Mutual synergy between catalase and peroxidase activities of the bifunctional enzyme KatG is facilitated by electron hole-hopping within the enzyme

Received for publication, April 18, 2017, and in revised form, September 22, 2017 Published, Papers in Press, September 27, 2017, DOI 10.1074/jbc.M117.791202

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Edited by Ruma Banerjee

KatG is a bifunctional, heme-dependent enzyme in the frontline defense of numerous bacterial and fungal pathogens against H2O2-induced oxidative damage from host immune responses. Contrary to the expectation that catalase and peroxidase activities should be mutually antagonistic, peroxidatic electron donors (PxEDs) enhance KatG catalase activity. Here, we establish the mechanism of synergistic cooperation between these activities. We show that at low pH values KatG can fully convert H2O2 to O2 and H2O only if a PxED is present in the reaction mixture. Stopped-flow spectroscopy results indicated rapid initial rates of H2O2 disproportionation slowing concomitantly with the accumulation of ferryl-like heme states. These states very slowly returned to resting (i.e. ferric) enzyme, indicating that they represented catalase-inactive intermediates. We also show that an active-site tryptophan, Trp-321, participates in off-pathway electron transfer. A W321F variant in which the proximal tryptophan was replaced with a non-oxidizable phenylalanine exhibited higher catalase activity and less accumulation of off-pathway heme intermediates. Finally, rapid freeze-quench EPR experiments indicated that both WT and W321F KatG produce the same methionine–tyrosine–tryptophan (MYW) cofactor radical intermediate at the earliest reaction time points and that Trp-321 is the preferred site of off-catalase protein oxidation in the native enzyme. Of note, PxEDs did not affect the formation of the MYW cofactor radical but could reduce non-productive protein-based radical species that accumulate during reaction with H2O2. Our results suggest that catalase-inactive intermediates accumulate because of off-mechanism oxidation, primarily of Trp-321, and PxEDs stimulate KatG catalase activity by preventing the accumulation of inactive intermediates.

Catalase-peroxidase (KatG)4 is a bifunctional heme-dependent enzyme found in bacteria and lower eukaryotes (1), which is integral to the defense of these organisms against H2O2 toxicity (2, 3). KatG function appears to carry especially important ramifications for plant and animal pathogens (4–6) because these organisms are likely to encounter excess H2O2 produced by their host’s immune response (e.g. oxidative burst). Among these enzymes, the function of KatG in Mycobacterium tuberculosis (MtKatG) is especially important because it is the only catalase-active enzyme carried by M. tuberculosis (7), and it activates the front-line antitubercular agent isoniazid (INH) (8). KatG-dependent oxidation of INH initiates formation of isonicotinyl-NAD, which inhibits an enoyl reductase (InhA) essential for mycolic acid biosynthesis (9). Accordingly, a large proportion of INH-resistant M. tuberculosis strains (>50%) produce an altered KatG (e.g. S315T) incapable of activating the drug (10, 11), rendering ineffective one of the most widely available and inexpensive antitubercular agents. The precise mechanism by which KatG activates INH and the molecular basis for how mutations to the katG gene interfere in said activation are the subjects of ongoing investigations (12–15).

KatG catalyzes H2O2 disproportionation to produce H2O and O2 (i.e. it is a catalase). It does so with an apparent second-order rate constant (~1 × 10−6 M−1 s−1) similar to typical (i.e. monofunctional) catalases (16, 17) even though it shares no structural homology with them. Instead, it is a member of the plant peroxidase-like superfamily with cytochrome P450 (CYP) peroxidase (Ccp), ascorbate peroxidase (APx), and horseradish peroxidase (HRP) (18, 19). Indeed, the active sites of Ccp, APx, and KatG are virtually superimposable (Fig. 1) (20–24). Not surprisingly, then, KatG also catalyzes H2O2 reduction to H2O concomitant with the oxidation of a structurally diverse range of exogenous electron donors (i.e. it is a peroxidase). Its peroxidase activity is well within the range of other plant peroxidase-like superfamily members (16, 17, 25), but KatG is the only member of the entire superfamily that possesses appreciable catalase activity.

The abbreviations used are: KatG, catalase-peroxidase; MtKatG, M. tuberculosis KatG; MYW, methionine–tyrosine–tryptophan covalent adduct; Ccp, cytochrome c peroxidase; APx, ascorbate peroxidase; LL1, large loop 1; LL2, large loop 2; ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonate); INH, isoniazid; PxED, peroxidatic electron donor; PAA, peracetic acid; μW, microwatt.

This work was supported in part by Grants MCB 1616059 and MCB 0641614 from the National Science Foundation (to D. C. G.) and National Institutes of Health Grant GM108988 (to A. L.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Despite their striking active-site similarities, KatG possesses structural features that distinguish it from other members of its superfamily. Among them are two large loops (LL1 and LL2), both of which are essential for KatG function (20–22, 26, 27). Both loops also contribute to a much narrower channel to the active-site heme, which among other things restricts access of many peroxidatic electron donors (PxEDs) to the heme edge (22, 27–29). Additionally, LL1 bears an invariant tyrosine (Tyr-229 by \( Mt\) KatG numbering) that participates in a unique methionine–tyrosine–tryptophan (MYW) adduct that serves as a protein-derived cofactor (20–22, 30, 31). Substitutions of any of the residues of the MYW adduct consistently produce KatG variants that retain or increase their peroxidase activity but have negligible catalase activity (14, 29, 32–37).

Commensurate with an active site completely distinct from typical catalases, it is clear that KatG operates by a novel catalase mechanism, although the details remain a matter of debate (15, 38–47). The first step is common to all heme-dependent catalases and peroxidases and is widely agreed upon for KatG; \( H_2O_2 \) oxidizes the ferric heme of KatG to form a ferryl-oxo porphyrin \( \text{Fe}^{IV}=O[\text{porphyrin}^{\cdot\cdot}] \) known as compound I (Fig. 1, inset). At this point, KatG diverges from the canonical catalase mechanism by reducing the porphyrin radical via an intramolecular electron transfer from the MYW radical, generating the KatG-unique compound I* (i.e. \( \text{Fe}^{IV}=O[\text{MYW}^{\cdot\cdot}] \)). Two paths have been proposed for the subsequent reaction of compound I* with \( H_2O_2 \) and return to the resting state. One posits formation of a ferric-superoxide complex known in peroxidase vernacular as compound III. Because of the presence of the MYW radical, this is referred to as compound III* (i.e. \( \text{Fe}^{III}=O[\text{MYW}^{\cdot\cdot}] \)). Intramolecular electron transfer from the \( \text{Fe}^{IV}=O \) heme to the MYW radical is proposed to return the enzyme to its ferric state along with the release of \( O_2 \) (38–40). Alternatively, others have proposed that \( H_2O_2 \) is oxidized to the peroxyl radical by the \( \text{Fe}^{IV}=O \) heme center of compound I*. Deprotonation of the indole of the MYW radical then permits formation of an MYW perhydroxy–indole intermediate followed by a bridged \( \text{Fe}^{III}=O-\text{MYW} \) complex, which then decomposes to release \( O_2 \) and return to the starting state (44).

