The Met-Tyr-Trp Crosslink in Mycobacterium tuberculosis Catalase-Peroxidase (KatG): Autocatalytic Formation and Effect on Enzyme Catalysis and Spectroscopic Properties
Reza A. Ghiladi, Giselle M. Knudsen, Katalin F. Medzihradszky, and Paul R. Ortiz de Montellano

From the Department of Pharmaceutical Chemistry, University of California, San Francisco, California, 94143-2280

Running Title: The Met-Tyr-Trp Crosslink in Mtb Catalase-Peroxidase (KatG)

Address Correspondence to: Paul R. Ortiz de Montellano, Department of Pharmaceutical Chemistry, University of California, San Francisco, 600 16th St. San Francisco, CA 94143-2280, Tel. 415-476-2903; Fax. 415-502-4728; E-Mail: ortiz@cgl.ucsf.edu

Catalase-peroxidases (KatGs) are bifunctional enzymes possessing both catalase and peroxidase activities. Three crystal structures of different KatGs revealed the presence of a novel Met-Tyr-Trp crosslink which has been suggested to impart catalatic activity to the KatGs. HPLC separation of the peptide fragments resulting from tryptic digestion of recombinant Mycobacterium tuberculosis WT KatG identified a peptide with unusual UV-visible spectroscopic features attributable to the Met255-Tyr229-Trp107 crosslink, whose structure was confirmed by mass spectrometry. WT KatG lacking the Met-Tyr-Trp crosslink was prepared, making possible studies of its formation under oxidizing conditions that generate either compound I (peroxyacetic acid, PAA) or compound II (2-methyl-1-phenyl-2-propyl hydroperoxide, MPPH). Incubation of this ‘crosslink-free’ WT KatG with PAA revealed complete formation of the Met-Tyr-Trp structure after six equivalents of peracid were added, whereas MPPH was unable to promote crosslink formation. A mechanism for Met-Tyr-Trp autocatalytic formation by KatG compound I is proposed from these studies. Optical stopped-flow studies of WT KatG and KatG(Y229F), a mutant in which the crosslink cannot be formed, were performed with MPPH and revealed an unusual compound II spectrum for WT KatG, best described as (P•)FeIII, where P• represents a protein-based radical. This contrasts with the oxoferryl compound II spectrum observed for KatG(Y229F) under identical conditions. The structure-function-spectroscopy relationship in KatG is discussed with relevance to the role that the Met-Tyr-Trp crosslink plays in the catalase-peroxidase mechanism.

Catalase-peroxidases (KatG) are bifunctional hemoproteins belonging to Class I of the peroxidase superfamily (plants, fungi, and bacteria) and exhibit both catalatic (H2O2 $\rightarrow$ H2O + 1/2O2) and peroxidatic (2AH + H2O2 $\rightarrow$ 2A• + 2H2O) activities (1). KatGs are anomalies: they have a high sequence homology with prokaryotic peroxidases, including fungal cytochrome c peroxidase and plant ascorbate peroxidase (2), and as such possess substantial peroxidase activity, yet also exhibit catalatic activity equivalent to that of the monofunctional catalases despite having low sequence homology with the latter (3). Additional enzymatic functions for KatGs have been described, including Mn2+-dependent peroxidase (4,5), cytochrome P450-like oxygenase (6), and peroxynitritase activities (7).

There has been considerable interest in KatG due to its role in activating the pro-drug isoniazid (INH) (8-10), the frontline antibiotic used to treat tuberculosis. Although the details are still being investigated, the consensus opinion is that KatG oxidizes isoniazid to an isonicotinoyl radical, which then couples to NADH (11-15). The resulting INH-NADH adduct is a potent inhibitor of InhA, an enoyl acyl-carrier-protein involved in Mycobacterium tuberculosis (Mtb) cell wall biosynthesis (11,16). The origins of isoniazid drug-resistance in Mtb can be traced to point mutations in KatG that inhibit this ‘INH-oxidase’ activity (17-20), thereby raising the need to better understand the catalase-peroxidase mechanism and function.

Interest in the catalase-peroxidases has also been spurred by the publication of three crystal structures of KatG from different sources: Haloarcula marismortui (21), Burkholderia pseudomomas (22), and Mycobacterium...
tuberculosis (23). A common feature in each crystal structure is the presence of two covalent bonds between three amino acid side chains, Trp107, Tyr229, and Met255 (Mtb numbering) located on the distal side of the heme active site (Figure 1). The consistent observation of a Met-Tyr-Trp ‘crosslink’ suggests that it is a characteristic common to all KatGs, and, as it is not found in the monofunctional peroxidases, implies that this structural element may impart catalytic activity to the KatGs (23). Indeed, several mutagenesis studies have confirmed that the crosslink is required for catalytic, but not for peroxidatic, activity (24-27).

The existence of the Met-Tyr-Trp crosslink adds the catalase-peroxidases to a growing list of metalloenzymes which have aromatic amino acids covalently modified in their active sites: cytochrome c oxidase (His240-Tyr244; heme$_{c_3}$-Cu$_{b}$) (28-30), catalase HPII (His392-Tyr415; heme) (31), galactose oxidase (Tyr272-Cys228; copper) (32,33), catalase-I (Cys356-Tyr379; heme) (34), and amine oxidase (2,4,5-trihydroxyphenylalanine quinone; copper) (35) are but a few examples. Formation of the covalent bonds in these systems is believed to occur through metal-mediated autocatalytic processes, with the resulting modified amino acids possessing altered redox and $pK_a$ properties that effect enzyme chemistry (36-39).

Quantitative studies addressing the formation of the Met-Tyr-Trp crosslink in KatG, however, are still lacking. Is bond formation an autocatalytic process, and if so, are high-valent iron-oxo species (i.e., compounds I and/or II, the catalytically competent intermediates responsible for substrate oxidation) involved? What role does the crosslink play in the formation of these intermediates, and what is the effect on their spectroscopic signatures? Can the presence of this structural element correlate to both enzyme function and spectroscopy? To address these and other questions, we describe here our biochemical and spectroscopic investigations of the role which the Met-Tyr-Trp crosslink plays in Mycobacterium tuberculosis KatG. Specifically, we have been able to express wild-type enzyme lacking the Met-Tyr-Trp crosslink, and followed its formation under conditions known to generate either compound I (oxoferryl porphyrin $\pi$-cation radical) or compound II (oxoferryl) intermediates. Additionally, we have employed stopped-flow UV-visible spectroscopy to compare the spectroscopic signature of the compound II intermediate generated by reaction of 2-methyl-1-phenyl-2-propyl hydroperoxide (MPPH) with WT KatG to that of KatG(Y229F), whose tyrosine-to-phenylalanine mutation prevents crosslink formation, and have shown that a significant departure from the classical ‘oxoferryl’ spectrum is observed in WT KatG. Definitive assignment of the compound II spectrum is necessary for complete identification of all species observed during enzyme turnover in optical stopped-flow studies, and may facilitate a better understanding of how catalase-peroxidases oxidize substrates in general, and specifically in studies involving the KatG-catalyzed oxidation of isoniazid. The relevance of these results is discussed with respect to the catalase-peroxidase mechanism.

**EXPERIMENTAL PROCEDURES**

**Materials** – Buffer salts and acetonitrile (HPLC grade) were purchased from Fisher Scientific. All other reagents and biochemicals, unless otherwise specified, were of the highest grade available from Sigma-Aldrich. 2-methyl-1-phenyl-2-propyl hydroperoxide (MPPH) was synthesized according to the published procedure (40,41).

**Plasmid Preparation, Protein Expression and Purification** – Recombinant WT KatG was overexpressed in *E. coli* as previously described, using the plasmid pMRLB11 (Colorado State University, NIAID Contract N01 AI-75320). Mutagenesis [melt (94 °C, 90 s), anneal (57 °C, 90 s), extension (68 °C, 7 min); 35 cycles] was performed directly on pMRLB11 using the GeneAmp XL PCR kit (Applied Biosystems) using the following mutagenic primers: 5’- CGGTGCAGATGGGGCTGATC TTC GTGAAC CCGGAGGCGCC-3’, 3’-GATCAGCCCCATCT-GCACCGCGCCAGCGG-5’. PCR samples were treated with DPN1 (New England Biolabs) to digest parental DNA, purified by gel extraction (0.7% agarose gel electrophoresis) using the QIAquick PCR Purification Kit (Qiagen), and circularized using the Quick Ligation Kit (New England Biolabs), resulting in pKatG(Y229F). Sequencing of double-stranded plasmid DNA by the Sanger method was used to confirm the desired
nucleotide substitution and the absence of secondary mutations (Biomolecular Resource Center, University of California – San Francisco). KatG(Y229F) was overexpressed and purified under identical procedures as those employed for WT KatG.

