A Low pKₐ Cysteine at the Active Site of Mouse Methionine Sulfoxide Reductase A*

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**Background:** The active site cysteine of methionine sulfoxide reductases has been reported to be 9.5.
**Results:** The pKₐ of methionine sulfoxide reductase is 7.2.
**Conclusion:** Methionine sulfoxide reductase has an active cysteine at its catalytic center.
**Significance:** Methionine sulfoxide reductase is readily oxidized by low concentrations of hydrogen peroxide, supporting both antioxidant and redox signaling functions of the enzyme.

Methionine sulfoxide reductase A is an essential enzyme in the antioxidant system which scavenges reactive oxygen species through cyclic oxidation and reduction of methionine and methionine sulfoxide. Recently it has also been shown to catalyze the reverse reaction, oxidizing methionine residues to methionine sulfoxide. A cysteine at the active site of the enzyme is essential for both reductase and oxidase activities. This cysteine has been reported to have a pKₐ of 9.5 in the absence of substrate, decreasing to 5.7 upon binding of substrate. Using three independent methods, we show that the pKₐ of the active site cysteine of mouse methionine sulfoxide reductase is 7.2 even in the absence of substrate. The primary mechanism by which the pKₐ is lowered is hydrogen bonding of the active site Cys-72 to protonated Glu-115. The low pKₐ renders the active site cysteine susceptible to oxidation to sulfenic acid by micromolar concentrations of hydrogen peroxide. This characteristic supports a role for methionine sulfoxide reductase in redox signaling.

A précis to methionine sulfoxide reductase A (msrA) is in the Introduction of the accompanying paper (1). Crystallographic and solution structures of msrA from various microorganisms and the cow established that their active sites are virtually identical (2–7). These structures and detailed mechanistic studies by Bosch-Muller et al. have elucidated the common catalytic cycle of the msrA and msrB (8, 9). A cysteine is always at the active site, and oxidation to the sulfenic acid is essential for both reductase and oxidase activities (9, 10). The pKₐ of the active site cysteine in msrA from Neisseria meningitidis was reported to be ~9.5 in the absence of methionine sulfoxide, decreasing to 5.7 upon binding of the substrate (11). A high pKₐ in the absence of methionine sulfoxide could protect the enzyme from adventitious oxidation by reactive oxygen and nitrogen species (11), but it would also prevent msrA from participating in redox signaling mediated by hydrogen peroxide (12, 13). In the course of studies on mouse msrA, we observed that the active site cysteine, Cys-72, was readily oxidized to the sulfenic acid by micromolar concentration of hydrogen peroxide. We therefore measured its pKₐ by three methods, spectrophotometric titration, oxidation by hydrogen peroxide, and NMR.

**EXPERIMENTAL PROCEDURES**

Preparation of the msrA was described in the accompanying paper (1). The E115Q mutant of wild-type msrA was constructed by recombinant PCR (14). The sulfenamide form of msrA was prepared by incubation of 10 mM protein with 10 mM methionine sulfoxide at room temperature for 5 min (15) followed by acidification to pH 1.6 by addition of 1/40 volume of 20% (v/v) trifluoroacetic acid. Acid conditions were chosen to stabilize the sulfenamide, and cleavage was effected by pepsin (Sigma P-7012) at a concentration of 4 μg/μL. The sample was incubated at 37 °C for 18 h at which time HPLC-tandem mass spectrometry was performed as described (15) except that the mass spectrometer was an Agilent model 6200.

**Determination of the pKₐ of Cys-72 by Spectrophotometric Monitoring of Its Ionization**—To avoid complications from oxidation of the other three cysteine residues or of Met-229, we studied the oxidation of Cys-72 with the previously described C107S/C218S/C227S/D228→233 (10).

Ionization of the cysteine thiol to its thiolate causes an increase in its molar absorbity of ~4,000 M⁻¹ cm⁻¹, providing a direct method for determining pKₐ (16). Spectra were recorded on a Shimadzu model 2501 double monochromator spectrophotometer at 20 °C. Stock msrA was incubated with dithiothreitol to assure reduction of Cys-72 and then dialyzed into 50 mM sodium phosphate buffer, pH 7.5, with 1 mM DTPA. A 100-μL aliquot was mixed with 200 μL of 60 mM potassium phosphate, 60 mM sodium pyrophosphate, pH 6.0, to give a solution of pH 6.2. The msrA concentration was 68 μM. One

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2 The abbreviations used are: msrA, methionine sulfoxide reductase A; DTPA, diethylenetriaminepentaacetic acid; HSQC, heteronuclear single-quantum correlation.

