Role of a bacterial organic hydroperoxide detoxification system in preventing catalase inactivation

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Abbreviations: H. pylori, Helicobacter pylori; AhpC, alkyl hydroperoxide reductase; PCR, polymerase chain reaction; EPR, electron paramagnetic resonance spectroscopy; t-BOOH, t-butyl hydroperoxide.

Running title: Organic hydroperoxide reductase protects catalase
Summary

In the gastric pathogen *Helicobacter pylori*, catalase (KatA) and alkyl hydroperoxide reductase (AhpC) are two highly abundant enzymes that are crucial for oxidative stress resistance and survival of the bacterium in the host. Here we report a previously unidentified connection between the two stress-resistance enzymes. We observed that the catalase in *ahpC* mutant cells, in comparison to the parent strain, is partially (about 50%) inactivated. The decrease of catalase activity is well correlated with the perturbation of heme environment in catalase as detected by electron paramagnetic resonance spectroscopy (EPR). To understand the reason for this catalase inactivation, we examined the inhibitory effects of hydroperoxides on *H. pylori* catalase (either present in cell extracts or added to the purified enzyme) by monitoring the enzyme activity and the EPR signal of catalase. *H. pylori* catalase is highly resistant to its own substrate, without loss of enzyme activity by treatment with a molar ratio of 1:3000 H$_2$O$_2$. However, it is inactivated by lower concentrations of organic hydroperoxides (the substrate of AhpC). Treatment with a molar ratio of 1:400 t-butyl hydroperoxide resulted in about 50% inactivation of catalase. UV-visible absorption spectra indicated that the catalase inactivation by organic hydroperoxides is due to the formation of a catalytically incompetent compound II species. To further support the idea that organic hydroperoxides which accumulate in the *ahpC* mutant cells are responsible for the inactivation of catalase, we measured the level of lipid peroxidation in *ahpC* mutant cells compared to wild type cells. The results showed that the total amount of extractable lipid hydroperoxides in the *ahpC* mutant cells is about 3 times that in the wild type cells. Our findings reveal a novel role of the organic hydroperoxide detoxification system in preventing catalase inactivation.
The ability of pathogenic bacteria to resist oxidative stress is crucial for their infection and pathogenesis in the host (1, 2). To defend against reactive oxygen species (ROS) that can cause protein oxidation, lipid peroxidation and DNA damage, living organisms rely on a series of enzymatic machinery. Superoxide dismutase (SOD), catalase, and alkyl hydroperoxide reductase (AhpC) are virtually ubiquitous enzymes conferring oxidative stress resistance (3, 4, 5, 6). SOD dismutates the superoxide anion into hydrogen peroxide ($\text{H}_2\text{O}_2$) and molecular oxygen ($\text{O}_2$), and catalase breaks down $\text{H}_2\text{O}_2$ into water and $\text{O}_2$. AhpC reduces organic hydroperoxides (ROOH, also extended to include HOOH) into the corresponding non-toxic alcohol (ROH). Elimination of organic hydroperoxides is particularly important for living cells since they can initiate lipid peroxidation chain reaction and consequently propagate free radicals, leading to DNA and membrane damage (7). AhpC is a component of a large family of thiol-specific antioxidant (TSA) proteins whose roles are generally not well understood (8, 9).

As a highly successful human bacterial pathogen, *Helicobacter pylori* infection induces a strong inflammatory response within the host thereby releasing a high level of host-derived toxic oxygen species; but *H. pylori* can survive and colonize persistently in the harsh conditions of gastric mucosa (10, 11, 12). To account for this capability, *H. pylori* possess SOD, KatA, and AhpC enzymes (11). In addition, some other factors have been identified that play important roles in oxidative stress resistance. NapA, a ferritin-like iron-binding protein, is involved in oxidative stress resistance probably through sequestering free iron in the cells (13, 14). Also, an NADPH quinone reductase (MdaB) confers oxidative stress resistance by maintaining the quinone pool of the cell in the reduced state (15). Disruption of each individual gene for SOD, KatA, AhpC, or MdaB severely affects the bacterium’s ability to colonize the host stomach (15,
16, 17, 18), demonstrating the importance of these enzymes in oxidative stress resistance and host colonization.