Spectroscopic Studies – Optical spectra were recorded on a Hewlett-Packard 8452A Diode Array Spectrophotometer equipped with a thermostated cell holder at 25 °C. Protoheme content was measured by the pyridine hemochrome assay using $\Delta\varepsilon_{557} = 20.7 \text{mM}^{-1}\text{cm}^{-1}$ (reduced - oxidized) for iron protoporphyrin IX (42,43).

Enzyme Assays – All measurements were performed in octiplet using a SpectraMax Plus384 UV-visible plate reader equipped with 96-well plates. Assays were carried out at 37 °C in 100 mM KP, buffer (pH 7.5) containing 5 µM EDTA (200 µL total volume). Catalase activity was measured spectrophotometrically by following the decrease over 60 s (linear least-squares fittings) of the hydrogen peroxide concentration (1.5, 5, 10, 15, 30, and 60 mM) at 240 nm ($\varepsilon_{240} = 43.6 \text{M}^{-1}\text{cm}^{-1}$) (44). Enzyme concentrations were as follows: WT, 10 nM; KatG(Y229F), 20 µM. Peroxidase activity was measured by following the increase (linear least-squares fittings) in absorbance for 1.0 mM ABTS ($\varepsilon_{405} = 36.8 \text{mM}^{-1}\text{cm}^{-1}$) (45) in the presence of tert-butyl hydroperoxide (0.25, 2.5 and 25 mM) over 60 seconds. Enzyme concentrations were as follows: WT, 500 nM; KatG(Y229F), 100 nM. Kinetic parameters ($K_m$, $V_{max}$) were obtained from non-linear regression (least squares fitting) of Michaelis-Menton plots using the GraphPad Prism kinetics software package (http://www.graphpad.com/).

Tryptic Digests and HPLC Analysis – 250 µL of a 50 µM solution of KatG (~1 mg protein) in 100 mM KPi (pH 7.5) was incubated with 5 µg (200:1 protein:protease ratio) of sequencing grade modified trypsin (Promega) for 3 h at 37 °C. Following proteolytic digestion, the peptide fragments were separated using HPLC [ProSphere (Alltech) C$_{18}$, 5µ, 100 Å; buffer A: H$_2$O + 0.1% trifluoroacetic acid (TFA); buffer B: MeCN + 0.1% TFA; linear gradient from 0-60% B over 4 h at 0.5 mL/min; UV-visible spectroscopic monitoring (diode array): 200-600 nm]. Fractions (0.5 mL) were collected, concentrated (SpeedVac), and submitted for MALDI-TOF MS analysis.

Mass Spectrometry – KatG was first purified by SDS-PAGE, followed by in-gel digestion with side-chain protected porcine trypsin (Promega) as previously described (http://donatello.ucsf.edu/ingel.html). Fractionation of the digest was performed on a nanoHPLC system, consisting of an Eksigent nanopump and a Spark autosampler (column: C-18, 75µm x 150 mm; solvent A: 0.1% formic acid in H$_2$O; solvent B: 0.1% formic acid in acetonitrile; flow rate: ~300 nL/min; linear gradient: 5-50% B over 30 min), and a QSTAR XL (Applied Biosystems) quadrupole-orthogonal-acceleration-time-of-flight hybrid tandem mass spectrometer served as detector in an information-dependent manner: 1 sec MS acquisitions were followed by computer-controlled 3 sec CID experiments.

Continuous 5 sec CID experiments on the 7+ charged ion of the cross-linked species were performed also under LC/MS conditions. The collision energy was manually adjusted. MS/MS/MS experiments were performed on an LTQ linear ion trap mass spectrometer (Thermo Finnigan) equipped with a nanoelectrospray source. The unfractionated digest was infused at a flow rate of ~300 nL/min, the solution contained ~35 % acetonitrile.

Mass spectrometric analysis of the collected HPLC fractions was performed on a Voyager DE STR MALDI-TOF mass spectrometer (Applied Biosystems) in reflectron mode. The matrices used were alpha-cyano-4-hydroxycinnamic acid and 2,5-dihydroxy-benzoic acid.

Overexpression of ApoKatG and Heme Reconstitution – WT KatG deficient in heme was overexpressed in a similar fashion to holoenzyme, but with important modifications. Deionized water (Millipore) was used in the preparation of the media and to rinse (5x) all glassware. Starter cultures, prepared as previously described, were used to inoculate 2.8 L Fernbach flasks, each containing iron-free medium prepared in the following manner: 10 g casamino acids (Difco, low iron content), 5 g yeast extract (QBioGene), 10 g sodium chloride and 1.5 L H$_2$O were treated with 50 g Chelex for 2 h to remove trace metals. Chelex was removed via filtration, the media was autoclaved, allowed to cool to RT, and...
supplemented with the following filter sterilized solutions: MgSO₄ and CaCl₂ (3 mL and 150 µL, respectively, of 1 M stock solutions), vitamin mix (15 mL of a 100x stock: 10 mg each of riboflavin, niacinamide, pyridoxine monohydrate, and thiamine in 100 mL H₂O), iron-free trace metals mix (15 mL of a 100x stock: 0.5 g EDTA, 5 mg each of ZnCl₂, CuCl₂, CoCl₂, H₃BO₃, 160 mg MnCl₂, 50 mg NiSO₄, 100 mL H₂O). Antibiotics and growth conditions were followed as previously described, with the exception that the incubation time prior to protein induction with IPTG was increased to 9 h. The iron-chelator 2,2′-dipyridyl was added to a final concentration of 300 µM (from a 300 mM stock solution in ethanol) two hours prior to IPTG addition. Cells were harvested, lysed, and apo-KatG purified from the lysate according to the literature reference.

Apo-KatG was denatured via dilution (1:10) into 50 mM CAPS buffer (pH 11) in the presence of 1% N-lauroylsarcosine to a final concentration of 50 µM. Refolding was accomplished via overnight dialysis of the protein solution (10 mL) at 4 °C against 1 L of 50 mM NaCl, 0.05% N-lauroylsarcosine, 5 µM EDTA and 125 µM hemin. The reconstituted KatG (KatG) was applied to a 5 mL HisTrap HP column (Ni-sepharose, Amersham Biosciences), washed with 5 column volumes of 50 mM phosphate buffer (pH 7.5) containing 300 mM NaCl, 0.05% N-lauroylsarcosine, 5 µM EDTA and 125 µM hemin. The reconstituted KatG (KatG) was applied to a 5 mL HisTrap HP column (Ni-sepharose, Amersham Biosciences), washed with 5 column volumes of 50 mM phosphate buffer (pH 7.5) containing 300 mM NaCl and 20 mM imidazole, and eluted batchwise upon application of this buffer at 75 mM imidazole. Buffer exchange and removal of the imidazole from the heme-reconstituted KatG (KatG) was accomplished via two successive applications of a PD-10 column (Sephadex G-25 M, Amersham Biosciences) with 100 mM KPi (pH 7.5) containing 5 µM EDTA, and the protein was concentrated using an Amicon Diaflow concentrator equipped with a 50 kDa membrane (BioMax-50).

**RESULTS**

UV-visible Spectroscopy of WT KatG and KatG(Y229F). – The electronic absorption spectra of WT KatG and KatG(Y229F) are shown in Figure 2, and relevant spectral features and analysis are presented in Table 1. The optical purity ratio (Reinheitzahl or Rₚ, defined as A₅₈₀/A₄₁₀) for WT KatG was found to be 0.62, while that for KatG(Y229F) was 0.59, both being consistent with literature values (46,47). Pyridine hemochrome assays yielded 0.97 – 1.04 heme/monomer, indicating complete holoenzyme formation for both KatGs.

Although not the focus of the present study, as a detailed analysis by EPR and/or resonance Raman spectroscopies is required for sufficient quantitation, analysis of the electronic absorption spectrum can provide some basic insight into the coordination environment and spin-state of the heme present in KatG. As described elegantly by Magliozzo and co-workers (48), analysis of the two ratios A₅₈₀/A₄₁₀ and A₆₄₅/A₆₄₅ reveals the relative populations of 6-c vs. 5-c HS heme: overall, 5-c HS heme species exhibit a slightly blue shifted and lower extinction coefficient Soret band than their 6-c HS counterparts, as well as a shoulder at 380 nm. Additionally, the CT1 feature from 30-36% of buffer B over 25 min was employed to elute the peptide fragment containing the Met-Tyr-Trp crosslink (CLPF). Peptide formation was determined via peak integration (area under the curve from 41-45 min), with 10 and 25 equivalents normalized to 100%. The experiment was repeated in triplicate, and the data reported as avg. ± S.D.