3 The msrA gene encodes both the mitochondrially targeted form and the cytosolic form. We follow the usual practice in the field and number residues beginning with Met-1. Studies in this paper were performed with the cytosolic form whose amino-terminal residue is Gly-22.

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sample was titrated with 200 mM NaOH, and its pH was measured after each addition of base. It was important to measure the pH with msrA present in the buffer because the protein altered pH by up to 0.2 unit compared with buffer alone. A second sample was placed in the spectrophotometer, and spectra were measured after each addition of NaOH. The observed absorbances were corrected for any light scatter and for dilution by the NaOH. Scatter was calculated from a line fit to the log of the absorbance measured at 350 and 400 nm (17).

**Determination of the pKₐ of Cys-72 by Its Rate of Oxidation**—Hydrogen peroxide oxidizes the thiolate form of cysteine and not the thiol form (18). The rate of oxidation of cysteine thus depends on the hydrogen peroxide concentration, the pH, and the pKₐ of the cysteine. As expected, Regino and Richardson found that the rate of oxidation at pH 8.0 of cysteine, glutathione, and N-acetylcysteine varied in accordance with their pKₐ variation (19). However, the rates were the same when calculated according to the concentration of thiolate present. They determined the second order rate constant for oxidation of the thiolate to be 17 M⁻¹ s⁻¹ at 25 °C. Using the same assay with cysteine as Regino and Richardson, we found the rate constant at 37 °C to be 32 M⁻¹ s⁻¹, consistent with a simple Q₁₀ effect. Given this rate constant and a measurement of the pseudo-first order rate of oxidation of msrA, the pKₐ of the active site cysteine can be calculated by measuring the rate of oxidation of msrA at a single pH from Equation 1 (19),

\[ K_a = \frac{k_{observed} \times [H^+]}{k - k_{observed}} \]  
(Eq. 1)

where the rate constant \( k = 32 \text{ M}^{-1} \text{s}^{-1} \) and \( k_{observed} \) is the observed rate of oxidation by hydrogen peroxide of the active site Cys-72. This method of determination of pKₐ requires that the oxidizing agent employed have unhindered access to the active site cysteine, a condition that should readily be met by hydrogen peroxide.

The C107S/C218S/C227S-Δ228–233 utilized in the spectrophotometric study was also used for the oxidation studies. The E115Q mutant of this protein was constructed with a QuikChange Site-directed Mutagenesis kit (Stratagene) using pET17b-C107S/C218S/C227S-Δ228–233 msrA vector as template. The primers used were 5’-ACTGGCCACGCCAA-GTCGTCCGGGTT-3’ and 5’-AACCCGGACGACTTGG-GCGTGGCCAGT-3’.

Oxidations were carried out at 37 °C in 50 mM sodium phosphate with 1 mM DTPA. The msrA concentration was 10 μM, and that of hydrogen peroxide was 100 μM. At desired times, an aliquot of 20 μl was withdrawn and the oxidation stopped by immediate mixing with 40 μl of 50% (v/v) acetic acid.

A convenient method for following the rate of oxidation is reverse phase chromatography, as the native form elutes later than the oxidized form. The instrumentation and separation conditions were as described (10), except that the acetonitrile gradient was altered to optimize separation. It was brought to 30% (v/v) in 5 min, then to 40% (v/v) over 12.5 min. The native protein eluted at 14 min with this program. The amount of native msrA was determined from the area of its peak at 210 nm.

**RESULTS**

**Determination of the pKₐ of Cys-72 by Spectrophotometric Monitoring of Its Ionization**—The ionization of Cys-72 was followed by the increase in absorbance at 240 nm and gave a typical Henderson-Hasselbalch plot with pKₐ = 7.1 (Fig. 1). The maximal increase in molar absorbity from the fit curve was...
4,400 M$^{-1}$ cm$^{-1}$, in good agreement with the value of $\sim$4,000 M$^{-1}$ cm$^{-1}$ estimated by Donovan (16).

