Synergistic Roles of Helicobacter pylori Methionine Sulfoxide Reductase and GroEL in Repairing Oxidant-damaged Catalase*

Manish Mahawar†, ViLinh Tran‡, Joshua S. Sharp§, and Robert J. Maier††

From the †Department of Microbiology and §Complex Carbohydrate Research Center, University of Georgia, Athens, Georgia 30602

Hypochlorous acid (HOCl) produced via the enzyme myeloperoxidase is a major antibacterial oxidant produced by neutrophils, and Met residues are considered primary amino acid targets of HOCl damage via conversion to Met sulfoxide. Met sulfoxide can be repaired back to Met by methionine sulfoxide reductase (Msr). Catalase is an important antioxidant enzyme; we show it constitutes 4–5% of the total sulfoxide can be repaired back to Met by methionine sulfoxide reductase (Msr). Catalase is an important antioxidant enzyme; we show it constitutes 4–5% of the total helicobacter pylori protein levels. msr and kата strains were about 14- and 4-fold, respectively, more susceptible than the parent to killing by the neutrophil cell line HL-60 cells. Catalase activity of an msr strain was much more reduced by HOCl exposure than for the parental strain. Treatment of pure catalase with HOCl caused oxidation of specific MS-identified Met residues, as well as structural changes and activity loss depending on the oxidant dose. Treatment of catalase with HOCl at a level to limit structural perturbation (at a catalase/HOCl molar ratio of 1:60) resulted in oxidation of six identified Met residues. Msr repaired these residues in an in vitro reconstituted system, but no enzyme activity could be recovered. However, addition of GroEL to the Msr repair mixture significantly enhanced catalase activity recovery. Neutrophils produce large amounts of HOCl at inflammation sites, and bacterial catalase may be a prime target of the host inflammatory response; at high concentrations of HOCl (1:100), we observed loss of catalase secondary structure, oligomerization, and carbonylation. The same HOCl-sensitive Met residue oxidation targets in catalase were detected using chloramine-T as a milder oxidant.

H. pylori colonizes over one-half of the human population, and it is the leading cause of gastric ulcers and many types of gastric cancers (1). In the epithelium, this bacterium induces an intense inflammatory response, including oxidative toxic molecule production. In response to H. pylori infection, activated neutrophils are the primary sources of reactive oxygen/nitrogen species (2). These cells respond to H. pylori antigens like NapA and CagA by expressing NADPH oxidases that catalyze the formation of superoxides. Dismutation of superoxides generates hydrogen peroxide (H$_2$O$_2$), while inducible nitric-oxide synthase produces nitric oxide. The combination of H$_2$O$_2$ with nitric oxide results in the formation of highly toxic peroxynitrite (ONOO$^-$). In addition, H$_2$O$_2$ is the substrate for production of other reactive oxygen species; these include highly reactive hydroxyl radicals and HOCl. HOCl is reported to be 100-fold more toxic than H$_2$O$_2$ (2), and its concentration can reach up to 5 mm at inflammatory sites (3). The battery of oxidants described above are able to oxidize virtually all large molecules, including DNA, RNA, proteins, and lipids (4, 5). To combat these potent oxidants, H. pylori is equipped with an array of antioxidant and macromolecule repair enzymes (5).

Because of their abundance and reactivity, proteins are a primary target for oxidative damage. Two types of oxidative protein modifications have been described, covalent modifications of amino acids and loss of secondary structure. Although a variety of covalent modifications to amino acids have been reported, Met and Cys residues are known to be primary oxidative targets under physiological conditions (6). Met residues in particular have high reactivity to HOCl, with a second-order rate constant reported to be 3.8 $\times$ 10$^7$ M$^{-1}$ s$^{-1}$ (6). Oxidation of Met leads to formation of Met-SO$_2$, and further oxidation results in Met sulfone (7). In some instances, Met-SO$_2$ formation was found to be associated with changes in surface polarity and subsequent unfolding of proteins (8, 9). The peptide repair enzyme Msr can reductively repair Met-SO$_2$ to Met (10). Two roles for Msr-mediated repair have been described. First, continual oxidation and reduction of surface Met residues in proteins are thought to act as a turnover sink to quench oxidants (11, 12). Second, Msr-mediated repair results in return of enzyme function (13, 14).

In bacteria, two types of Msrs (MsrA and -B) have been described that use S and R epimers of Met-SO as substrates, respectively (15). In H. pylori, MsrA and -B are fused and constitute a single protein. An H. pylori msr strain is highly sensitive to oxidative stress and is deficient in stomach colonization (16). Moreover, using a cross-linking approach, site-specific recombinase, GroEL, and catalase were identified as Msr-interacting proteins in H. pylori (17). In addition, a tripartite complex formed of catalase, Msr, and GroEL was observed (17). Catalase is a key antioxidant protein that dissipates H$_2$O$_2$ by decomposing it to H$_2$O and O$_2$. H$_2$O$_2$ itself is not highly toxic, but as it is produced in large amounts during the inflammatory cell respiratory burst and is a precursor for hydroxyl radical and peroxynitrite; trx, thioredoxin1; trxR, thioredoxin reductase; MRM, multiple reaction monitoring.
HOCI formation, catalase enhances survival of nearly all animal pathogens. In *H. pylori*, catalase is one of the most abundant proteins (constitutes ~4–5% of the total proteins, described herein), and it has some unique properties. Its unusual characteristics include a high pI (>9.0) (18) and high resistance to H$_2$O$_2$ (19). Although an *H. pylori* catalase mutant (katA) strain is viable in vitro under nonoxidative stress conditions, catalase activity has been shown to be associated with the resistance of the bacterium to professional phagocytes and macrophages (20, 21). Notably, an *H. pylori* catalase-deficient mutant strain is compromised in persistent mouse stomach colonization ability (22).

