannot be reduced by the thioredoxin recycling system; and 5) the presence of the Cys-198/Cys-206 disulfide bond does not prevent a second turnover with formations of a sulfenic acid on Cys-51 and of a second molecule of Met.

However, as shown in Table II, the percentage of errors are such in the estimation of the free sulfhydryl content that our data cannot totally exclude an alternative mechanism in which Cys-206 would play a similar role as Cys-198, *i.e.* an attack of Cys-206 on the Cys-51 sulfenic intermediate followed by formation of a disulfide bond with Cys-198 via a nucleophilic attack of Cys-198 on the Cys-51/Cys206 disulfide bond.

The fact that the four Cys were reactive under native conditions toward DTNB shows that they are readily accessible. Moreover Cys-198/Cys206 disulfide bond was easily reduced by thioredoxin and disulfide bonds can be formed between Cys-51/Cys-198, Cys-198/Cys-206 and eventually Cys-51/Cys-206. This

indicates that Cys-198 and Cys-206 are located near the surface of the protein and that the distance between Cys-51, Cys-198, and Cys-206 should be compatible with the formation of intra-disulfide bonds at least after the formation of the sulfenic acid intermediate on Cys-51. The fact that a k_{cat}/K_m value of $10^3 \text{ s}^{-1} \text{ M}^{-1}$ (deduced from a k_{cat} value of 1 s^{-1} and a K_m value for MetSO of 1 mM; data not shown) was observed indicates a rather efficient release of the water molecule, when Cys-198 attacks the oxidized Cys-51. This is largely higher than the value determined on chemical model ($\sim 10^{-5} \text{ s}^{-1} \text{ M}^{-1}$) (23). Thus a protonation of the sulfenic acid by catalytic assistance within the active site should probably occur. In that context the knowledge of the x-ray structure of wild type MsrA from *E. coli*, which will be soon available, will be very informative not only to confirm the accessibility of Cys-51, Cys-198, and Cys-206 and to define the structural factors involved in Cys-51 and Cys-51 sulfenic acid activations but also to positioning the Cys-198 and Cys-206 relative to that of Cys-51 (24).

As indicated before, a variable protein population of non-modified form and of sulfenic acid derivative was always observed from mass spectrometry analyses upon MetSO treatment. The percentage of sulfenic acid was estimated to vary from 40 to 60%, depending on the sample preparations. The situation was similar for C86S/C198S/C206S triple mutant when treated with a stoichiometric amount of H₂O₂. However, in all cases, further dimedone treatment always led to about 100% of adduct population. Therefore, this strongly suggested

an instability of the sulfenic acid under the experimental conditions used for preparing protein samples for spectrometry analyses. Moreover, the fact that a mass decrease of 4 ± 2 Da was observed for the wild type nonmodified population, compared with the mass of the native enzyme (data not shown), strongly suggests the formation of at least one disulfide bond, probably between Cys-198 and Cys-206.

The involvement of both Cys-198 and Cys-206 in the mechanism is in agreement with the data presented by Lowther *et al.* (13). However, some apparent contradictions with our results remain. In particular, their estimate of the free sulfhydryl content before and after treatment with MetSO, which was determined by mass spectrometry with methyl methanethio-sulfonate as cysteine probe, is not in agreement with the decrease in free thiols expected from our proposed mechanism. For instance, two free sulfhydryls for the bovine wild type enzyme were deduced to be lost, whereas three sulfhydryls are expected to be lost if one assumes the formation of a sulfenic acid intermediate. In fact, re-estimating the decrease in free thiols by taking into account the formation of sulfenic acid on Cys-72 and the formation of possible intra-disulfide bonds leads to conclusions in accord with our proposal. This is the case for the wild type and all the mutants except the Cys-218 mutant. For instance, the theoretical mass decrease for wild type is 127, which is in good agreement with the 111–120 values they determined experimentally. For the mutants in which Cys-227 is mutated, the theoretical and experimental mass decreases are also similar: 1) C227S single mutant: 96 *versus* 104 and 109; 2) C107S/C227S double mutant: 96 *versus* 95 and 106; 3) C218S/C227S double mutant: 31 *versus* 22 and 26; and 4) C107S/C218S/C227S triple mutant: 31 *versus* 21 and 33. In contrast, mass decreases determined experimentally for the C218S single mutant and the C107S/C218S double mutant can only be explained if a disulfide bond is formed between Cys-72 and Cys-227. This is an indication that Cys-227 can play a similar role as Cys-218 in the catalytic mechanism.

It was clearly shown that, *in vivo*, thioredoxin is the reductant (9–11). The fact that 2 mol of Met/mol of enzyme are formed *in vitro* with wild type *E. coli* enzyme in the absence of reductant raises the question of where thioredoxin enters the mechanism *in vivo*. Is it just after the formation of Cys-198/Cys-206 disulfide bond but before the reduction of a second molecule of MetSO by Cys-51, or is it after the reduction? In the latter case, three successive steps involving two molecules of thioredoxin must occur: 1) reduction of the Cys-198/Cys-206 disulfide bond by thioredoxin; 2) regeneration of the free thiol of Cys-51 via the formation of a disulfide bond between Cys-198 and Cys-206; and 3) a second reduction of Cys-198/Cys-206 disulfide bond by thioredoxin. With the currently available data, it is difficult to discriminate between these two routes. However, it is probable that thioredoxin reduction would be operative after every cycle of formation of Cys-198/Cys-206 disulfide bond.

Although the *E. coli* and the bovine enzymes should share the same chemical mechanism, they can behave differently in some aspects. A disulfide bond can be formed in the bovine enzyme between Cys-72 and Cys-227 at least in the absence of Cys-218, whereas we have no strong experimental evidence supporting the formation of a disulfide bond between Cys-51 and Cys-206 in the *E. coli* enzyme. More important is the remaining thioredoxin recycling activity observed with the bovine enzyme on the single mutants C218S and C227S and on the double mutant C218S/C227S. Such a behavior supports the idea that MsrA bearing only Cys-72 (51 in our case) is active *in vivo* with the thioredoxin-dependent recycling system. The fact that in our hands mutants of *E. coli* MsrA, in which Cys-198 or

Cys-206 are changed into Ser, only showed activity with DTT but no significant activity with the *E. coli* thioredoxin recycling system contradicts the above assumption. Thus, if activity is observed *in vivo* with MsrAs bearing only one Cys, this would imply an alternative efficient recycling thiol system. Based on our results obtained *in vitro* (data not shown), involvement of glutathione can be excluded.

The importance of protein-sulfenic acids in enzyme catalysis and redox regulation was recently described in a review by Claiborne *et al.* (25). The mechanism proposed in the present study for the MsrA catalysis recalls the mechanism of alkyl hydroperoxide reductase (26, 27) and of NADH oxidase (28) in which the enzyme sulfenic intermediates were shown to be reduced via formation of a disulfide bond. Such mechanisms can also be compared with those of enzymes like glutathione peroxidase, which has an essential selenocysteine instead of a cysteine (29–32). In that case, reduction of the hydroperoxide leads to formation of a selenenic acid enzyme intermediate, which is chemically similar to a sulfenic acid. The only difference is the recycling system, which involves two molecules of glutathione instead of an internal cysteine recycling system.

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A Sulfenic Acid Enzyme Intermediate Is Involved in the Catalytic Mechanism of Peptide Methionine Sulfoxide Reductase from *Escherichia coli*

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