Catalase-peroxidases (KatG), which belong to Class I heme peroxidase enzymes, have high catalase activity and substantial peroxidase activity. The Y229F mutant of Mycobacterium tuberculosis KatG was prepared and characterized to investigate the functional role of this conserved residue unique to KatG enzymes. Purified, overexpressed KatG[Y229F] exhibited severely reduced steady-state catalase activity while the peroxidase activity was enhanced. Optical stopped-flow experiments showed rapid formation of Compound (Cmpd) II (oxyferryl heme intermediate) in the reaction of resting KatG[Y229F] with peroxyacetic acid or chloroperoxybenzoic acid, without detectable accumulation of Cmpd I (oxyferryl heme \(\sigma\)-cation radical intermediate), the latter being readily observed in the wild-type enzyme under similar conditions. Facile formation of Cmpd III (oxyferryl enzyme) also occurred in the mutant in the presence of micromolar hydrogen peroxide. Thus, the lost catalase function may be explained in part because of formation of intermediates that do not participate in catalatic turnover. The source of the reducing equivalent required for generation of Cmpd II from Cmpd I was shown by rapid freeze-quench electron paramagnetic resonance spectroscopy to be a tyrosine residue, just as in wild-type KatG. The kinetic coupling of radical generation and Cmpd II formation was shown in KatG[Y229F]. Residue Y229, which is a component of a newly defined three amino acid adduct in catalase-peroxidases, is critically important for protecting the catalase activity of KatG.

Mycobacterium tuberculosis catalase-peroxidase (KatG) is a dimeric dual-function Class I heme peroxidase (1) of special interest in the field of tuberculosis research because of its role in the activation of the important antibiotic isoniazid and in the origins of resistance to this drug because of mutations in KatG. KatGs have high homology to yeast cytochrome c peroxidase (CcP), including conserved distal arginine, tryptophan, and histidine residues and conserved proximal histidine and its hydrogen bonding partner, asparagine (2). Because these conserved residues are of key importance in governing catalytic mechanisms, strong functional homology would be expected among the Class I enzymes. Nevertheless, catalase activity is absent from CcP and ascorbate peroxidase but is very robust in KatGs. The origin of the high catalase activity of KatGs must, therefore, reside in features of these enzymes beyond the conservation of the key active site residues. Mutation of the conserved tryptophan residue gave rise to mutant Escherichia coli and Synechocystis enzymes with poor catalase function (3–5). The origin of this change was assigned to altered binding of the second molecule of hydrogen peroxide required for reduction of compound (Cmpd) I in the catalase cycle. Other mutations have also been shown to interfere with catalytic activity (6–10).

With the first reports of the three-dimensional x-ray crystal structures of two bacterial catalase-peroxidases came the discovery of an unusual three-amino acid adduct involving covalently linked distal side tryptophan, tyrosine, and methionine residues (11, 12). The presence of this adduct, then, may be the key feature that distinguishes KatGs from their homologues (Fig. 1).

Mutation of the tyrosine residue in the amino acid adduct was reported to completely eliminate Synechocystis KatG catalase activity (13). Thus, clues that the adduct plays a unique functional role in KatGs were emerging, and an interesting challenge to explain its special role in catalytic mechanisms was presented. Replacement of the redox active tyrosine with phenylalanine would prevent formation of the adduct in \(M.\) tuberculosis KatG. Any resulting changes in mechanism in this mutant could be probed by us in both optical stopped-flow and rapid freeze-quench EPR experiments to gain insights into mechanistic details absent from current literature on catalase-peroxidases.

Previous studies (14) of \(M.\) tuberculosis KatG have shown that Cmpd I, the classical oxyferryl iron-protoporphyrin IX/\(\pi\)-cation radical species, is formed and is significantly less stable than the Cmpd I formed in plant peroxidases such as horseradish peroxidase. Cmpd II could not be detected, but Cmpd III (oxyperoxidase) is formed when KatG reacts with a large excess of \(H_2O_2\). Similar results are reported for other KatGs (3–5). The formation of Cmpd III in the presence of \(H_2O_2\) indicates that KatG enzymes must cycle through Cmpd II, because the expected pathway to oxyferrous enzyme is through an initial single-electron reaction of Cmpd II with hydrogen peroxide (15). The formation of Cmpd II from Cmpd I in \(M.\) tuberculosis KatG involves an electron transfer-producing tyrosyl radical (16).