*H. pylori* express abundant levels of catalase and AhpC proteins (19). The genetic and biochemical characterization of *H. pylori* catalase and AhpC have been performed in different laboratories (13, 20, 21, 22, 23, 24). *H. pylori* catalase is a homotetrameric protein, with each subunit having a molecular mass of 59 kDa. It is a monofunctional catalase without peroxidase activity. A unique property of *H. pylori* catalase is that it has an isoelectric point (pI) of >9. Another property of *H. pylori* catalase distinct from other typical catalases is its stability at very high concentrations of H$_2$O$_2$ (20). *H. pylori* cells were shown to be resistant to high concentration (~100 mM) of H$_2$O$_2$ and this resistance was abolished in *katA*" mutants (24). *H. pylori* AhpC is a major component of the thioredoxin-dependent peroxiredoxin system (AhpC-Trx-TrxR) that catalyzes reduction of hydroperoxides including H$_2$O$_2$ and organic hydroperoxides (22, 25), as well as reduction of peroxynitrite (26). It was extremely difficult to obtain an *ahpC* knock-out mutant (21, 22); eventually the mutant was obtained by screening transformants at very low O$_2$ (1% partial pressure) condition (13). AhpC mutant cells exhibited severe growth sensitivity to hydroperoxides and to the superoxide-generating agent paraquat (13).

We studied the relationship between these two important antioxidant proteins and discovered that the organic hydroperoxide reductase (AhpC) plays a previously unidentified role in protecting catalase from inactivation by organic hydroperoxides. For the first time, it is shown that loss of AhpC function leads to a significant increase of organic hydroperoxides within the cells, and that these hydroperoxides are potent inhibitors of catalase.
Experimental Procedures

H. pylori strains and growth conditions. H. pylori strains ATCC43504 or the isogenic mutants were cultured on Brucella agar (Difco) plates supplemented with 5% fetal bovine serum (called BA plates). Cultures of H. pylori were grown microaerobically at 37°C in an incubator containing 5% CO₂ and 2% oxygen. Chloramphenicol (50 µg/ml) or kanamycin (40 µg/ml) was added in the medium, for culturing mutants.

DNA techniques. All DNA manipulations were performed as described (27). Chromosomal DNA was extracted from H. pylori with the Aquapure genomic DNA extraction kit (Bio-rad). Plasmid DNA preparations were carried out with the QiaPrep Spin mini kit (Qiagen). DNA fragments or PCR products were purified from agarose gels with the Qiaquick gel extraction kit (Qiagen). PCR was performed in a Perkin-Elmer 2400 thermal cycler with Taq or Pfu DNA polymerase (Fisher). Oligonucleotide primers were synthesized by Integrated DNA Technologies, Coralville, Iowa.

Construction of H. pylori mutants. To construct a katA mutant, primers katAF (5’-TCCATAAGAGAACAACGCGC-3’) and katAR (5’-CAACAATGTGATTACGCGC-3’) were used to PCR amplify a 953-bp fragment containing the H. pylori katA gene (HP0630) with genomic DNA from strain ATCC43504 as template. The PCR fragment was directly cloned into pGEM-T vector (Promega) according to the manufacturer’s instruction, to generate pGEM-katA. The host strain used for cloning was E. coli DH5α. Subsequently, a chloramphenicol acetyl transferase (CAT) cassette was inserted at the unique HindIII site within the katA sequence of pGEM-katA. The recombinant plasmid was then introduced into H. pylori by natural transformation via allelic exchange, and chloramphenicol resistant colonies were isolated. The
disruption of the gene in the genome of the mutant strain (43504 katA:Cm) was confirmed by PCR showing an increase in the expected size of the PCR product.

With a similar procedure, the ferritin mutant strain (43504 pfr:Kan) was constructed as follows. A 1213-bp fragment containing *H. pylori* pfr gene was PCR amplified using primers pfrF (5’-TGGCTAGTTTTAAGGGCATG-3’) and pfrR (5’-AAGCGCAAAATTGGCAAGCG-3’), and cloned into pGEM-T vector. Subsequently, the 301-bp *Hind*III fragment within *pfr* gene was replaced by a kanamycin resistance cassette (Kan).

The construction of other mutant strains (*sodB*, *mdaB*, *ahpC1*, *ahpC2*, *ahpCnapA*, *napA*) was described previously in our laboratory (13, 15, 18). An *H. pylori* 43504 katAahpC double mutant was constructed in this study by transforming *ahpC*:Kan (type I) mutant strain with plasmid pGEM-katA:Cm.

**Cell-free extract and membrane fraction.** Plate-grown *H. pylori* cells were harvested and suspended in phosphate-buffered saline (PBS). The cells were collected by centrifugation (10,000 xg for 10 min), resuspended in PBS, and broken by two passages through a French pressure cell at 18,000 lb/in². Crude extracts were then cleared of unbroken cells by centrifugation at 10,000 xg for 10 min. The supernatant (cell-free extract) was then subject to ultracentrifugation (45,000 xg for 60 min) to obtain the membrane fraction (the pellet).