Stopped Flow UV-Visible Spectrophotometry – Experiments were performed on a Hi-Tech SF-61 DX2 Double Mixing Stopped-Flow System employing a diode array spectrophotometer, and were carried out at 25 °C in 100 mM KPi (pH 7.5) containing 5 µM EDTA. The initial (pre-mixing) concentrations were: [KatG] = 20 µM, [MPPH] = 20, 50, or 200 µM, [PAA] = 20, 50, or 200 µM. Data was collected (300 scans) over 0.6, 6, 60, and 600 seconds using the KinetAsyst software package (Hi-Tech), and analyzed using the Specfit Global Analysis System software package (Spectrum Software Associates) as pseudo-first order reactions.
in a 5-c HS heme is found at ~640 nm (or higher), while that of a 6-c HS heme is generally closer to 630 nm. Furthermore, LS heme systems exhibit a red-shifted Soret, the absence of a CT1 feature, and visible features at 565 and 580 nm. Thus, as can be seen from the data in Table 1, WT KatG has a greater amount of 5-c HS heme present than KatG(Y229F), and both lack a detectable (by optical spectroscopy) low-spin heme component. These results are consistent with previous observations for these two enzymes (47,48).

Catalase and Peroxidase Activities of WT KatG and KatG(Y229F) – Kinetic parameters ($k_{cat}$, $K_m$, and catalytic efficiency, $k_{cat}/K_m$) for the catalase and peroxidase activities of WT KatG and KatG(Y229F) are presented in Table 2. As catalases do not follow typical Michaelis-Menten kinetics (lack of a detectable enzyme-substrate complex and inability to reach saturation with $H_2O_2$ before inactivation), kinetic constants reported here for catalase activity are ‘apparent’ values (49,50). Both enzymes exhibited saturable catalase activity under the conditions employed for the kinetic study. The $k_{cat}$ [WT KatG, 6000 s$^{-1}$; KatG(Y229F), 0.1 s$^{-1}$] and $K_m$ values [WT KatG, 2.5 mM; KatG(Y229F), 40 mM] are within the ranges reported for these two enzymes (47,51), with minor differences being attributed to either different assay conditions (pH, temperature, buffer) or differences in the recombinant enzymes used in the various studies. Overall, however, the result that catalase activity is severely disrupted for KatG(Y229F) versus that in WT KatG (6000 s$^{-1}$, ~10$^6$ lower) is consistent with literature observations (25,27,47) that the Met-Tyr-Trp crosslink is essential for catalatic activity.

Peroxidase activities (saturable) were measured for the one-electron oxidation of 2,2'$\text{-}$azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) to the corresponding radical cation, $\text{ABTS}^*$, by KatG in the presence of tert-butylhydroperoxide. KatG(Y229F) exhibited an increase in $k_{cat}$ (0.843 s$^{-1}$) and decrease in $K_m$ (2.7 mM) as compared to WT KatG (0.062 s$^{-1}$ and 8.4 mM, respectively). The increase in peroxidase activity for $Mtb$ KatG(Y229F) has been noted previously by Magliozzo and co-workers (47), who suggested that upon loss of the covalent adduct, the increase in peroxidase activity (with concomitant loss of catalase function) is due to enhanced formation and/or increased stabilization of the compound II intermediate, which plays a role in the peroxidase, but not catalase, cycle.

Tryptic Digestion of WT KatG and KatG(Y229F) – The HPLC chromatograms (A$_{220}$ monitoring) of the tryptic digests of WT KatG and KatG(Y229F) are shown in Figure 3. At least 42 peptide fragments are well resolved, representing a significant percentage of the 72 theoretical fragments calculated for KatG proteolytic cleavage by trypsin, and an even higher percentage of the 54 fragments of size $n>4$ ($n$ = # of amino acid residues). Both digests exhibit peptide elution patterns which are very similar, with notable exceptions: First, the presence of a large peptide cluster in the region of 185-190 minutes in WT KatG is absent in KatG(Y229F) (Figure 3, boxed area). This difference was highly suggestive of the presence of a Met-Tyr-Trp crosslinked peptide fragment in WT KatG (Figure 4), predicted (from trypsin cleavage sites) to incorporate (henceforth referred to as the crosslinked peptide fragments, CLPF), which would be unable to form in KatG(Y229F) due to the Tyr→Phe mutation. Second, several additional peaks, which were not further characterized, were also observed in KatG(Y229F) but were absent in WT KatG, and may represent the above uncrosslinked peptides.

The HPLC chromatograms of the tryptic digest of WT KatG, monitored at $\lambda$ = 220, 280, and 330 nm, are shown in Figure 5A. Peptides typically exhibit absorbance maxima below 220 [arising from the peptide backbone and/or $\pi\rightarrow\pi^*$ (E$_2$) aromatic ring absorptions] and at 280 nm [attributed primarily to tryptophan & tyrosine ($\pi\rightarrow\pi^*$ ‘B’)] and cysteine ($n\rightarrow\sigma^*$) ($\varepsilon_{280} = 5690, 1280$, and 120 M$^{-1}$cm$^{-1}$, respectively), with little to no absorbance of light above ca. 300 nm (minor Trp shoulder) (52,53). The CLPFs eluting at $\sim$185 min, however, exhibit an unusual absorption even at 330 nm (Figure 5A, top). The UV-visible spectrum of this peptide is shown in Figure 5B, and is contrasted against the absorption spectrum of ‘typical’ peptides observed in this tryptic digest (specifically shown is the peptide eluting at t=$195$ min). Here, a clear red shift of the CLPF protein absorption to $\lambda_{max} = 300$ nm is observed, with an
additional absorption at $\lambda = 254$ nm. If the crosslinks were absent in the three peptides, the calculated absorption spectrum (based on the primary amino acid sequence comprising the CLPF) (52) would have a single feature at $\lambda_{\text{max}} = 280$ (8250 M$^{-1}$cm$^{-1}$, arising from 1 tryptophan and two tyrosines), and no prominent shoulder at 254 nm.

In the context of the Met-Tyr-Trp crosslink, the origin of these bathochromic shifts can be explained by the Woodward- Fieser rules (54-58) for the $E_2$ and $B$ bands of Tyr229 and Trp107. Specifically, the covalent bond between the phenol ring of Tyr229 and the indole ring of Trp107 creates an extended pi-system [supported by the near coplanarity of Tyr229 and Trp 207 in the crystal structure of KatG (23)], resulting in a red shift of the $\pi \rightarrow \pi^*$ transition. The theoretical shifts of the $E_2$ band, from a calculated 210 nm [contributions from both indole (209 nm, $\varepsilon =6730$ M$^{-1}$cm$^{-1}$) and phenol (211 nm, $\varepsilon =6200$ M$^{-1}$cm$^{-1}$) rings] to 254 nm ($\varepsilon_{\text{sum}}=12930$ M$^{-1}$cm$^{-1}$), and of the $B$ band, from a calculated 280 nm [indole (280 nm, $\varepsilon =5690$ M$^{-1}$cm$^{-1}$) and phenol (274 nm, $\varepsilon =1400$ M$^{-1}$cm$^{-1}$)] to 300 nm ($\varepsilon_{\text{sum}}=7090$ M$^{-1}$cm$^{-1}$) (58), match well with the observed shifts (Figure 5B). The theoretical $\varepsilon_{300}/\varepsilon_{254}$ (1.82) is consistent with the observed $A_{300}/A_{254}$ (1.72), further supporting these assignments and the existence of the Tyr229-Trp107 covalent bond in the CLPF.

The C-S$^\oplus$ covalent bond between Met255 and Tyr229 is predicted from theory (58) to have little to no effect on either the $E_2$ or $B$ bands. Normally, formation of a C-S (neutral) bond on the phenol ring of Tyr229 would be described as an auxochromatic substitution, where the lone pairs of the S heteroatom are shared with the $\pi$-electron system of the ring, resulting in a red shift of the $\pi \rightarrow \pi^*$ transition due to the $n-\pi$ conjugation. In this particular case, however, a C-S$^\oplus$ bond is formed, and the sulfonium ion is predicted to have little $n-\pi$ contribution due to the presence of the positive charge on the sulfur, thereby essentially negating any substituent-induced bathochromic shift. This has been observed in other systems (58), notably for C-N bond formation (aniline is bathochromic with respect to benzene, but anilinium cations are not). Although UV-visible spectroscopic evidence for the presence of the covalent bond between Met255 and Tyr229 in the CLPF was not observed, convincing evidence is provided by mass spectrometry (see below).