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**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents**—All standard chemicals and reagents, including Luria-Bertani (LB) medium, were purchased from Fisher. Isonicotinic acid hydrazide (INH) (Sigma) was recrystallized from methanol before use. PAA (Sigma) (32%) was diluted to 10 mM in potassium phosphate buffer and incubated with 780 units/ml catalase (Roche Applied Science) for 4 h at 37 °C to remove hydrogen peroxide, followed by removal of the enzyme by ultrafiltration. Restriction nucleases, polymerase kinase, DNA ligase, and the Klenow fragment of DNA polymerase were obtained from New England Biolabs, Inc.

**Construction, Expression, and Purification of the Y229F Mutant of KatG**—The plasmid pKAT II was used as an overexpression vector for KatG (17) and as the source for mutagenesis. XL-Blue (recA endA gyrA96 thi-1 hsdR17 supE44 relA1 lacI97 proAB lacZΔM15 Tn10 (Tet’)/Tn5) (Stratagene) was used in the mutagenesis steps. *E. coli* strain UM262 (recA katG::Tn10 pro leu rpsL hsdS30 end3 lacY1) (18) was used for overexpression of both wild-type and mutated KatG proteins. UM262 and pKAT II were both gifts from Stewart Cole (Institut Pasteur, Paris). Mutagenesis was performed by using the QuickChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). This method allows direct mutagenesis of KatG in the expression vector without subcloning in an intermediate vector. The pairs of complementary oligonucleotides (synthesized and purified by Qiagen) were designed to introduce the required mutation. The oligonucleotide pairs were 5′-672GATGGGGCGATGATCTGAGGCACCGGAGG700-3′ and 5′-700CCTCCGGGTTCAGCAGATGCCCATC672-3′ for KatG(Y229F) (a → t mutation, mutated codons are in bold). Mutagenesis was performed according to the manufacturer’s protocol, and the reaction products were transformed into the *E. coli* XL-Blue strain. Sequencing of the mutated katG gene confirmed that only the desired nucleotide substitution occurred (Gene Wiz, Inc.), and the mutated plasmid was transformed into *E. coli* strain UM262 for protein expression. Overexpression of recombinant WT KatG and mutated KatG(Y229F) enzymes and their purification was achieved (as described previously, Ref. 9) in potassium phosphate buffer, pH 7.2. The pure enzymes had optical purity ratios (A<sub>407</sub>/A<sub>570</sub>) greater than or equal to 0.5. SDS gel electrophoresis was carried out under denaturing (SDS-PAGE) and non-denaturing (native PAGE) conditions using an Amer sham Biosciences PhastGel system.

**Enzyme Assays**—Protein concentration, expressed as heme concentration, was determined by using a heme extinction coefficient at 407 nm equal to 100 m<sup>2</sup> m<sup>-1</sup> cm<sup>-1</sup> (19). Catalase and peroxidase activities were determined spectrophotometrically in replicate analyses using initial rates of reactions with hydrogen peroxide or t-butyl hydroperoxide plus o-dianisidine (peroxidase activity) (19, 20). Spectrophotometric measurements were performed on an NT14 UV-Vis spectrophotometer (Aviv Associates, Lakewood, NJ).

**Stopped-flow Optical Measurements**—A rapid scanning diode array stopped-flow apparatus (HiTech Scientific Model SF-61DX2) was used for single- and double-mixing kinetics experiments. Data acquisition and analyses were performed using the KinetAsyst software package (HiTech Scientific). The formation and decomposition of Compounds I, II, and III were followed at 25 °C in reactions of resting WT KatG and KatG[Y229F] with freshly prepared solutions of PAA, CPBA, or H<sub>2</sub>O<sub>2</sub>, all in 20 mM potassium phosphate buffer, pH 7.2. Double-mixing stopped-flow experiments were performed to follow the reaction of Compound II with H<sub>2</sub>O<sub>2</sub> or reducing substrates, as described previously (14).