**Protein concentration determination and gel electrophoresis.** Protein concentrations were determined with a bicinchoninic acid protein assay kit (Pierce, Rockford, Ill.). For SDS-PAGE, 5 µg of cell extract was placed into SDS buffer, boiled for 5 min, and applied to a denaturing 12.5% acrylamide gel. With densitomeric measurement of all the protein bands on SDS gel, the portion (%) attributed to a specific protein (KatA or AhpC) was calculated. Based
on this percentage, the molecular weight of the protein, and the total protein concentration, we estimated the molar concentration of KatA or AhpC in the cell extract.

**Purification of catalase.** Native *H. pylori* catalase was purified following the method similar to that described by Radcliff et al. (28). Briefly, *H. pylori* cells grown on plates were harvested by suspension in 0.1 M sodium phosphate buffer (pH7.5). After centrifugation, the cell pellet was resuspended in the buffer. Cells were disrupted by 3 cycles through a French pressure cell at 18,000 lb/in$^2$, and the lysate was centrifuged at 28,000xg for 10 min to remove the cell debris. The supernatant was collected and subjected to ultracentrifugation at 100,000xg for 45 min. The supernatant was applied to a SP Sepharose cation-exchange column (Amersham) that had been equilibrated with 25mM sodium phosphate buffer (pH7.5), and proteins were eluted by the creation of a gradient with 1 M NaCl in 25mM sodium phosphate buffer (pH7.5). Catalase positive fractions were selected by checking for oxygen-reducing activity in 3% H$_2$O$_2$. The pooled catalase positive fractions were further purified by gel filtration chromatography using Sephacryl S-200 column (Amersham) and eluted with 25mM sodium phosphate buffer (pH7.5). The purified catalase was then filter sterilized, stored at 4°C, and protected from light.

**Determination of catalase activity.** Quantitative catalase activity of *H. pylori* cell extract or purified catalase protein was determined following the method described by Hazell et al. (20). Briefly, catalase activity was measured spectrophotometrically at 25°C by following the decrease in absorbance at 240 nm ($\varepsilon_{240nm} = 43.48$ M$^{-1}$ cm$^{-1}$) of 13 mM H$_2$O$_2$ in PBS. All assays were repeated to give 12 rate determinations for the first minute of reaction. One unit was defined as the amount of enzyme that catalyzes the oxidation of 1 µmol H$_2$O$_2$ min$^{-1}$ under the assay condition.
**Determination of lipid hydroperoxides.** The amount of lipid hydroperoxides within *H. pylori* cell extracts or the membrane fractions was determined with Lipid Hydroperoxide Assay Kit (Cayman Chemical, Ann Arbor, MI) following the manufacturer’s instruction. Briefly, lipid hydroperoxides within a sample were first extracted into chloroform, which eliminates any interference caused by hydrogen peroxide or endogenous ferric ions in the sample. The lipid hydroperoxides in the extracted sample were directly used in the assay by reacting them with ferrous ions. The resulting ferric ions are detected using thiocyanate ion as the chromogen by measuring absorbance at 500nm ($\varepsilon_{500\text{nm}} = 16,667$ M$^{-1}$ cm$^{-1}$). Ethanolic solution of 13-hydroperoxy octadecadienoic acid was used as lipid hydroperoxide standard.

**Determination of organic peroxide reductase activity in cell extract.** t-butyl hydroperoxide (tBOOH) was added to *H. pylori* WT cell extract to a final concentration of 1 mM. At various time points, an aliquot of the sample was removed and assayed for the remaining amount of tBOOH using Lipid Hydroperoxide Assay Kit as described above. The organic peroxide reductase activity was expressed as the decrease of tBOOH amount (nmol) per min per mg total protein in the cell extract.

**EPR spectroscopy.** In this study, EPR was applied to whole cells, cell extracts, or purified catalase, in order to monitor changes of the catalase heme environment. For whole cell samples, a 5 ml cell suspension in PBS (OD$_{600\text{nm}} = 8$) was incubated with 20 mM desferrioxamine at 37°C for 15 min. The cells were then centrifuged, washed with cold 20 mM Tris-HCl (pH 7.4), resuspended in a final volume of 0.4 ml of the same buffer, and frozen in 3-mm quartz EPR tubes by immersion in liquid nitrogen. For cell extracts or purified catalase, the samples were incubated (for 30 min at room temperature) with different concentrations of
hydroperoxides as indicated, and immediately frozen in EPR tubes. Samples were stored at -78 °C for EPR analysis.