In this study, we sought to evaluate the role of Msr in repairing HOCI-oxidized Met residues in catalase and to further assess its functional significance. By using tandem MS/MS along with other biochemical techniques, we show that HOCI damages *H. pylori* catalase by oxidation of specific Met residues, leading to structural changes and loss of catalytic activity. However, Msr and GroEL function in a cooperative manner to repair oxidatively damaged catalase and restore some enzyme activity.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions—**msr and catalase gene deletion mutant strains (msr and katA strains) were constructed in *H. pylori* strain SS1 (SS1) background. The genes were inactivated by inserting antibiotic resistance cassettes (kanamycin and chloramphenicol for Msr and catalase, respectively) as described previously (16, 19). *H. pylori* strains were grown in Brucella agar (BD Biosciences) plates containing 10% Bacto tryptone (BD). Plates were incubated microaerobically in a humidified chamber at 37 °C grown in Brucella agar (BD Biosciences) plates containing 10% Bacto tryptone (BD). Plates were incubated microaerobically in a humidified chamber at 37 °C under 5% CO$_2$, 20% O$_2$ with the balance of gas as N$_2$. After incubation for 4 h at 37 °C, cell pellets were harvested and washed in 0.1 M NaCl and centrifuged at 13,000 g for 5 min. Saponin (0.1% w/v in PBS) was used to lyse HL-60 cells to obtain surviving *H. pylori* (21). It was added to the cell pellet and incubated for 5 min at 37 °C. Bacterial suspensions were serially diluted and plated on blood agar plates (24). Colonies were enumerated after 5–7 days of incubation (24).

**Exposure of SS1 and msr Strains to HOCI and Measurement of Catalase Activity—**Less than 2-day grown SS1 and msr strain cultures were suspended in MHB (+ 6% FBS) and adjusted to 10$^8$ colony-forming units/ml. The cultures were then exposed to 2.5 mM HOCI in sealed serum bottles at 2% O$_2$, 10% H$_2$, 5% CO$_2$, and the balance of gas was N$_2$. After incubation for 4 h at 37 °C, cells were lysed, and catalase activity was determined.

**Oxidation of Purified Catalase—**Many studies have employed H$_2$O$_2$ (9, 13, 25) to oxidize Met residues in proteins, and to study subsequent Mr-s mediated repair, we previously observed that *H. pylori* catalase can tolerate huge amount of H$_2$O$_2$ without activity loss (19). Therefore, we used HOCI, ChT, and ONOO$^-$ to oxidize catalase. HOCI (available as sodium salt-free chlorine ≤4%) and ChT were purchased from Sigma. ONOO$^-$ was procured from EMD Biosciences. Stocks of HOCI and ONOO$^-$ were diluted in 0.1 N NaOH, and concentrations were determined by monitoring absorbance at 290 and 302 nm, respectively (molar extinction coefficient 350 and 1670 liter mol$^{-1}$ cm$^{-1}$ for OCl$^-$ and ONOO$^-$, respectively) (26, 27). The oxidation treatment was carried out in Buffer A (50 mM sodium phosphate, pH 7.5, 50 mM KCl, 10 mM MgCl$_2$), and 10 mM of catalase was mixed with various fold excess of oxidants as indicated in the figures. Following 15 (HOCI) or 30 (ONOO$^-$/ChT) min of incubation at room temperature, oxidants were quenched by excess Met (5 mM final) for 10 min (14). To address possible ONOO$^-$ breakdown over the 30 min, catalase was also treated with a total of 200-fold excess of ONOO$^-$ in 10 equal aliquots (20-fold excess each time) injected at 3-min intervals (28). Other control experiments were conducted whereby catalase was incubated with various fold excesses of H$_2$O$_2$, or sodium nitrite as indicated in the figures.

**Repair of Catalase—**DTT was procured from Pierce. ATP and NADPH were obtained from Sigma. HOCI-treated samples were repaired with Msr (1-fold molar excess relative to catalase) and/or GroEL/ES (2-fold molar excess relative to catalase) or buffer in the presence of 400 μM NADPH, 5 μM trx, 100 μM trxR, 5 mM ATP, and 100 μM DTT at 37 °C for 1 h.
Mass Spectrophotometry-Trypsin or Asp-N Endoproteinase (Asp-N) Digestion—Catalase was incubated in trypsin (50 mM ammonium bicarbonate, 5 mM DTT, pH 7.8, at 80 °C for 30 min) or Asp-N (50 mM Tris-HCl, 0.5 mM zinc acetate, pH 8.0, 10 mM DTT, at 60 °C for 45 min) digestion buffer. The samples were then cooled to room temperature and digested with sequencing grade modified trypsin (Promega) or Asp-N (Pierce) overnight at 37 °C. Proteases were used at a ratio of 1:100 (protease/catalase). After digestion, samples were stored at −20 °C until analysis.