**EPR Spectroscopy**—Rapid freeze-quench EPR samples were prepared as described previously (16) using an Update Instrument, Inc. Model 1000 chemical-freeze-quench apparatus. WT KatG or KatG[Y229F] (typically 100 μM heme) and peryoxacyclic acid (300 μM) were mixed and aged for the indicated time periods (all in 20 mM potassium phosphate buffer, pH 7.2), followed by freeze-quenching of reaction mixtures in isopentane at −130 °C. Frozen sample powders were packed into precision bore quartz EPR tubes immersed in an isopentane bath and examined with a Varian EPR spectrometer operating at X-band. A finger Dewar inserted into the EPR cavity was used for recording spectra at 77 K. A liquid helium cryostat and Heli-Tran liquid helium transfer system (Advanced Research Systems, Inc., Allen town, PA) were used for EPR at 6–10 K. EPR data acquisition and manipulation were performed as described previously (14). CuII/EDTA in 50% ethylene glycol was used as a standard for spin quantification by double integration of EPR signal intensities. The signal to noise ratio in EPR spectra was improved by signal averaging when necessary. The estimation of spin concentration in freeze-quench samples included application of a packing factor of 0.5 to account for sample dilution by isopentane.

**Isothermal Titration Calorimetry**—The binding of INH to KatG(Y229F) was evaluated by using a MicroCal isothermal titration calorimeter apparatus, as described previously (10).

**RESULTS**

**Characterization of Purified KatG(Y229F)**—The WT KatG and KatG[Y229F] mutant enzymes were produced in *E. coli* using a previously described overexpression system carrying the wild-type *M. tuberculosis* katG gene or the mutated gene (9). Purified mutant enzyme was indistinguishable from WT KatG on SDS-PAGE and in native polyacrylamide electrophoresis gels (data not shown). The optical purity ratio, A<sub>405</sub>/A<sub>280</sub>, of purified KatG[Y229F] was 0.5, similar to WT KatG. The optical spectrum of resting KatG[Y229F] contained shallow features near 540 and 580 nm, which is characteristic of either six-coordinate low spin ferric heme or oxyferrous enzyme, neither of which is evident in the spectrum of WT KatG. The small red shift of the Soret peak (408 nm versus 405 nm in the freshly isolated WT enzyme) (16) is also consistent with a small contribution from low spin heme species (data not shown).

Low temperature EPR spectra of KatG[Y229F] were recorded to gain additional insight into heme structural features in this mutant. The freshly purified mutant enzyme has an EPR spectrum dominated by a rhombic signal with g values of 6.0 and 5.6 (Fig. 2, top spectrum) previously assigned in WT KatG to a six-coordinate heme species (16). In contrast to this, the EPR spectrum of the WT enzyme is dominated by a broader rhombic signal (g<sub>1</sub> = 6.3; g<sub>2</sub> = 5.14; g<sub>3</sub> = 2.0) previously assigned to the major, five-coordinate heme species (Fig. 2, bottom spectrum). A small amount of a second rhombic signal (g<sub>1</sub> = 6.6; g<sub>2</sub> = 5.2; g<sub>3</sub> = 2.0) was observed in both WT and mutant enzymes. For both enzymes, an evolution toward an
axial signal characteristic of six-coordinate high spin heme iron occurred when the enzyme was examined after storage for a few weeks (data not shown), but this phenomenon is not explored further here (16).

The spectrum of freshly purified KatG[Y229F] was also recorded under conditions useful for observation of rhombic low spin heme iron signals (microwave power = 0.1 mW, temperature = 10 K). Under these conditions, no signal characteristic of low spin heme iron was found. This result suggests that the origin of the α and β bands detected in the optical spectrum of KatG[Y229F] is oxyferrous enzyme (Cmpd III; see below), which is non-paramagnetic.

