J|A|C|S COMMUNICATIONS

Published on Web 05/27/2010

A Radical on the Met-Tyr-Trp Modification Required for Catalase Activity in Catalase-Peroxidase Is Established by Isotopic Labeling and Site-Directed Mutagenesis

Xiangbo Zhao,[†] Javier Suarez,[†] Abdelahad Khajo,[†,§] Shengwei Yu,[†] Leonid Metlitsky,[†] and Richard S. Magliozzo*,[†,§]

Department of Chemistry, Brooklyn College, 2900 Bedford Avenue, Brooklyn, New York 11210, and Departments of Chemistry and Biochemistry, The Graduate Center of the City University of New York, 365 Fifth Avenue, New York, New York 10016

Received April 19, 2010; E-mail: rmaglioz@brooklyn.cuny.edu

Mycobacterium tuberculosis (*Mtb*) catalase-peroxidase (KatG) has attracted great interest because of its role in the activation of the antitubercular prodrug isoniazid (INH) and because of widespread resistance to INH due to KatG mutations in the tuberculosis pathogen.[1,2] In addition to broad peroxidase activity, KatG enzymes exhibit robust catalase activity ($k_{cat}/K_M \approx 10^6$ M^{-1} s^{-1}),[3] dismutating hydrogen peroxide into dioxygen and water via a nonscrambling mechanism despite a lack of sequence homology with monofunctional heme catalases.[4] One key structural feature that differentiates KatG from other heme peroxidases is the conserved distal-side three amino acid adduct Met255-Tyr229-Trp107 (MYW) (Figure 1), a post-translational modification essential for catalase activity.[5−9] What makes KatG such an efficient catalase is intriguing because peroxidases are generally very poor catalases.

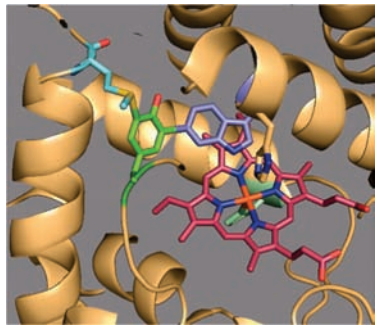


Figure 1. Distal-side Met-Tyr-Trp adduct in *Mtb* KatG (PDB entry 2CCA).

We recently reported that a transient tyrosyl-like radical forms during the reaction of KatG with H_2O_2 and persists during the time interval for turnover of excess peroxide.[10] Mutations that disrupt MYW adduct formation (e.g., M225A, Y229F, or W107F) abolish this radical species and cause complete loss of catalase activity without interfering with peroxidase activity.[10] According to the correlation of electron paramagnetic resonance (EPR) spectral parameters for this radical with structural features of tyrosine residues in *Mtb* KatG and with density functional theory (DFT) calculations of the electronic structure of a neutral MYW radical modeled after the crystal structure of *Mtb* KatG, the tyrosyl-like radical was tentatively assigned to the MYW adduct. Confirmation of the radical site is essential for understanding KatG function. For the present study, in an attempt to identify the site of the catalytically competent radical, 21 *Mtb* KatG tyrosine mutants were prepared by site-directed mutagenesis, and two types of deuterium-labeled

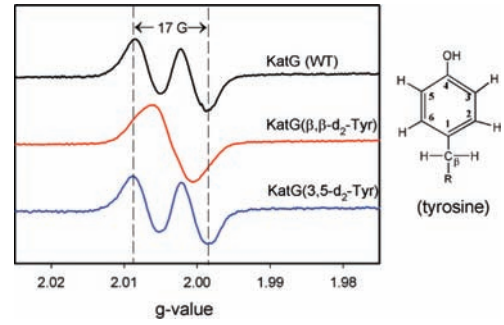


Figure 2. The X-band EPR spectrum (77 K) of the radical formed during catalase turnover in *Mtb* KatG becomes an apparent singlet for enzyme labeled with β-methylene-deuterated tyrosine but is unchanged in KatG labeled with 3,5-deuterotyrosine. Enzymes were manually mixed with an 8000-fold molar excess of H_2O_2 at pH 8.5 and rapidly frozen in liquid N_2.

wild-type (WT) KatG with ^2H at the β-methylene positions or the 3- and 5-positions of the ring were also prepared.