MS Pseudo-MRM—Digested catalase samples were analyzed using the LTQ front end of an LTQ-FT mass spectrometer (Thermo Scientific) coupled to an Agilent 1100 HPLC system with CaptiveSpray ionization using an Advance Ion Source for Thermo MS (Michrom Bioresources Inc.) Reversed phase LC was performed using an in-line C18 trapping cartridge to desalt, followed by separation on a capillary C18 column (Michrom Bioresources, 0.2 × 50 mm, 3 μm, 200 Å) using a linear gradient from 95% Buffer A (water, 0.1% formic acid) and 5% Buffer B (acetonitrile, 0.1% formic acid) to 40% Buffer A, 60% Buffer B. The flow rate was 3 μl/min for 70 min. Transitions for quantitation by pseudo-MRM analysis were determined by multiple runs of data-dependent LC-MS/MS (nine MS/MS events per survey scan, a maximum of four fragmentation events per precursor mass, and an exclusion window of 5 min), followed by screening with Mascot (Matrix Science) to detect catalase peptides, manual interpretation of tandem MS/MS spectra, and manual selection of transitions to maximize selectivity and sensitivity in the pseudo-MRM experiments.

For pseudo-MRM analysis, the precursor ions for each transition were entered into an include list for data-dependent acquisition in Xcalibur. The LTQ was configured to continue scanning in MS mode until it detected a precursor ion on the included list with a normalized signal level of at least 500 counts/spectrum, at which point the LTQ would isolate and fragment up to nine such precursor ions before performing another MS survey scan. The normalized collision energy for all MS/MS acquisitions was set at 35 V. Post-acquisition, the data were filtered by precursor scan for each transition, and the selected ion chromatogram for the appropriate product ion for the selected precursor was plotted to generate the pseudo-MRM chromatograms. To analyze for the percent of oxidation for a specific methionine residue, the area of the oxidized peak in a base peak chromatogram was divided by the total area of the oxidized and unoxidized peaks. In all cases, the full MS/MS spectra were manually inspected to ensure that the quantitated peak represented the appropriate peptide, as well as to verify the site of oxidation to the suspected methionine residue.

Circular Dichroism (CD) Spectroscopy—CD spectra were acquired on a Jasco J-715 circular dichroism spectrophotometer using a 1-mm path length quartz cuvette (Starna Cells, Inc.). Spectra were obtained at room temperature in an O2-free environment (chamber was continuously flushed with N2). After oxidation and quenching, catalase samples were dialyzed against 5 mM sodium phosphate buffer, pH 7.5, and adjusted to 200 μg/ml. Blank spectra were obtained using the 5 mM sodium phosphate buffer, pH 7.5. Samples were scanned from 190 to 250 nm (far-UV CD) at a speed of 100 nm/min and analyzed by manual comparison of CD spectra using the Jasco Spectra Manager software.

Size Exclusion Chromatography—Catalase samples were loaded onto a HiLoad™ 16/60 Superdex™ 75 gel filtration column (GE Healthcare). The column was developed at a flow rate of 0.4 ml/min; chromatograms were integrated and analyzed using Unicorn 5.01 software. Molecular weights were determined by running a standard molecular weight determination kit (Sigma).

Detection of Carbonyl Groups—Carbonyl groups were detected by OxyBlot™ protein oxidation detection kit (Millipore Corp., Billerica, MA) with slight modifications. Briefly, carbonyls in the catalase were derivatized with 2,4-dinitrophenylhydrazine. About 0.6 μg of derivatized catalase was separated on an SDS gel and transferred to nitrocellulose membrane. Following blocking, the membranes were incubated in rabbit anti-dinitrophenyl antibody (1:150 in PBS-T). After several washes, membranes were incubated in goat anti-rabbit IgG (H+L) conjugated to alkaline phosphatase (Bio-Rad). After 2 h of incubation, blots were washed extensively and developed with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (29).

Quantitation of Catalase in H. pylori Crude Lysate—Less than 2-day grown SS1 cultures were suspended in phosphate-buffered saline and lysed by sonication. Ten micrograms of protein lysates (determined by Pierce BCA assay kit) containing all cell proteins were loaded onto SDS gels. Relative quantity of the catalase band was calculated by loading known amounts of pure enzyme to adjacent lanes and comparing bands by densitometry after staining with Coomassie Brilliant Blue.

RESULTS

Combating oxidative stress is important to H. pylori survival both in vivo and in vitro. Consistent with this, we found that catalase composed about 4–5% of total cell proteins. Also, studies on gene deletion mutants indicate that both Msr and catalase are important for mouse stomach colonization by H. pylori (16, 22).

Sensitivity of msr and katA Strains to HL-60 Cells—Neutrophils are the primary phagocytes recruited in response to H. pylori infection (2), and they produce HOCl as a principal antimicrobial oxidant (3, 30). Because of a deficiency in the ability to repair Met-SO in proteins, including catalase, an msr strain might show hypersensitivity to neutrophil-mediated killing. Differentiated HL-60 cells were used as the source of oxidant in the killing assays. These cell lines are rich in the key HOCl-generating enzyme, myeloperoxidase, which constitutes about 5% of the total HL-60 cellular proteins (31). Moreover, in terms of physical characteristics as well as in biological activities, myeloperoxidase from these cells is similar to human neutrophils (31). To determine the relative contribution of catalase versus its repair enzyme Msr, we included both a katA and an msr strain to compare with the parent, in these killing assays. As presented in Fig. 1, HL-60 cells killed most of the msr strain (~94%) and likewise the bulk of the katA strain (~81%) within 15 min, whereas ~16% of parent SS1 died by the same HL-60 exposure. After 60 min of incubation, most of the mutant bacteria were not viable (~98% of msr and ~92% of katA), whereas
Msr and GroEL Protect Catalase

FIGURE 1. Survivability of SS1, msr, and katA derivative strains from SS1 to differentiated HL-60 cells. Cells were infected with bacteria at a multiplicity of infection of 1:50. At the times indicated, the HL-60 cells were lysed, and the suspensions were serially diluted and plated onto blood agar plates. Colonies were enumerated after incubation of the plates at 2% O2 for 5–7 days. Data are presented as mean ± S.D. (n = 6). The two mutant strains were significantly less viable than SS1 at both 15 and 60 min post-exposure (p < 0.001), although the mutant strains differed from each other at p < 0.04.

