Reduced Affinity for Isoniazid in the S315T Mutant of *Mycobacterium tuberculosis* KatG Is a Key Factor in Antibiotic Resistance*

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Catalase-peroxidase (KatG) from *Mycobacterium tuberculosis* is responsible for the activation of the antibiotic drug isonicotinic acid hydrazide (INH) and is important for survival of *M. tuberculosis* in macrophages. Characterization of the structure and catalytic mechanism of KatG is being pursued to provide insights into drug (INH) resistance in *M. tuberculosis*. Site-directed mutagenesis was used to prepare the INH-resistant mutant KatG[S315T], and the overexpressed enzyme was characterized and compared with wild-type KatG. KatG[S315T] exhibits a reduced tendency to form six-coordinate heme, because of coordination of water to KatG[S315T] in reaction mixtures to which no peroxide or other catalytic rates, poor binding of the drug to the enzyme limits drug activation and brings about INH resistance.

Reduced Affinity for Isoniazid in the S315T Mutant of *Mycobacterium tuberculosis* KatG is a Key Factor in Antibiotic Resistance

Tuberculosis (TB) infection is the leading cause of death due to a single infectious agent, causing over 2 million deaths annually (1). *Mycobacterium tuberculosis* is the causative agent of this infection, and antibiotics have been used to treat the infection since the 1940s. Although Isoniazid (isonicotinic acid hydrazide, INH) is still one of the most effective antibiotics against tuberculosis (2), the number of INH and other drug-resistant strains has increased dramatically. Outbreaks of multidrug-resistant *M. tuberculosis* make the solution of this worldwide health problem even more urgent. The mechanism of action of INH is not fully understood, but it has been clearly demonstrated that the catalase-peroxidase of *M. tuberculosis*, encoded by the katG gene, is required for bacterial sensitivity to INH (3, 4). The katG gene alone can restore INH susceptibility to resistant mutants (*katG<sup>S315T</sup>*) of *Mycobacterium smegmatis* and *M. tuberculosis* (4, 5), and its expression can make certain strains of *Escherichia coli* sensitive to INH (3, 4). INH is a pro-drug that requires activation by KatG leading to its ultimate bactericidal effects that target mycobacterial biosynthesis (6–8). Mutations in the katG gene are the major cause of INH resistance in clinical isolates (5, 9–11).

*M. tuberculosis* KatG, a catalase-peroxidase containing 80-kDa subunits with one heme per subunit in the dimer, is homologous to yeast cytochrome c peroxidase in its N-terminal region, especially in the distal and proximal heme regions (12–18). Catalase-peroxidases are classified as Class I peroxidases (19) though structural and mechanistic differences from this class of enzymes continue to emerge from spectroscopic and functional studies. For example, an unstable but catalytically competent oxyferryl iron-protoporphyrin IX<sup>+</sup>-cation radical intermediate (Cmpd I) is formed in KatG and reacts with INH (20), whereas Cmpd II has not been identified in reactions of Cmpd I with single electron reducing substrates including INH, ascorbate, or ferrocyanide (20, 21). Therefore, INH activation by KatG can proceed at least in part via a classical mechanism requiring the formation of Cmpd I (20–22). Also, tyrosyl radical is readily formed in reactions of the resting enzyme with peroxide, though its function is not yet assigned (21).

The replacement of amino acid Ser-315 in KatG is one of the most commonly encountered substitutions in clinical INH-resistant strains (5, 23–31). For example, more than half of INH-resistant clinical isolates carry the mutation S315T, which usually results in a 20–200-fold increase in minimal inhibitory concentration (MIC) for INH in *vivo*. The KatG[S315T] mutant strain is virulent in the mouse model, indicating that this frequent mutation does not result in a significant loss of bacterial fitness, nor does this mutation lead to a major loss of catalase activity or peroxidase activity measured with artificial substrates in *vitro* (32–34). Recent studies addressing the function of purified KatG[S315T] in INH oxidation (34) showed that it was only moderately less efficient than WT KatG in reaction mixtures to which no peroxide or other initiator of peroxidase activity was added, and this mutant was...
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more significantly defective in INH oxidation compared with WT KatG in a reaction pathway initiated by superoxide generated in the presence of ferric enzyme but not with addition of t-butyl hydroperoxide (35). The latter study pointed toward a faulty reaction between ferric KatG(S315T) and superoxide as the origin of INH resistance. In another study, a subtle change in INH binding to KatG(S315T) was suggested, yet equivalent binding sites for INH in KatG(S315T) and WT KatG were suggested from NMR relaxation data (36, 37). Here, our approach to the problem of understanding INH resistance relies on examination of both the interaction between the resting KatG(S315T) enzyme and the drug and the reaction of the drug with catalytically competent enzyme intermediates such as Cmpd I.