**Mass Spectrometry** – Initially, the CLPF was characterized by tryptic digestion of recombinant *Mtb* WT KatG without isolation, allowing for protein sequence confirmation and purity assessment. The KatG protein was purified by SDS-PAGE, in-gel digested with trypsin, and an aliquot of this digest was subjected to LC/MS/MS analysis in an information-dependent manner. Most of its sequence corresponding to NCBI entry gi|12744172, i.e. AAK06518 was verified from collision-induced dissociation (CID) data. Peptides

$^{105}$MAWHAGTYR$^{114}$, $^{215}$DLENPLAAYQMGL1YVNPEGPNPDPM

$^{249}$AAAVIDIR and $^{274}$MAMNDVETAAIVGGHTFGK

were proposed to form the CLPF (cross-linked amino acids are in highlighted, Figure 4). The calculated neutral (zwitter ionic) mass of this structure is 6880.31 Da. Indeed, we identified a digest component of such molecular mass, represented by ions at m/z 1147.67(6+), 983.87(7+), 861.01(8+) and 765.45(9+). The monoisotopic neutral mass determined from these values (6879.99) is in good agreement with the calculated value. In order to prove the identity of this molecule, the most abundant ion representing it (m/z 984; see inset Figure 6) was selected as the precursor ion for a CID experiment (Figure 6a). The results (Figures 6b & 6c, Table 3) provided all the necessary proof needed, though fragment assignment represented a challenge until the favored fragmentation step was determined: the base peak of the CID spectrum, m/z 671.99(3+) is the result of a bond cleavage between the $\gamma$-carbon and the sulfur atom of Met255, with the charge retained on the “remains” of the [255-274] peptide. The m/z 1217.77(4+) peak represents the other half of the structure formed by this cleavage (cross-linked [105-114] and [215-249] peptides, with a CH$_3$S-group still attached to Tyr229) (Figure 6c).$^2$ The identity of these fragments was confirmed by MS/MS/MS experiments performed in an ion trap (data not shown). The presence of the two longer peptides is indicated by extensive y and b-ion series. The mass of b-ions formed from the [255-274] peptide are shifted reflecting the Met side-chain fragmentation mentioned above. The shortest peptide produced only a few fragments. However, its presence can be
confirmed from these \( y \) ions as well as from \( y \)- and \( b \)-type fragments of the longest peptide, [215-249], that contains the cross-linked Tyr, the CH_{3}-S- group and the [105-114] peptide attached to it.

The HPLC-isolated fraction proposed to contain the CLPF (Figure 3, boxed area) was analyzed by MALDI-TOF-MS. The results (data not shown) were unequivocally consistent with the LC/MS/MS analysis, indicating that the isolated fraction did indeed contain the CLPF, as suggested by the UV-visible spectroscopic data.

**Autocatalytic Formation of the Met-Tyr-Trp Crosslink in \(^8\) WT KatG** – Recombinant WT KatG exhibited the presence (minimum 95% fully-formed, data not shown) of the Met-Tyr-Trp crosslink as confirmed by tryptic digestion and HPLC (UV-visible spectroscopic detection of the CLPF) as well as by mass spectrometry (*vide supra*). This was possibly due to its autocatalytic formation during overexpression in *E. coli* owing to the presence of hydrogen peroxide or alkylhydroperoxides in the *in vivo* system, although the intervention of a cellular assembly system could not be excluded. Thus, in order to study the formation of the Met-Tyr-Trp crosslink under controlled conditions *in vitro*, it was necessary to first isolate WT KatG which had not undergone *in vivo* catalysis. The simplest strategy for accomplishing this was to overexpress heme-deficient KatG (and therefore incapable of undergoing any autocatalytic process that might be required to form the Met-Tyr-Trp crosslink), isolate the protein, and reconstitute the apoprotein with heme in the absence of any peroxide.

Modification of the overexpression medium to limit iron content allowed for the successful production of apoprotein owing to the lack of iron for heme biosynthesis. This was achieved in two ways: i) a growth medium similar to that of LB broth, yet lacking free iron (due to Chelex treatment of the components) was employed, and ii) 2,2’-dipyridyl, a membrane-permeable iron-chelator (59), was added to the culture flasks prior to protein induction. WT KatG grown under these low-iron conditions exhibited an \( R_z \) value of 0.05 (Figure 7), indicating only 8% holoenzyme (when compared to an \( R_z \) of 0.62 for normal growth conditions). Chelex treatment or 2,2’-dipyridyl alone reduced the heme content of KatG (\( R_z \) values of 0.28 and 0.20, respectively), but not to the level achieved by their combination. Tryptic digestion and HPLC analysis of this heme-deficient KatG revealed only 7.3 ± 2.6% of the CLPF formed.

Reconstitution of apo-KatG with heme was accomplished using denaturing conditions to expose the heme-binding pocket, followed by refolding of the protein in the presence of hemin, (protoporphyrin-IX)Fe^{III}-Cl. The resulting reconstituted WT KatG (henceforth referred to as \( ^8 \) WT KatG), exhibited an \( R_z \) value of 0.57, indicating 92% holoenzyme (when compared to a maximum \( R_z \) of 0.62 for normal WT KatG) (Figure 7). Catalase and peroxidase kinetic parameters for \( ^8 \) WT KatG are given in Table 2. \( K_m \) values for substrate binding were essentially unchanged. The \( k_{cat} \) values, however, for \( ^8 \) WT KatG are slightly slower than for WT KatG, but consistent with the slightly lower \( R_z \) value exhibited by \( ^8 \) WT KatG. This is borne out in the similarity of \( k_{cat} \) and \( R_z \) ratios for \( ^8 \) WT KatG/WT KatG: catalase, 0.94; peroxidase, 0.96; \( R_z \), 0.92.

Incubation of \( ^8 \) WT KatG with up to 6 equivalents PAA (Figure 8A), a two-electron oxidant which yields compound I formation in WT KatG (60), led to an increase in the amount of CLPF observed after tryptic digestion and HPLC analysis. Above 6 equivalents, however, CLPF amounts remained essentially constant (up to 25 equivalents PAA added/heme). By contrast, in the absence of PAA, only 7.0 ± 2.9% (normalized to the amount of CLPF observed at 25 equivalents of PAA) of the CLPF was detected, an amount nearly identical to that found for apoKatG, indicating that heme reconstitution alone does not contribute to CLPF formation. Furthermore, incubation of WT KatG with 0 and 25 equivalents PAA yielded 96.2 ± 2.9 and 97.3 ± 2.2%, respectively, of the CLPF detected in \( ^8 \) WT KatG when undergoing the same treatment, suggesting that the maximum amount CLPF formed in \( ^8 \) WT KatG is equivalent to that present in WT KatG.

In order to determine if compound II is involved in Met-Tyr-Trp crosslink formation, \( ^8 \) WT KatG was incubated with an excess (25 equivalents) of 2-methyl-1-phenyl-2-propyl-hydroperoxide (MPPH), a reagent which undergoes homolytic O-O bond cleavage (61) preferentially yielding compound II rather than compound I (see stopped-flow UV-visible discussion below). Only a minor increase (11.3 ± 4.2%) in CLPF was observed, suggesting that
compound II is incapable of directly contributing to the formation of the crosslink. Thus, it appears that only compound I is capable of Met-Tyr-Trp formation. The sinusoidal lineshape of the data in Figure 8B suggests that four or more oxidizing equivalents (i.e. two equivalents or more of PAA) are necessary before an increase in CLPF is detected, consistent with the formation of two covalent bonds. The apparent inefficiency in the formation of the Met-Tyr-Trp crosslink, as demonstrated by the extra oxidizing equivalents needed above the obligatory four necessary for two covalent bonds, suggests that additional amino acids may be oxidized other than those in the Met-Tyr-Trp crosslink; protein radical formation in residues other than Met255, Tyr229, and Trp255 has been previously demonstrated (25,47,62-64).

Characterization of Compound II in WT KatG and KatG(Y229F) – Stopped-flow UV-visible spectroscopic methods were employed to detect the compound II intermediate of both WT KatG and KatG(Y229F) (Figures 9A & 10A, respectively, and Figure 11). Upon rapid mixing (2 ms) of a solution of ferric WT KatG [UV-visible spectrum: 408 (Soret, $\varepsilon$=107 mM$^{-1}$cm$^{-1}$), 496, 630 nm] with MPPH, a new species was observed [UV-visible spectrum: 410 (Soret, $\varepsilon$=118 mM$^{-1}$cm$^{-1}$), 628 nm] (Figure 9B) whose spectral features matched neither those of WT KatG compound I [iron-oxo porphyrin $\pi$-cation radical; UV-visible spectrum: 411 (Soret displays 40% hypochromicity vs. resting), 550, 590, 655 nm] or compound III [ferrous-dioxygen/ferric-superoxo complex; UV-visible spectrum: 418 (Soret), 545, 580 nm] (60). The spectral features, however, were highly consistent (with respect to $\lambda_{max}$ and extinction) to the previously characterized compound II intermediates of Synechocystis PCC 6803 KatG [UV-visible spectrum: 407 (Soret), 626 nm] (65) and Anacystis nidulans KatG [UV-visible spectrum: 406 (Soret), ~625 nm] (66). The values of $k_{obs}$ for formation of this new species were linearly dependent on [MPPH] (2.5 – 10 fold excess per heme), giving a bimolecular rate constant of (4.8 ± 0.4) x $10^{4}$ M$^{-1}$s$^{-1}$. The intermediate was also found to be unstable at pH 7.5 and 25 °C, with a slow decay (0.095 s$^{-1}$) resulting in re-formation of resting (ferric) enzyme, coconitant with a slight bleaching (~5%) of the heme Soret band. MPPH has been previously shown to undergo homolytic O-O bond cleavage (61), predominantly at pH values above 6.0 (67), resulting in compound II formation (as opposed to compound I intermediates which are derived from heterolytic cleavage of the O-O bond in alkylhydroperoxides). Based on these UV-visible spectroscopic observations and on the known chemical reactivity of the MPPH oxidant, we assign the new species detected here as Mtb WT KatG compound II. The similarity in rates of formation for compound II derived from MPPH vs. compound I derived from PAA (~$10^{8}$ M$^{-1}$s$^{-1}$) is likely attributed to similar values in the rates of peroxide binding to the heme and subsequent cleavage of the oxygen-oxygen bond, and is most likely independent of the specific nature (homolytic vs. heterolytic) of O-O bond cleavage.