~61% of parent SS1 was not viable. However, both msr and katA strains were more susceptible (p < 0.001, Student’s t test) to killing by HL-60 cells than the parent SS1; still the difference in sensitivity between the two mutant strains (msr versus katA) was statistically significant (p < 0.04). Therefore, Msr plays some other roles in aiding H. pylori survival in addition to catalase repair. This is expected as other repair targets should be present in H. pylori (17).

Catalase Activity in SS1 and msr Strains following HOCl Treatment—We previously observed that catalase activity of an msr strain is more susceptible to O2-related damage (17). We speculated that Msr might play an important role in maintaining catalase activity when H. pylori is under HOCl stress. Thus, we incubated SS1 and msr strains with HOCl (in a low O2 atmosphere) and then measured the resulting catalase activities from crude extracts. The activity of the msr strain was significantly decreased (to ~60%) following HOCl treatment, whereas catalase activity was not damaged in the parent SS1 strain (Fig. 2).

Catalase Activities in Oxidized and Repaired Samples—Various oxidants were tested for their ability to damage H. pylori catalase. First, we evaluated the effect of HOCl on purified catalase. As presented in Fig. 3A, HOCl destroyed catalase activity in a dose-dependent fashion. After incubation of 10 μM catalase with 30-, 60-, and 100-fold (i.e. 300, 600, and 1000 μM) molar excess of HOCl for 15 min, the remaining catalase activities were ~66, 44, and 5%, respectively, compared with untreated samples. ONOO− is another key nitrosative stress agent produced by inflammatory cells, and ChT is a milder oxidant but one that preferentially oxidizes Met residues (32). As shown in Fig. 3A, these oxidants were not as effective at inhibiting catalase as HOCl; a higher molar excess of ONOO− or ChT as well as a longer incubation were required to adversely affect catalase activity. After 30 min of incubation with 200- and 400-fold molar excess of ONOO− and ChT, the remaining catalase activities were ~48 and 70%, respectively, compared with untreated enzyme. ONOO− is unstable at neutral pH, so stock solutions were maintained in NaOH. After 30 min of catalase incubation with 200-fold excess ONOO−, the pH was 9.2. Nevertheless, possible oxidant breakdown was addressed; sequential multiple aliquot (as described under “Experimental Procedures”) injections of the oxidant to ensure that “fresh” ONOO− was continuously present were done, similar to the procedure of Lancellotti et al. (28). Under this regime, treatment of catalase with ONOO− gave almost the same levels of catalase inactivation as the single shot method (data not shown). Therefore, breakdown of ONOO− is not the reason for the original observation of lower catalase sensitivity. Commercial ONOO− is often contaminated with peroxide and nitrite, so controls are important to interpret oxidation effects of these potential contaminants (33). However, treatment of catalase with H2O2 or sodium nitrite did not affect enzyme activity (Fig. 3A).

As we observed the largest damaging effect on catalase activity by HOCl, and Met residues are the primary targets of this oxidant, we investigated a role of Msr in repair of HOCl-damaged catalase. We examined the potential role of Msr in conjunction with GroEL/ES (in the presence of trx-trxR system) to reactivate HOCl-damaged catalase (Fig. 3B). Incubation of oxidized catalase with Msr components (Msr, NADPH, trx, trxR, and DTT) or GroEL/ES components (GroEL/ES, ATP, and DTT) alone had little ability to recover enzyme activity (from many experiments less than 5% activity recovered compared with the oxidant-damaged enzyme, data not shown). However, Msr components in combination with GroEL/ES augmented the activity recovery (Fig. 3B). Compared with the oxidized

FIGURE 2. Catalase activity and expression in SS1/msr strains following HOCl treatment. A, parent strain SS1 or the msr derivative from SS1 was incubated with HOCl for 4 h at 2% O2, and catalase activities were determined in crude lysates. Catalase activity of SS1 + PBS was considered as 100%. Data are presented as mean ± S.D. (n = 4). B, expression of catalase following 4 h of HOCl treatment. Lysates (10 μg of protein each) were subjected to SDS-PAGE followed by Western blotting using anti-catalase antibodies. The immunoreactive band is indicated by the arrow.
sample (but still containing the repair mixture of all the Msr and GroEL/ES components, taken as 100%), the activity recoveries were 82 and 62%, respectively, in the case of 30- (30×) and 60-fold (60×) HOCl treatments, respectively. These values represent modest but significant (24 and 41%, respectively) activity recovery compared with the oxidant-damaged enzyme. However, the Msr plus GroEL components were not able to restore activity of the most damaged (100-fold excess HOCl treated) catalase.