In this paper, we report on the generation of the mutant enzyme KatG(S315T), its overexpression, purification, and characterization. Biochemical and spectroscopic studies using electron paramagnetic resonance (EPR) and optical stopped-flow spectrophotometry, as well as isothermal titration calorimetry (ITC) measurements, were used to determine the consequences of the mutation and the special role of Ser-315 in enzyme function. Our results suggest that a dramatic change in INH binding in KatG(S315T) is a major component in the loss of function under conditions relevant to use of this antibiotic for TB therapy.

EXPERIMENTAL PROCEDURES

Materials—INH (Sigma-Aldrich) was re-crystallized from methanol before use. PAA (Sigma-Aldrich) (32%) was diluted to 10 m in potassium phosphate buffer and was incubated with 780 units/ml catalase (Roche Molecular Biochemicals) for 4 h at 37 °C to remove hydrogen peroxide, followed by removal of the enzyme by ultrafiltration. Restriction nuclease, polynucleotide kinase, DNA ligase, and the Klenow fragment of DNA polymerase were obtained from New England Biolabs, Inc. Other chemicals and reagents, including LB medium, were purchased from Fisher Scientific.

Mutagenesis—Phagemid pBluescript II KS− (pKS II−) from Stratagene was used for cloning, mutagenesis, and sequencing. The plasmid pKAT II (a gift from Stewart Cole, Institute Pasteur, Paris) was used as an overexpression vector for KatG (18) and as the source of the katG gene that was cloned into pKS II− to generate pSY15 used for mutagenesis. E. coli strain DH5α (F−, d80lacZD15MDlacZAYaargU196degolRecAendAhsR17 (m−k+poAAsuE441 thi-lgyrA96v-l) was used as a host for the plasmids and cloning procedures, and B. subtilis matS thi enteric Δlac-proAD matS-Tn10 [F−, proA B−, lacZAM151]. was used in the mutagenesis step (Clontech, Palo Alto, CA). E. coli strain UM262 (recA katC::Tn10 pro leu rpsL hsdM hsdR endl lacY) was the host for overexpression. Mutagenesis was performed using the Transformer™ site-directed mutagenesis kit from Clontech (Palo Alto, CA). The 1.0-kb ClaI-XhoI fragment of the katG gene was subcloned into the pKS II− vector in two steps to generate pSY15 in which site-directed mutagenesis was performed. The mutagenesis primer was CGATCAACCGGCCATGAGGT (S315T); the selection primer was CTGTTGACTTTGATGATTTCAACAGGC. A unique restriction site (ScaI) was replaced with a new unique restriction site (BglII). Bold sequence represents mutated bases whereas the underlined portions represent the restriction enzyme site after conversion of double-stranded plasmid DNA by the Sanger method (39) was used to confirm the desired nucleotide substitution (Gene Link, Inc., Hawthorne, NY). The confirmed mutated katG insert was excised from the pKS II− vector using NheI and XhoI endonucleases. This NheI-XhoI fragment containing the mutation was ligated into the pKATH vector to replace the corresponding wild-type fragment, generating the pSY31 vector.

Purification of M. tuberculosis Catalase Peroxidase—The catalase peroxidases (KatG and mutant KatG(S315T)) used in this study were isolated and purified from an overexpression system in E. coli UM262 strain carrying pKAT II or pSY31 vectors. Bacteria were grown in LB medium plus ampicillin (100 µg/ml) at 28 °C, in 0.5- or 1-liter flasks. Protein expression was induced by the addition of 3-β-indoleacrylic acid (40 mg/l) when the optical density of cultures reached 0.9–1. Delta-aminolevulinic acid (150 µM) was added to cultures to maximize the yield of holoenzyme (20). Cells were harvested 6–18 h post-induction. Purification followed a published procedure using 20 mM potassium phosphate buffer, pH 7.2, instead of TEA-Cl buffer throughout (17). Optimal purity ratios (A407/A280) were ~0.6 for WT KatG and 0.45 for KatG(S315T).