The arginine switch is invariant among KatGs (Arg-418 in \( Mt\) KatG), and its conformation is pH-dependent (48–52). Structures solved for a bacterial KatG at pH 8.5 show the guanidinium moiety of the switch forms a salt bridge with the tyrosyl phenoxide anion of the MYW cofactor (in Fig. 1). At pH 4.5, the arginine side chain is oriented away from the MYW adduct and toward the surface of KatG (out Fig. 1). At pH 6.5, corresponding roughly to the optimum pH for catalase activity, this arginine equally populates both conformational states (48, 49). Substitution of Arg-418 with Leu, Ala, or Asn, but not Lys, sharply diminishes the catalase activity of KatG (39, 49, 50, 52), and as has been recently shown with \( Magnaporthe grisea \) extracellular KatG, it eliminates the pH dependence of catalase activity (52). The mechanism by which Arg-418 facilitates catalatic turnover is still under investigation. In general, its occupation of the out conformation is connected with MYW oxida-

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**Figure 1.** Superposition of KatG and CcP active sites as well as a putative superposition of the catalase and peroxidase catalytic cycles of KatG. KatG is shown with carbons in cyan, and CcP is shown with carbons in gray. The active-site waters (red spheres) and heme are from KatG, and the two conformations (in and out) of the arginine switch (R418) are shown. KatG numbering is according to the \( M. tuberculosis \) enzyme \( Mt\) KatG. This image was generated using MacPyMOL 1.6.0.0 (77) using coordinates from Protein Data Bank code 2CCA (78) and 2CYP (24) for \( Mt\) KatG and yeast CcP, respectively. Putative superposition of the catalase and peroxidase cycles of KatG is shown in the inset. The reaction common to both (compound I formation) is shown with the bold gray arrow. Additional steps of classical peroxidase activity are shown with thin arrows. The additional steps of catalase activity are shown in bold arrows, including two proposed mechanisms (red versus blue) for the return of compound I* to the starting state.
tion, whereas the in conformation is connected with reduction of the MYW radical (39, 44, 45, 49, 53). Interestingly, a recent computational study suggests that Arg-418 facilitates the rotation of the Tyr and Trp aromatic rings with respect to one another, helping enable reduction of the MYW radical by the FeIII–O2•− heme (45).

What then is the place of peroxidase activity and PxEDs in KatG catalysis? As both catalase and peroxidase catalytic mechanisms involve the H2O2–dependent formation of compound I, the only difference between the two activities is the route by which the enzyme returns to the ferric state. As outlined above, catalase turnover requires the oxidation of a second H2O2; however, with peroxidases an exogenous (usually aromatic) electron donor is oxidized instead. Typically, compound I is reduced by one electron to produce compound II (FeIV=O) and the corresponding PxED radical (Fig. 1, inset). A second single-electron transfer returns the enzyme to the ferric state and produces a second equivalent of the PxED radical. According to this model, one would anticipate that peroxidase and catalase activities should be mutually antagonistic, and in particular, PxEDs should inhibit catalase activity. Indeed, the first published report on a catalase-peroxidase showed that the classical peroxidase electron donor o-dianisidine did inhibit catalase activity at pH 7 (54). However, we have recently shown that a number of PxEDs can stimulate the catalase activity of KatG by over an order of magnitude (55). Interestingly, this synergistic effect is most prominent at lower pH (i.e. ~pH 5) and ~1 mM H2O2, conditions that coincide with antimicrobial defenses like the neutrophil-based oxidative burst. Clearly, the inter-relationship between the catalatic and peroxidatic mechanisms of KatG is more complex than has been previously appreciated.

In this report, we investigate the mechanism by which PxEDs stimulate the catalatic activity of KatG. At low pH and in the absence of a PxED, O2 production ceases well short of the expected catalase stoichiometry, and only the addition of more enzyme is able to restart O2 generation. There is also a substantial lag between the conclusion of H2O2 consumption and the return of the enzyme to its ferric state, which suggests that catalase-inactive intermediates accumulate during multiple turnovers. Proposed KatG catalase mechanisms require precise intramolecular electron transfer from the MYW cofactor (44, 45). We surmised that inactivation may occur due to off-catalase electron transfer, a problem that could be resolved by inclusion of a PxED. Furthermore, because the enzyme’s proximal tryptophan (W321, Fig. 1) has shown a propensity (observed experimentally and computationally) toward oxidation to form a radical intermediate (53, 56–59), we also surmised that the proximal Trp is a likely participant in off-pathway electron transfers leading to catalase-inactive intermediates. To test these hypotheses, we replaced the proximal tryptophan with non-oxidizable phenylalanine (W321F KatG) and compared it with wild-type KatG (wtKatG). Optical stopped-flow, rapid freeze-quench EPR, and steady-state kinetic analyses of both proteins were carried out in the presence and absence of PxEDs. Our results suggest that catalase-inactive intermediates accumulate due to off-mechanism oxidation, primarily of Trp-321, and PxEDs stimulate KatG catalase activity by preventing the accumulation of inactive inter-

![Figure 2: Effect of ABTS (a PxED) on the rate and amount of catalatic O2 production by KatG.](image)

**Results**

**Inactivation of KatG during catalatic O2 production**

We monitored O2 production by KatG upon reaction with 0.5 mM H2O2 at pH 5 (Fig. 2), the optimum for PxED-stimulated KatG catalase activity. Notably, the amount of O2 produced (~0.1 mm, ~40% yield) was substantially below that anticipated from the 2 H2O2 to 1 O2 stoichiometry of the catalase reaction. Addition of another 0.5 mM H2O2 at the conclusion of O2 production did not result in any new O2 generation, but a second addition of KatG produced a new burst of O2 evolution. In contrast, inclusion of 0.1 mM ABTS (a PxED) in the reaction caused 10-fold higher rates of O2 production as well as the H2O2 to O2 stoichiometry expected from catalase activity. Furthermore, an additional injection of enzyme generated no new O2, but addition of another 0.5 mM H2O2 resulted in new O2 production. Thus, in the absence of ABTS, KatG was inactivated during turnover, leaving a substantial portion of H2O2 unreacted, but in the presence of ABTS, KatG remained active such that the H2O2 added to the reaction was completely depleted.