**MS/MS Identification of Met Residues in Catalase**—Catalase samples were digested with trypsin or separately with Asp-N, and the resulting peptide mix was then subjected to LC-MS/MS. A representative MS/MS spectrum is displayed in Fig. 4A; it shows b- and y-type product ions of the tryptic peptide 369–392 of 60× HOCl-treated catalase. Fragment ions shifted by the mass of one oxygen are indicated by a +O annotation, which can be mapped to a single oxidation event on Met372. Individual Met residues on each peptide were analyzed for their native or oxidized (+O) status, and percent oxidations were quantitated by calculating the peak area from pseudo-MRM transitions specific to the peptide. Although many Met residues (Met117, Met331, Met489, and Met490) or trypsin (Met162, Met193, and Met220) digestions. Therefore, the use of these two enzymes augmented Met-containing peptide detection, enhancing overall sequence coverage. At the same time, we were able to rigorously assess the oxidation status of Met residues that were recovered by both proteases.

Out of 14 Met residues in catalase, we detected 11 residues. We were not able to identify N-terminal Met (possibly because of post-translational processing) and the two most C-terminal Met residues (Met493 and Met498) (probably because of the very small size of the peptides in both tryptic and Asp-N digests). Fractional oxidations for individual Met residues are presented in Fig. 4B. In native “as purified” catalase, oxidations of these Met residues were negligible (near to baseline). Following 60× HOCl treatment, oxidations of Met162, Met220, Met292, Met489, and Met490 reached to ~90–100%. Met372 was ~50% oxidized, and other Met residues were not heavily oxidized. All identified Met residues were either distributed on different peptides or distinguishable by prominent fragmentation ions between the two Met residues except for Met489 and Met490; these were located on the same peptide (amino acid 472–491) in the Asp-N digest, and the product ion spectra of this peptide revealed no abundant fragmenta-
tion product occurring in the one peptide bond between the two Met residues, so only their collective oxidation status could be measured. Incubation of oxidized catalase with the Msr in the presence of 10 mM DTT (DTT can act as an in vitro reductant for Msr) (34) reduced most of the oxidized Met residues close to the baseline nonoxidant treated levels (Fig. 4B). In the case of 400-fold HOCl treatment, we observed the oxidation and repair of the same Met residues that were oxidized by HOCl, but their overall oxidation was about 20–40% less than with HOCl (data not shown). In the ONOO−-treated sample, we did not observe significant Met oxidation (data not shown).

**FIGURE 4.** A, MS/MS spectrum of peptide 369–392 following trypsin digestion of previously 600-fold HOCl-treated catalase. Fragmentation pattern of peptide 369–392 is displayed. The designation +O on b- and y-ions represents addition of 16 Da to the predicted product ion of the unoxidized peptide, consistent with the addition of one oxygen atom to Met372. B, Identification of Met residues after oxidation and repair of catalase. 10 μM catalase was incubated with 600-fold HOCl for 15 min, and excess oxidant was quenched by adding excess L-Met. Following dialysis-based removal, oxidized samples were incubated with Msr in the presence of DTT. Samples were digested separately with trypsin or Asp-N, and Met residues were identified and quantified by LC-MS/MS.

**TABLE 1**

| Met residue | Trypsin | Asp-N | Met residue | Trypsin | Asp-N |
|-------------|---------|-------|-------------|---------|-------|
| Met-1       | +       | −     | Met-292     | +       | +     |
| Met-115     | +       | +     | Met-331     | −       | +     |
| Met-162     | +       | −     | Met-372     | −       | +     |
| Met-181     | +       | −     | Met-489     | −       | +     |
| Met-193     | +       | −     | Met-490     | −       | +     |
| Met-220     | +       | +     | Met-493     | −       | −     |
| Met-265     | +       | +     | Met-498     | −       | −     |

**TABLE 1**

**MS/MS sequence coverage of Met residues in catalase following trypsin and Asp-N digestion**

Met residue number is the location of Met in the catalase sequence, and + means that particular Met was observed from the corresponding enzymatic digestion.
Next, we attempted to explore the correlation between surface exposure of Met residues in catalase and their susceptibility to oxidation. We calculated surface accessibility of the sulfur center of all of the Met residues based on the x-ray crystal structure of H. pylori catalase (Protein Data Bank code 1QWL) (35). We observed that Met<sup>162</sup>, Met<sup>292</sup>, Met<sup>372</sup>, Met<sup>489</sup>, and Met<sup>490</sup> are calculated to be the most surface-exposed Met residues in catalase (Table 2) as well as the ones most vulnerable to oxidation (Fig. 4B). Nonexposed Met residues showed little or no oxidation. The only exception was Met<sup>220</sup>, as this residue was oxidized but not predicted to be localized on the catalase surface. This apparent discrepancy may be due to protein dynamics in the region of Met<sup>220</sup>, which are not probed by a static crystal structure, causing Met<sup>220</sup> to be more solvent-exposed than the crystal structure would indicate.

**Structural Changes in Catalase following HOCl Treatment**—Several studies have demonstrated unfolding (36, 37) or oligomerization (38–40) of proteins following oxidant treatment. For example, a recent study described secondary structure destabilization and unfolding of apolipoprotein A-1 as a consequence of Met oxidation (9). CD spectra of catalase following HOCl treatment is presented in Fig. 5A. Our deconvoluted secondary structure composition of nonoxidized (not HOCl treated) catalase was 29% α-helix and 21% β-sheet. The observed secondary structure pattern is in good agreement with the reported x-ray crystal structure of H. pylori catalase (Protein Data Bank code 1QWL), which has 32% helical and 16% β-sheet. For protein stability assessments, CD is typically followed at 220 nm, whereby an increase in ellipticity (millidegree) indicates loss of secondary structure. After treatment with increasing concentrations of HOCl, we observed an increase in the ellipticity at 220 nm, which is near a local minimum for both α-helix and β-sheet but near a maximum for random coil (Fig. 5A). This progressive increase in ellipticity at 220 nm nicely correlated with the added HOCl amount (Fig. 5B), suggesting a dose-dependent loss of catalase structure. Furthermore, we have noticed a strong positive correlation between secondary structure content as measured by negative CD values at 220 nm (Fig. 5B) and catalase activity (Fig. 3A).