Enzyme Assays—Protein concentration was determined using the heme extinction ε407 nm = 100 m M−1 cm−1. Catalase activity was measured spectrophotometrically by following the decrease in H2O2 concentration at 240 nm (ε383 = 43.8 m M−1 cm−1). One unit of catalase activity is defined as the amount of enzyme that decomposes 1 µmol of H2O2 in 1 min in a 25 mM H2O2 solution at pH 7.2 at 25 °C (32). Peroxidase activity was also assayed spectrophotometrically by following the absorbance change for 0.1 mM o-dianisidine in 50 mM sodium acetate buffer, pH 5.5, in the presence of 23 mM t-BOOH (ε340 = 11.33 m M−1 cm−1) for the dianisidine oxidation product at 25 °C (17). One unit of peroxidase activity is defined as the amount of enzyme that forms the oxidation product of 1 µmol of product/min. Spectrophotometric measurements were obtained using an NT14 UV-Vis spectrophotometer (Aivis Associates, Lakewood, NJ). SDS gel electrophoresis was carried out under denaturating (SDS-PAGE) and non-denaturing conditions using an Amersham Biosciences PhastGel system.

Stopped-flow Optical Measurements—A double mixing stopped-flow apparatus (HiTech Scientific Model SF-61DX2) was used to measure optical spectra during Cmpd I formation from resting (ferric) enzyme and for the reaction of Cmpd I with INH. Data acquisition and analysis utilized Kinet-Ayst software (HiTech Scientific), and all reactions were performed in potassium phosphate buffer, pH 7.2, at 25 °C, as described by Chouchane et al. (20).

Isothermal Titration Calorimetry—Isothermal titration calorimetry was performed using a MicroCal VP ITC calorimeter. Baseline correction, peak integration, and dilution corrections were performed using the ORIGIN analysis software supplied with the instrument. WT KatG and KatG(S315T) samples were prepared in 20 mM potassium phosphate buffer, pH 7.2. Substrate solutions were prepared in the same buffer to ensure minimal background from buffer mismatch. Reliable titration data were obtained using 20 µM enzyme in the sample cell (1.4 ml) and an INH concentration in the ligand delivery syringe that gave an INH-to-heme ratio of either 1.41 or 30:1 (for KatG(S315T) only) at the end of the titration. Typically, 20 injections (10 µl per injection) were made, at 12–15-min intervals, and the heat of reaction per injection (microcalories per second) was determined by integration of the peak areas. Heat of dilution of INH was separately determined by injection of INH into buffer only and was subtracted from the observed heat of binding prior to data analysis. The Origin software provided the best-fit values of H (enthalpy of binding), the stoichiometry of binding (n) per mole of heme, and the dissociation constant (Kd) from plots of heat evolved per mole of substrate injected versus the drug/heme molar ratio.

EPR Spectroscopy—EPR spectra were recorded at X-band with a Varian E-12 spectrometer equipped with a liquid helium cryostat and He-Tran liquid helium transfer system (Advanced Research Systems, Inc, Lakewood, PA) for recording spectra below 77 K. WinEPR software was used for data acquisition (20). Rapid freeze-quench EPR sample preparation was performed using an Update Instrument, Inc. MOD-EL1000 chemical-freeze quench apparatus according to a published procedure (21).