**Determination of $pK_a$ of Cysteines by NMR**—The cysteine $pK_a$ values of msrA were determined from the pH dependence of their $\beta$ resonances, measured by $^{13}$C constant-time HSQC (Fig. 2). Fitting to the Henderson-Hasselbalch equation yielded transitions at 7.2 and 9.7 for Cys-$\beta$ and 7.2, 8.4, and 9.6 for Cys-107, Cys-218, and Cys-227, respectively. The sample precipitated below pH 6 and was denatured above pH 10.5. The cysteine backbone amide resonances for $^{15}$N-labeled myristoylated and nonmyristoylated msrA were also examined. These exhibited similar chemical shifts and pH dependence (Fig. 3) from pH 6.5 to 8.5, suggesting that the cysteine $pK_a$ values for the myristoylated and nonmyristoylated forms are comparable. With a limited amount of myristoylated $^{13}$C/$^{15}$N-labeled msrA available, $\beta$ resonance pH dependence was measured for the nonmyristoylated form only.

The pH dependence for $\beta$ and the backbone amide of Cys-$\beta$ both show two transitions, one at pH 7.2, and a larger one at 9.7 (Figs. 2 and 3). Such pH profiles are expected if, in addition to ionization of its thiol, a cysteine residue interacts with another titratable group such as a tyrosine, aspartate, or glutamate (20). We cannot a priori assign the thiol ionization to one of the two transitions, but the spectrophotometric method and the oxidation method do allow us to assign the first transition to Cys-$\beta$ ionization.

We note that Cys-$\beta$ lies near the carboxylate group of Glu-115. This carboxylate group is hydrogen-bonded to the backbone amide of the following residue, Phe-$\beta$. Examination of the backbone amide pH dependence curves of Phe-$\beta$ and Glu-115 shows a clear transition at pH $\sim$7.2 for both (Fig. 4). The carboxylate of Glu-115 is $\sim$5 Å from the SH group of Cys-$\beta$, and coupling of the Cys-$\beta$ and Glu-115 titratable groups could cause the pH 7.2 feature in the Cys-$\beta$ pH dependence curve (21). This possibility was investigated experimentally (see later subsection). The thiol of Cys-107 also lies near Glu-115, $<8$ Å distant, so the transition seen for Cys-107 $\beta$ at pH 7.2 may also be influenced by Glu-115. The curve for Cys-107 also shows an increase in slope at the highest pH point (10.5), so that it could have a $pK_a >$10.5, a possibility that cannot be ruled out at present.

The backbone amide signal of Trp-$\beta$ shows a strong transition at pH 9.7, with perhaps a weaker transition at approximately pH 7.2 (Fig. 4). A rotation of the Cys-$\beta$ side by $\sim$60 degrees brings the Cys-$\beta$ sulfur within hydrogen-bonding distance ($\sim$2 Å) of the Trp-$\beta$ backbone amide hydrogen, suggesting that Cys-$\beta$ in the thiolate form can facilitate a hydrogen bond with the Trp-$\beta$ backbone amide. To investigate this possibility we took advantage of the ability of the sulfenic acid form of Cys-$\beta$ to form a sulfinamide (10). The nitrogen participating in sulfinamide formation is probably a peptide bond amide because there are no amines near Cys-$\beta$. The Cys-$\beta$ sulfur is
within hydrogen bonding distance of the amide nitrogens of the adjacent Phe-73 and Trp-74, making them the most likely candidates to form the sulfenamide.

We determined the site of sulfenamide formation by peptic mapping. In the nonoxidized control msrA, pepsin cleavage occurred after Phe-68 and Phe-73, placing Cys-72 in the peptide G107C108S/C218S/C227S (calculated mass = 513.1716, observed = 513.1731). As expected, this peptide was not observed in the sulfenamide form of msrA. Trp-74 in the control msrA was located in the peptic peptide W74G75A76E77 (calculated mass = 461.1911, observed = 461.1939), and it was also lost in the sulfenamide-containing msrA. If Phe-73 formed the sulfenamide, a peptide of mass 511.1716 should be observed. If Trp-74 formed the sulfenamide, then the two linked peptides would have mass 972.3471. The sulfenamide msrA did not have a 511.1716 Da peptide, but it did have a new peak at 972.3471, establishing that the sulfenamide was formed between Cys-72 and Trp-74. This was confirmed through sequencing by tandem mass spectrometry (data not shown). We conclude that the thiolate of Cys-72 is stabilized by a hydrogen bond with the amide of Trp-74.