When WT KatG reacts with a large excess of H_2O_2, a narrow doublet EPR signal is found (peak-to-trough line width of 17 G, $g = 2.0034$; Figure 2), which we previously identified as a tyrosyl-like radical on the basis of the *g*-value anisotropy evaluated using high-field EPR.[10] Here, isotopically labeled *Mtb* KatG was overexpressed in *Escherichia coli* BL21 grown in M9 minimal medium supplemented with either of two deuterium-labeled tyrosine molecules. Purified enzymes were reacted with excess H_2O_2 and immediately frozen. For KatG containing β-methylene-deuterated tyrosine, a singlet signal with a line width of 11 G is seen (Figure 2). Replacement of hydrogen with deuterium reduces electron−nuclear hyperfine interactions because of the ~6-fold smaller *g* factor for ^2H versus ^1H. The change in the X-band EPR spectrum demonstrates that the narrow doublet signal in WT KatG is associated with a tyrosine residue, as we previously assigned. In contrast to this, the EPR signal from KatG labeled with ring-deuterated tyrosine (3,5-d_2-Tyr) is *unchanged* relative to unlabeled KatG. Replacement of these two ring hydrogens with deuterium in a tyrosyl radical would also alter the hyperfine splittings and/or reduce the line width of the EPR signal, neither of which was observed. The absence of a change suggests that the radical species lacks 3,5-ring hydrogens. Alternatively, the unchanged narrow doublet could indicate unsuccessful labeling of KatG in the case of the ring-deuterated tyrosine. That the KatG used here was in fact labeled with 3,5-d_2-Tyr was demonstrated by taking advantage of our previous observations that in reactions of KatG with alkyl peroxides,[11,12] tyrosyl radical(s) remote from the MYW adduct are formed. As shown in Figure 3, the line width of the singlet signal typical of WT KatG treated

[†] Brooklyn College.
[§] The Graduate Center of CUNY.

10.1021/ja103311e

with peracetic acid (PAA) for 5 s (using a rapid freeze−quench technique) was reduced from 23.0 to 18.8 G for the 3,5-d_2-Tyr-labeled enzyme. These observations prove that 3,5-d_2-Tyr was successfully incorporated into KatG. For enzyme labeled with Tyr deuterated at the β-methylene sites, the line width was reduced to 14.1 G, again demonstrating that the majority species giving these EPR signals upon turnover with PAA is a tyrosyl radical(s) [and that the radical(s) formed using PAA is different from that formed during catalase turnover[13]].

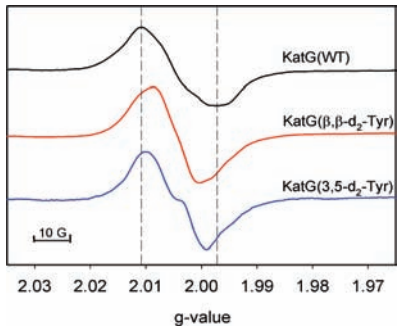


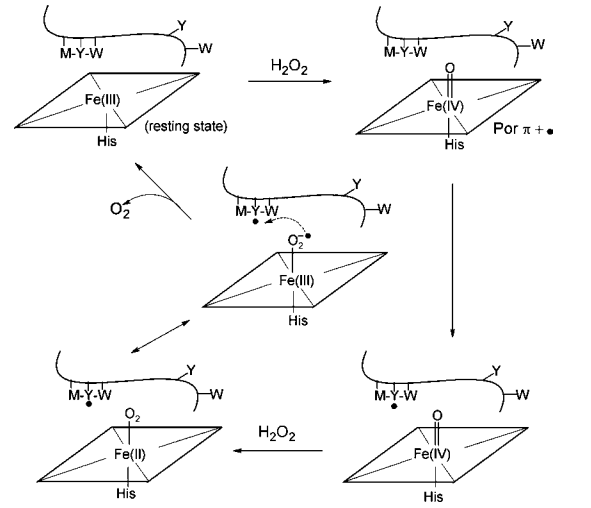
Figure 3. The EPR spectrum (77 K) of the radical formed in *Mtb* KatG reacted with peracetic acid is narrowed for enzyme containing β-methylene- or 3,5-ring-deuterated tyrosine. Enzymes were mixed with a 3-fold molar excess of peracetic acid and frozen after 5 s using a rapid freeze−quench apparatus.

In an attempt to explore the possible roles of specific tyrosine residues in radical formation and the catalase mechanism, we mutated each of the 21 tyrosine residues in *Mtb* KatG. As shown in Table S1 in the Supporting Information, except for Tyr229Phe, each mutant enzyme exhibited the narrow doublet EPR signal upon reaction with H_2O_2 and had close to normal catalase activity. Do these observations directly confirm residue 229 as the radical site? It is conceivable that Tyr229 or an intact MYW adduct is required for formation of a tyrosyl radical(s) on some other site or sites during catalase turnover. However, the observation of the same narrow doublet in each mutant would then mean that upon mutation of one of the residues other than Tyr229, *yet another* Tyr radical that exhibits the same EPR signal is formed. Furthermore, a scenario in which different tyrosyl residues beyond Y229F could be radical sites is unreasonable given the conserved catalase activities, since a catalytic function of the radical in the active site containing oxyferrous heme[9,14] must be maintained in each functional mutant. Taken together, these observations—the unchanged narrow doublet EPR signal found during catalase turnover in the 3,5-d_2-Tyr labeled enzyme and the presence of the narrow doublet in each mutant except Y229F—are then best explained by assigning the catalytic radical to the MYW adduct in which the tyrosine ring lacks hydrogens at the 3- and 5-positions. These hydrogens are replaced by sulfur or carbon in the cross-links to neighboring Met225 and Trp107 side chains (Figure 1).