RESULTS

The KatG(S315T) protein used in these studies was produced in E. coli using an overexpression system carrying the mutated M. tuberculosis katG gene. The purified mutant enzyme consistently exhibited a reduced optical purity ratio compared with WT KatG purified under identical conditions (A407/A280 = 0.45 (mutant) versus 0.60 (WT)). One reason for this could be the presence of heme-deficient enzyme. We reported previously that WT KatG isolated and purified after overexpression in M. tuberculosis katG using an overexpression system carried the mutated M. tuberculosis katG gene. The purified mutant enzyme consistently exhibited a reduced optical purity ratio compared with WT KatG purified under identical conditions (A407/A280 = 0.45 (mutant) versus 0.60 (WT)). One reason for this could be the presence of heme-deficient enzyme. We reported previously that WT KatG isolated and purified after overexpression in E. coli grown in LB medium, in the absence of the heme biosynthetic precursor δ-aminolevulinic acid (δ-ALA), contains a nearly equal yield of heme-deficient enzyme and holoenzyme, whereas in the presence of δ-ALA, the heme-deficient enzyme is eliminated. For KatG(S315T) grown in LB medium without δ-ALA, the yield of holoenzyme was extremely low at best. A notable improvement in yield of holoenzyme was found after growth in the presence of δ-ALA, though purified KatG(S315T) still had a low optical purity ratio compared with WT KatG. Also contributing to this lower optical ratio is the greater abundance of 5-c heme in the purified mutant enzyme, in which...
conversion to 6-c enzyme is less extensive than in WT KatG (see below). The 6-c enzyme formed by coordination of a molecule of water to heme iron has a higher Soret extinction coefficient than 5-c enzyme. This is reflected in the optical purity ratio for the wild-type enzyme, which ranges from 0.55 in samples containing mostly 5-c heme, to 0.65 or greater in samples containing a majority of 6-c heme (40). The purity ratio for KatG[S315T] ranges from 0.44 to 0.46 in our preparations, which contain mostly 5-c heme; therefore, assuming that the extinction coefficients for 5-c heme in WT and mutant enzymes are the same, and that the optical purity ratio for the purified mutant should also be 0.55, 18% of the purified mutant enzyme lacks heme. Because enzyme concentrations and activities are calculated based on heme active site concentration rather than total enzyme, the apoenzyme is not accounted for in our experiments on KatG[S315T]. The apoenzyme would not be expected to contribute catalase or peroxidase activity, which resides solely in the heme cofactor according to inhibition by cyanide and azide (17).

In addition to the difference in optical purity ratio, a small red shift of the Soret maximum (1–2 nm) and a blue shift of the CT1 band (645 versus 635) were usually noted in the purified mutant compared with WT KatG (see Fig. 1 and Table I). These small differences are also consistent with a low abundance of 6-c heme in purified KatG[S315T].

Catalase and peroxidase activities of purified KatG[S315T] are reduced by nearly 2-fold and 4-fold, respectively (Table I), compared with the wild-type enzyme prepared using the same procedure (20). Similar reductions in catalytic ability have been reported by others (5, 32, 34). Because these steady-state measurements do not provide insight into the kinetics of the separate reactions in the multi-step catalytic cycles, optical stopped-flow experiments were pursued.

Stopped-flow Spectrophotometry—KatG[S315T] maintains significant levels of catalytic activity. Therefore, formation of Cmpd I and/or other peroxidase intermediates was considered important to investigate, especially in the context of potential INH resistance mechanisms. We reported previously (20, 22) second order rate constants for formation of Cmpd I in WT KatG and another mutant (KatG[W321F]) using various peroxides and also reported that INH reacted with this intermediate in KatG and HRP. Examination of the formation of Cmpd I in the mutant KatG[S315T] revealed some interesting differences from the wild-type enzyme. For example, neither CPBA (up to 5 mM) nor t-BOOH (250–500 mM) gave a detectable yield in conversion of the resting mutant enzyme to Cmpd I under conditions clearly shown to produce this intermediate in WT KatG.

Evidence for Cmpd I formation was only demonstrated in reactions of KatG[S315T] (10 μM) with PAA (30–100 μM), evidenced by a decreased Soret intensity and characteristic features in the visible region (peaks at 550 and 590 nm, along with a shoulder at 655 nm). Fig. 2 shows optical stopped-flow data for 10 μM KatG[S315T] mixed with 100 μM PAA (similar changes but with lower yield of Cmpd I were obtained using 3-fold or 5-fold excess PAA). The apparent yield of Cmpd I did not increase using higher concentrations of PAA. The total initial Soret absorbance decrease for the mutant is smaller than that observed for WT KatG under similar conditions, possibly because of differences in extinction coefficients for both resting enzyme and Cmpd I in these two enzymes under the conditions used here. Similar to WT KatG Cmpd I, KatG[S315T] Cmpd I decays to the resting enzyme in the absence of added substrates, after a steady-state interval that depends on the total peroxide concentration. This behavior was interpreted to represent consumption of peroxide during cycling of the enzyme through hypervalent intermediates and its return to the resting (ferric) state when peroxide becomes unavailable (20, 22). Under the same conditions, the duration of this interval in the mutant is 20% longer than that for WT KatG (128 s versus 106 s) (Fig. 3) consistent with slower cycling reactions, which are not investigated further at this time.