Compared with WT KatG, the catalase activity of KatG[Y229F] was reduced by 1400-fold (2.7 ± 0.3 versus 3800 ± 300 units/mg) under assay conditions using millimolar concentrations of H₂O₂, whereas its peroxidase activity was 3-fold greater than that of WT KatG (2.8 ± 0.4 versus 0.9 ± 0.1 units/mg). The k_{cat}/K_m value for the catalase reaction was reduced from 1 × 10^6 in WT KatG to 4 × 10^2 in KatG[Y229F]. Specific mechanistic insights were provided by the stopped-flow kinetic analyses presented below.

Stopped-flow experiments were used to examine the formation of intermediates and to provide insights into the alterations observed in the steady-state measurements. In these experiments, resting enzyme was mixed with peroxides, and the reaction was followed by monitoring changes in the optical spectrum. Values for k_{obs} were determined by fitting hyperbolic exponential functions to changes in absorbance at a particular wavelength either in the Soret region or at a maximum absorbance in the visible region. For WT KatG, we previously reported the second-order rate constants for Cmpd I formation based on the observed rates using varying concentrations of CPBA (14) or PAA (9). The rate constants were in the range of 10^4 M⁻¹ s⁻¹ for M. tuberculosis WT KatG and two mutants associated with resistance to the antibiotic INH (9, 10, 14).

In WT KatG, the decay of Cmpd I and the return of the enzyme to the resting state were seen without other intermediates detected (14).

For KatG[Y229F], rapid turnover of Cmpd I to Cmpd II and/or Cmpd III was observed. Fig. 3 shows an example of the spectral changes for KatG[Y229F] (10 μM) reacted with PAA (30 μM). The initial spectrum is that of resting enzyme; the intermediate spectrum is characterized by the expected decrease in intensity of the Soret peak consistent with generation of Cmpd I (14). The final spectrum is characterized by a red-shifted Soret peak (416 nm) and the appearance of features near 530 and 560 nm; the CT1 band (near 640 nm) characteristic of the resting enzyme is nearly abolished. The features in the final spectrum are common to those found in horseradish peroxidase Cmpd II or CcP Cmpd I, both of which contain oxyferryl heme but not the porphyrin-π-cation radical (21). Examination of the time courses at 409 nm or 416 nm allowed for evaluation of k_{obs} for formation of Cmpd I and Cmpd II, respectively. Values of k_{obs} for formation of Cmpd I were linearly dependent on the concentration of PAA (3- to 10-fold excess over heme concentration). The second-order rate constant for Cmpd I formation was estimated to be 4 × 10^6 M⁻¹ s⁻¹, which is 330-fold more rapid than the rate for WT KatG (1.21 × 10^5 M⁻¹ s⁻¹) (14) but very similar to the rate reported for Synechocystis KatG[Y249F] (8.0 × 10^6 M⁻¹ s⁻¹) (13).

The rate of Cmpd I formation was also evaluated using CPBA from pH 5.5 to 8.8. For WT KatG, a 3-fold decrease in the second-order rate constant with increasing pH was observed (5.6 × 10^4 versus 2.1 × 10^4 M⁻¹ s⁻¹); for KatG[Y229F], the decrease was more dramatic (6-fold) in the same pH range (5.6 × 10^6 versus 1.0 × 10^6 M⁻¹ s⁻¹). Whether the steeper dependence is associated with a specific change in the contribution of a distal residue to Cmpd I formation remains to be proven.

After Cmpd I formation, the rate of appearance of the features characteristic of Cmpd II was only slightly increased, going from 3-fold to 10-fold excess of PAA concentration (k_obs =

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2 An artificial signal caused by Cu(II) (g = 2.3, g⊥ = 2.1) was occasionally seen under such conditions for the mutant enzyme and for WT KatG.
12.7–14.2 ± 0.5 s⁻¹). The spectral features of Cmpd II persisted for at least 6 min (see below) under these conditions. The spontaneous and rapid evolution of Cmpd I to Cmpd II contrasts with the behavior of the WT enzyme, in which Cmpd II is not seen (14) and is different from the behavior of Synechocystis KatG[Y249F], in which the endogenous conversion to Cmpd II was 23-fold slower (k_{obs} 0.6 ± 0.1 s⁻¹) (13).