We used gel permeation chromatography to estimate the size of catalase protein fractions upon oxidation. As presented in Fig. 6A, HOCl induced oligomerization of catalase in a dose-dependent manner. Native as purified catalase gave one peak with a molecular mass of ~190–200 kDa. This observed molecular mass represents the catalase tetramer and is in agreement with an earlier reported mass of H. pylori catalase (18). HOCl treatment gave an additional peak at ~400 kDa. In addition, after 30 and 60× HOCl treatments, about 8% of the total catalase eluted at a size of >2000 kDa. Following 30, 60, and 100× HOCl treatments, the remaining native catalase tetramer (~190–200 kDa) was sequentially diminished to ~68, 54, and 0%, respectively, of the nonoxidized samples. Interestingly, ChT did not induce oligomerization of catalase (Fig. 6B). We also ran the oxidized samples on SDS gels under reducing conditions. In nonoxidized samples, catalase migrated at about 55 kDa. Treatment of catalase with increasing concentrations of HOCl led to its progressive oligomerization (i.e. loss of 55-kDa species). In 100× HOCl-treated samples the 55-kDa monomeric band was nearly absent, and large molecular weight species appeared (data not shown). These results indicate that the HOCl-induced oligomerization of catalase is probably due to a noncovalent cross-linking event that does not solely consist of disulfide bond formation.

**TABLE 2**
Solvent accessibility of individual catalase Met residues

All solvent accessibility measurements are based on the average of the two subunits in the x-ray crystal structure, Protein Data Bank code 1QWL. SASA means solvent-accessible surface area. Average oxidation is the fraction of oxidized Met residues as identified by MS/MS following incubation of catalase with 60× HOCl.

| Met residue | Average sulfur SASA (Å<sup>2</sup>) | Average oxidation | Met residue | Average sulfur SASA (Å<sup>2</sup>) | Average oxidation |
|-------------|--------------------------------------|-------------------|-------------|--------------------------------------|-------------------|
| Met-115     | 0                                    | 0.03              | Met-292     | 14.25                                | 0.89              |
| Met-162     | 1.59                                 | 0.79              | Met-331     | 0.165                                | 0.01              |
| Met-181     | 0.47                                 | 0.2               | Met-372     | 38.55                                | 0.45              |
| Met-193     | 0                                    | 0.12              | Met-489     | 7.81                                 | 1                 |
| Met-220     | 0                                    | 0.89              | Met-490     | 1                                    |                   |
| Met-263     | 0                                    | 0.17              |             |                                      |                   |

**FIGURE 5.** Far-UV CD spectra of catalase treated with various fold excesses of HOCl. A, catalase was incubated with the indicated molar fold excess of HOCl for 15 min, and oxidant was quenched by addition of excess l-Met. Following dialysis, samples were adjusted to 200 μg of protein/ml (see text) and scanned from 195 to 250 nm. B, correlation between HOCl concentrations and CD ellipticity values (millidegree) at 220 nm.
HOCl treatment of catalase also produced carbonylation that was detected by OxyBlot stains of SDS gels. 60 and 100× HOCl-treated catalase yielded about equal total carbonylation that was evident among all the separated species (i.e. 55 kDa and oligomers, data not shown).

**DISCUSSION**

Successful colonization by *H. pylori* depends in part upon the ability of the bacterium to combat oxidative stress. The repertoire of antioxidant proteins together constitutes a significant proportion of the total *H. pylori* protein profile (5). Mutant strains in most of the antioxidant genes are viable *in vitro* under nonoxidative conditions but are defective in mouse stomach colonization abilities (5). Among the possible macromolecule targets, proteins are a primary one for oxidative damage (6). Although degradation followed by replacement via new synthesis (protein turnover) is an obvious direct approach to replenish oxidatively damaged proteins, repair of damaged proteins is expected be less energetically expensive and a more rapid process than *de novo* synthesis. Thus protein repair systems can provide a means to survive under stress conditions, and pathogens in particular are adept at repair processes.

Met residues are considered to be primary targets of many oxidants, including the oxidants produced by host inflammatory cells (6). The role of Msr in higher organisms in repairing Met-rich proteins (13, 41–44) is well established, but few Msr targets have been reported in bacteria. They include GroEL and Ffh (12, 14, 25). Interestingly, we have observed up-regulation of Msr following subjection of *H. pylori* to oxidative stress, and three proteins that interact with Msr are catalase, GroEL, and SSR (17). A peptide repair system is especially important in some pathogens such as *H. pylori* as the bacterium is known both for its persistence and ability to elicit a robust inflammatory response.