Catalase activity could be recovered by adding ABTS after H2O2; however, the longer KatG reacted with H2O2 in the absence of ABTS, the less activity could be recovered upon addition of ABTS (Fig. 2, inset). These data suggest that PxEDs intervene relatively early in the process of inactivation and prevent the accumulation of irreversibly inactivated KatG.

**Conclusion of H2O2 consumption versus return of resting KatG**

Optical stopped-flow reactions of 3 μM KatG with 667 molar eq of H2O2 at pH 5.0 showed that H2O2 consumption (240 nm)
ceased at about 6 s, but more than 50 s were required for the ferric (i.e. resting) enzyme (408 nm) to completely reemerge. This slow return of the ferric state ($k_{\text{obs}} \sim 0.055$ s$^{-1}$) was not kinetically competent to account for the more than 500 molar eq of H$_2$O$_2$ consumed within the first 6 s of the reaction. These data indicate that catalase-inactive intermediates accumulate during turnover and that they are slow to return to the resting state.

**wtKatG versus W321F: H$_2$O$_2$ consumption and corresponding heme states**

Under typical catalase assay conditions (i.e. pH 7.0), our W321F KatG showed kinetic parameters nearly identical to the wild-type enzyme (Table 1). However, at pH 5.0, W321F KatG showed greater catalase activity, particularly at higher H$_2$O$_2$ concentrations. Reaction of W321F KatG with 2 mM H$_2$O$_2$ resulted in the same initial rate of H$_2$O$_2$ consumption as that observed for wtKatG (Fig. 3, inset). However, the rate of peroxide consumption decreased more rapidly for W321F KatG than the W321F variant such that it took wtKatG roughly four times longer than W321F to consume a comparable quantity of H$_2$O$_2$. Furthermore, the return to the ferric state after H$_2$O$_2$ was consumed was nearly 10-fold faster for W321F ($k_{\text{obs}} \sim 0.47$ s$^{-1}$) than for wtKatG ($k_{\text{obs}} \sim 0.055$ s$^{-1}$) (Fig. 3).

Wild-type and W321F KatG showed nearly identical spectra 2.5 ms after mixing with 2.0 mM H$_2$O$_2$, including a Soret $\lambda_{\text{max}}$ at 416 nm and Q band maxima at 542 and 578 nm (Fig. 4, *blue traces*). A notable feature of the spectrum relative to the enzyme’s resting, ferric state is the lack of absorbance above 600 nm. The charge-transfer band, a hallmark of the ferric state, is absent and is not replaced by another absorption feature in spectra captured during KatG’s most rapid catalytic H$_2$O$_2$ decomposition.

For wtKatG at the time corresponding to H$_2$O$_2$ depletion (6 s), the most obvious spectral change was a broad and substantial increase in absorption at wavelengths above 520 nm (Fig. 4A) with bands evident at 546 and 583 nm and a slight deflection at $\sim 650$ nm. There was also a small decrease in Soret band intensity at 416 nm. This same spectral progression has been observed across a range of KatGs upon reaction with H$_2$O$_2$ (38, 42). Therefore, a species whose features most closely resemble a Fe$^{IV}$-O$^{\cdot}$O$^{\cdot}$ state dominates during the most rapid catalytic H$_2$O$_2$ consumption, and this gives way to a ferryl-like species that dominates at the time of H$_2$O$_2$ depletion. Indeed, a species with identical spectral features to the latter has been generated directly in several KatGs by reaction with peracetic acid (PAA) (42, 60, 61). This has been assigned through a combination of EPR and X-ray crystallographic studies as a ferryl-oxo (most likely protonated) species with a protein-centered radical where the proximal Trp has been identified as a prominent site of oxidation (i.e. Fe$^{IV}$–OH[Trp-321]$^\dagger$ by MtKatG numbering) (49, 56, 57).

Spectra observed for W321F KatG at its time of H$_2$O$_2$ depletion (Fig. 4B) were distinct from the wild-type enzyme. There was a substantial decrease in Soret intensity along with a blue-shift to about 410 nm. Similar to wtKatG, there was a broad non-descript increase in absorption intensity above 520 nm, but there were no distinct absorption bands at 546 and 583 nm, and the shoulder near 650 nm was more pronounced. These
features have been observed in *M. tuberculosis* W321F KatG (and the equivalent variant from other species) following reaction with organic hydroperoxides (e.g. PAA) (60, 62). These features are consistent with a FeIV=O[porphyrin]± (i.e. compound I) species (25).

**wtKatG protein-based radicals upon reaction with H₂O₂ alone**

The EPR spectrum of wtKatG freeze-quenched after a 10-ms reaction time with H₂O₂ showed a complete loss of signals corresponding to the ferric heme (Fig. 5A) concomitant with formation of an intense doublet radical signal (Fig. 5B). Taking into account the partial power saturation of the radical signal at 4.5 K and 1 milliwatt of microwave power (Fig. 6A), this radical is in agreement with the so-called narrow doublet assigned by others as the cation radical of the MYW (38–40). This is in agreement with the so-called narrow doublet assigned by others as the cation radical of the MYW (38–40). This is in agreement with the so-called narrow doublet assigned by others as the cation radical of the MYW (38–40). This is in agreement with the so-called narrow doublet assigned by others as the cation radical of the MYW (38–40). This is in agreement with the so-called narrow doublet assigned by others as the cation radical of the MYW (38–40). This is in agreement with the so-called narrow doublet assigned by others as the cation radical of the MYW (38–40). This is in agreement with the so-called narrow doublet assigned by others as the cation radical of the MYW (38–40). This is in agreement with the so-called narrow doublet assigned by others as the cation radical of the MYW (38–40). This is in agreement with the so-called narrow doublet assigned by others as the cation radical of the MYW (38–40). This is in agreement with the so-called narrow doublet assigned by others as the cation radical of the MYW (38–40). This is in agreement with the so-called narrow doublet assigned by others as the cation radical of the MYW (38–40). This is in agreement with the so-called narrow doublet assigned by others as the cation radical of the MYW (38–40).

At the time of H₂O₂ depletion (6 s), the ferric state was still absent (Fig. 5A), but the doublet radical was replaced by a singlet signal (Fig. 5B). In contrast to the narrow doublet, this signal was very broad (>300 G) (Fig. 6B), suggesting that there was exchange coupling with the heme iron. A very similar exchange-coupled radical species has been observed with KatG from *M. tuberculosis* and *Burkholderia pseudomallei* upon their reactions with PAA. In both cases, this radical has been assigned to the proximal Trp (Trp-321 and Trp-330, respectively) (56, 57).