The evidence for a radical associated with the MYW adduct and catalase activity in catalase-peroxidase is supported by the observation that the EPR signal intensity can be as high as 0.5 spins per heme in rapid freeze−quench samples frozen after a few milliseconds of reaction with H_2O_2 (data not shown). According to DFT calculations, the majority of unpaired spin density is contained within the tyrosyl ring in this structure.[10]

Unlike other peroxidases, KatG forms Compound III (oxyferrous heme) upon reaction with excess H_2O_2 yet rapidly turns over massive excesses of peroxide. In mutants such as M255A, Y229F, and W107F, the formation of a stable Compound III requires only a very small excess of H_2O_2, and similar to the case of monofunctional peroxidases, no catalase turnover occurs.[7,9,10] The rapid catalase activity of KatG therefore depends on efficient recycling of Compound III. Consistent with this idea is our earlier evidence that the radical must participate in a reaction with oxyferrous heme, enabling dioxygen release and yielding ferric KatG,[9,10] as proposed by Jackopitsch et al.[14] Therefore, it is reasonable that superoxide rather than dioxygen is dissociated from oxyferrous heme and that the unpaired spin is quenched by the MYW radical during catalase turnover by KatG (Scheme 1).

Scheme 1. Formation of the Adduct Radical and Its Possible Role in the Catalase Activity of KatG [The Cartoon Depicts Heme, The Distal Met-Tyr-Trp (MYW) Adduct/Radical, and Protein Sites (Y, W Residues) Where Other Amino Acid Radicals May Form]



Acknowledgment. This work was supported by NIH Grant RO1-AI060014 (NIAID) to R.S.M. The authors thank Margarita Vasserman for performing activity assays.

Supporting Information Available: Materials and detailed experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Zhang, Y.; Heym, B.; Allen, B.; Young, D.; Cole, S. *Nature* **1992**, *358*, 591.
(2) Rozwarski, D. A.; Grant, G. A.; Barton, D. H. R.; Jacobs, W. R., Jr.; Sacchettini, J. C. *Science* **1998**, *279*, 98.
(3) Nagy, J. M.; Cass, A. E.; Brown, K. A. *J. Biol. Chem.* **1997**, *272*, 31265.
(4) Vlasits, J.; Jakopitsch, C.; Schwanninger, M.; Holubar, P.; Obinger, C. *FEBS Lett.* **2007**, *581*, 320.
(5) Ghiladi, R. A.; Knudsen, G. M.; Medzihradszky, K. F.; Ortiz de Montellano, P. R. *J. Biol. Chem.* **2005**, *280*, 22651.
(6) Jakopitsch, C.; Auer, M.; Ivancich, A.; Ruker, F.; Furtmuller, P. G.; Obinger, C. *J. Biol. Chem.* **2003**, *278*, 20185.
(7) Yu, S.; Girotto, S.; Zhao, X.; Magliozzo, R. S. *J. Biol. Chem.* **2003**, *278*, 44121.
(8) Ghiladi, R. A.; Medzihradszky, K. F.; Ortiz de Montellano, P. R. *Biochemistry* **2005**, *44*, 15093.
(9) Zhao, X.; Yu, S.; Ranguelova, K.; Suarez, J.; Metlitsky, L.; Schelvis, J. P. M.; Magliozzo, R. S. *J. Biol. Chem.* **2009**, *284*, 7030.
(10) Suarez, J.; Ranguelova, K.; Jarzecki, A. A.; Manzerova, J.; Krymov, V.; Zhao, X.; Yu, S.; Metlitsky, L.; Gerfen, G. J.; Magliozzo, R. S. *J. Biol. Chem.* **2009**, *284*, 7017.
(11) Chouchane, S.; Girotto, S.; Yu, S.; Magliozzo, R. S. *J. Biol. Chem.* **2002**, *277*, 42633.
(12) Ranguelova, K.; Girotto, S.; Gerfen, G. J.; Yu, S.; Suarez, J.; Metlitsky, L.; Magliozzo, R. S. *J. Biol. Chem.* **2007**, *282*, 6255.
(13) Trp radicals are also formed under these conditions, but the contribution to the EPR signal from any additional species is small. Moreover, for the deuterium-labeled enzymes, the changes in line width are readily observed, as the majority species giving the signals is tyrosyl radical(s). See: (a) Colin, J.; Wiseman, B.; Switala, J.; Loewen, P. C.; Ivancich, A. *J. Am. Chem. Soc.* **2009**, *131*, 8557. (b) Singh, R.; Switala, J.; Loewen, P. C.; Ivancich, A. *J. Am. Chem. Soc.* **2007**, *129*, 15954.
(14) Jakopitsch, C.; Vlasits, J.; Wiseman, B.; Loewen, P. C.; Obinger, C. *Biochemistry* **2007**, *46*, 1183.

JA103311E