Measurement of the rate of Cmpd I formation (20) allowed estimation of second-order rate constants equal to 2.93 × 10^4 M⁻¹ s⁻¹ at 25 °C and 2.21 × 10^4 M⁻¹ s⁻¹ at 5.5 °C, similar to WT KatG (Table I). (Linear plots (R-squared coefficients of determination above 0.996) were generated from duplicate measurements of pseudo-first order rates as a function of three or four PAA concentrations.) This result is important in light of the very poor reaction of the bulky peroxides with resting mutant enzyme.

For WT KatG, Cmpd III (oxygenferrous enzyme) is formed by addition of a large excess of H₂O₂ (mM range) to the resting enzyme, though this intermediate is not stable. In the case of KatG[S315T], small to moderate excesses of H₂O₂ did not elicit any change in optical spectrum (not shown), as reported for WT KatG (20). However, even large excesses of peroxide (5 mM) did not elicit the optical spectrum of Cmpd III in the mutant. Instead, the only detectable change in optical spectrum was a

![Fig. 1. Optical spectra of resting WT KatG and KatG[S315T].](image1)

![Fig. 2. Spectral changes upon addition of PAA to resting catalase-peroxidase observed using stopped-flow spectrophotometry. 100 μM PAA was added to 10 μM resting catalase-peroxidase in 20 mM phosphate buffer, pH 7.2. The initial, final, and two intermediate spectra are shown. 1, resting enzyme KatG[S315T]; 2, KatG[S315T] Cmpd I. Inset, visible region of initial and final spectra.](image2)
small increase in intensity of the Soret peak (with a small red shift) along with a blue shift of the CT1 band. These changes do not represent formation of Cmpd III (Soret peak near 418 nm, H9251 and H9252 bands at 570 and 540 nm) but instead are consistent with transient coordination of H2O generated as a product of the catalase reaction. Direct evidence for this was found in stopped-flow experiments presented below.

Reaction of Cmpd I with INH—KatG Cmpd I was reported previously (20) to be catalytically competent in INH oxidation. Here, we again used double mixing stopped-flow spectrophotometry to investigate this reaction further in both WT KatG and the mutant. In single mixing experiments, using 10 μM KatG or KatG[S315T] mixed with 100 μM PAA, formation of Cmpd I was observed (see Figs. 2 and 3), and we thereby established conditions under which Cmpd I could be pre-formed for double mixing experiments. In the double mixing protocol, KatG or KatG[S315T]-Cmpd I formed during an initial reaction (2-s delay) was mixed with INH in a range of concentrations (5, 10, 20, 50, 100, 200, and 500 μM final), and absorbance changes were then followed. INH accelerated the return of Cmpd I to the resting state in both enzymes, in a concentration-dependent manner (Fig. 4, A and B). The optical changes reflect a combination of reactions that presumably include reduction of Cmpd I to Cmpd II and then Cmpd II to the resting enzyme, though Cmpd II is not detected. Note that previous results (20) demonstrated that in the case of HRP, INH cleanly produces Cmpd II from Cmpd I, confirming the drug’s function as a single-electron reducing substrate in the classical peroxidase cycle. We currently believe that Cmpd II is very unstable in KatG, preventing its accumulation under these conditions.