When CPBA was used to generate Cmpd I, formation of Cmpd II also was observed, but it decayed within 5 s, unlike the results with PAA (data not shown). These results are consistent with CPBA serving not only to generate Cmpd I, but also as a single electron-reducing substrate for Cmpd II. Single electron processes have been reported previously in reactions of CPBA with horseradish peroxidase (22) but have not been explicitly shown for WT KatG. We show below that Cmpd II in KatG[Y229F] is also reduced in the presence of other substrates, such as ascorbate or INH.

To focus on issues relating to the poor catalase activity of KatG[Y229F], stopped-flow experiments were conducted using H₂O₂. When resting enzyme was mixed with one or two stoichiometric equivalents of H₂O₂, the features of Cmpd II were again detected (data not shown), demonstrating a similar reaction pathway to that found using PAA. With higher peroxide concentrations (100–1000 μM H₂O₂), Cmpd III rapidly developed (Fig. 4A). These features, which include a decreased and red-shifted Soret peak (416 nm) and shoulders at 543 and 580 nm, are closely related to those of horseradish peroxidase Cmpd III and other oxyperoxidases (14, 23–25). To investigate whether the formation of Cmpd III was the result of reaction of H₂O₂ with Cmpd II, a double-mixing protocol was used. Here, KatG[Y229F]-Cmpd II was allowed to form in the first mixing phase from KatG[Y229F] plus PAA (B). Final concentrations were: KatG[Y229F], 10 μM; PAA, 100 μM; H₂O₂, 100 μM.

The catalytic competence of Cmpd II in the mutant enzyme was demonstrated using ascorbate or INH as reducing substrates. Addition of ascorbate or INH in a double-mixing experiment after the generation of Cmpd II in the first mixing phase (resting enzyme plus PAA incubated for 1 s), followed by the addition of H₂O₂ (Fig. 4B). Optical features consistent with the formation of Cmpd III again appeared.

The reaction of INH with Cmpd I in WT and mutant KatGs was shown previously (9, 10, 14). Therefore, the antibiotic serves as a reducing substrate for both peroxidase intermediates in KatG, and the activation of this pro-drug through the peroxidase pathway in M. tuberculosis KatG is substantiated. The mechanisms for such activation have often invoked reaction paths different from the classical peroxidase cycle (26–28).

**Rapid Freeze-quench EPR**—In WT KatG, the kinetics and
stoichiometry of tyrosyl radical formation was studied using rapid freeze-quench-EPR spectroscopy (16). Quenching of this radical by small reducing molecules including INH was also shown. The evolution of the initially formed radical from a structure that exhibited a doublet EPR signal to one that exhibited a singlet was also described. Kinetic evidence suggested formation of a unique tyrosyl radical from an intermediate, assumed to be Cmpd I. Although it is reasonable that electron transfer reduces Cmpd I to Cmpd II concomitant with radical formation, such a correlation could not be followed in optical stopped-flow experiments with WT KatG because Cmpd II was not detected. Here, the optical evidence for accumulation of Cmpd II in KatG[Y229F] suggested that it might result from rapid endogenous electron transfer from tyrosine to Cmpd I, and thus the rapid formation of tyrosyl radical should be evident. This was, in fact, observed.