Catalase is a key antioxidant protein that degrades H₂O₂. For example, we have observed much more carbonylation of *H. pylori* proteins (data not shown) as well as DNA damage (45) in a *katA* strain versus its parent upon exposure of both strains to oxidative stress. In *H. pylori*, catalase is expressed in relatively large amounts (4–5% of total protein), and the enzyme does not undergo up-regulation during oxidative stress (Fig. 2B). *H. pylori* has two described mechanisms devoted to maintaining catalase activity; in addition to the Msr-mediated repair described herein, we previously reported a role for the peroxiredoxin AhpC in protecting catalase against organic hydroperoxide-mediated inactivation (19). Our previously reported interaction of catalase with Msr was found to be dependent on the oxidation state of catalase, whereby previously oxidized enzyme (Met residues likely converted to Met-SO) interacted more readily with Msr (17). The Met content in catalase is unusually high (14 out of 505 residues, representing ~3%) thus making it a good target for Met repair. We identified (Fig. 4B) six Met residues susceptible to HOCl oxidation and subsequent Msr-mediated repair. Interestingly, almost all (five out of these six) heavily oxidized Met residues are predicted to be exposed on the catalase surface (Table 2), consistent with previous data indicating that the rate of reaction of Met residues with various reactive oxygen species is directly dependent upon solvent accessibility of the reactive site (11, 46, 47). For other Msr repair targets, 12 (14) and 4 (25) Met residues were shown to undergo repair within the bacterial proteins GroEL and Ffh (a component of signal recognition particle), respectively.

At inflammatory sites, neutrophils produce high levels (up to 5 mM) of HOCl (3). Among the oxidants tested, HOCl caused the most damaging effect on catalase activity (Fig. 3A). The second-order rate constants (M⁻¹ s⁻¹) of HOCl for Met and Cys residues are ~3.8 × 10⁷ and 3.0 × 10⁴, respectively (6). This high reactivity may explain the dramatic effect of HOCl on catalase activity and likely makes this oxidant a potent antibacterial agent generated by phagocytes. The high amounts of both catalases made by *H. pylori* and likewise the HOCl produced by the host may illustrate the evolving struggle between host and pathogen.

Although ONOO⁻ destroyed catalase activity (Fig. 3A), much higher oxidants and a longer exposure time were required to achieve levels of inactivation (~50%) similar to that achieved by HOCl (Fig. 3A). The second-order rate constant (M⁻¹ s⁻¹) of ONOO⁻ is ~10² and 10³ for Met and Cys residues (48) and is lower than of HOCl by a magnitude of ~10⁶. Interestingly, we did not observe any significant Met-SO in ONOO⁻-treated catalase. Cys and Tyr residues were found to be modified in several ONOO⁻-inactivated enzymes (48). Nitration of Tyr predominates over Met oxidation following exposure of proteins to ONOO⁻ under physiological conditions (49, 50). Sequence analysis revealed that *H. pylori* catalase has 25 Tyr and 2 Cys residues, so the major result of ONOO⁻-mediated catalase inactivation would be expected to be via Tyr nitration. In comparison with the parent strain SS1, the msr strain was much more susceptible to killing by HOCl than by ONOO⁻ (data not shown), also indicating that Met residue oxidation may not be a primary ONOO⁻ stress target in *H. pylori*.

Loss of catalase secondary structure was associated with catalase activity damage. This likely explains the requirement of GroEL in recovering the activity of Msr-repaired oxidized catalase (Fig. 3B). Other studies reported oxidation-induced loss of secondary structure in several proteins (9, 36, 51, 52). A recent study suggested oxidative unfolding and consequent aggregation as a major mechanism of HOCl-mediated protein inactivation (40). As sulfoxides are more hydrophilic than Met residues, their formation can change the overall surface charge of a protein (8, 38). This destabilizing effect can lead to unfolding of the protein. Unless a chaperone for refolding comes into play, the damaged Met-SO-rich protein would be expected to be subject to aggregation and subsequent degradation. It is likely that Met-SO in catalase first needs to be repaired before the process of chaperone-mediated refolding can be accomplished;

---

**FIGURE 6. HOCl treatment causes oligomerization of catalase.** Gel filtration chromatography of catalase following HOCl (A) or ChT (B) treatments. After oxidation and quenching, catalase samples were subjected to gel filtration chromatography. Chromatograms were integrated and analyzed using Unicom 5.01 software.
thus the three component system is required (see Fig. 7). GroEL/ES and DnaK/DnaJ are primary chaperones dedicated to protection against stress-induced protein aggregation (53). GroEL has been shown to protect bovine catalase against thermal stress (54). When plant catalase was expressed in E. coli, its activity level was augmented when accompanied with GroEL/ES coexpression (55). GroEL interacts with an estimated 300 E. coli proteins; many are essential ones for survival (56). Interestingly, GroEL is also a target of Met oxidation and Msr-mediated repair (14). Therefore, Msr apparently serves to protect catalase directly as well as indirectly via repair of the chaperone.

We observed a significant number of Met-SO residues that undergo repair (Fig. 4B) and a significant activity recovery of 30 and 60% HOCl-treated catalase by adding a combination of both the Msr and GroEL systems (Fig. 3B). Still the activity recovery was partial suggesting some other (severe) modifications of catalase as a result of HOCl treatment. Although we observed HOCl-mediated carbonyl formation (data not shown) and oligomerization of catalase (Fig. 6), we did not observe any significant heme modification based on spectral assays (data not shown). HOCl-induced protein radicals, carbonyl formation, and subsequent oligomerization of eukaryotic catalase was reported (39); although heme was postulated to be a primary site to promote oxidative damage, the heme group itself was not significantly modified following prolonged exposure of catalase to HOCl (39). A separate study observed severe carbonylation and oligomerization of heme-depleted apomyoglobin following HOCl treatment (38), indicating that the heme group may not be necessary for HOCl-mediated carbonylation and oligomerization.