X-band (~9.6 GHz) EPR spectra were recorded on a Bruker ESP-300E EPR spectrometer equipped with an ER-4116 dual-mode cavity and an Oxford Instruments ESR-9 flow cryostat. The intensity of the EPR signals was normalized to the OD of whole cell samples and the protein absorption band of cell-free extracts and purified samples.

**Results and Discussion**

**Monitoring KatA and AhpC proteins in *H. pylori* cell extract by SDS-PAGE.**

Both KatA and AhpC are major proteins expressed in *H. pylori* cells. Hazell et al. (20) reported that catalase accounts for ~1% of the cell’s total protein in *H. pylori*. The proteome analysis of Jungblut et al. (19) showed that AhpC (TsaA) is the third most abundant protein in *H. pylori*. The expression level of these proteins in *H. pylori* cells is easily visualized on SDS-PAGE. We identified the specific protein bands by (a) expected molecular weight, (b) loss of the band in the corresponding mutant strain, and (c) direct N-terminal sequencing of the protein band. Regulation mechanisms for the expression of KatA and AhpC in *H. pylori* are currently unclear, but they may involve regulation at both transcriptional and post-translational levels.

Using SDS-PAGE, we measured the net expression level of the proteins. There are some variations in the protein expression levels by different strains (not shown). The profiles of the total proteins in the parent strain ATCC43504 and the isogenic mutant strains of *katA*, *ahpC*, or *napA* are shown in Fig.1. Compared to the wild type strain, the corresponding protein band of KatA, AhpC, or NapA is missing in the respective mutant strains. As previously shown, there are two types of *ahpC* mutants, with the type I mutant overexpressing NapA (13). Based on
densitometric measurement of the protein bands on the gel, KatA and AhpC each constitute 2-3% of the total proteins in the wild type cell. Compared to other bacteria, for example Camplobacter jejuni (29), the expression level of KatA and AhpC is much higher in H. pylori.

Decreased catalase activity in ahpC mutant cells.

H₂O₂ is a general agent of oxidative stress to the cells. To determine the relative contributions of H. pylori SOD, KatA, AhpC, NapA, or MdaB in resistance to H₂O₂, we determined the H₂O₂-decomposing activity of each mutant strain, compared to the wild type strain. As shown in Fig. 2A, wild type H. pylori exhibited a high level of catalase activity (~3000 units / mg of total protein); whereas, knock-out mutants in katA (catalase gene) resulted in complete loss of catalase activity. As expected, disruption of other antioxidant genes such as sodB (the gene for superoxide dismutase) or mdaB (the gene for NADPH quinone reductase) did not significantly affect catalase activity of the cells. To our surprise, however, the catalase activity in gene-targeted ahpC mutant cells was determined to be approximately half of that in the wild type cells (Fig. 2A).

It was observed in Pseudomonas aeruginosa that catalase activity is affected by mutation of a separate gene (30); a bfrA mutant of P. aeruginosa had only 47% the KatA activity of wild type strain, despite possessing wild type expression level of KatA. BfrA, composed of 24 subunits capable of binding 700 iron atoms, is the major iron storage protein in P. aeruginosa (31). The results of Ma et al. (30) suggested that BfrA is required as a source of iron for the heme prothetic group of KatA. H. pylori possess two iron-storage proteins, NapA and Pfr. NapA is a homologue of bacterial DNA-protecting proteins (Dps), and its molecular structure has been determined (32, 33). It has a dodecameric structure (12 subunits) capable of binding up to 500 iron atoms (32). Pfr is the major iron storage protein in H. pylori (34). As a typical ferritin, Pfr
forms a 24mer structure and binds more than 2000 iron atoms (35). As shown in Fig.2A, H. pylori KatA activity is not significantly affected by loss of either NapA or Pfr. Therefore, the observation that H. pylori KatA activity is affected by loss of AhpC is an unusual phenomenon, unrelated to iron storage, which is different from the modulation of catalase activity by BfrA in P. aeruginosa.

As shown in Fig.1, the resolution of KatA protein band and its density relative to the total protein pattern allows it to be unambiguously monitored. Comparison of the protein profiles from crude extracts of the wild type and various mutant strains (Fig.1) indicated that knock-out of AhpC did not significantly change the level of catalase protein expression. Hence a regulatory role for AhpC in catalase expression was ruled out. Therefore, we hypothesized that the catalase in ahpC mutant cells might have undergone certain structural changes leading to its partial inactivation.