Similarly, rapid mixing of a solution of ferric KatG(Y229F) [UV-visible spectrum: 406 (Soret, $\varepsilon$=101 mM$^{-1}$cm$^{-1}$), 504, 638 nm] with MPPH yielded a new species [UV-visible spectrum: 417 (Soret, $\varepsilon$=108 mM$^{-1}$cm$^{-1}$), 531, 561 nm] (Figure 10B), whose spectral features did not match those of either compound I or compound III (vide supra). The spectral features were consistent, however, with the previously assigned spectrum for Mtb KatG(Y229F) compound II [UV-visible spectrum: 416 (Soret), 530, 560 nm] as described by Magliozzo and co-workers (47) for the reaction of PAA with KatG(Y229F), with the compound II spectrum of Synechocystis PCC 6803 KatG(Y249F) [UV-visible spectrum: 418 (Soret), 530, 558 nm] (27), as well as for the classical oxoferryl compound II intermediates observed in HRP and other peroxidases (68). The values for $k_{obs}$ for KatG(Y229F) compound II formation were linearly dependent upon [MPPH] (2.5 – 10 fold excess per heme), with a biomolecular rate constant of (5.8 ± 0.7) x $10^{8}$ M$^{-1}$s$^{-1}$, similar to the previously determined rates [4 x $10^{6}$ M$^{-1}$s$^{-1}$ and 8 x $10^{6}$ M$^{-1}$s$^{-1}$ for Mtb KatG(Y229F) (47) and Synechocystis PCC 6803 KatG(Y249F) (27), respectively] for compound I formation, supporting the notion that peroxide binding to the heme, and not the nature of O-O bond cleavage (homolytic vs. heterolytic) is rate-determining. Decay of compound II to resting (ferric) enzyme was ~50-fold more rapid (5.5 s$^{-1}$) than that of WT KatG, suggesting that the Y229F mutation either i) destabilizes the iron-oxo intermediate by disrupting H-bonding to the o xo moiety, ii)
directly reduces compound II to the resting enzyme, resulting in a tyrosyl radical, or iii) facilitates electron transfer from a nearby redox active protein sidechain (i.e., W107, which is no longer involved in the Met-Tyr-Trp crosslink in the Y229F mutant).

**Compound II Is Observed During Slow Decay of Compound I in WT KatG** – Upon rapid mixing of a solution of WT KatG with PAA, formation of compound I [UV-visible spectrum: 412 (Soret, \( \varepsilon = 63 \text{ mM}^{-1}\text{cm}^{-1}) \), 550, 590, 650 (sh) nm; \( k_{\text{app}} = (1.5 \pm 0.2) \times 10^4 \text{ M}^{-1}\text{s}^{-1} \), in agreement with literature precedents (60)] was observed (Figure 11). The compound I intermediate was unstable under the conditions employed (pH 7.5, 25 °C), and decayed slowly (0.0767 s\(^{-1} \)) to a new species whose UV-visible spectral features [410 (Soret, \( \varepsilon = 112 \text{ mM}^{-1}\text{cm}^{-1}) \), 625 nm] are nearly identical to those observed for WT KatG compound II formed using MPPH (~5% lower intensity observed is attributed to heme bleaching). The compound II intermediate detected here is itself unstable, with a slow conversion back to the resting enzyme (0.023 s\(^{-1} \)), which also exhibited a minor amount of heme bleaching. Given the high degree of spectral similarity between resting enzyme and compound II in wild-type *Mtb* KatG (this work), *Synechocystis* PCC 6803 KatG (65), and *Anacystis nidulans* KatG (66), it is not surprising that previous attempts to observe compound II formation during the decay of compound I were unsuccessful. This is further complicated by the fact that the rates of formation and disappearance for compound II are both relatively slow and nearly equal, thus making global analysis more difficult. The results here, however, do suggest that compound I reduction can occur in two discreet 1 electron steps, proceeding through compound II before returning to the ferric resting state.

**DISCUSSION**

We employed tryptic digestions of KatG in combination with high-pressure liquid chromatography and mass spectrometry to identify the presence of a peptide fragment containing the Met-Tyr-Trp crosslink (cross-linked peptide fragment, CLPF). Previous studies have successfully identified crosslinked amino acid side chains by similar means, including the Met-Tyr-Trp crosslink in KatG from both *Bulkholderia pseudomonas* (22,69) and *Synechocystis* PCC 6803 (26), the cysteine-tryptophylquinone linkage in quinohemoprotein amine dehydrogenase from *Paracoccus denitrificans* (70), the cysteine-tyrosine crosslink in catalase-1 from *Neurospora crassa* (34), and the histidine-tyrosine linkage in cytochrome c oxidase from *Thermus thermophilus* (30). One notable difference between these studies and our own attempts in identifying the Met-Tyr-Trp crosslink in WT KatG is our isolation and analysis of the CLPF by HPLC prior to mass spectrometric characterization, which allows for quantitation as well as confirmation of its presence.

Identification of a candidate for the CLPF was simplified by comparison of the HPLC chromatograms obtained for the tryptic digest of WT KatG to that of KatG(Y229F), which was predicted to lack the CLPF. A peptide fragment in the former but not latter digest was identified that exhibited an unusual UV-visible spectrum for a polypeptide, with large bathochromic shifts of the major absorption bands. This alone suggested the presence of the Met-Tyr-Trp crosslink, as shifts for comparable aromatic-ring substitutions are predicted from theory (58) and observed in similar small molecule analogs, most notably for the cross-linked histidine-phenol mimic of cytochrome c oxidase (71), which exhibits an ~20 nm red-shift of the phenol B (tyrosine) band (from 280 to ~300 nm).

Isolation of this CLPF candidate, and subsequent characterization by mass spectrometry conclusively demonstrated the existence of the Met-Tyr-Trp crosslink in WT KatG. Furthermore, structure confirmation for the CLPF is provided for the first time (for any of the KatGs) by CID and MS/MS/MS analyses. The unusual masses observed for the fragmentation of the CLPF were attributed to a unique \( \beta \)-elimination of the Met-Tyr bond, and further supported the existence of the crosslinked amino acid side chains in the CLPF.

Overexpression of apoKatG, followed by *in vitro* reconstitution with heme in the absence of exogenously added peroxide, yielded WT KatG, a holoenzyme lacking the Met-Tyr-Trp crosslink normally found in the recombinant wild-type enzyme. This allowed us to study crosslink formation under oxidizing conditions that proceed through either compound I (PAA) or compound II (MPPH) intermediates; hydrogen peroxide was
specifically avoided due to complications arising from the catalase activity attributed to the presence (~7%) of background crosslink in \(^5\)WT KatG. Incubation studies between \(^5\)WT KatG and peroxyacetic acid (0-10 equivalents/heme) revealed a reaction stoichiometry (for full CLPF formation) of six equivalents PAA per KatG (monomer). Given that PAA is a two-electron oxidant, the disparity between the twelve oxidizing equivalents needed for what is formally a four-electron oxidation (formation of the two covalent bonds in the Met-Tyr-Trp crosslink), leads us to conclude that under the conditions studied, crosslink formation is only ~33% efficient. We suggest that additional oxidizing equivalents may generate protein radicals outside the heme active site, as has been previously observed for both \(\textit{Mtb}\) (47,62,72) and \(\textit{Synechocystis}\) PCC 6803 (25,64) KatGs. Alternatively, higher efficiency may occur \textit{in vivo} in the presence of bound substrate. Incubation of \(^5\)WT KatG with a large excess of MPPH, which preferentially oxidizes KatG to compound II over compound I (as demonstrated by stopped-flow UV-visible spectroscopic studies), led to a negligible increase in CLPF present. This suggests that both oxidizing equivalents must be present simultaneously to achieve bond formation.