Fig. 6 shows the results of a rapid freeze-quench-EPR experiment in which KatG[Y229F] was mixed with PAA and frozen at incubation times from 10 ms to 5 s. An initial doublet (hyperfine splitting ≈ 15 G, line-width ≈ 26 G) was observed only at 10 ms or earlier, and a singlet signal, centered at $g = 2.004$, appeared immediately after. The apparent singlet exhibited a progressive line-width reduction from $-20$ G to $-17$ G, most likely because of the disappearance of the doublet species, the contribution of which broadens the signal. In the analogous experiments performed with WT KatG, the same initial doublet signal appeared, but at a slower rate, and persisted through hundreds of milliseconds before evolution to the singlet (16). Fig. 7 presents a plot of the yield of tyrosyl radical (expressed as spins/heme) in KatG[Y229F] reacted with PAA, along with an optical trace at 560 nm showing the increase in concentration of Cmpd II during the same time interval. The yield of radical was evaluated by double integration of EPR signal intensities, whereas the observed change in absorbance at 560 nm obtained in the related optical stopped-flow experiment shows the formation of Cmpd II. The radical concentration increased very rapidly during the initial phase after mixing with peroxide, reaching a maximum of 0.09 spins/mol heme at 100 ms. The EPR signal intensity decreased after this time point. Noteworthy for KatG[Y229F] is the correlation of the rates that demonstrate the kinetic coupling of Cmpd II formation with tyrosyl radical formation. The low yield of tyrosyl

\[ \text{Fig. 5. Reduction of KatG[Y229F] Cmpd II by ascorbic acid or INH. Optical stopped-flow spectra were recorded in double-mixing experiments. A, initial spectrum (1) was recorded after mixing resting enzyme (10 \mu M) with PAA (100 \mu M), } \ t = 1 \text{ s; final spectrum (2) was recorded after a second mixing step in which } 100 \text{ \mu M ascorbate was added (t = 375 s). B, initial spectrum (1) recorded as in A; final spectrum (2) was recorded after a second mixing step in which 500 \mu M INH was added (t = 37.5 s).} \]
radical could result from a relatively rapid quenching of the radical by secondary electron transfer processes that do not affect the stability of Cmpd II.

**Effect of Mutation on Isoniazid Binding**—The affinity of the mutant enzyme for isoniazid was examined by using isothermal titration calorimetry to contribute to our understanding of this interaction and how specific residues in or around the active site affect the drug-binding site. A 50-fold decreased affinity of KatG[Y229F] for INH was evaluated (K_d = 133 μM ± 8 versus 2.5 μM for WT KatG); it was similar to KatG[S315T], a drug-resistant mutant examined previously (K_d = 400 μM) (10).

**DISCUSSION**

We have presented evidence for alterations in the catalytic cycle of *M. tuberculosis* KatG in its Y229F mutant that provides insights into unique relationships between structure and catalytic function. The mutated tyrosine residue is found in a loop of polypeptide in catalase-peroxidases not found in other Class I peroxidase sequences (29). Our results reveal the requirement for this residue and the adduct described above (Fig. 1) in preventing Cmpd II formation and thereby protecting catalase function in the WT enzyme. Loss of catalase function was also reported for *Synechocystis* KatG[Y249F] (13) which carries the tyrosine mutation we explored in the *M. tuberculosis* enzyme. Mutation of the conserved tryptophan residue of the covalent adduct also gives rise to a loss of catalase activity in catalase-peroxidases (3–5). As these tryptophan mutants also seem to enable facile formation of Cmpd III according to the published optical spectra in the reports from other laboratories (3–5) and in our preliminary results on *M. tuberculosis* KatG(W107F), we suggest the more general idea that mutations eliminating formation of the covalent adduct in KatG enzymes lead to enhanced peroxidase and lost catalase activity as an indirect result of facilitated formation and/or stabilization of Cmpd II. Cmpd II is inactive for catalase function but is active in peroxidase cycles. Furthermore, the presence of Cmpd II enhances the production of Cmpd III when hydrogen peroxide is present. The facile formation of Cmpd III also contributes to decreased catalase function, as this intermediate reduces the availability of the obligatory resting enzyme or Cmpd I. In the wild-type enzyme, similar reaction paths are likely to operate after the formation of Cmpd I, though the instability of Cmpds II and III allow it to function as a catalase over a wide range of H_2O_2 concentrations.