Purified H. pylori AhpC was shown to be able to reduce H₂O₂ in vitro at a similar rate as its t-butyl hydroperoxide (tBOOH) reducing activity (22). However, the H₂O₂-decomposing activity in the katA mutant (AhpC+) cells was undetectable (our result Fig. 2A and ref. 23, 24). Because AhpC requires thioredoxin (Trx) and thioredoxin reductase (TrxR) for its activity, failure to detect the H₂O₂-decomposing activity of AhpC could be due to a limited amount of available Trx-TrxR proteins. Therefore, we tested whether the WT cell extract has the activity to reduce organic peroxides. Using tBOOH as a substrate, and without adding reductant, the WT cell extract showed reductase activity, with the specific activity being 15.6 ± 2.3 nmol (tBOOH reduced) per min per mg total protein in the cell extract. If AhpC has a similar rate of H₂O₂-reducing activity, it would be far below the detection level of the assay system for catalase. Thus,
the decrease of catalase activity observed in *ahpC* mutant cells could not be attributed to the loss of the H$_2$O$_2$-decomposing activity of AhpC, but to perturbation of catalase itself.

**A perturbed heme environment associated with catalase in *ahpC* mutant cells.**

Catalase contains a heme active site, which can be detected by electronic paramagnetic resonance (EPR) spectroscopy in ferric oxidation states (36). Purified *H. pylori* catalase exhibits a characteristic near-axial high-spin ferric EPR signal with effective $g$-values = 6.36, 5.35 and 1.98 (37). Considering the high abundance of catalase protein in *H. pylori*, and the high transition probability of the low-field components of axial high-spin ferric resonances, we applied EPR analysis to whole cells, in order to monitor any structural change attributable to the heme group of catalase (Fig. 2B). EPR studies of wild-type cells showed features at $g = 6.4$ and 5.4 characteristic of the low-field components of the catalase high-spin ferric heme resonance. Confirmation of this assignment was provided by the complete absence of this resonance in the *katA* gene knock-out strain. No significant change on the catalase EPR signal was observed in the *sodB, mdaB, napA* or *pfr* mutant cells. However, the EPR signals were altered (compared to the parent strain) in the cells of all three mutants of *ahpC* (strains *ahpC* type I, *ahpC* type II, and the double mutant *ahpCnapA*), with an approximate 50% decrease of the amplitude of the $g = 6.4$, 5.4, 2.0 resonance and a concomitant slight increase in a high-spin ferric heme resonance at $g = 6.8$, 5.1, 2.0. The decrease of the $g = 6.4$, 5.4, 2.0 resonance correlates well with the decrease in catalase activity (Fig.2A,B), despite no change in KatA protein expression level (Fig.1). The intensity of EPR signals was strictly normalized based on the protein concentration of each sample. These results suggested that disruption of AhpC in the cells results in a modification of the catalase heme environment, leading to the partial inactivation of the catalase.
At present, the identity of the heme structural perturbation signal corresponding to the \( g \) value of 6.8, 5.1, 2.0 resonance is not clear, but the complete loss of these signals in the \( katA\text{a}hpC \) double mutant cells indicated that both resonances arise from catalase, not from other proteins that might have been overexpressed in the \( ahpC \) mutant cells. Since similar EPR signals with increased rhombicity have been observed in purified \( H.\ pylori \) catalase samples with formate or azide bound in the distal heme pocket (\( g \sim 6.6, 5.4, 2.0 \)) (37), the heme environment is likely to be modified by binding of a small molecule in close proximity to the heme iron.

**Inactivation of catalase in cell-free extracts by organic hydroperoxide.**

The primary function of AhpC is associated with the detoxification of organic hydroperoxides (8, 9). The active site of the AhpC enzyme can accommodate virtually any ROOH, including HOOH (H\(_2\)O\(_2\)) (22, 38). \( H.\ pylori \) AhpC mutant cells exhibited growth sensitivity to hydroperoxides and to the superoxide-generating agent paraquat (13). To test whether any of these oxidative agents is responsible for inactivation of catalase, we treated the cell-free extract of wild type \( H.\ pylori \) with various agents followed by measuring catalase activity and monitoring EPR signals (Fig. 3). Treatment with up to 0.5 M H\(_2\)O\(_2\) did not significantly affect catalase activity and did not change the catalase heme EPR signal, indicating \( H.\ pylori \) catalase is highly stable in the presence of a high concentration of its own substrate. Similarly, treatment with up to 0.1 M paraquat had no effect (data not shown). Since the whole cells of a \( sodB \) mutant strain (Fig. 2) also showed no effect on catalase (neither on the enzyme activity nor the EPR signal), it seems that \( H.\ pylori \) catalase is not sensitive to superoxide.