There are few literature precedents relating to covalent bond formation between tyrosine and either tryptophan or methionine. In the only other example of a Tyr-Trp bond, \(\textit{CcP}(\text{H52Y})\) (73), the histidine-to-tyrosine mutation allows for the formation of a C-N bond (as opposed to the C-C bond in \(\textit{Mtb}\) KatG) between the indolic-N (\(\text{Ne}1\)) of Trp51 and the \(\text{Ce}\) of Tyr52. Poulos and coworkers (73) argue that given the short lifetimes of protein radicals, two sequential one-electron oxidations by two compound I intermediates (resulting from two enzyme turnovers) yielding the Tyr-Trp bond is unlikely to occur in \(\textit{CcP}(\text{H52Y})\). Rather, they argue that any mechanism for bond formation between two protein side chains must support concurrent radical formation on both protein residues, and our data which demonstrates the lack of CLPF formation derived from KatG compound II (one oxidizing equivalent above the resting state), supports this notion. In light of our work here with PAA, we thus suggest that compound I is the two-electron oxidant in \(\textit{Mtb}\) KatG involved in crosslink formation. Similarly, in catalase-1 a mechanism has been proposed which implicates compound I (from reaction of \(\text{H}_2\text{O}_2\) with the resting enzyme) in the formation of the Cys-Tyr bond (34), and roles for autocatalytic bond formation by compound I have been described in other systems as well (74). Surprisingly, in \(\text{CcP}(\text{H52Y})\), the lack of a detectable compound I intermediate necessitated an alternative: an iron-bound peroxide was invoked to concurrently oxidize both Tyr and Trp residues via two simultaneous H-atom abstractions, thereby forming the Tyr-Trp crosslink.

To our knowledge, there are no examples of methionine-tyrosine side-chain crosslinks reported to date, with the exception of those occurring in the KatGs. However, several examples of the related cysteine-tyrosine linkage are known: galactose oxidase from \(\textit{Dactylium dendroides}\) contains a covalent bond found between Tyr272 (\(\text{Ce}\)1) and Cys228 (\(\text{Sy}\)) (33), and catalase-1 from \(\textit{Neurospora crassa}\) has a bond between the \(\text{C}\beta\) of Tyr379 and the sulfur atom of Cys356 (34). In both cases, the phenol oxygen of the tyrosine (as a phenolate) is bound to a redox active metal center (heme iron in catalase-1, copper in galactose oxidase). The difference between Cys-X (i.e. Cys-Tyr) and Met-X (i.e. Met-Tyr-Trp) linkages is the presence of the sulfonium ion in the latter. One consequence of this positive charge may be a raised redox potential for protein residues covalently bound to the methionine, as donation of an electron would create an unfavorable electrostatic interaction between the Met-S\(^+\) and the resulting radical cation. This suggests that the sulfonium ion, which is unique only to methionine-containing crosslinks, may hinder reduction of compound I by Tyr229 or Trp107, which is mechanistically significant as the product of such a hypothetical electron transfer would be oxoferryl compound II (plus a protein radical), a catalytically incompetent intermediate with respect to catalase activity. Indeed, this scenario was observed by Magliozzo and co-workers (47) in their reaction of \(\textit{Mtb}\) KatG(Y229F) with PAA, although the exact assignment of the protein radical, outside of it being attributed to a tyrosine residue, was not determined. While other roles for the sulfonium ion can be postulated, including altering the pK\(_a\) of the neighboring residues, or affecting the H-bonding network of the active site,
further studies are necessary to definitively resolve the role it plays in the catalytic cycle of the catalase-peroxidases.

Several factors must be considered when proposing a mechanism for the autocatalytic formation of the Met-Tyr-Trp crosslink. First, two covalent bonds are formed between three protein side chains, implying a sequential process. Indeed, studies by Obinger and co-workers (26) with mutants of Synechocystis catalase-peroxidase demonstrated that both Tyr and Trp were necessary for complete Met-Tyr-Trp crosslink formation, whereas mutation of the methionine to isoleucine still produced a Tyr-Trp bond despite the lack of a Met-Tyr linkage; this result is significant, as it suggests that the crosslink forms first between the Cη2 of Trp-107 and Cε1 of Tyr-229 (Mtb numbering), followed by bond formation between the Cε2 of Tyr-229 and Sδ of Met-255. Second, our results with PAA directly support compound I as the two-electron oxidizing species, and not an iron-bound peroxyde [as in CcP(H52Y)] or compound II alone (as demonstrated by the lack of crosslink formed in the presence of MPPH). Finally, protein radicals are short lived, necessitating a two-electron oxidant, such as compound I, to simultaneously generate radicals on both residues involved in the coupling reaction.

With these criteria in mind, we suggest that the formation of compound I (Figure 12, step a) either by a peracid (in vitro) or hydrogen peroxide (in vivo) leads to oxidation of both Tyr229 and Trp107 (step b). The crystal structure of Mtb WT KatG shows that the indole ring of Trp107 is nearly coplanar and stacked above the pyrrole-B ring of the heme cofactor at a distance of ~3.7 Å, while Tyr229 is also within ~5.4 Å (Cδ2 to pyrrole-A) of the heme prosthetic group (23). Both of these redox active residues are therefore within the threshold for long-range electron-transfer (75,76), and are capable of being oxidized by compound I with concomitant formation of Trp107• and Tyr229•. As both protein radicals are formed from a single turnover event, their short lifetimes are not an issue, and coupling of the two radicals (step c) occurs, resulting in the formation of the Tyr-Trp bond (step d). Formation of a second compound I intermediate (step e) results in further oxidation of the Tyr-Trp crosslink (steps f & g), leading to nucleophilic attack of the sulfur atom of Met255 on the quinone-like intermediate (step h), ultimately forming the Met-Tyr-Trp crosslink (step i).

The Met-Tyr-Trp crosslink has a marked effect on the UV-visible spectrum of the high-valent iron-oxo intermediates of Mtb KatG. This spectral dependency on the presence of the Met-Tyr-Trp crosslink correlates to enzyme function: WT KatG (Met-Tyr-Trp crosslink present) has both catalase and peroxidase activities, and the compound II species exhibits spectral features that are ‘catalase-peroxidase’-like (slight hyperchromicity of the Soret band, with little to no shift observed vs. ferric ‘resting’ enzyme, and the presence of a ~625 nm feature), while KatG(Y229F) (Met-Tyr-Trp crosslink absent) has only peroxidase activity, and exhibits the classical ‘peroxidase-like’ oxoferryl compound II spectrum (red-shift in the Soret band by ~10 nm, as well as prominent ~530 and 560 nm features). As to the origin of these spectral differences, several plausible explanations exist. Protein environment strongly influences the spectral properties of the heme prosthetic group, and the KatG(Y229F) mutation is expected to have a cascade effect on the overall protein architecture of the active site: the side chain of Tyr229 is covalently bound to the side chain of Trp107, which itself shares a hydrogen bond (through an active site water molecule, W427) with the catalytically indispensable distal histidine (H108). Such changes in the active site may affect π-stacking interactions between Trp107 and the heme, influence the geometry of the heme in its binding pocket, weaken or strengthen the iron-oxo bond, or disrupt distal-side hydrogen bonding networks, all of which could in turn influence the electronic absorption spectrum of compound II. In support of the latter, several KatG mutations have been shown to disrupt active site hydrogen bonding networks (24,77,78), and it is conceivable that such an alteration in H-bonding to the oxo moiety of compound II may alter its spectroscopic signature.

One interesting possibility is that the spectral differences in compound II may be explained in light of two resonance structures for a 1 e−-oxidized state of KatG: (KatG)FeIV=oxo • (KatG)FeIII-OH, where KatG• represents a protein radical not electronically coupled to the heme (68,79,80). The former represents the
‘classical’ oxoferryl species of the peroxidases, whereas the latter represents the ‘catalase-peroxidase’ compound II, whose structure has been suggested to have an absorption spectrum only slightly perturbed from that of resting enzyme (65,66,68). Our stopped-flow UV-visible characterization of compound II in both WT KatG and KatG(Y229F) correlate well with this proposal, but further characterization is necessary to conclusively make this assignment. As to the origin of the putative protein radical in (KatG•)Fe^{III}-OH, mutagenesis studies in conjunction with EPR spectroscopy on Synechocystis PCC 6803 KatG suggest it may be Trp106 (Trp91 in Mtb) (64): Trp91 is part of an integral H-bonding network that includes Trp107 of the Met-Tyr-Trp crosslink, several waters, the heme propionate arm, and the catalytically important distal histidine (His108) and arginine (R104) residues, making it a good candidate for the putative radical species of compound II in Mtb KatG.

The mechanistic advantage of (KatG•)Fe^{III}-OH over (KatG)Fe^{IV}=oxo is that while the latter is catalytically inactive with respect to catalase activity (formation of compound III ensuing upon reaction with a second equivalent of H_{2}O_{2}), the former may still posses activity. In (KatG•)Fe^{III}-OH, the heme is essentially in the ‘resting’ state, and therefore capable of reducing H_{2}O_{2} to yield compound I (the first step common to both catalase and peroxidase cycles). Thus, by avoiding by transferring the oxidizing equivalent to the protein, an oxoferryl intermediate can be avoided catalase activity is maintained. This possibility would be unique only to the KatGs, as preventing compound II from forming is a conundrum which only a bifunctional enzyme employing a common intermediate (i.e, a catalase-peroxidase using compound I in both catalytic mechanisms) must address: Catalases themselves lack peroxidase-substrate binding sites, and will therefore not undergo reduction of compound II to compound I by an exogenous electron source, a process which the KatGs must overcome. On the other hand, peroxidases are able to perform two sequential one electron oxidations, and the presence of a relatively stable ‘classical’ oxoferryl compound II in the absence of a second reducing substrate would inhibit any subsequent catalase activity in a KatG. Accordingly, the function of the crosslink may be to protect the catalase-peroxidase by promoting oxoferryl reduction with concomitant formation of a protein radical, thereby ensuring that the heme cofactor remains in a catalytically competent state for both enzyme activities during turnover.