The results for *M. tuberculosis* KatG[Y229F] reported here (summarized in Fig. 8) differ somewhat from those reported by Jakopitsch et al. (13) on *Synechocystis* mutant KatG[Y249F], in which Cmpd I formation was also accelerated compared with the wild-type enzyme, whereas the spontaneous evolution of Cmpd III proceeded slowly (0.6 s⁻¹) compared with the 23-fold more rapid reaction reported here (14 s⁻¹). This variation in rate of Cmpd II formation because of endogenous electron transfer may reflect differences between the number and location of tyrosine residues in the two enzymes. It is also likely that the origin of differences in behavior of the mutants results from an altered orientation of the tryptophan ring when the adduct is absent. The new orientation of the tryptophan (and the adjacent histidine) in the mutants apparently improves interactions with peroxides and oxygenous ligands within the distal pocket, leading to more rapid formation of Cmpd I and greater stability of Cmpds II and III. The large increase in the rate of Cmpd I formation in the mutants may be caused by a facilitated initial proton transfer required for peroxide binding. Improved hydrogen bonding between oxygenous iron ligands and the indole ring of a less constrained tryptophan in mutants that lack the adduct may also contribute to the stability of
Cmpds II and III. These speculations are consistent with the proposals by Miller et al. (30) concerning mechanistic details for Cmpd I formation and stabilization of other intermediates in Ccp. The enhancement of steady-state peroxidase activity in the mutants would be explained by the increased lifetime of Cmpd II, which compensates for a decreased availability of Cmpd I, especially because reaction of reducing substrates with Cmpd II is probably rate-limiting in the peroxidase cycles.

The formation of a tyrosyl radical in KatG[Y229F] is kinetically coupled to the reduction of Cmpd I. The electron transfer reaction allows for the detection of Cmpd II because of its rapid accumulation and also because of its enhanced stability compared with that in WT KatG. Tyrosyl radical is also formed in the wild-type enzyme but at a slower rate (16). The identity of the residue-forming tyrosyl radical is still under investigation, but we suggest that the same residue is oxidized in both WT and KatG[Y229F] enzymes. An obvious choice for the tyrosyl radical site in WT KatG would be residue Tyr-229, because it is the tyrosine closest to the active site (11, 12). Arguments against this idea come from preliminary spin trapping experiments and from the observation of weak hyperfine interactions due to 3\(^1\)H and 5\(^1\)H phenolic hydrogens in the tyrosyl radical EPR signal found in WT KatG (16). Such interactions would be absent from a tyrosyl radical on the phenol ring contained in the covalent adduct (11, 12). Radical localization on the same residue in both WT and mutant KatG enzymes is suggested based on the constancy of the principal hyperfine interaction in the doublet signals found for the mutant (Fig. 6) and for WT KatG, which is dependent upon a preserved orientation of the tyrosine ring plane with respect to the \(\beta\)-methylene hydrogens in both cases (16). These observations then suggest that mutation of Tyr-229 and the absence of the covalent adduct actually occur more rapidly electron transfer from the tyrosine to Cmpd I or, alternatively in WT KatG, the electron transfer pathway is inhibited. Protection of the catalase function in the wild-type enzyme is the most important consequence of this.

The covalent adduct may also be required for establishing the proper geometry of residues that contribute to formation of a small molecule binding site described in B. pseudomallei KatG (12). This conclusion is based on the finding of a significantly reduced affinity for INH in KatG[Y229F], suggesting a key structural relationship between two regions near the heme pocket.

The loss of catalase function in KatG[Y229F] does not reflect poor reaction of Cmpd I with a second molecule of hydrogen peroxide, as might be predicted from the combined observation of the low steady-state catalase activity and the enhanced rate of formation of Cmpd I reported here (and for Synechocystis KatG[Y243F]). Instead, it is the rapid endogenous electron transfer consuming Cmpd I that interferes with catalase activity in KatG[Y229F]. This interesting behavior raises questions about the relative stability of Cmpd I in KatG enzymes, the nature of electron transfer processes that could be related to production of a catalytically competent tyrosyl radical, and a role for this radical in processes that are potentially related to the activation of INH. These mechanistic issues are important in the development of our understanding of the catalytic function of KatG.

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