When the reaction was quenched 1 min after mixing with H₂O₂, a signal corresponding to the ferric state (g ~6) was clearly present (although at diminished intensity compared with the resting state) (Fig. 5A) along with a singlet protein-based radical (Fig. 5B). Some broadening, although considerably less prominent than the species observed at 6 s, was evident (Fig. 6B). These data in addition to the power and temperature of the signal at 6 s (Fig. 6C) are consistent with multiple species being observed in both the 6-s and 1-min samples. In the 6-s sample, a large fraction of the observed signal arose from the exchange-coupled Trp-321 radical, and in the sample quenched at 1 min, the Trp-321 radical had migrated to more remote oxidizable amino acid residue(s).

The power saturation profiles for the narrow doublet, 6-s sample, and 1-min sample are shown in Fig. 6D. As expected, the MYW ± is very easily saturated under these conditions with a smaller P½ of 6.4 μW. For the 6-s sample, the broad feature, measured at 3300 G, exhibits an extremely large P½ of 16 milliwatts, consistent with its assignment to an exchange-coupled radical. If the power saturation of the 6-s sample is measured from peak-to-trough, the P½ is ~12 μW, and the data show relatively poor fitting, suggesting that there are two overlapping signals here and that the 12-μW value should be regarded as an upper limit for P½ for the second signal. The P½ value of the sample quenched at 1 min is 7.5 μW, intermediate between the MYW ± and the organic radical seen in the 6-s sample and is indicative of a mixture of amino acid-based radicals.

In samples quenched 5 min after reaction with H₂O₂, a greater contribution from the ferric state (Fig. 5A) was observed along with a protein-based radical similar to that detected at 1 min albeit at lower intensity (Figs. 5B and 6B). Thus, freeze-quench EPR data are in good agreement with our stopped-flow results where the re-emergence of the ferric state is only observed after the depletion of H₂O₂ (at 6 s post-mixing), and the rate of return is too slow to account for the preceding catalytic consumption of >500 eq of H₂O₂. These data suggest that the primary catalase-inactive intermediate to accumulate contains a protein-based radical distinct from the MYW ± narrow doublet. The exchange coupling observed from this species is consistent with the proximal tryptophan (Trp-321) as the site of protein radical accumulation with subsequent radical migration away from the active site.

**W321F KatG protein-based radicals formed upon reaction with H₂O₂ alone**

Reaction of W321F KatG with the same concentration of H₂O₂ produced a nearly identical doublet radical spectrum at 10 ms (Fig. 5C). Similar to wtKatG, the narrow doublet was replaced by a protein-based singlet radical species at the time anticipated for the completion of H₂O₂ consumption (1.6 s for W321F versus 6 s for wtKatG). However, in contrast to the wild-type enzyme, the radical generated by W321F lacked any broadening that would indicate exchange coupling with the heme iron (Fig. 7A). Although a radical species was detected nearly a full minute later, its intensity in the variant was far less than that observed for the wild-type enzyme at the same time.
Proximal tryptophan and inactivation of KatG catalase

Figure 5. EPR spectra of freeze-quenched samples from reaction of WT and W321F KatG with H$_2$O$_2$. High-spin ferric species typical of the wtKatG resting state ($g = 6$) are shown in A, the $g$, component of the resting state as well as protein-based radicals ($g = 2$) of wtKatG and W321F KatG are shown in B and C, respectively. The molar proportions of enzyme to H$_2$O$_2$ used for stopped-flow experiments (1:667) were maintained for these experiments. Consequently, ferric enzyme (150 $\mu$M after mixing) was reacted with H$_2$O$_2$ (100 mM after mixing) for the time indicated prior to freeze-quenching. Reactions were carried out at 25 °C in 100 mM acetate, pH 5.0. All spectra were recorded at 4.5 K. Spectrometer settings were as described under “Experimental procedures.” Freeze-quenched samples were generated at least twice with protein from different purifications. Spectra for a given time point were indistinguishable across both experiments.

Effect of PxEDs on wtKatG and W321F

Inclusion of the PxED, ABTS, stimulated the consumption of H$_2$O$_2$ as well as the subsequent return of the ferric state of WT and W321F KatG; however, the effect was far more pronounced for WT (Fig. 8). Consistent with these data, we observed that ABTS increased the wtKatG catalase $k_{cat}$ by 8-fold compared with only 3-fold for the W321F variant, with the same trend observed for $k_{cat}/K_m$ (Table 1). We have noted previously that several PxEDs (e.g. 3,3′,5,5′-tetramethylbenzidine) are able to stimulate wtKatG catalase activity to a level that equals or exceeds ABTS (55). These PxEDs all produced a significantly lower stimulation of W321F KatG catalase activity (data not shown).

To examine the effects of PxEDs on the intermediates formed for wild-type and W321F KatG, similar freeze-quench EPR experiments were performed as those presented above. With PxED (ABTS/ascorbate) included in the reaction mixture, WT and W321F KatG both showed the same narrow doublet 10 ms after mixing with H$_2$O$_2$ as was observed without PxED (Fig. 9, A and B). Spectra recorded for samples quenched at the time anticipated for H$_2$O$_2$ depletion or any time thereafter showed minimal, if any, contribution from protein-based radicals for either wild-type or W321F KatG (Fig. 9, C and D).

Discussion

Off-pathway electron transfer through Trp-321

Our data suggest that inactivation of KatG during catalatic consumption of H$_2$O$_2$ occurs due to off-pathway intramolecular electron transfer. Several lines of evidence suggest the proximal Trp (Trp-321 in MtKatG) is a prominent site to initiate these radical transfer events. An exchange-coupled radical consistent with that previously assigned as Trp-321$^{+}$ (56, 57) accumulates and is the dominant radical as H$_2$O$_2$ consumption ceases. This signal is not observed when W321F KatG, an enzyme with robust catalase activity, is reacted with H$_2$O$_2$. The proximal Trp has been shown in several other contexts to be susceptible to oxidation. Reactions of KatGs with PAA routinely generate compound I species, which include a radical at this position (e.g. Fe$^{IV}$=O[Trp-321]$^{+}$ in MtKatG) (56, 57). Calculations have shown that KatG compounds I (i.e. two-electron oxidized KatG) have substantial spin density associated with the proximal Trp, a result that is especially prominent when the arginine switch is oriented away from the MYW adduct (see R418 out in Fig. 1) (53). Interestingly, the out conformation for the arginine switch is known to increasingly dominate as the pH decreases below 6; this is where KatG has the greatest tendency toward inactivation and where we have observed that inclusion of a PxED shows the greatest stimulatory effect on KatG catalase activity. Finally, CcP, one of KatG’s closest superfamily relatives, forms the well-established compound ES (i.e. Fe$^{IV}$=O[Trp-191]$^{+}$) upon reaction with H$_2$O$_2$ (58, 59), where Trp-191 is CcP’s proximal Trp (see Fig. 1).