There are multiple phases in the absorbance versus time traces shown in Fig. 4, for which data collection is shown after the initial 2-s delay. In the first phase (from 0 to 4 s; inset), a rapid increase in absorbance at 407 nm took place, probably as a consequence of binding of INH to Cmpd I. No significant changes in the visible region of the spectrum were seen during this phase. This observation may be related to the pre-equilibrium between Cmpd I and CPBA reported for HRP (41). After this initial absorbance increase, a slower reaction subsequently regenerated the spectrum of the resting enzyme evident in the...
changes in both the Soret and visible regions. Also evident in the stopped-flow data is the formation of an intermediate having a higher absorbance than the final product. This phenomenon is notable at the highest concentrations of INH (Fig. 4A), after the initial rapid rise in absorbance, and is consistent with formation of 6-c enzyme after completion of the peroxidase cycle, followed by its conversion to 5-c enzyme as INH rebinds to the ferric enzyme. The optical change recorded during this phase is also dependent on the concentration of INH. For the mutant, similar behavior occurs (Fig. 4B), but to a much lesser extent than in WT KatG. Here, both the low affinity for the drug and the mutant’s preference for 5-c heme likely contribute to this difference from WT enzyme. Conversion of 6-c to 5-c heme because of the dissociation of water upon INH binding to WT KatG has been described in separate titrations of the enzyme with the drug (36, 40).

Relative to its lifetime after mixing with buffer alone, the decay of Cmpd I in the presence of INH (evaluated as the time required for the absorbance to reach its maximum value after the second mixing step) was accelerated 2.5-fold (100 μM INH), 3-fold (200 μM INH), and 5-fold (500 μM INH) for WT KatG (Fig. 4A). KatG[S315T] Cmpd I reacted much more slowly with INH, and even at the drug concentration of 500 μM, this decay was only accelerated 2-fold compared with the rate with buffer alone (Fig. 4B). Ascorbate also accelerated the rate of decay of Cmpd I in the mutant (data not shown), as shown previously for WT KatG (20). Here again, no intermediates such as Cmpd II could be detected in the double mixing stopped-flow experiments.

The inset (Fig. 4, A and B) shows the concentration dependence of the time course for formation of the Cmpd I-INH complex in WT KatG. This initial absorbance increase was not seen in the reaction of mutant Cmpd I with INH except at very high drug concentration. These results point to poor interaction between the drug molecule and Cmpd I of the mutant enzyme. The concentration dependence of the rates for the pre-equilibrium and the subsequent reactions are non-linear, and second order rates were not calculated.

**ITC**—Optical difference spectroscopy was used by us and in other laboratories (22, 36) to estimate affinity constants for the binding of INH to KatG. This approach relies on the fact that titration of KatG with drug elicits spectral changes in the resting enzyme consistent with conversion of 6-c heme into 5-c heme. When the concentration of 6-c enzyme in the enzyme is low, no optical change can be detected upon addition of INH, as we reported for freshly isolated, partially purified KatG in which 5-c heme predominates (40). The low abundance of 6-c heme in purified KatG[S315T] suggested that a more useful approach for evaluation of INH affinity would be a direct thermodynamic method such as ITC. In this technique, a small molecule ligand is titrated into a solution of the enzyme, and the heat evolved during each step in the titration is calculated. A binding curve is generated from this raw data, and values for the dissociation constant, number of binding sites, enthalpy and entropy of binding are evaluated. Results for WT KatG demonstrate complete titration of the enzyme (saturation) with only a small excess of INH (molar ratio of INH to heme, −1.5) (Fig. 5A). A K of $4 \times 10^5$ M$^{-1}$ ($K_d = 2.5 \mu M$) and n values close to 0.5 (and $\Delta H = -1 \times 10^5$ cal/mole, and $\Delta S = -330$ cal/mole/deg) were found. The micromolar dissociation constant correlates with the drug concentration range relevant to the MIC for wild-type M. tuberculosis (0.05 μM or 0.36 μM). Under similar conditions, a featureless binding titration curve was found for KatG[S315T]. Titration of KatG[S315T] with large excesses of INH (Fig. 5B) allowed estimation of a dissociation constant ($K_d$) of $400 \mu M$, hundreds of times greater than that for WT KatG. Thus, the binding of INH to the mutant is demonstrated to occur in the range of the MIC (10 μg/ml or 70 μM) for the INH-resistant M. tuberculosis S315T strain in vivo (5, 24). This is the first direct demonstration of correlations between MIC values and the affinities of purified KatG enzymes for the drug molecule in vitro.