To test whether organic hydroperoxides are responsible for inactivation of catalase, we used t-butyl hydroperoxide (tBOOH), a small molecule of organic hydroperoxide that is soluble in aqueous solution, to treat an \( H.\ pylori \) cell extract. By treatment with various concentrations of
tBOOH, we observed progressive inactivation of catalase and a concomitant decrease in the $g = 6.4, 5.4, 2.0$ resonance and a slight increase in the $g = 6.8, 5.1, 2.0$ resonance (Fig. 3). For example, after treatment with 150 mM tBOOH, about 70% of catalase activity was lost and the $g = 6.4, 5.4, 2.0$ resonance was decreased to approximately one third of the intensity in untreated cell-free extract. Treatment of wild-type cell extract with approximately 70 mM tBOOH reduced the catalase activity to one half of the control level, and the EPR signal mimicked that of the whole cells of the $ahpC$ mutant strain (Fig. 2B). As a control, the cell-free extract of the $ahpC$ mutants without treatment (not shown) gave rise to an EPR signal similar to that of whole cells of $ahpC$ mutant strain.

For the purpose of EPR analysis, the concentration of cell extract used in these experiments had to be extremely high. Accordingly, high concentrations of hydroperoxides were used to observe the inhibiting effect. Under the experimental condition, the molar ratio of 70 mM tBOOH to AhpC and to catalase present in the cell extract was estimated (See Experimental Procedures) to be 20,000:1 and 40,000:1, respectively. The actual amount of tBOOH that exerts damage on catalase is unknown, because part of the added tBOOH was consumed by AhpC in the cell extract. Nonetheless, at the same experimental condition, the catalase (in the cell extract) is stable with 500 mM $H_2O_2$, while it is inhibited 50% with 70 mM tBOOH. These results indicated that an organic hydroperoxide is able to induce inactivation and perturbation of the catalase heme environment in cell-free extracts analogous to those induced by the absence of AhpC in the $ahpC$ mutant cells.

**Inactivation of purified *H. pylori* catalase by organic hydroperoxide.**

To closely examine the sensitivity of catalase to hydroperoxides in vitro, *H. pylori* catalase was purified to near homogeneity (Fig. 4). Compared to the *H. pylori* catalase used in the study of
Loewen et al. (37) which was expressed in *E. coli*, we purified native *H. pylori* catalase directly from *H. pylori* cell extract (lane 1) by cation exchange (lane 3) and gel filtration (lane 4).

In addition to a high isoelectric point (pI > 9), *H. pylori* catalase has another property distinct from other typical catalases in that it is stable at very high concentrations of H$_2$O$_2$ (20). In the presence of excess of H$_2$O$_2$, typical catalases can form an intermediate [compound II (Fe(IV))] as a result of one electron oxidation, and this intermediate does not react with H$_2$O$_2$ and thus the accumulation of compound II leads to the deactivation of catalase (39). In contrast, *H. pylori* catalase can withstand very high concentrations of H$_2$O$_2$ (molar ratio = 1:3000) without loss of enzyme activity (Fig. 5). However, purified *H. pylori* catalase showed sensitivity to organic hydroperoxides, confirming the results observed for cell-free extracts. Treatment with increasing concentrations of tBOOH resulted in progressive decrease in the catalase $g$ = 6.4, 5.4, 2.0 EPR signal and concomitant decrease in the catalase activity (Fig. 5). Under these experimental conditions, pre-treatment of purified catalase with tBOOH in a molar ratio of approximately 1:400 caused the loss of 50% of catalase activity, and an approximate 50% decrease in the catalase EPR signal.

Parallel absorption studies (Fig.6) indicate that addition of a 800-fold excess of tBOOH to purified catalase results in the immediate and near complete one-electron oxidation to yield the Fe(IV) compound II species as evidenced by the shift in the Soret-band maximum from 405 to 430 nm (36). The Fe(IV) compound II species is EPR silent and is not catalytically competent for dismutation of hydrogen peroxide into water and oxygen. Hence the inhibition and loss of catalase EPR signal on addition of tBOOH is attributed to the formation of compound II species.

Compared to the results observed for the whole cells (Fig.2) or cell extracts (Fig.3), $g$ = 6.8, 5.1, 2.0 EPR signals are not evident with the purified catalase after tBOOH treatment. The
binding of an organic hydroperoxide in the distal heme pocket is a good candidate for the origin of the \( g = 6.8, 5.1, 2.0 \) EPR signal in the cells or cell extracts. But this binding may require one or more additional small molecules that are not present in the purified enzyme sample. We also purified the catalase from *H. pylori* \( ahpC \) mutant cells, and examined its sensitivity to hydroperoxides. The results (not shown) were the same as those for the catalase from the wild type strain.