The pH-dependent shifts for Gly-71, Gly-75, and Ala-76, as well as the side chain amide of Trp-74, show relatively small changes (<0.2 ppm) and no significant broadening, suggesting that no local unfolding of the helix containing residues 71–76 is occurring in the pH range studied.

**Determination of the pK_a of Cys-72 by Its Rate of Oxidation**—Cysteine pK_a values in proteins have often been estimated by determining their rate of reaction with a reagent that is specific for the thiolate form such as alkylation by iodoacetamide. The pK_a measured by such methods requires that pseudo-first order rates be determined and that the reagent have unhindered access to the cysteine residue of interest, conditions that are often not met. Hydrogen peroxide generally has access to all solvent-exposed residues, and Cys-72 is such a residue (2–7). We measured the rate of oxidation of Cys-72 by hydrogen peroxide under pseudo-first order conditions and, as expected, the rate was linear when displayed on a semilogarithmic plot (Fig. 5). As noted under “Experimental Procedures,” this method allows determination of the pK_a from the oxidation rate at a single pH. To provide additional confidence in this approach, it was measured at varying pH with the same result (Fig. 5, inset). The pK_a of Cys-72 was 7.4, in good agreement with 7.1 and 7.2 measured by spectrophotometric and NMR titration. The agreement among the three methods supports the conclusion that Cys-72 is an “active cysteine” with a pK_a of 7.2, the average of the three methods.

**Role of Glu-115**—Glu-115 is involved in substrate binding (2, 3) and could also lower the pK_a of Cys-72 if Glu-115 were protonated and formed a hydrogen bond with Cys-72 (22, 23). Because we observed that Glu-115 and Cys-72 share a transition with pK_a of 7.2, we studied the susceptibility to hydrogen peroxide of the E115Q mutant because the hydrogen bond would be weaker. The pK_a of this mutant is 8.2 (Fig. 6), confirming that Glu-115 lowers the pK_a of Cys-72.

Our assays of activity of msrA are routinely performed at pH 7.5. When the E115Q was assayed at this pH, its activity was only ~2% that of the Glu-115 wild-type form. If the reduced activity were due simply to the higher pK_a of Cys-72, then the pH optimum would likely simply shift to a higher pH. However, if Glu-115 were also important in substrate binding, then activity would be lowered across the pH activity curve. Fig. 7 shows that the pH activity profiles of the wild-type and mutant are superimposable, but the mutant has only ~2% of the activity of the wild-type at all pH values. We conclude that Glu-115 is essential both for lowering the pK_a of Cys-72 and for substrate binding, as suggested by Ref. 11.

**DISCUSSION**

Biochemical, mutational, and structural studies have firmly established that the Cys-72 thiolate is the active site nucleophile of msrA which attacks the sulfoxide of the substrate. Ionization of Cys-72 at physiological pH requires that the typical cysteine pK_a of 8.5–8.8 be lowered. “Active” or low pK_a cysteines at the catalytic center of enzymes are well known, and the lowering of...
the pKa is often effected by nearby basic residues which facilitate deprotonation. None of the structures of msrA from a number of species has this feature at their active site. Cys-72 is located at the amino-terminal end of an α-helix. The two groups reporting the first structures of msrA, one from Escherichia coli (3) and the other from the cow (2), both suggested that this placement could decrease the pKa of msrA. The lowering of the pKa occurs because the amino-terminal side is the positive end of the α-helix dipole (24, 25). A protonated carboxyl group can also lower the pKa of cysteine by stabilizing the thiolate through hydrogen bonding. This less common structural mechanism for lowering the pKa is well documented to occur at the active site of the DJ-1 family of proteins (23). It also functions to lower the pKa for protein splicing by inteins (22).

The increase of pKa from 7.4 to 8.2 in the E115Q msrA demonstrates that hydrogen bonding of protonated Glu-115 is the major mechanism by which the pKa of Cys-72 is lowered.