Scavenging of off-catalase KatG protein-based radicals by PxEDs

A variety of PxEDs do not inhibit but instead substantially stimulate KatG catalase activity (55). Accordingly, we show here that inclusion of a PxED does not diminish (if anything, it enhances) the formation of the MYW adduct radical associated with catalase turnover, not only in wtKatG, but also in the W321F variant. This confirms what has been surmised from KatG’s very narrow active-site access channel (20–22). Specifically, PxEDs like ABTS cannot directly access the active site, but rather must act from some exterior site(s).

Strikingly, PxEDs essentially abrogate the accumulation of all other protein radical species. This includes the exchange-coupled Trp-321$^{+}$ radical; those that are observed well after H$_2$O$_2$...
consumption has ceased, and the alternative radical species that are detected when W321F KatG reacts with H$_2$O$_2$. Combined with its inability to reduce the MYW, this implies that PxED (e.g., ABTS) oxidation occurs by a through-protein electron hole-hopping mechanism. Consequently, PxED oxidation (and by extension peroxidase activity) requires a preceding off-catalase radical transfer event, the first step of which appears to be oxidation of the proximal Trp. This mechanism produces an interesting reciprocity between KatG’s catalase and peroxidase activities. Off-catalase electron transfers result in PxED oxidation (i.e., peroxidase activity), which simultaneously reduces protein radicals and prevents further protein oxidation. This ultimately preserves catalase activity to enable the full depletion of H$_2$O$_2$. Indeed, our data show that in the presence of ABTS, there is no loss of activity even following 50,000 turnovers per active site (Fig. 2). Interestingly, PAA-dependent isoniazid (INH) oxidation by MtKatG also appears to be mediated by an electron hole-hopping mechanism and to occur at or near the protein surface (56). This raises the intriguing possibility that in the presence of the physiological substrate...
H₂O₂ depletion) were recorded at 4.5 K. All spectrometer settings were as during H₂O₂ decomposition by KatG even in the absence of a KatG protein oxidation in the absence of a PxED. Such is evident in our EPR spectra as the exchange-coupled W321.

More than this, the structure of KatG is one that almost seems to invite through-protein radical migration. The active-site-bearing N-terminal domain has an extraordinarily high content of oxidizable amino acid side chains (18 Trp, 4.2%; 16 Tyr, 3.7%; 14 Met, 3.2%; 2 Cys, 0.5%; 50 total, 11.6%). Hole-hopping electron transfer has recently been proposed and explored as a broadly used strategy for preserving the activity of redox enzymes (63–65); it is one that KatG appears particularly well-equipped to utilize. Indeed, reactions of KatGs with peroxides are known to generate a variety of protein-based radicals and a range of advanced protein-oxidation products. As summarized in Fig. 10A, these include radicals identified in at least one KatG at the equivalent of Trp-91, Trp-135, Trp-321, and Trp-149 (56, 57, 66) as well as oxygenation of Trp-438, Trp-135, Met-377, Met-255/257, and Met-176 (61). Similarly, oxidative cross-linking/oligomerization has been observed, ostensibly arising from exterior Tyr- and/or Trp-based radicals (39, 67). It is reasonable to surmise that sacrificial KatG protein oxidation may substantially prolong catalytic H₂O₂ decomposition, but our data show that in the absence of a PxED, KatG eventually undergoes irreversible inactivation. Under the conditions of Fig. 2 (i.e. 0.5 mM H₂O₂, pH 5.0), 5 nM KatG generated 100 μM O₂ over the course of the reaction, indicating that irreversible inactivation occurred after roughly 20,000 turnovers.

Functional significance of redox chain overlap: KatG and CcP

Using the algorithms of Gray and Winkler (63), we note that the large majority of KatG residues known to undergo oxidative modification fit into one of four redox chains (Fig. 10A). In the context of this study, the chain that includes Trp-321 is particularly interesting. As one of KatG’s closest relatives, CcP is also rich in oxidizable amino acids (Trp + Tyr + Met + Cys = 27 or 9.2%). Nevertheless, there is a surprising lack of conservation of oxidation sites with only 11 in common between the two enzymes, including four instances where Trp replaces Tyr or vice versa (Fig. 10, B and D). Interestingly, seven of the conserved oxidation sites are found proximal to the active-site heme. Among these, an electron hole-hopping path can be discerned that connects to the enzyme’s surface and includes Trp-321 (heme–Trp-321–Met-377–Tyr-353 and/or Trp-341) (Fig. 10A). The proximal Trp has been observed in both proteins to be oxidized to a radical intermediate (56–58). Met-377 (Met-231 in CcP) lies 3.9 Å from the proximal Trp, and in both KatG (61) and CcP (65), this Met is oxygenated upon reaction with peroxides. Both Tyr-353 (Trp-223 in CcP) and Trp-341 (Trp-211 in CcP) form part of a shallow solvent-accessible cleft ~17 Å below the heme plane. In CcP, both Trp-223 and Trp-211 are oxygenated following the enzyme’s reaction with H₂O₂ (65), and in MtKatG, NO has trapped a radical centered at Tyr-353 (68).

The conservation of this electron transfer conduit points to use of a shared electron transfer structure to support divergent redox protein function. Of course, CcP is a monofunctional peroxidase, but it is unique in the peroxidase-catalase superfamily in that it uses a large protein-based substrate (ferrocytochrome c) as a PxED. This precludes direct access of the substrate to the CcP heme and necessitates through-protein radical transfer, which includes the enzyme’s proximal tryptophan (Trp-191). In the case of KatG, PxEDs are precluded from access to the heme because the channel to the active site is

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**Figure 9. Comparison of EPR spectra for protein-based radicals 10 ms after mixing with H₂O₂ (A and B) and at H₂O₂ depletion (C and D).** Spectra were recorded for wtKatG (A and C) and the W321F variant (B and D) in the absence (blue lines) and presence of ABTS/ascorbate (red lines). Reaction conditions, sample preparation, and evaluation were as described for Fig. 5. When included, the concentrations of ABTS and ascorbate were 1.0 and 2.0 mM, respectively. Spectra shown in A and B (10 ms after mixing with H₂O₂) were recorded at 77 K, minimizing power saturation of the radical observed at 4.5 K (see Figs. 5, B and C, and 6A). Spectra shown in C and D (at the time of H₂O₂ depletion) were recorded at 4.5 K. All spectrometer settings were as described under “Experimental procedures.”