**Heme Iron Coordination Number**—Here, we return in more detail to the issue of iron coordination number in KatG mentioned above. We have shown recently (40) that the relative abundance of 5-c and 6-c heme species is variable in WT KatG and depends on factors including choice of buffer used for purification and storage conditions after purification. The most significant observation for wild-type enzyme was that the majority 5-c heme species in the freshly isolated enzyme decreases whereas the abundance of 6-c heme increases as a function of time, over a period of weeks. Fig. 6 shows the low temperature EPR spectra of freshly isolated ferric WT KatG, KatG after storage for 3 weeks, and KatG[S315T] also after storage for 3 weeks. The spectrum of KatG[S315T] has two predominant signals: $r_1$ ($g_1 = 6.3; g_2 = 5.14; g_3 = 2$) and $r_2$ ($g_1 = 6.0; g_2 = 5.2; g_3 = 2$) similar to the wild-type enzyme examined directly after purification (before storage). These signals were assigned previously to 5-c and 6-c heme species, respectively. A small component, $r_3$ ($g_1 = 6.6; g_2 = 5.2; g_3 = -2$), can also be observed, which is attributed to a second 5-c high spin species. For the wild-type enzyme examined after storage, the $r_1$ signal is no
In the wild-type enzyme, we demonstrated the form of KatG. In the mutant, along with a greater stability of 5-c compared to results, which were confirmed at room temperature using Raman spectroscopy, demonstrate that the mutant is more resistant to conversion to the 6-c form. Our observations contrast with those showing abundant low spin heme in purified KatG[S315T] presented elsewhere (42).

DISCUSSION

We set out to study the catalytic function of KatG[S315T] in the context of what is already known about this prevalent drug-resistant mutant from biochemical and cellular studies. The information obtained points to the following facts: 1) strains carrying this mutation are viable and virulent pathogens (33, 43), 2) a binding site for the drug is present in the mutant at a distance from the heme iron equivalent to that in WT KatG (36, 37), 3) according to spin-trapping experiments reported elsewhere (44) KatG[S315T] in the presence of very large excesses of INH and the peroxidase cycle initiator t-BOOH produces nearly equal yields of drug-derived radicals as WT KatG over a period of hours, 4) the mutant apparently oxidizes INH more slowly than WT KatG in the absence of added peroxide or other peroxidase cycle initiator (34). Lower yields in drug oxidation by KatG[S315T] compared with WT KatG were also found in the presence of superoxide, though little difference was found when t-BOOH was added to ferric enzymes (35). Evidence for mildly reduced catalytic competence of the mutant from results in these other studies, but mechanistic changes in the peroxidase cycle, which we now believe is the most relevant path for drug activation in vivo, have not been identified. Although it had been reported that oxyferrous KatG might be catalytically competent in drug oxidation (45), the standard peroxidase cycle is now considered to be operative and relevant for INH activation based on our observation that KatG Cmpd I is kinetically competent and is reduced by INH and that INH acts as a single electron reductant in a classical peroxidase cycle in HRP (29).

The spectroscopic characterization of KatG[S315T] presented here demonstrated a slightly reduced affinity for heme in this mutant, along with a greater stability of 5-c compared with 6-c heme, the former defined elsewhere as the "native" form of KatG. In the wild-type enzyme, we demonstrated the evolution of 6-c heme from 5-c heme because of accumulation of iron-coordinated water during purification and storage under a variety of conditions (40). An extensive Raman analysis of KatG and this mutant to be reported elsewhere provided clear evidence for the predominance of 5-c heme in this mutant. Low temperature EPR results presented here are also consistent with this feature of the mutant. These observations suggest an interesting correlation between iron coordination number and the structure of the enzyme in a region remote from the distal pocket. Iron coordination number is commonly considered to be governed by side chain interactions within the distal pocket surrounding sixth ligands such as water. Serine 315 in M. tuberculosis KatG, by analogy to the conserved serine 305 in H. marismortui catalase-peroxidase, is located near a heme propionate side chain at which hydrogen bonding occurs among the carboxylate group, a molecule of water, and the hydroxyl group; thus, some small alteration in heme binding upon mutation of serine to threonine may reasonably be related to a poor geometry for this hydrogen bonding arrangement at the heme periphery. This change in the mutant must also interfere with the geometric adjustments in the heme pocket that accompany formation of 6-c heme. Consistent with the reduced tendency for water coordination in the mutant is its lower affinity for cyanide (36). These differences between WT KatG and the mutant studied here are not considered critically important in explaining INH resistance, because the rate of formation of KatG Cmpd I is not different for 5- and 6-heme (40).