We have been able to show that the structural element of the crosslinked Met-Tyr-Trp amino acids forms in an autocatalytic process, that it alters the spectral features of KatG compound II which may form during catalysis, and that this correlates well to changes in enzymatic function, particularly catalase activity. It is clear from this work that an enzyme structure-spectroscopy-activity relationship is readily observed in the catalase-peroxidases which is solely attributed to the presence of the Met-Tyr-Trp crosslink. However, many interesting questions still remain and need to be addressed further: How the Met-Tyr-Trp crosslink alters the electronic absorption spectrum of compound II is still unclear. Whether the presence of the sulfonium cation raises the potential of the crosslink such that electron-transfer to the Trp91 is favored, or if it simply prevents compound I reduction by a redox-active protein side chain [as is suggested for KatG(Y229F)], is unknown. The specifics of the H-bonding network with respect to the Met-Tyr-Trp crosslink and catalase activity are also poorly understood. Spectroscopic and biochemical studies addressing these questions, as well as other aspects of catalase-peroxidase mechanism, are in progress, and may shed light on these issues.

Acknowledgements – We thank Prof. Larry Que (University of Minnesota) for providing details on improving the MPPH synthetic protocol, and Jessica D. Tenenbaum for stimulating discussions on hydrogen bonding to the oxo-moiety of iron(IV)=O compound II intermediates. We also acknowledge the Colorado State University (NIH/NIAID Contract N01 AI-75320) for supplying the KatG-encoding plasmid pMRLB11.

This work was supported by NIH grant AI58524 (R.A.G. F32-Postdoctoral Fellowship), NCRR RR001614 and RR012961 (K.F.M., to the UCSF Mass Spectrometry Facility, director A.L. Burlingame) and GM56531 and GM32488 (P.O.M.).
The abbreviations used are: KatG, catalase-peroxidase; CeP, cytochrome c peroxidase; Mtb, Mycobacterium tuberculosis; WT, wild-type; MPPH, 2-methyl-1-phenyl-2-propyl hydroperoxide; KatG[Y229F], Y229F mutant of KatG; PAA, peroxyacetic acid; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate); TFA, trifluoroacetic acid; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; WT KatG, heme-reconstituted wild-type KatG; CLPF, crosslinked peptide fragment; Rz, Reinheitszal; EPR, electron paramagnetic resonance spectroscopy; HS, high-spin; LS, low-spin; CT1, charge transfer band 1; CID, collision-induced dissociation; Cmpd I, compound I, enzyme that is two equivalents oxidized above resting, (protoporphyrin-IX)iron(IV)=O π-cation radical; Cmpd II, compound II, enzyme which is one-electron oxidized above resting, ‘oxoferryl’ (protoporphyrin-IX)iron(IV)=O or (P•)Fe^{III} where P• represents a protein radical.

The basis for these assignments was a previous observation (Katalin F. Medzihradszky, unpublished results) that peptides with sulfonium ether-containing methionines (carboxymethyl derivatives) are prone to undergo a β-elimination-type fragmentation and lose the positively charged substituent of the γ-carbon.
Figure Legends

Figure 1: Active site of \textit{Mtb} KatG depicting the Met-Tyr-Trp crosslink and its proximity to the heme cofactor. Coordinates were obtained from the Protein Data Bank (1SJ2) and displayed using the Swiss PDB Viewer.

Figure 2: UV-visible spectra for WT KatG (black) and KatG(Y229F) (grey). Conditions: 100 mM potassium phosphate, pH 7.5, 25 °C.

Figure 3: HPLC chromatograms of the tryptic digests of WT KatG (bottom) and KatG(Y229) (top). The region ~185 min, corresponding to the CLPF, is highlighted.

Figure 4: Schematic representation of the CLPF. The three residues comprising the crosslink, Met255, Tyr229, and Trp107, are highlighted.

Figure 5: (A) HPLC chromatogram of the tryptic digest of WT KatG monitored at 330 (top), 280 (middle), and 220 (bottom) nm. The CLPF at ~185 min is highlighted. The strong absorption at 260 min corresponds to heme. (B) UV-visible spectrum of the CLPF (grey, t=185 min), compared to a ‘normal’ peptide (black; t=195 min).

Figure 6: (A) Low energy CID spectrum (MS/MS) of the CLPF. Ion intensities were magnified by a factor of ~7.5. The inset shows the precursor ion and illustrates the resolution afforded by the QSTAR mass spectrometer. The cleavage that produced the base peak and the ion at m/z 1217.77 is shown in C, while all the fragments are listed in Table 3. Fragment assignments from MS/MS (B) and MS/MS/MS (C) of the ion at m/z 984.4 are also shown. B-type ions are depicted above the corresponding amino acid residue, y-type ions are depicted below.

Figure 7: UV-visible spectra for apoKatG (dashed line) expressed under iron-free conditions, \textsuperscript{8}WT KatG (grey) formed from reconstitution of the apoprotein with heme, and recombinant WT KatG (black) expressed under normal conditions.

Figure 8: (A) HPLC chromatograms (CLPF region) of the tryptic digests of \textsuperscript{8}WT KatG after incubation with 0-10 equivalents of PAA prior to proteolytic digestion. (B) CLPF formation as a function of PAA. The data was normalized to 100% as represented by CLPF formation upon incubation with 25 equivalents PAA.

Figure 9: (A) Stopped-flow UV-visible spectroscopic monitoring of the reaction (300 scans, 6 sec) between WT KatG and MPPH. See experimental for details. (B) Calculated UV-visible spectra for both resting (black) and compound II WT KatG (grey) are shown; the rapid-scanning data from A were compiled and fitted to a single exponential reaction model using the Specfit global analysis program.

Figure 10: (A) Stopped-flow UV-visible spectroscopic monitoring of the reaction (300 scans, 0.6 sec) between KatG(Y229F) and MPPH. See experimental for details. (B) Calculated UV-visible spectra for both resting (black) and compound II KatG(Y229F) (grey) are shown; the rapid-scanning data from A were compiled and fitted to a single exponential reaction model using the Specfit global analysis program.

Figure 11: (A) Stopped-flow UV-visible spectroscopic monitoring of the reaction (300 scans, 600 sec) between WT KatG and PAA. See experimental for details. (B) Calculated UV-visible spectra for WT KatG resting (black), compound I (dashed line), and compound II (grey) are shown; the rapid-scanning data from A were compiled and fitted to a triple exponential reaction model using the Specfit global analysis program.

Figure 12: Proposed mechanism for the formation of the Met-Tyr-Trp crosslink in \textit{Mtb} KatG.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 7
Figure 10

A

Absorbance vs. Wavelength (nm)

B

Epsilon (mM·cm⁻¹) vs. Wavelength (nm)

KatG(Y229F) (ferric)
KatG(Y229F) Compound II

417 nm
531 561
406
504 531 561 638
Figure 11
Figure 12
Table 1: UV-visible Spectroscopic Data for WT KatG and KatG(Y229F)

<table>
<thead>
<tr>
<th></th>
<th>R_x (Soret)</th>
<th>( \lambda_{\text{max}} ) (Soret)</th>
<th>( \beta )</th>
<th>CT2 (LMCT)</th>
<th>CT1 (LMCT)</th>
<th>( \frac{A_{\text{Soret}}}{A_{380}} )</th>
<th>( \frac{A_{511}}{A_{545}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.63</td>
<td>408</td>
<td>496</td>
<td>540</td>
<td>630</td>
<td>1.79</td>
<td>1.05</td>
</tr>
<tr>
<td>Y229F</td>
<td>0.59</td>
<td>406</td>
<td>504</td>
<td>540</td>
<td>638</td>
<td>1.68</td>
<td>0.85</td>
</tr>
</tbody>
</table>
Table 2: Kinetic Parameters for Catalase and Peroxidase Activities

<table>
<thead>
<tr>
<th></th>
<th>WT KatG</th>
<th>KatG(Y229F)</th>
<th>0.5WT KatG</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Catalase Activity</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{cat}$, s$^{-1}$</td>
<td>6000 ± 70</td>
<td>0.1 ± 0.05</td>
<td>5660 ± 90</td>
</tr>
<tr>
<td>$K_{m}$, mM</td>
<td>2.5 ± 0.2</td>
<td>39.8 ± 6.4</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>$k_{cat}/K_{m}$ (M$^{-1}$s$^{-1}$)</td>
<td>(2.40 ± 0.03) x 10$^6$</td>
<td>2.5 ± 1.3</td>
<td>(2.26 ± 0.05) x 10$^6$</td>
</tr>
<tr>
<td><strong>Peroxidase Activity</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{cat}$, s$^{-1}$</td>
<td>0.062 ± 0.001</td>
<td>0.843 ± 0.056</td>
<td>0.060 ± 0.002</td>
</tr>
<tr>
<td>$K_{m}$, mM</td>
<td>8.4 ± 0.5</td>
<td>2.7 ± 0.7</td>
<td>8.5 ± 0.4</td>
</tr>
<tr>
<td>$k_{cat}/K_{m}$ (M$^{-1}$s$^{-1}$)</td>
<td>7.3 ± 0.4</td>
<td>316 ± 39</td>
<td>7.1 ± 0.3</td>
</tr>
</tbody>
</table>
Table 3: Fragment assignments from the CID spectrum (Figure 6) of m/z 983.87(7+).