**Higher levels of lipid hydroperoxides in \( ahpC \) mutant cells.**

Unsaturated fatty acids have been repeatedly found as a constituent of lipids in *H. pylori*, and the growth of *H. pylori* displayed sensitivity to addition of unsaturated free fatty acids due to their incorporation into phospholipids and subsequent membrane destruction (40, 41). Thus, under the physiological (oxidative stress) condition, there might be a steady flow of lipid hydroperoxides present within *H. pylori* cells; this situation would require an abundant organic peroxide reductase activity to remove the damaging organic hydroperoxides.

The results presented above suggested that loss of AhpC function leads to accumulation of organic peroxides, which is responsible for inactivation of catalase. To further support this notion, we measured the levels of lipid hydroperoxides in wild-type and \( ahpC \) mutant *H. pylori* cells (Table 1). The total amount of lipid hydroperoxides (in the entire cell extract) in \( ahpC \) mutant cells was determined to be about 3 times that of wild type cells. The majority of lipid hydroperoxides within the cells was present in the membrane fraction. When considering only the membrane fraction, the extent of lipid peroxidation in \( ahpC \) mutant cells was about 4 times that of wild type cells. This result indicated that organic hydroperoxides indeed accumulated to a significantly greater extent in the \( ahpC \) mutant cells compared to the wild type cells.
The above result also suggests that under physiological conditions the major function of *H. pylori* AhpC is to reduce organic hydroperoxides. Knock-out of AhpC (an abundant protein) to create *ahpC* mutants enabled us to detect a significant increase of lipid hydroperoxides in the cells. To our knowledge, this is the first direct demonstration of lipid peroxidation in bacterial cells. The detoxification of organic hydroperoxides in *H. pylori* seems quite different from that in *E. coli*. It is reported that *E. coli* lacks the polyunsaturated fatty acids necessary for lipid peroxidation (42), and AhpC is not such an abundant protein in *E. coli* as in *H. pylori*. In accordance with these, the recent study of Seaver and Imlay (38) suggested that the major physiological substrate of *E. coli* AhpC is H$_2$O$_2$ rather than organic hydroperoxides.

We showed that in vitro treatment of purified catalase with tBOOH in a molar ratio of approximately 1:400 caused 50% inactivation of catalase (Fig.5). To complement this result, we found that the *ahpC* mutant cells accumulated much more lipid hydroperoxides than the wild type (Table 1). However, it is difficult to figure out the molar ratio of catalase to lipid hydroperoxides within the *ahpC* mutant cells. Also, the lipid hydroperoxides present within *H. pylori* cells could have different levels of effectiveness in inactivating catalase. Furthermore, the inactivation of catalase by organic hydroperoxides within the cells might take place in a local environment (e.g. periplasm) where the lipid hydroperoxides could be much more concentrated. In this regard, it is of note that about half of the total catalase is located in the periplasm of *H. pylori* cells (43).

In summary, our results showed that lipid hydroperoxides accumulate in *ahpC* mutant cells, and lipid hydroperoxides inhibit catalase activity via the formation of a catalytically incompetent compound II species. This observation indicates a novel physiological role of the AhpC system...
in protecting catalase, thereby demonstrating a previously unrecognized connection in the function of two major proteins in a pathogenic bacterium.

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Figure legends

**Fig. 1** Profiles of total proteins in *H. pylori* cells detected by SDS-PAGE. Five micrograms of crude extract from *H. pylori* wild type strain ATCC43504 and the isogenic mutant of *katA*, *ahpC* or *napA* was loaded into each lane. Lane M is the low-range markers of molecular weight indicated on left. The protein bands corresponding to KatA, AhpC, or NapA are marked with arrows on right.

**Fig. 2** Catalase activities and the EPR signals in various *H. pylori* strains. The cells of the wild type (WT) strain ATCC43504 and the isogenic mutants (as labeled on left; *ahpC1* and *ahpC2* indicate *ahpC* type I and type II strain respectively) were grown and harvested at late log-phase. (A) The catalase activities (units/mg protein) were determined from cell-free extract and
the data were obtained from 3 independent experiments (12 readings in each experiment) with standard deviation indicated. (B) EPR was detected with the whole cells, and a representative example from repeated experiments is shown. The EPR intensity has been normalized based on the optical density of the samples, and the scales for three \( ahpC \) mutant strains (\( ahpC1, ahpC2, ahpCnapA \)) are presented as two times (2x) of those for other strains. EPR spectra were recorded at 4.2 K with 10 mW microwave power, 0.63 mT modulation amplitude, and a microwave frequency of 9.6 GHz.