KatG[S315T] has lower catalase activity (2259 units/mg) than KatG (3771 units/mg) and also lower peroxidase activity (0.26 units/mg) compared with WT KatG (0.9 units/mg) when measured using t-BOOH. Note that we did not observe formation of Cmpd I in the mutant using t-BOOH in stopped-flow experiments. That peroxidase activity can be initiated using t-BOOH, however, indicates that Cmpd I is generated in its presence, yet no accumulation of this intermediate was detected under a range of concentrations in stopped-flow experiments. These observations are consistent with low-level production and relatively rapid decay of Cmpd I under these conditions. Poor turnover with t-BOOH is likely to be related to the location of the serine 315 residue at a narrow point in an access channel from the surface of the enzyme (46), which in the Ser-Thr mutant is occupied by a bulkier side chain. Thus, increased steric constraints on heme access contribute to the poorer steady-state peroxidase activity in the mutant because of poor access of t-BOOH to iron. On the other hand, rates of turnover of resting enzyme with PAA to give Cmpd I are not reduced compared with WT KatG, consistent with unhindered access of this small peroxide. Suggestions about this mutation being related to heme access had been put forth in earlier work on KatG[S315T] (32).

Other peroxides, including H₂O₂ or CPBA, did not produce the expected intermediates in the mutant, but for different reasons. For example, Cmpd III could not be identified in the mutant when it was treated with large excesses of hydrogen peroxide under conditions that led to Cmpd III in WT KatG. Because the mutant retains catalase activity, Cmpd I must be formed in the presence of hydrogen peroxide. To form Cmpd III, hydrogen peroxide must react with Cmpd II, assuming KatG follows plant peroxidase reaction paths (47). The absence of Cmpd III in the mutant suggests either that a very fast rate for reaction of Cmpd I with hydrogen peroxide prevents accumulation of Cmpd II in other reaction paths, or a highly unstable Cmpd III is formed in the mutant. Arguing against the former idea is the finding of lower catalase activity in the mutant, rather than higher, compared with WT KatG. In favor of the second idea are the observations of Wengenack et al. (35), showing that reactions initiated by superoxide acting on the ferric mutant enzyme KatG[S315T] are disfavored compared

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* S. Kapetanaki and J. P. M. Schelvis, personal communication.
with similar reactions in WT KatG. This would be explained by a reduced affinity of the ferric mutant enzyme for superoxide. Note that for WT KatG, Cmpd III (which can be formulated as oxyferrous heme or ferric-superoxy heme) decays spontaneously to resting enzyme, consistent with dissociation of superoxide. The increased stability of 5-c-heme in the mutant also argues in favor of an unstable Cmpd III in KatG[S315T]. The poor reaction of KatG[S315T] with CPBA likely reflects steric effects similar to those responsible for blocking access of tBOOH to the heme pocket.

The results of ITC experiments demonstrate the low affinity of the resting mutant enzyme for the drug. This observation alone would strongly suggest an origin for drug resistance because of this mutation. However, it was conceivable that the catalytically relevant intermediate Cmpd I could overcome the poor binding in the resting enzyme if drug binding to Cmpd I had a rapid on-rate. We rule out this possibility based on our stopped-flow results showing neither a more rapid binding reaction nor an increased turnover rate of KatG[S315T] Cmpd I with the drug. This set of observations shows that INH affinity for the resting enzyme is correlated with reactivity of the drug with KatG Cmpd I and suggests a common drug binding site in both enzyme states.

In conclusion, KatG[S315T] in both the resting state and in its Cmpd I intermediate exhibit very low affinity for INH, which is likely the source of decreased susceptibility to INH in strains carrying this mutation. Also, these results suggest that redesign of the INH molecule to improve drug binding may be a viable approach to overcome resistance in KatG[S315T]. Our results fit into a model in which KatG[S315T] mutation does not compromise survival or virulence yet provides moderate drug resistance in clinical settings significant enough to cause a worldwide problem in TB treatment.

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Reduced Affinity for Isoniazid in the S315T Mutant of Mycobacterium tuberculosis KatG Is a Key Factor in Antibiotic Resistance
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