H₂O₂. INH activation may be facilitated by the synergistic cooperation of KatG’s catalase and peroxidase activities.

**KatG protein oxidation in the absence of a PxED**

Of course, through-protein electron hole-hopping occurs during H₂O₂ decomposition by KatG even in the absence of a PxED. Such is evident in our EPR spectra as the exchange-coupled W321⁺ observed at the time of H₂O₂ depletion is replaced at later reaction times by radicals that lack a close interaction with the heme iron. Similarly, at temperatures where the exchange-coupled W321⁺ signal is not detected (e.g. 77 K), EPR spectra reveal that more remote protein-based radicals are also present at the time of H₂O₂ depletion (Fig. 6C, inset). An essentially identical phenomenon has been observed when KatG is reacted with PAA (56). It is important to bear in mind that PAA is a hydroperoxide that cannot support the full catalase cycle, and as such it makes off-catalase electron transfer unavoidable.

More than this, the structure of KatG is one that almost seems to invite through-protein radical migration. The active-site-bearing N-terminal domain has an extraordinarily high content of oxidizable amino acid side chains (18 Trp, 4.2%; 16 Tyr, 3.7%; 14 Met, 3.2%; 2 Cys, 0.5%; 50 total, 11.6%). Hole-hopping electron transfer has recently been proposed and explored as a broadly used strategy for preserving the activity of redox enzymes (63–65); it is one that KatG appears particularly well-equipped to utilize. Indeed, reactions of KatGs with peroxides are known to generate a variety of protein-based radicals and a range of advanced protein-oxidation products. As summarized in Fig. 10A, these include radicals identified in at least one KatG at the equivalent of Trp-91, Trp-135, Trp-321, and Trp-149 (56, 57, 66) as well as oxygenation of Trp-438, Trp-135, Met-377, Met-255/257, and Met-176 (61). Similarly, oxidative cross-linking/oligomerization has been observed, ostensibly arising from exterior Tyr- and/or Trp-based radicals (39, 67). It is reasonable to surmise that sacrificial KatG protein oxidation may substantially prolong catalytic H₂O₂ decomposition, but our data show that in the absence of a PxED, KatG eventually undergoes irreversible inactivation. Under the conditions of Fig. 2 (i.e. 0.5 mM H₂O₂, pH 5.0), 5 nM KatG generated 100 μM O₂ over the course of the reaction, indicating that irreversible inactivation occurred after roughly 20,000 turnovers.

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The conservation of this electron transfer conduit points to use of a shared electron transfer structure to support divergent redox protein function. Of course, CcP is a monofunctional peroxidase, but it is unique in the peroxidase-catalase superfamily in that it uses a large protein-based substrate (ferrocytochrome c) as a PxED. This precludes direct access of the substrate to the CcP heme and necessitates through-protein radical transfer, which includes the enzyme’s proximal tryptophan (Trp-191). In the case of KatG, PxEDs are precluded from access to the heme because the channel to the active site is
particularly narrow. As mentioned above, this permits the mutually synergistic or reciprocal operation of the catalase and peroxidase activities of KatG. With either enzyme, in the absence of an appropriate PxED, the protein itself becomes an electron source to forestall, for a limited time, irreversible inactivation.

**Integrating KatG catalysis: catalase, peroxidase, inactivation, and PxED-based recovery**

A model summarizing our observations is presented in Fig. 11. Following its formation (paths a), the dominant pathway for reaction of compound I (i.e. FeIV = O[porphyrin]²⁺) is formation of compound I⁺ (i.e. FeIV = O[MYW]⁺) and the subsequent catalytic oxidation of H₂O₂ (paths b). Occasionally (about once every 140 turnovers), the porphyrin radical is instead reduced by the proximal Trp (Trp-321) to form FeIV = O[Trp-321]⁺ (reactions c). Indeed, with relatively few equivalents of H₂O₂ (~650), Trp-321⁺ accumulates and is the dominant radical species at the time of H₂O₂ depletion (see Figs. 5B and 6B). This is mirrored by a previous report showing a decrease in MYW⁺ signal with time during reactions with H₂O₂ (38). KatG’s catalase activity exceeds its peroxidase activity by about 2 orders of magnitude. We have noted that KatG “peroxidase activity” may be more accurately described as a PxED-dependent stimulation of catalase activity, a mechanism that requires relatively infrequent PxED oxidation (e.g. 0.007 ABTS⁺ generated for every H₂O₂ consumed) (55). Based on our present results, we suggest that each PxED oxidation event (e.g. ABTS⁺) reports correction of off-catalase radical transfer. As such, we estimate that the frequency of catalatic H₂O₂ oxidation (paths b) versus off-catalase radical transfer (paths c) is about 140 to 1.
Radical transfer away from the active site toward the KatG surface (paths d) regenerates the ferric heme, which can then engage in additional catalase turnover (paths a and b). Consistent with this notion, remote protein-based radicals are observed at later times as the ferric state slowly returns (Fig. 5B and 6B). Additional turnover produces additional iterations of off-pathway electron transfer, resulting in advanced protein oxidation products. In our reaction scheme, this is represented as a vertical descent via repeated iterations c and d.

Our data suggest that it is a ferryl heme from which the ferric state returns upon depletion of H$_2$O$_2$. The spectrum that progressively develops as catalatic H$_2$O$_2$ consumption proceeds (Fig. 4A) is indistinguishable from the Fe$^{IV}$-OH[protein]$^*$ state that is directly generated when KatGs are reacted with organic hydroperoxides (42, 49, 56, 57, 60). The slow recovery of the resting state from these ferryl states is anticipated, particularly as protein-based electron sources become depleted through oxygenation and other oxidative modifications (i.e. vertical descent in Fig. 11 through repeated exploitation of paths c and d). With the relatively low H$_2$O$_2$ concentration examined by stopped-flow (667 molar eq), the ferric state returns with a $k_{obs}$ of about $5.5 \times 10^{-2}$ s$^{-1}$ (see Fig. 3). Notably, when a PxED is present during H$_2$O$_2$ consumption, little if any ferryl state accumulates, instead, the Fe$^{III}$--O$_2^*$-like spectrum of the catalase active state reverts rapidly and directly to ferric KatG (55).