<table>
<thead>
<tr>
<th>Mass Detected</th>
<th>Assign.</th>
<th>Mass Calculated</th>
<th>Assign.</th>
<th>Mass Calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>147.11</td>
<td>y₁**</td>
<td>147.11</td>
<td>y₁*, y₁^</td>
<td>175.12</td>
</tr>
<tr>
<td>201.12</td>
<td>a₂*</td>
<td>201.13</td>
<td>y₂**</td>
<td>204.14</td>
</tr>
<tr>
<td>213.09</td>
<td>PD^</td>
<td>213.09</td>
<td>EN-28^</td>
<td>216.12</td>
</tr>
<tr>
<td>229.12</td>
<td>b₁^</td>
<td>229.12</td>
<td>244.09</td>
<td></td>
</tr>
<tr>
<td>288.2</td>
<td>y₂^</td>
<td>288.2</td>
<td>322.69(2+)</td>
<td>y₂^</td>
</tr>
<tr>
<td>338.17</td>
<td>y₃*</td>
<td>338.18</td>
<td>351.19</td>
<td>y₃**</td>
</tr>
<tr>
<td>358.17</td>
<td>b₁^</td>
<td>358.16</td>
<td>401.71(2+)</td>
<td>y₅**</td>
</tr>
<tr>
<td>403.22</td>
<td>y₅^</td>
<td>403.23</td>
<td>413.67(2+)</td>
<td>b₅-H₂O**</td>
</tr>
<tr>
<td>422.67(2+)</td>
<td>b₅**</td>
<td>844.35</td>
<td>449.18(2+)</td>
<td>b₅-H₂O**</td>
</tr>
<tr>
<td>452.25</td>
<td>y₆*</td>
<td>452.25</td>
<td>458.2(2+)</td>
<td>b₆**</td>
</tr>
<tr>
<td>472.24</td>
<td>b₆^</td>
<td>472.2</td>
<td>472.24(2+)</td>
<td>y₇^</td>
</tr>
<tr>
<td>493.71(2+)</td>
<td>b₆**</td>
<td>986.43</td>
<td>502.29</td>
<td></td>
</tr>
<tr>
<td>514.79(2+)</td>
<td>y₈**</td>
<td>1028.59</td>
<td>528.31</td>
<td>from y₁₀**</td>
</tr>
<tr>
<td>550.25(2+)</td>
<td>b₁₀**</td>
<td>1099.51</td>
<td>573.32</td>
<td>y₁₀</td>
</tr>
<tr>
<td>578.28(2+)</td>
<td>y₁₀^</td>
<td>1155.58</td>
<td>589.31</td>
<td>y₁₀**</td>
</tr>
<tr>
<td>606.79(2+)</td>
<td>b₁₂**</td>
<td>1212.59</td>
<td>614.25</td>
<td>b₁₂**</td>
</tr>
<tr>
<td>644.37</td>
<td>y₆^</td>
<td>644.37</td>
<td>646.32</td>
<td>y₆**</td>
</tr>
<tr>
<td>647.62(3+)</td>
<td>from</td>
<td>665.96(3+)</td>
<td>y₁₀-H₂O**</td>
<td>1996</td>
</tr>
<tr>
<td>671.99(3+)</td>
<td>y₁₀**</td>
<td>2014</td>
<td>682.35</td>
<td>b₆</td>
</tr>
<tr>
<td>684.83(2+)</td>
<td>b₆**</td>
<td>1368.68</td>
<td>703.33</td>
<td>y₆**</td>
</tr>
<tr>
<td>715.39</td>
<td>y₆^</td>
<td>715.41</td>
<td>736.34</td>
<td>b₆-NH₃^</td>
</tr>
<tr>
<td>743.28</td>
<td>b₅**</td>
<td>743.3</td>
<td>753.37</td>
<td>b₅</td>
</tr>
<tr>
<td>769.35(2+)</td>
<td>y₁₁^</td>
<td>1337.74</td>
<td>781.87(2+)</td>
<td>y₁₁**</td>
</tr>
<tr>
<td>784.4</td>
<td>y₅</td>
<td>784.41</td>
<td>797.87(2+)</td>
<td>y₁₁**</td>
</tr>
<tr>
<td>H₂O**</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>802.4</td>
<td>y₆**</td>
<td>802.42</td>
<td>807.37</td>
<td>b₅-NH₃</td>
</tr>
<tr>
<td>824.4</td>
<td>b₅**</td>
<td>824.42</td>
<td>832.4(2+)</td>
<td>b₅**</td>
</tr>
<tr>
<td>846.41</td>
<td>y₆^</td>
<td>846.45</td>
<td>864.9(2+)</td>
<td>y₁₁**</td>
</tr>
<tr>
<td>897.37</td>
<td>b₅</td>
<td>897.37</td>
<td>905.91(2+)</td>
<td>b₅**</td>
</tr>
<tr>
<td>H₂O**</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>910.89(2+)</td>
<td>y₁₁^</td>
<td>1820.86</td>
<td>915.48</td>
<td>y₁₁**</td>
</tr>
<tr>
<td>923.46</td>
<td>b₅^</td>
<td>923.48</td>
<td>943.48</td>
<td>y₆**</td>
</tr>
<tr>
<td>970.95(2+)</td>
<td>from</td>
<td>986.52</td>
<td>b₁₀**</td>
<td>986.43</td>
</tr>
<tr>
<td>y₁₀**</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1028.51</td>
<td>y₁₀</td>
<td>1028.59</td>
<td>1100.25(4+)</td>
<td>y₁₁^</td>
</tr>
<tr>
<td>1128.48(4+)</td>
<td>y₁₁^</td>
<td>4501.15</td>
<td>1155.54</td>
<td>y₁₁</td>
</tr>
<tr>
<td>1213.55(4+)</td>
<td>X-NH₃</td>
<td>4851.28</td>
<td>1217.77(4+)</td>
<td>X</td>
</tr>
<tr>
<td>1238.18(3+)</td>
<td>b₂^</td>
<td>3713.73</td>
<td>1309.13(3+)</td>
<td>b₂^</td>
</tr>
<tr>
<td>1348.91(3+)</td>
<td>y₂**</td>
<td>4044.9</td>
<td>1372.60(3+)</td>
<td>y₂**</td>
</tr>
<tr>
<td>1396.32(3+)</td>
<td>y₂**</td>
<td>4186.97</td>
<td>1466.67(3+)</td>
<td>y₂**</td>
</tr>
</tbody>
</table>

¹Fragment ions b and y are formed via peptide bond cleavages, when the charge is retained on the N-terminal or on the C-terminal fragment, respectively, and are numbered from the corresponding termini. [Peptide fragmentation nomenclature: K. Biemann, Methods Enzymol., 193, 886 (1990).] Fragments of peptide [105-114] are labeled with *. Fragments of peptide [255-274] are labeled with **, and all the b** ions as well as y₁₀** feature a 48 Da lower mass value than fragments of a nonmodified peptide, reflecting the β-elimination of CH₃SH from the side-chain of the N-terminal Met. Fragments formed along the backbone of peptide [215-249] are labeled with ^. Fragments that contain the Tyr residue also contain the full length covalently linked [105-114] peptide, but only the CH₃S-part of the side-chain of Met-255. Similarly fragment X consists of the full length [105-114] and [215-249] peptides cross linked, with only the CH₃S-group attached to the Tyr-ring.

²Only singly charged fragment masses were calculated.
The Met-Tyr-Trp crosslink in mycobacterium tuberculosis catalase-peroxidase (KatG): Autocatalytic formation and effect on enzyme catalysis and spectroscopic properties

Reza A. Ghiladi, Giselle M. Knudsen, Katalin F. Medzihradszky and Paul R. Ortiz de Montellano

J. Biol. Chem. published online April 18, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M502486200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2005/04/18/jbc.M502486200.citation.full.html#ref-list-1