**Fig. 3** Effect of hydroperoxides on *H. pylori* catalase in cell-free extract. Wild type strain ATCC43504 cell extract was pre-treated with different concentrations of hydroperoxides (\( \text{H}_2\text{O}_2 \) or \( \text{tBOOH} \)) for 30 min at room temperature. Part of each sample was immediately used for determining the catalase activity and the remaining sample was frozen in EPR tubes for EPR analysis. The catalase activities were determined from 3 independent experiments (12 readings in each experiment), averaged with standard deviation, and presented as a percentage compared to the control sample without hydroperoxide treatment. For the EPR results, a representative spectrum from repeated experiments is shown. Note that the EPR scales for three \( \text{tBOOH} \) treatment samples are presented as two times (2x) of that for the control. The conditions used for EPR measurements are the same as those used in Fig. 2.

**Fig. 4** Purification of *H. pylori* catalase. A 12% polyacrylamide gel containing 1% SDS stained with Coomassie Brilliant Blue included the following samples: cell extract from *H. pylori* strain ATCC43504 (lane 1), flow-through sample from SP Sepharose cation-exchange column (lane 2), fraction 30 eluted from SP Sepharose cation-exchange column (lane 3) and fraction 47
eluted from S-200 gel filtration column (lane 4). The protein bands corresponding to KatA as well as AhpC are marked with arrows on left. Lane M is the low-range markers of molecular weight indicated on right.

Fig. 5  Effect of hydroperoxides on the purified *H. pylori* catalase. Purified *H. pylori* catalase was pre-treated with hydroperoxides (H$_2$O$_2$ or tBOOH) at different molar ratios (catalase:hydroperoxide) for 30 min at room temperature prior to measurement of catalase activity and freezing for EPR analysis. The conditions used for catalase assays and for EPR measurements are the same as those used in Fig. 3.

Fig. 6  UV-visible absorption spectra of purified *H. pylori* catalase before (solid line) and after (broken line) addition of 800-fold excess of tBOOH.
Fig. 1

The figure shows a gel with various lanes labeled M, WT, katA-, ahpC- type I, ahpC- type II, and ahpC- napA-. The molecular weights are marked at the left side: 97.4, 66.2, 45, 31, 21.5, and 14.4 kDa. The bands for KatA, AhpC, and NapA are indicated by arrows.
Fig. 2

(A) Catalase activity
(B) EPR signal

- WT
- katA
- sodB
- mdaB
- ahpC1
- ahpC2
- ahpCnapA
- napA
- pfr
- katAahpC
### Treatment, Catalase Activity (%), and EPR Signal

| Treatment       | Catalase Activity (%) | EPR Signal |
|-----------------|-----------------------|------------|
| Control         | 100                   |            |
| + 500 mM H$_2$O$_2$ | 98 ± 7                |            |
| + 50 mM tBOOH | 64 ± 6                |            |
| + 100 mM tBOOH | 35 ± 4                |            |
| + 150mM tBOOH | 28 ± 4                |            |

![Graph showing EPR signal with g-values](image-url)
Fig. 4

1  2      3   4   M

97.4
66.2
45
31
21.5
14.4

KatA

AhpC
### Fig. 5

| Treatment          | Catalase activity (%) | EPR signal |
|--------------------|-----------------------|------------|
| Control            | 100                   |            |
| + 1:3000 H$_2$O$_2$| 97 ± 8                | ![EPR signal](image1) |
| + 1:300 tBOOH      | 62 ± 5                | ![EPR signal](image2) |
| + 1:600 tBOOH      | 29 ± 4                | ![EPR signal](image3) |
| + 1:900 tBOOH      | 26 ± 3                | ![EPR signal](image4) |

*Note: The EPR signals show g-values of 5.4 and 6.4.*
Fig. 6
Table 1. Total lipid hydroperoxides (nmol / $10^{10}$ cells) in *H. pylori* cells

| Strain      | Cell extract | Membrane fraction |
|-------------|--------------|-------------------|
| 43504 WT    | 4.10 ± 0.52  | 2.52 ± 0.37       |
| 43504 *ahpC* | 11.61 ± 1.06 | 9.75 ± 1.18       |

*H. pylori* cells (WT or *ahpC* mutant) were grown to late log phase and harvested, from which the cell extract or membrane fraction were prepared. The total amount of lipid hydroperoxides were determined and calculated as the amount (nmol) present in the samples from $10^{10}$ cells. The data are averaged from 3 independent experiments with standard deviation.
Role of a bacterial organic hydroperoxide detoxification system in preventing catalase inactivation
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