A PxED, when present, does not directly access the active site. Rather a radical transfer pathway(s) delivers oxidizing equivalents via Trp-321 to an exterior site(s) where electron transfer from the PxED to protein radicals (Trp, Tyr, etc.) can occur (Fig. 11, paths c to d to e). Consequently, a PxED is effective at remediating the early stages of KatG protein oxidation, preventing progression to advanced/irreversibly oxidized states. Indeed, the capacity of a PxED to recover KatG catalase activity upon its addition diminishes exponentially the longer KatG reacts with H$_2$O$_2$ alone (see Fig. 2, inset).

All our data point toward the proximal tryptophan (Trp-321) as the primary starting point for off-catalase electron transfer. Although substantially less than wild type, PxEDs do show a modest stimulatory effect on W321Fcatalase activity upon its addition diminishes exponentially the longer KatG reacts with H$_2$O$_2$ alone (see Fig. 2, inset).

In conclusion, the KatG proximal Trp-321 is a starting point for off-catalase radical transfer, the continuation of which even-

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**Figure 11. Proposed mechanism for inactivation of KatG catalase and its prevention by a PxED.** The KatG catalase cycle is initiated by H$_2$O$_2$ oxidation of ferric heme to the compound I (i.e. Fe$^{III}$=O[porphyrin]$^-$) state (reactions a). The catalatic return of compound I to the ferric state (abbreviated from Fig. 1) proceeds by paths b, the first step of which is the oxidation of the MYW adduct to its radical cation state (MYW$^+$) (not shown). Alternative off-catalase oxidation of the proximal Trp (W$_{321}$) is shown by reactions c. The relative thickness of the arrows for b versus c indicates that MYW oxidation and subsequent completion of the catalase cycle (b) occurs with a much greater frequency than Trp-oxidation (c) ($\sim$140:1). The return of ferric heme following off-catalase electron transfer occurs by hole-hopping (i.e. radical transfer indicated by red half-arrows) to KatG-oxidizable residues more distant from the active site (i.e. P$_{ext}$), producing the corresponding protein-based radicals (P$_{ext}^*$) (paths d). Horizontal transitions correspond to catalase activity. Each vertical transition is a step in the progressive oxidation of the KatG protein. In the early stages protein-based radicals are the dominant oxidation intermediates, as vertical steps continue, more advanced oxidation products (P$_{ext}^3$) and (P$_{ext}^P$) accumulate, including oxygenated Trp and Met, cross-linked Tyr, oxidative protein aggregates, etc. Conceivably, catalase activity can still be maintained even though the KatG protein has sustained some level of exterior oxidation, but eventually, enzyme inactivation results. Inclusion of a PxED intercepts and reduces protein-based radicals on the KatG surface (e), preventing/reversing vertical progression toward inactivation.
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tually produces advanced protein oxidation and irreversible inactivation. However, with a PxED present, radicals transferred by electron hole-hopping via the proximal Trp to the enzyme surface are reduced. This indefinitely preserves KatG catalase activity and simultaneously produces KatG peroxidase activity, greatly increasing the capacity of the enzyme to degrade H$_2$O$_2$. Given the wide use of KatGs by plant and animal pathogens and the pivotal role that PxEDs could play in KatG-dependent detoxification of host-derived H$_2$O$_2$, this phenomenon may hold important implications for understanding host–pathogen interactions.

**Experimental procedures**

**Materials**

Ampicillin, H$_2$O$_2$ (30%), hemin, imidazole, calcium chloride hydrate, sodium dithionite, ABTS, 3,3'-dimethoxybenzidine (o-dianisidine), N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride, 3,3',5,5'-tetramethylbenzidine dihydrochloride hydrate, chlorpromazine, pyrogallol, and L-ascorbic acid hydrate, sodium dithionite, ABTS, 3,3'-tetramethylbenzidine dihydrochloride hydrate, chlorpromazine, pyrogallol, and L-ascorbic acid were from Sigma. Mono- and dibasic sodium phosphate, Benzonase nuclease, and nickel-nitrilotriacetic acid resin were from Agilent (La Jolla, CA). All restriction enzymes and Phusion High Fidelity PCR master mix with GC Buffer were purchased from Invitrogen. All oligonucleotide primers were obtained from Bio-Rad. Centrifugal filtration columns (10DG) were purchased from Bio-Rad. All buffers and media were prepared using water purified through a Barnstead EASYpure II UV ultrapure water system (18.2 megohms/cm resistivity).

Site-directed mutagenesis was carried out by applying the “Round-the-Horn” approach (69) to the construct we use for the expression of wild-type MtKatG. This construct, pMRB11, is a PET23b-derived plasmid bearing the *M. tuberculosis* katG gene and was obtained from the TB Vaccine Testing and Research Materials Contract at Colorado State University. The sense strand primers designed for W321F substitution (5’-GAGGTGTTAATTACAGAACCACCAGCAGAAATGG-GAC-3’) included a site for codon replacement (bold) as well as mutations designed to introduce diagnostic restriction digest sites for screening (italics). This approach allowed us to generate reverse primers without substitutions for W321F 5’-GAT-GCCGCTGTTAGCGTCCTTACC-3’. Both primers were modified by 5’-phosphorylation to allow for blunt-end ligation of PCR products. PCR for generation of the variant was carried out using Phusion High Fidelity polymerase (New England Biolabs, Beverly, MA) in GC Buffer–containing master mix and 3% DMSO. The PCR products were treated with DpnI to eliminate the starting template and ligated using T4 DNA ligase. The ligation products were used to transform *E. coli* (XL-1 Blue) by a standard heat-shock procedure. Transformants were selected using ampicillin-containing media, and candidate plasmids were screened by BsaAI restriction digest. Successful candidates were sent for full DNA sequence analysis (Davis Sequencing, Davis, CA) to verify that the intended mutations were present and that no unintended mutations were generated.

**Protein expression and purification**

*E. coli* C41(DE3) cells bearing the heme protein expression plasmid pHPEX3 (70) were transformed with the appropriate expression construct, and transformants were selected on the basis of tetracycline/ampicillin resistance. Expression of wild-type MtKatG and all variants was carried out as described previously (55). As with wild-type MtKatG, the W321F variant was expressed in a soluble form. Thus, purification was carried out as reported previously (55), with the exception that lysis was carried out by sonication. A Branson 250 sonifier (Danbury, CT) fit with a standard tip was set to constant output and 3.5 duty. Sonication was carried out in eight cycles (42 s on, 42 s off). Benzonase nuclease (250 units) was added to the lysate following sonication.