During our earlier studies with murine msrA, we consistently observed facile oxidation of the active site Cys-72 by hydrogen peroxide at neutral pH. This observation implied that its pKa was low even in the absence of substrate. With the recognition that murine msrA is also a stereospecific protein methionine oxidase (10), a reinvestigation of the pKa of Cys-72 became important. If hydrogen peroxide in the absence of substrate could react with msrA to form the sulfenic acid, then the enzyme could participate in redox signaling. We therefore measured the pKa by spectrophotometric titration, by NMR titration, and by the rate of oxidation by hydrogen peroxide. Each method demonstrated a low pKa, with an average of 7.2. NMR indicated that Cys-72 and Glu-115 appear to be coupled titratable groups, thus the first transition at 7.2 corresponds to loss of the first proton of this two-proton system. Additional investigations will be required to elucidate the structural basis for the second transition at pH 9.7, which could be due to loss of the second proton or may be related to ionization of nearby tyrosines.

FIGURE 7. pH profile for reduction of methionine sulfoxide by wild-type (■) and E115Q (●) msrA. The wild-type activity is plotted against the left ordinate and the E115Q activity against the right ordinate.

The apparent pKa of msrA from N. meningitidis has been studied by two methods (11). The first was the rate of reaction of the active site cysteine with dipyridyl disulfide which gave a pKa of ~9.5. The authors cautioned that restricted access of the reagent to the active site cysteine might yield an inaccurate estimate. They therefore also estimated the pKa from the pH-dependent change in absorbance at 240 nm of msrA and again obtained a high pKa of 9.7. The increase in molar absorbivity observed with increasing pH was >23,000 M⁻¹ cm⁻¹. The authors proposed that the high pKa of the active site cysteine was lowered by 3.7 units to 5.7 upon binding of the sulfoxide substrate.

Donovan first reported the spectrophotometric titration at 240 nm of cysteines in aldolase (16). He found that the molar absorbivity increased by ~4,000 M⁻¹ cm⁻¹ upon ionization. However, he also pointed out that tyrosine residues also show an increase in molar absorbivity at 240 nm upon ionization, and that increase was approximately 11,000 M⁻¹ cm⁻¹. The N. meningitidis msrA with only the active site cysteine also had 16 tyrosine residues. The reported increase in absorbivity of 23,000 M⁻¹ cm⁻¹ must therefore have been due primarily to tyrosine ionization. The observed pKa of 9.7 is typical for tyrosine residues.

The first crystal structures of msrA led to the proposal that Glu-115 was important in binding and orientation of the sulfoxide substrate (2, 3). Subsequent investigations by Branlant and colleagues provided evidence that Glu-115 was also involved in the activation of Cys-72, but only after binding of substrate (11). The present studies demonstrate that binding of substrate is not required to lower the pKa of Cys-72.

Signaling and regulation by redox modification of proteins are now well established physiological mechanisms (12, 13). Recently, msrA was found to catalyze the oxidation of protein methionine residues to methionine sulfoxide in addition to catalyzing reduction of the sulfoxide, and a structural basis for regulation of this bifunctional enzyme was proposed (10). The oxidizing agent was millimolar methionine sulfoxide, a nonphysiological concentration. Our present study demonstrates that the active site cysteine of msrA can be converted to its sulfenic acid form at physiological pH by micromolar hydrogen peroxide. Thus, msrA may participate in a regulatory system that is dependent on hydrogen peroxide.

Considerable experimental evidence supports a role for msrA in oxidative defense by scavenging reactive oxygen and nitrogen species through cyclic oxidation and reduction of methionine and methionine sulfoxide (26–35). The presumed pathway of scavenging is via direct oxidation of protein methionine residues by the reactive species, following which msrA reduces the methionine sulfoxide back to methionine. Whereas hypochlorous acid reacts well with methionine at physiological pH (36, 37), hydrogen peroxide does not, although the rate can be accelerated by the bicarbonate/carbon dioxide present in vivo (38). Acting in the oxidase direction, msrA can react with low concentrations of hydrogen peroxide to form sulfenic acid on the active site Cys-72, which can then stereospecifically oxidize methionine residues in proteins (10). Subsequently, acting in the reductase direction, msrA can utilize thioredoxin to reduce the protein methionine sulfoxides back to methionine. The net result is that the low pKa allows msrA to scavenge hydrogen peroxide, thus contributing to oxidative defense.
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