Our data demonstrate that HOCl rapidly oxidized six Met residues in catalase, introduced structural changes, and subsequently caused activity loss (Figs. 3A, 4B, 5, and 6). Msr repaired these Met-SO residues, but activity recovery required the GroEL system (Figs. 3B, 4B, and 7). Our experiments also suggest that both Msr and catalase enhance survival of H. pylori against HOCl-producing neutrophils. Although Msr aids H. pylori survival by protecting catalase, it plays additional protective roles that aid bacterial survival in the presence of neutrophils. Identifying the importance of these repair targets in comparison with the catalase target will help us to further understand the roles of Msr.

Acknowledgments—We thank Dr. S. Benoit for help in protein purification and G. Wang for providing katA strain. We also thank Dr. Jeffrey Urbauer for use of the circular dichroism spectrophotometer. J. S. S. acknowledges support for analytical instrumentation used in this study from National Institutes of Health Grant RR005351 from the NCRR.
Radic. Biol. Med. 48, 446–456
29. Harlow, E., and Lane, D. (1988) Antibodies: A Laboratory Manual, p. 598, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
30. Rosen, H., Klebanoff, S. J., Wang, Y., Brot, N., Heinecke, J. W., and Fu, X. (2009) Proc. Natl. Acad. Sci. U.S.A. 106, 18686–18691
31. Hope, H. R., Remsen, E. E., Lewis, C., Jr., Heuvelman, D. M., Walker, M. C., Jennings, M., and Connolly, D. T. (2000) Protein Expr. Purif. 18, 269–276
32. Su, Z., Limberis, J., Martin, R. L., Xu, R., Kolbe, K., Heinemann, S. H., Hoshi, T., Cox, B. F., and Gintant, G. A. (2007) Biochem. Pharmacol. 74, 702–711
33. McLean, S., Bowman, L. A., Sanguinetti, G., Read, R. C., and Poole, R. K. (2010) J. Biol. Chem. 285, 20724–20731
34. Lowther, W. T., Weissbach, H., Etienne, F., Brot, N., and Matthews, B. W. (2002) Nat. Struct. Biol. 9, 348–352
35. Loewen, P. C., Carpena, X., Rovira, C., Ivancich, A., Perez-Luque, R., Haas, R., Odenbreit, S., Nicholls, P., and Fita, I. (2004) Biochemistry 43, 3089–3103
36. Melkani, G. C., Zardeneta, G., and Mendoza, J. A. (2002) Biochem. Biophys. Res. Commun. 294, 893–899
37. Hawkins, C. L., and Davies, M. J. (2005) Chem. Res. Toxicol. 18, 1600–1610
38. Chapman, A. L., Winterbourn, C. C., Brennan, S. O., Jordan, T. W., and Kettle, A. J. (2003) Biochem. J. 375, 33–40
39. Bonini, M. G., Siraki, A. G., Atanassov, B. S., and Mason, R. P. (2007) Free Radic. Biol. Med. 42, 530–540
40. Winter, J., Ilbert, M., Graf, P. C., Ozcelik, D., and Jakob, U. (2008) Cell 135, 691–701
41. Moskovitz, J. (2005) Biochim. Biophys. Acts 1703, 213–219
42. Tsverkov, P. O., Ezraty, B., Mitchell, J. K., Devred, F., Peyrot, V., Derrick, P. J., Barras, F., Makarov, A. A., and Lafitte, D. (2005) Biochimie 87, 473–480
43. Brennan, L. A., Lee, W., Giblin, F. J., David, L. L., and Kantorow, M. (2009) Biochim. Biophys. Acts 1790, 1665–1672
44. Grimaud, R., Ezraty, B., Mitchell, J. K., Lafitte, D., Briand, C., Derrick, P. J., and Barras, F. (2001) J. Biol. Chem. 276, 48915–48920
45. Wang, G., Conover, R. C., Olczak, A. A., Alamuri, P., Johnson, M. K., and Maier, R. I. (2005) Free Radic. Res. 39, 1183–1191
46. Vogt, W. (1995) Free Radic. Biol. Med. 18, 93–105
47. Levine, R. L., Mosoni, L., Berlett, B. S., and Stadtman, E. R. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 15036–15040
48. Alvarez, B., and Radi, R. (2003) Amino Acids 25, 295–311
49. Berlett, B. S., Levine, R. L., and Stadtman, E. R. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 2784–2789
50. Tien, M., Berlett, B. S., Levine, R. L., Chock, P. B., and Stadtman, E. R. (1999) Proc. Natl. Acad. Sci. U.S.A. 96, 7809–7814
51. Sharp, J. S., Sullivan, D. M., Cavanagh, J., and Tomer, K. B. (2006) Biochemistry 45, 6260–6266
52. Venkatesh, S., Tomer, K. B., and Sharp, J. S. (2007) Rapid Commun. Mass Spectrom. 21, 3927–3936
53. Georgopoulos, C. (2006) Genetics 174, 1699–1707
54. Hook, D. W., and Harding, J. J. (1997) Eur. J. Biochem. 247, 380–385
55. Mondal, P., Ray, M., Sahu, S., and Sabat, S. C. (2008) Protein Pept. Lett. 15, 1075–1078
56. Lund, P. A. (2009) FEMS Microbiol. Rev. 33, 785–800