**UV-visible spectra and activity assays**

Following purification, UV-visible spectra for WT and W321F MtKatG (3 μM each) were evaluated in 100 mM phosphate, pH 7.0, as described previously (55). Molar absorptivities were determined using the pyridine hemichrome assay (71). For WT and W321F KatG, optical purity ratios $A_{348}/A_{291}$ (i.e. $R_2 = 0.62$), the absorption maxima for the Soret band ($\lambda_{max} = 408$ nm), and the two charge-transfer bands (CT2 $\lambda_{max} = 500$ nm; CT1 $\lambda_{max} = 633$ nm) were indistinguishable.

Peroxidase activity was evaluated as described previously (55) by monitoring ABTS (the PxED oxidation to ABTS$^*$ ($\epsilon_{417} = 34.7\text{ mm}^{-1}\text{ cm}^{-1}$) (72). To evaluate the effect of H$_2$O$_2$ concentration on peroxidase activity, ABTS was held constant at 0.1 mM. To determine the effect of ABTS concentration, H$_2$O$_2$ concentration was held constant at 1.0 mM. All peroxidase assays were carried out at room temperature in 50 mM acetate, pH 5.0. The concentration of the enzyme used in all assays was 20 nm.

Unless otherwise specified, catalase activity was evaluated by monitoring O$_2$ production over time using a Clark-type O$_2$-sensitive electrode (Hansatech, Pentney, Norfolk, UK) as described previously (55). In specified experiments, catalase activity was evaluated spectrophotometrically by monitoring a decrease in H$_2$O$_2$ concentration over time at 240 nm ($\epsilon_{240} = 39.4\text{ M}^{-1}\text{ cm}^{-1}$) (73). Analyses of steady-state kinetic data were carried out as described previously (55). As before, wtKatG catalase activity showed a two-component response to H$_2$O$_2$ at pH 5, necessitating the use of Equation 1,

$$v_0 = \frac{k_{cat}[H_2O_2]}{k_{cat}/k_{on} + [H_2O_2]} + k_{app}[H_2O_2]$$

(Eq. 1)

which permits determination of the apparent second-order rate constant ($k_{app}$) for a “high $K_m$” response to H$_2$O$_2$. In addition,
data were fit to directly estimate $k_{\text{cat}} / K_m$ (indicated as $k_{\text{on}}$ in Equation 1) and $k_{\text{cat}}$. In all other instances, a second "high $K_m$" component was not observed, and data were fit to Equation 2.

$$v_o / [E]_o = \frac{k_{\text{cat}} [H_2O_2]}{k_{\text{cat}} / K_m + [H_2O_2]}$$  \hspace{1cm} (Eq. 2)

**Stopped-flow**

Heme intermediates formed by WT and W321F MtKatG under steady-state conditions were observed using a PC-upgraded SX18.MV rapid reaction analyzer from Applied Photo-physics (Leatherhead, UK). As described previously (55), to more clearly observe absorption due to heme intermediates, we included ascorbate to scavenge the radical oxidation products of PxDs (e.g. ABTS$^+$) (55, 74, 75). Single-mixing experiments were set up such that 6 μM wild-type or W321F KatG was placed in syringe A in 5 mM phosphate buffer, pH 7.0. Syringe B contained 0.2 mM ascorbate, 0.2 mM ABTS, and varying concentrations of H$_2$O$_2$ in 100 mM acetate buffer, pH 5.0.

**Freeze-quench preparation of EPR samples**

Wild-type and W321F KatG were each concentrated to ~300 μM using an Amicon Ultra-4 centrifuge filter (cutoff at 50 kDa). One syringe contained ~300 μM enzyme in 5 mM phosphate, pH 7.0, and the other syringe contained 667 molar eq of H$_2$O$_2$ in 100 mM acetate buffer, pH 5.0. Reactions testing the impact of PxD included ABTS (2.0 mM) and ascorbate (4.0 mM) along with H$_2$O$_2$.

EPR samples were prepared by mixing equal volumes of solution from each syringe. Each reaction was quenched by freezing after the appropriate time following mixing. Samples frozen less than 1 s after mixing were quenched by spraying the reaction mixture directly into liquid ethane (~130 °C) by a standard rapid quench procedure using a System 1000 Chemical/Freeze Quench Apparatus (Update Instruments, Inc.), and the reaction time was determined by the length of the aging loop and velocity of the motor driving the syringes. For reactions between 1 and 30 s, the samples were still quenched with liquid ethane; however, a modified flow-pause-flow freeze-quench procedure was used in which the quenching time was determined by the pause duration. For reaction times longer than 30 s, reactions were initiated by hand mixing. The samples were centrifuged to remove excess bubbles from O$_2$ production, transferred to quartz EPR tubes, and quenched manually in cold isopentane (~130 °C). All samples were stored in liquid N$_2$ until analyzed by EPR spectroscopy.

**EPR measurements**

All X-band (9 GHz) EPR spectra were collected using either a Bruker ER200D or an EMX spectrometer operating in perpendicular mode at 100 kHz modulation frequency in a 4119HS resonator. The spectrometer was equipped with an ESR910 liquid helium cryostat and an ITCl035 temperature controller (Oxford Instruments), and spectra were recorded at 4.5 K and/or 77 K. For spectra recorded at 4.5 K instrument parameters, unless otherwise indicated, were as follows: microwave frequency, 9.393 GHz; modulation amplitude, 2 G; modulation frequency, 100 kHz; microwave power, 1 milliwatt; time constant, 163.84 ms; sweep time 335.54 s; number of scans, 1; conversion, 327.68 ms; resolution, 1024 point; harmonic 1st; receiver gain, 1.0 × 10$^3$; and phase, 0 degrees. For spectra recorded at 77 K, all parameters were the same except the modulation amplitude was 1 G, the receiver gain was 1.0 × 10$^3$, and the microwave power was 15.84 μW.

Power saturation for select species was examined by fitting normalized signal intensities using Equation 3,

$$(I/I_o) / (I/I_o)_0 = 1 / (1 + P/P_{1/2})b / 2$$  \hspace{1cm} (Eq. 3)

where $P_{1/2}$ is the microwave power at half-saturation, and $b$ describes the contribution from inhomogeneous broadening (76). For the signal we observed for wtKatG in reactions quenched 6 s after mixing with H$_2$O$_2$, signal intensities at 3300 G as well as peak-to-trough were evaluated. The former was to isolate the contribution of the exchange-coupled species.

**Acknowledgments**—We thank Dr. Jay Winkler for assistance with the Protein Electron Transfer Database. We thank our colleagues Drs. Holly Ellis and Steve Mansoorabadi for helpful discussions, and Dr. Evert Duin for assistance with the EMX EPR spectrometer.

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